MOLECULAR MECHANISMS IN DNA REPLICATION & RECOMBINATION

Organizers: Harrison Echols and Anna Marie Skalka

January 25 - February 1, 1992

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Keynote Address

F 001 REPLICATION AND RECOMBINATION OF A EUKARYOTIC VIRAL CHROMOSOME, Robert Lehman, Paul Boehmer, Robert Bruckner, Mark Dodson, Rebecca Ellis Dutch, Thomas Hernandez, Byeong Doo Song and Boris Zemelman, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305-5307.

We have chosen the linear 153 kb genome of herpes simplex 1 virus (HSV-1) as a model for the replication and recombination of a eukaryotic chromosome.

The products of seven HSV-1 genes are necessary for HSV-1 DNA replication *in vivo*. These proteins, which have been obtained in near homogeneous form, include a DNA helicase that binds specifically to an HSV-1 origin, a heterotrimeric helicase-primase, a DNA polymerase with its associated processivity enhancing factor and a single-stranded DNA binding protein. Our recent analyses of these enzymes will be presented as will our attempts to reconstitute replication of HSV-1 origin containing plasmids *in vitro*.

During the course of infection, elements of the HSV-1 genome undergo inversion through the viral *a* sequences. To investigate the mechanism of this recombinational event, we have developed an assay that detects the deletion of DNA segments flanked by directly repeated *a* sequences in plasmids transiently maintained in Vero cells. With this assay, we have observed a high frequency of recombination (approximately 8%) in plasmids that undergo replication in HSV-1 infected cells, and a low level of recombination between *a* sequences in plasmids introduced into uninfected cells, and in unreplicated plasmids in HSV-1 infected cells. We have also observed a low frequency of recombination between *a* sequences promoted by partially purified extracts of HSV-1 infected and uninfected Vero cells.

DNA Structure and Topology

F 002 STRUCTURAL INFORMATION RELEVANT TO TRANSCRIPTION, TRANSVERSION AND FRAMESHIFT MUTATIONS, FROM X-RAY DIFFRACTION STUDIES, Olga Kennard, Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge, UK

The development of techniques of oligonucleotide synthesis has opened up the possibility of crystallising DNA fragments containing mismatched base pair sequences as well as sequences characteristic of mutational hot-spots. The analysis of such crystals by X-ray diffraction has yielded experimental structural models relevant to an understanding of mechanisms involved in certain mutational events.

Base pair mismatches have been studied in a variety of DNA conformations. These studies were designed to explore questions such as the nature of the hydrogen bonding between mismatched base pairs, the influence of nearest neighbours and DNA conformation on mispair formation, the effect of mismatches on the structure of the double helix, the recognition of mispairs and factors influencing the stability of mismatched base pair. (For a recent review see O.Kennard and W.Hunter, Angewandte Chemie 1991 in Press).

Recently the crystal structures of two dodecamer were solved, one containing the sequence (GGCGCC), the mutational hot-spot of the restriction enzyme *Nar* I. It was analysed at two different temperatures the temperature change unexpectedly leading to the propagation, along the helix axis, of a locally altered conformation. The second structure has a a closely related sequence, which corresponds to the codon 10-13 of the *c-Ha-ras* protooncogene. The dodecamers exhibit a novel type of interaction between the functional groups of specific bases and backbones of adjacent helices (Y.Timsit, E.Westhof, R.P.P. Fuchs, D.Moras, Nature, 341, 459-462, 1989 and Y.T.Timsit E.Vilbois and D.Moras, Nature, in press and personal communication). The interaction appears to be sequence dependent, and can serve as a model for a possible structural mechanism involved in frameshift mutations.

The paper will discuss the scope and limitations of the X-ray diffraction methods and the way in which models derived from the X-ray analyses can contribute ideas to our understanding of the transfer of genetic information.

Polymerase Mechanism

F 003 ON VARIOUS ASPECTS OF THE DNA POLYMERASES, Stephen J. Benkovic, Michael Dahlberg, James Peleska, Michelle West, Chris Lively, Todd Capson, Bryan Eger, and Barbara Kaboord, The Pennsylvania State University, Department of Chemistry, 152 Davey Lab., University Park, PA 16802.

The kinetic sequences used for extension of the primer template substrate by various DNA polymerases including the Klenow, beta and T4 polymerases will be reviewed. The contributions individual steps make to the overall fidelity of the replication will be examined. Particular attention will be paid to the T4 polymerase enzyme and the integration between the polymerase and exonuclease activities required to achieve high fidelity in its replication. On the basis of pre-steady-state and steady-state experiments a kinetic sequence has been formulated for the T4 polymerase enzyme that can be compared and contrasted to other polymerases. This kinetic sequence will serve as a basis for examining the role of accessory proteins in the fidelity or the processivity of the T4 polymerase enzyme.

F 004 **ASYMMETRIC DIMERIC REPLICATIVE COMPLEXES: STRUCTURE, MECHANISM AND INTERSUBUNIT COMMUNICATION**
C. McHenry, J. Reems, M. Griep, J. Carter, M.Franden, J. You, R. Thimmig, T. Knauf, and R. Aebersold. Dept. Biochem., Biophys. & Genet., Univ. of Colorado Health Sciences Center, Denver CO 80262

DNA polymerase III holoenzyme, the replicative complex of *E. coli*, is a ten-subunit asymmetric dimeric enzyme with putative functionally distinguishable leading and lagging strand halves that act coordinately at the replication fork. To understand the role of the holoenzyme subunits in establishing this asymmetry and in coordinating the two halves, it is important to establish the structure of the enzyme at the level of subunit organization and subunit-template-primer interactions. To accomplish this, we are taking a tripartite approach. The linear arrangement of subunits along the template-primer is being determined by photocross-linking, distances between sites are being determined by fluorescence energy transfer and subunit-subunit contacts will be determined by chemical cross-linking. DNAse I protection experiments have established that the holoenzyme occupies a 30-nucleotide footprint on primers. To determine the position of holoenzyme subunits within the footprint, we have placed photoreactive nucleotide analogs (using the methods of Benkovic and colleagues) in a series of primers at unique positions within the footprinted region. Photocross-linking establishes which subunit contacts the photoreactive position. For example, we have determined that γ occupies the -3 position (relative to the 3'-primer terminus) in preinitiation complexes (complexes containing the auxiliary subunits but lacking polymerase) but that a rearrangement occurs bringing the polymerase (α) subunit in contact upon initiation complex formation. Fluorescence energy transfer from a donor attached to the -3 position to an acceptor attached to cys_{333} of the β subunit indicates that it resides 65Å away, two full helical turns from the primer terminus.

The structural genes for only five of the ten holoenzyme subunits (α, τ, γ β and ϵ) have been identified, cloned and overproducing strains for th corresponding subunits constructed. The lack of overproducers for th remaining subunits has hampered more sophisticated enzymologica approaches. To overcome this obstacle, we have taken a "reverse genetic approach to isolate the remaining holoenzyme genes. The sequences of and ψ corresponded to partially sequenced open reading frames alread in databases (15 and 96 min., respectively). The structural gene for δ' i distinct from δ and maps at 24.5 min.

The generality of the asymmetric dimer hypothesis is being tested in retroviral system to determine whether the two halves of the structurall asymmetric HIV p51/p66 reverse transcriptase possesses differen functional properties, consistent with distinct (+) and (-) stran polymerases. To enable this, the known components of the HI replicative system have been produced by a strategy that does no employ the use of infectious particles. Recombinant HIV p51 and p6 reverse transcriptase subunits and HIV nucleocapsid protein have bee produced with termini identical to those present in the viral protein The human $tRNA^{lys}_3$ primer and model templates containing HIV LTR have been made *in vitro* using T7 RNA polymerase. It has been foun that the p51 and p66 subunits have strikingly different properties in term of salt sensitivity and template preference. These findings are consisten with our hypothesis.

F 005 MECHANISM OF DNA POLYMERASE III HOLOENZYME,
Mike O'Donnell, Rene Crombie, Ziming Dong, Rene Onrust, Maija Skangalis, P. Todd Stukenberg, Patricia Vaughan and Hui Xiau, Howard Hughes Medical Institute, Microbiology Department, Cornell University Medical College, NYC, NY 10021

The chromosomal replicase of E. coli, DNA polymerase III holoenzyme (holoenzyme), is comprised of at least 10 subunits ($\alpha,\epsilon,\theta,\tau,\gamma,\delta,\delta',\chi,\psi,\beta$). The holoenzyme is rapid (500 nucleotide/s) and highly processive (>8kb). The high processivity of the holoenzyme derives from its accessory proteins. Reconstitution studies show that the accessory proteins assemble a "preinitiation complex" on primed M13mp18 ssDNA. This preinitiation complex confers onto the core polymerase ($\alpha\epsilon\theta$) the processive synthesis characteristic of the holoenzyme. We have examined the mechanism and structure of the preinitiation complex. We find the γ complex ($\gamma\delta\delta'\chi\psi$) recognizes primed DNA and couples ATP to clamp the β subunit onto DNA. The γ complex acts catalytically in this process, much like a chaperonin in using ATP to assemble a complex (β·DNA). Once the β dimer is fastened onto DNA it behaves like a washer on a steel rod, free to slide back and forth on duplex DNA. β also binds directly to the α, the DNA polymerase subunit. Hence, the β clamp confers high processivity onto the polymerase by tethering it to DNA for extensive synthesis. X-ray analysis of β is in progress in collaboration with Dr. John Kuriyan.

We have also examined the mechanism by which holoenzyme cycles from a completed template to a new primed DNA. Rapid cycling of holoenzyme is anticipated from its low cellular abundance yet the need to extend thousands of RNA primers in discontinuous synthesis of the lagging strand. However, in vitro, the holoenzyme requires several minutes to cycle from a completed DNA to a new primed template. We have developed an experimental system in which the holoenzyme cycles rapidly. The key feature which promotes rapid cycling is formation of a β clamp on the new template. We have used ^{3}[H] subunits to examine the mechanism and fate of subunits during cycling. We find the new β clamp induces disaggregation of the holoenzyme. The core is taken up by the new β clamp; the old β clamp is left behind. The holoenzyme only disaggregates after complete replication of the template. This feature may ensure that the holoenzyme at a replication fork would replicate an Okazaki fragment to completion before being disaggregated by a new β clamp.

To elucidate the function of each holoenzyme subunit we have identified the genes of the remaining five subunits for which no gene had been identified. These genes for $\delta,\delta',\chi,\psi,\theta$ lie in distinct reading frames. These subunits have been overproduced and purified by molecular cloning of their genes. We hope to fully reassemble the holoenzyme from its component parts and, in so doing, determine the actual structure of the molecule.

F 006 COMPARISON OF THE STRUCTURES OF HIV REVERSE TRANSCRIPTASE AND THAT OF THE KLENOW FRAGMENT OF DNA POL I BOUND TO DUPLEX DNA,
Thomas A. Steitz, Lori Kohlstaedt, Jimin Wang, Jonathan Friedman, Phoebe Rice and Lorena Beese, Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University, New Haven, CT 06511

Both the reverse transcriptase (RT) from HIV and the Klenow fragment (KF) of DNA polymerase I from E. coli are DNA polymerases, the former accepting either RNA or DNA as template and primed initially by $tRNA^{Lys}$, and the latter accepting only DNA as template. We have determined the crystal structures of KF with bound duplex DNA and dNTP substrates as well as that of HIV RT. RT is a heterodimer of a 66 kD subunit containing a polymerase and an RNase H domain and a 51 kD subunit containing only the polymerase domain. A 3.5 Å resolution map of the heterodimer crystallized in space group C2 with a=122.6, b=70.2, c=106.4 Å, β=105.6° shows that the two subunits are not related by a simple rotation axis and the two polymerase domains have strikingly different conformations in spite of having identical sequences. The structures of the polymerase domains of these two enzymes are clearly related though not identical. The site for duplex DNA bound to KF is in an unexpected location that is, however, consistent with the relative positions of the RNase H and polymerase domains of RT, assuming that these two enzymes function similarly.

Multiprotein Replication and Recombination Complexes

F 007 THE RECA PROTEIN AS A RECOMBINATIONAL REPAIR SYSTEM, Michael M. Cox, University of Wisconsin, Madison, WI.

The *Escherichia coli* RecA protein plays a central role in homologous genetic recombination, recombinational repair, and several other processes in bacteria. *In vitro*, an extended filament involving thousands of RecA monomers promotes a reaction in which individual DNA strands switch pairing partners (DNA strand exchange). This reaction has been extensively studied as a paradigm for the central steps in recombination. Because the strand-exchange reaction is relatively simple and isoenergetic, the complexity of the RecA system that carries it out has led to controversy about the functional significance of many fundamental properties of RecA. Filamentous protein structures involving thousands of RecA monomers, which hydrolyze 100 ATPs per base pair of heteroduplex DNA formed, are hard to rationalize in the context of recombination between two homologous DNAs. The thermodynamic barriers to strand exchange are much too small. These molecular features of the system can be easily rationalized, however, by shifting the focus to DNA repair.

F 008 MULTI-PROTEIN INTERACTIONS INITIATING λ DNA REPLICATION AND REPLICATIVE BYPASS OF DNA LESIONS, H. Echols, H. Hoffmann, S. Lyman, C. Vasilikiotis, M. Dodson, M. Rajagopalan, S. Kwack, and C. Lu, University of California, Berkeley, CA 94720.

Initiation of DNA replication by phage λ proceeds through the assembly and disassembly of a specialized nucleoprotein structure at the origin (*ori*λ) sequence. This pathway serves to position the DnaB helicase at the origin and initiate the localized DNA-unwinding reaction required for specific initiation. By studying the protein-protein interactions with physical and biochemical methods, we have suggested the following order of events. The assembly reaction proceeds by association of P·DnaB with O·*ori*λ, after which DnaJ is added by interaction with DnaB. In the presence of high levels of DnaK and ATP, a partial disassembly reaction releases much of the P and DnaJ, allowing DnaB to function as a helicase. The disassembly pathway probably involves an ATP-dependent three-protein interaction between DnaJ, DnaK, and P. If GrpE is added to the complete system, the disassembly reaction requires less DnaK, and DNA-unwinding becomes about 50% bidirectional, a closer approximation to the replication pattern *in vivo* than the DnaJ·DnaK reaction.

We have also been studying the role of the UmuC and UmuD proteins in DNA lesion-induced mutagenesis, which is likely to involve a multi-protein interaction at the replication-blocking damage, facilitating bypass by DNA polymerase III. A partially purified UmuC protein enhances the bypass of pyrimidine dimers and abasic lesions, but the individual interactions have not yet been characterized.

F 009 MAPPING PROTEIN BRIDGES IN THE HIGHER-ORDER COMPLEXES OF LAMBDA SITE-SPECIFIC RECOMBINATION, Arthur Landy[1], Sunghoon Kim[2], Lina Moitoso de Vargas[3], Sok Hong Kho[1], Simone Nunes-Duby[1], [1]Division of Biology and Medicine, Brown University, Providence, RI 02912, [2]Dept. of Biology, MIT, Cambridge, MA 02139, [3]New England Medical Ctr., Boston, MA 02111.

The bivalent DNA binding protein Int and the sequence-specific DNA bending proteins IHF, Xis and FIS comprise the key structural elements of higher-order protein-DNA complexes mediating synapsis and strand exchange in lambda site-specific recombination. Protein-protein and protein-DNA interactions in the attR and attL complexes of excisive recombination have been studied using suicide recombination substrates, nuclease protection, gel shift titrations, quantitative immuno-blotting and patterns of synergism among pairs of mutants with marginally impaired recombination function.

λ excisive recombination occurs between the two prophage sites, attL and attR, that form the junctions between an integrated provirus and E. coli chromosomal DNA. The products of this recombination, attP and attB, are themselves substrates for the integrative recombination that generates the provirus. Strand exchange is executed by the phage-encoded protein Integrase (Int), which has two autonomous DNA binding domains: the amino-terminal domain binds with high-affinity to "arm-type" sites distant from the region of strand exchange; the carboxy-terminal domain, which also contains the DNA nicking and ligating activity, binds with low-affinity to "core-type" sites, where strand exchange takes place. All the binding sites for the three DNA bending proteins are located between the arm- and core-type Int binding sites.

Current and earlier work indicate that the DNA bending proteins facilitate long-range tethering of arm-type and core-type DNA sites by the bivalent Int protein. Both the attR and attL complexes contain Int molecules with unsatisfied DNA binding domains that have the potential to form intermolecular bridges between the recombination partners. A specific map is proposed for the intra- and intermolecular Int bridges mediating a higher-order synaptic complex.

Each of the four Int molecules participates through a different set of interactions. The two Ints forming intermolecular arm-core bridges each have their high affinity binding site on a different partner. One Int forms an intramolecular arm-core bridge and seems to be especially critical for recombination. The fourth Int is bound only to a core site and might join the complex at a later stage of the reaction. Protein-protein interactions comprise another prominent feature of the complexes that will also be discussed.

F 010 MECHANISM OF DNA TRANSPOSITION REACTIONS: THE CHEMICAL STEPS AND THE PROTEIN DNA ARCHITECTURE,
Kiyoshi Mizuuchi, Tania A. Baker, Robert Craigie, Alan Engelman, and Michiyo Mizuuchi, Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892.

Bacteriophage Mu, like other transposons studied in detail, generates a critical transposition intermediate through two chemical steps. The first of these steps is the site specific endonucleolytic cleavage at the two ends of the Mu sequence. Two single strand cleavages expose the 3'-OH ends of the Mu sequence. The second strand at each end remains uncut to maintain the continuity between the Mu sequence and the flanking sequence. This is in contrast to the initial cleavage step of transposons that transpose exclusively non-replicatively. These elements generate double strand cuts at the transposon ends. In the second chemical step, the target DNA is cleaved and covalently joined to the donor DNA 3'-ends (DNA strand transfer) in a concerted reaction. This step generates the strand transfer product containing a pair of forked DNA junctions between the donor and target DNAs. The strand transfer product serves as a critical transposition intermediate which can be resolved either by DNA replication, or by nucleolytic cleavage and gap repair.

The two chemical steps are mechanistically separable, and both of the steps take place in the absence of any high energy co-factor. This is true for all the DNA transposition reactions studied biochemically up to date, including the retroviral DNA integration reaction. Thus, the phosphodiester bond energy of the target DNA must be conserved through the DNA strand transfer process. The stereochemical course of DNA strand transfer was determined for both Mu transposition and retroviral DNA integration. Inversion of the phosphate chirality at the reactive phosphate supports a one-step transesterification model in which the 3'-OH of the donor ends directly attack the target phosphodiester bonds. The two-step transesterification model involving one protein-target DNA covalent intermediate is excluded. The chemical mechanism of the endonuclease step and general implications of these mechanisms will also be discussed.

The two chemical steps described above take place within a context of high order protein-DNA architecture. Such an arrangement not only achieves temporal coordination of the events taking place at the two ends of the element, but also provides mechanisms for selectivity and control that serve to eliminate physiologically detrimental reactions.

We have identified a number of protein-DNA complexes that function as intermediates in Mu transposition. Structural and functional characterization of these complexes have revealed an overall picture as to how the physiologically critical reaction selectivities are achieved. Special interactions between the Mu transposase (MuA) and the accessory MuA binding sites on the Mu donor DNA are required to achieve a structural transition of MuA to form the stable synaptic complex containing the two Mu ends. This process not only assures selection of properly oriented two Mu ends, but it also accommodates the mechanism of direct regulation of Mu transposition by the Mu repressor at this early reaction step. The assembly of the protein-DNA architecture is also influenced by MuB protein which binds to the target DNA. Results from a combination of kinetic experiments and perturbation of the reaction conditions point to the importance of the delicate balance among kinetic parameters for the assembly steps. Such a balance is essential for the orchestration of the overall process to avoid destruction of the Mu DNA by a poor choice of the target sites.

Initiation and Termination of Prokaryotic Replication

F 011 CONTROL of INITIATION of *E. COLI* MINICHROMOSOMES, A. Kornberg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305-5307

The mechanisms of the start and elongation of DNA chains include the assembly of the seven, cloned, distinctive subunits of the primosome and some of their properties. Interaction of the primosome and the dimeric, asymmetric DNA polymerase III holoenzyme may generate the putative "replisome," effective in the concurrent, coordinated replication of both chains at a replication fork. Numerous parameters affect initiation of the *E. coli* chromosome at its unique origin (*oriC*). These include: I. DnaA Protein: its nucleotide form (ATP vs. ADP) abundance, aggregation, and membrane interactions; II. Accessory Factors: binding proteins (e.g. HU, IHF), specificity proteins (e.g. RNase H, TopoI), and transcriptional activation; III. Effectors and Signals: negative effectors (e.g. IciA protein), other *oriC* binding proteins (e.g. FIS, 35 kDa), and anticipated positive signals from increasing cell mass; and IV. DNA: superhelical density, bendability, unwindability (thermodynamic and kinetic), modification (e.g. methylation), and membrane attachment. Mechanisms that arrest helicase action block movement of the chromosomal forks and may thereby provide time for topological operations needed to complete and segregate the two daughter chromosomes.

More detailed presentation of the studies that form the basis for this review can be found in Abstracts authored by Masahiro Akiyama *et al.*, George C. Allen, Jr., Celina Castuma, Elliott Crooke, Deog Su Hwang, Tsutomu Katayama, Eui Hum Lee, Kirsten Skarstad and Beat Thöny.

F 012 PROTEIN-PRIMED REPLICATION OF BACTERIOPHAGE Ø29 DNA. Margarita Salas, Luis Blanco, Manuel Serrano, María A. Blasco, José A. Esteban, Juan Méndez, Antonio Bernad, José M. Lázaro, María S. Soengas, Raimundo Freire, Alicia Bravo, José M. Sogo[+], Crisanto Gutiérrez and José M. Hermoso. Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain and [+]ETH-Hönggerberg, Zürich, Switzerland.

Bacteriophage Ø29 DNA replication takes place by a protein-priming mechanism in which the viral DNA polymerase catalyzes both the covalent linkage of dAMP to the OH group of serine 232 in the terminal protein (TP) and the elongation of the DNA chain by strand-displacement. Electron microscopic analysis has shown that the same type of replicative intermediates (RIs) observed *in vivo* are found in the *in vitro* system using highly purified proteins. In addition, we showed that type II RIs (full-length DNA partially double-stranded and partially single-stranded) are formed when two growing DNA chains, running from opposite ends, merge. The formation of the initiation complex (TP-dAMP) is strongly stimulated (about 100-fold) when Mn^{2+} is used instead of Mg^{2+}, due to an increase in the affinity for dATP. Formation of the initiation complex between the TP and any of the four dNMPs was detected in the absence of template, although the affinity for the nucleotide was greatly reduced. Otherwise, this template-independent initiation reaction showed similar requirements as the reaction in the presence of template. Single-stranded oligonucleotides with the sequence corresponding to the right 3'-terminus of Ø29 DNA were active templates for the formation of the TP-dAMP complex and their further elongation. These results suggest a model in which the 3' end of the template strand is made single-stranded as a prerequisite for the initiation of replication. The results using single-stranded oligonucleotides with mutations in the 3' terminal bases of the Ø29 DNA sequence will be also reported. In addition to initiation and polymerization activities the Ø29 DNA polymerase has pyrophosphorolytic and 3'→5' exonuclease activities. The characteristics of the latter enable the Ø29 DNA polymerase to act as a proofreading enzyme. Amino acid sequence comparison of 32 DNA-dependent DNA polymerases belonging to the two main superfamilies, *E. coli* DNA polymerase I-like and DNA polymerase α-like, showed significant sequence similarity both in the N-terminal portion, shown to contain the 3'→5' exonuclease domain, and in the C-terminal portion, that contains the polymerization domain. By site-directed mutagenesis in the Ø29 DNA polymerase we have mapped three regions involved in the 3'→5' exonuclease activity and four regions involved in the initiation, polymerization and pyrophosphorolytic activities. The viral protein p6 activates the initiation of replication by forming a nucleoprotein complex at the replication origins. The requirements for activation of initiation and a refined model of protein p6-DNA complex will be discussed. The viral protein p5 is a SSB protein and stimulates Ø29 DNA replication at incubation times when the replication in the absence of p5 levels off. Other SSB proteins can functionally substitute for protein p5.

F 013 THE FUNCTION OF *E. COLI* HEAT SHOCK PROTEINS IN PLASMID P1 DNA REPLICATION, Sue Wickner[1], Dorota Skowyra[1], Joel Hoskins[2] and Keith McKenney[2], [1]Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 and [2]Center for Advanced Research in Biotechnology, National Institute of Standards and Technology, Gaithersburg, MD 20899.

We have been interested in the initiation and regulation of replication of plasmid P1. The P1 replicon exists with a copy number of about one per cell and consists of an origin of replication, a gene coding for the initiator protein, RepA, and a control locus. P1 plasmids containing only the P1 origin and the *repA* gene are maintained at a copy number of about 8. The replication *in vitro* of *ori*P1 DNA requires many host proteins, including DnaA initiator protein, DnaB helicase, DnaC, DnaG primase, DNA polymerase III holoenzyme, DNA gyrase and RNA polymerase, in addition to RepA, which binds specifically to five 19-bp direct repeats in the P1 origin region and to nine similar repeats in the plasmid copy control locus. Thus RepA functions both in initiation of replication and regulation of replication. We found that three *E. coli* heat shock proteins, DnaJ, DnaK and GrpE, are also involved in *ori*P1 DNA replication and have been studying their role. We showed that RepA exists in a stable protein complex with DnaJ containing a dimer each of RepA and DnaJ. DnaK, in a reaction dependent on DnaJ and ATP, activates the *ori*P1 DNA binding function of RepA by about 100-fold. We found that RepA is the only protein of the three required for the reaction that is detected with the DNA. We isolated and characterized the activated form of RepA. Our results demonstrate that DnaK in conjunction with DnaJ activates RepA by converting RepA dimers to monomers. This activation may be involved in the regulation of the rate of initiation of P1 DNA replication.

Initiation and Termination of Eukaryotic Replication

F 014 BIOCHEMICAL ANALYSIS OF THE VIRUS-ENCODED PROTEINS INVOLVED IN HERPES SIMPLEX VIRUS DNA SYNTHESIS
Mark D. Challberg, Daniel Fierer, John Gottlieb, and Glenn Sherman, Laboratory of Viral Diseases, NIH, Bethesda, MD 20892

Genetic experiments have shown that seven HSV genes are both necessary and sufficient to support the replication of viral DNA in infected cells. Ongoing biochemical analyses in several laboratories support the idea that the products of these seven genes all participate directly in viral DNA synthesis. We are currently using both biochemical and molecular genetic approaches to understand the function of these polypeptides in detail.

The HSV DNA polymerase purified from infected HeLa cells consists of a stable complex of two polypetides: UL30, the catalytic subunit, and UL42, an accessory subunit. Several lines of evidence support the view UL42 increases the efficiency of the DNA polymerase by increasing its processivity. Using a novel synthetic model primer-template, we have characterized the interaction of the HSV DNA polymerase with its nucleic acid substrate. Our results suggest that UL42 increases the processivity of DNA polymerase by acting as a sliding clamp, reducing the probability that the polymerase will dissociate from the elongating DNA chain after each cycle of catalysis.

The UL5, UL8, and UL52 polypeptides form a three protein complex that has both helicase and primase activities. Purification of the isolated subunits and subcomplex associations of subunits has demonstrated that UL5 and UL52 act together to catalyze both helicase and primase activities (1). On the basis of sequence motifs, we presume that UL5 has the active site for helicase function, but UL5 is not active as a helicase in the absence of UL52. Although UL8 is required for neither helicase nor primase activities, it is required for the efficient utilization of primers by DNA polymerase in a model system for lagging strand synthesis. We suggest that the role of UL8 is to stabilize the association of nascent primers with the template, increasing the probabilty that primers are elongated by DNA polymerase. Work is underway to test this model.

1. Dodson, M. S. and I. R. Lehman (1991) Proc. Natl Acad Sci, USA 88:1105-9

F 015 INITIATION OF MITOCHONDRIAL DNA REPLICATION, Jeffrey L. Bennet, Kinlin L. Chao, Sunjoo Jeon- Yu, Melissa A. Parisi, Mark E. Schmitt, Baoji Xu, and David A. Clayton, Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305-5427.

Human mitochondrial transcription factor 1 (mtTFl) is required for accurate and efficient transcriptional initiation at human mitochondrial DNA (mtDNA) promoters and, consequently, for leading-strand mtDNA replication priming. The sequence of this nucleus-encoded DNA-binding protein reveals no similarities to any other DNA-binding proteins except for the existence of two domains that are characteristic of high mobility group (HMG) proteins. Human mtTF1 is most related to a DNA-binding HMG-box region in hUBF, a human protein known to be important for transcription by RNA polymerase I. Mitochondrial and RNA polymerase I transcription proteins may share a common ancestry that could extend to other HMG box-containing proteins. Interestingly, a similar protein can be isolated from yeast mitochondria. Current information points to a functional relationship between the two, thereby implying possible conserved features of mtDNA replication priming in the two systems.

RNase MRP is a site-specific endoribonuclease that processes primer RNA from the leading-strand origin of mammalian mtDNA replication. It is present in active form as isolated from the nucleus, suggesting a bipartite cellular location and function. Analysis of the mode of RNA cleavage by the more abundant nuclear RNase MRP revealed the surprising and unprecedented ability of the endonuclease to process RNA at multiple discrete locations. Substrate cleavage is dependent upon the presence of a previously described G-rich sequence-specific element adjacent to the primary site of RNA processing. Downstream cleavages occur in a distance- and sequence-specific manner. This feature (multisite cleavage) is conserved in RNase MRP from several vertebrate sources. The positions of multisite cleavage of mitochondrial RNA substrates from the leading-strand origin are consistent with previously mapped sites of transition of RNA to DNA synthesis *in vivo*. Molecular data on RNase MRP from several organisms permit some basic insights on structure and function of RNase MRP and RNase MRP RNA.

F 016 EXAMINATION OF DISCRETE STEPS IN THE REPLICATION OF DNA CONTAINING THE SV40 ORIGIN,
Jerard Hurwitz, Anthony Amin, Mei Chen, Frank Dean, Suk-Hee Lee, Yota Murakami, Zhen-Qiang Pan, and Yeon-Soo Seo, Memorial Sloan-Kettering Cancer Center, Program in Molecular Biology, 1275 York Avenue/Box 97, New York, NY 10021.

The replication of SV40 ori+ DNA can be divided into discrete reactions which can be studied individually *in vitro*. These reactions include: 1. The site specific ATP-dependent binding of T ag at the core origin. 2. The ATP-dependent polymerization of T ag at the core origin to a double hexamer. 3. The T ag mediated untwisting of the DNA and alteration of the origin region. 4. The topo I, human SSB (HSSB and ATP-dependent unwinding of the duplex by the T ag helicase activity. 5. The pol α-primase complex catalyzed initiation of oligoribonucleotides from the lagging template of both strands. 6. The extension of oligonucleotide primers (8-15 nt long) by pol α to chains 35 nt in length. 7. The further extension of these chains by the action of the PCNA-dependent pol to mature Okazaki fragment (150-300 nt in length). 8. The elongation of the first formed Okazaki fragments by pol δ holoenzyme generating leading strand chains. 9. The combined action of RNase H, a 5'-3' exonuclease, topo II and DNA ligase leading to RFI products.

The reactions leading to the synthesis of oligoribonucleotides are highly specific; only T ag, pol α and HSSB support the DNA primase catalyzed synthesis of oligonucleotides from SV40 ori+ DNA Subsequent elongation of oligoribonucleotides can be carried out with other polymerases. A model for the control of lagging and leading strand synthesis, dependent on the pol α-primase and pol δ holoenzyme action on the movement of T antigen, will be discussed.

Conservative Site-Specific Recombination

F 017 MOLECULAR ANALYSIS OF V(D)J RECOMBINATION,
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We previously described a rapid assay for the study of V(D)J recombination in lymphoid cells by the use of extrachromosomal substrates. This assay helped us to find unexpected products of the reaction: hybrid joints, where a new junction between a signal sequence and coding region DNA is made, and open-and-shut joints, which rejoin the original signal and coding partners and are recognized because the DNA ends have been processed. We also found a specific defect in the joining of coding ends (but not signal ends) in cell lines derived from scid mutant mice. These results suggest an intermediate structure where coding and signal ends are mutually accessible, but coding ends require additional functions to be joined.

More recently, we have developed a gel assay that detects V(D)J recombination of extrachromosomal substrates in DNA re-isolated from lymphoid cells following transfection. The plasmid DNA is found in different sub-cellular fractions as a function of time. Soon after transfection, most of the DNA is in an insoluble chromatin fraction. Freely diffusible plasmid DNA is found only after 17-20 hours, and all of this DNA has been replicated. The results also suggest that recombination occurs mainly in the insoluble fraction, with completed recombinants later being released.

Further study of pre-B and pre-T scid cell lines shows that the formation of coding joints, though always low, can be increased (to 10-20 fold below normal cells) by changing culture conditions. By restriction and sequence analysis, we found some coding joints that appeared normal and some that were highly deleted, as are most coding joints in the immune system of scid mice. Though coding joints could easily be found with substrates that formed only this junction, inversional recombinants that required the formation of coding and signal joints on the same DNA were still extremely rare.

F 018 GIN-FIS MEDIATED SITE-SPECIFIC RECOMBINATION,
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Gin catalyzed site-specific DNA inversion in phage Mu is stimulated by the E.coli FIS protein and a recombinational enhancer sequence. Only negatively supercoiled DNA substrates are recombined and only sites in inverted repeat configuration yield recombination products with high efficiency. Gin and FIS have to assemble the recombination sites in a topologically unique synaptic complex since DNA inversion results in unknotted products and proceeds with a defined change in linking number. In order to determine how the FIS-enhancer complex contributes to the assembly of the defined synaptic intermediate, we analyzed gin mutants which have a FIS independent phenotype. These mutants map in a central region of the protein. We suggest that the FIS independent phenotype is caused by an altered quality of the contacts between the two monomers within the dimer. This is supported by the properties of a gin mutant, which lies in the same domain of the protein but is defective for recombination. The phenotype of this particular mutant can either be suppressed by secondary mutations in Gin or by changing the reaction conditions in vitro. We have isolated another set of mutations in Gin, which suppress the FIS independent phenotype, they restore FIS dependence. These mutations cluster in a different region of the protein, for which we propose an involvement in higher order contacts between Gin dimers during recombination. We present a biochemical analysis of the mutant proteins and discuss implications for the mechanism of the reaction.

F 019 SYNAPSIS AND STRAND EXCHANGE IN LAMBDA SITE-SPECIFIC RECOMBINATION,
Howard Nash, Alex Burgin, Steven Goodman, Andrew Granston and Anca Segall, Laboratory of Molecular Biology, NIMH, Bethesda.

Integration and excision of the DNA of bacteriophage lambda into and out of the E. coli chromosome involve DNA loci called attachment sites. The bacterial site, attB, is the only recombination locus that has a simple structure; it consists of a pair of properly spaced binding sites for the phage-encoded recombinase, Int. The other attachment sites have additional binding sites for Int as well as sites for IHF, a bacterial protein that serves as an accessory factor for integration and excision.

IHF is a sequence-specific DNA binding protein that sharply bends the DNA to which it binds. We have previously shown that one of the three IHF binding sites in the phage attachment site, attP, can be functionally replaced with modules that bend DNA. However, recombination of these bend-swap chimeras still requires IHF, presumably to occupy the remaining IHF sites of attP. We now show that the requirement for IHF in excisive recombination between the prophage sites attL and attR can be eliminated by suitable placement of artificial bends. The observation of a substantial level of IHF-independent recombination in this system completes the proof that a principal role for IHF is simply to deform DNA.

Previous work has indicated that IHF interacts with DNA primarily via the minor groove, an unusual mode for a sequence-specific DNA binding protein. To learn what residues may be involved in this interaction, we have isolated mutants of IHF that are defective in DNA binding but retain the tertiary structure of the protein. For the alpha subunit of IHF most of our mutants lie in the arm region of the protein, a segment of about 25 amino acids that, by analogy to the known structure of the related HU protein, is thought to form a two-stranded anti-parallel beta ribbon. We have noted a similarity between the amino acid sequence of IHF alpha in the region of the arm that is highlighted by our mutants and a segment of the yeast TATA binding protein, TFIID, that is also implicated in DNA binding by mutagenesis studies.

Although synapsis between attachment sites seems an essential part of any hypothetical reaction pathway, stable juxtaposition between attachment sites has not been observed in the lambda system. We have now found that Int protein can hold two attL sites in a stable non-covalent complex. This complex is strictly bimolecular: only two attLs can be accommodated. To be included in a complex, each attachment site must have two kinds of targets for Int binding, arm sites and core sites. Int is the only protein required for complex formation; IHF protein can inhibit complex formation. We are studying the structure of the complex and are determining its relationship to the in vivo and in vitro recombination that occurs between attL sites.

Cleavage of DNA by Int topoisomerase involves nucleophilic attack by tyrosine342 on phosphodiester bonds of the substrate. In a complete recombination reaction, four phosphodiester bonds must be broken. Although it is clear that some of the breakage is accomplished by Int topoisomerase, it is not known if all of the bonds are attacked in this way. We are using altered DNA substrates and altered Int proteins to address this question.

F 020 RAG-1, RAG-2 AND RECOMBINATION IN THE DEVELOPING IMMUNE SYSTEM,
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The ordered rearrangement of immunoglobulin and T cell receptor V, D and J gene segments to produce the genes that encode functional immune receptors is a central process of lymphoid development. The recombination reaction responsible for the assembly of these genes is known as V(D)J recombination and is the only site-specific recombination reaction known in vertebrates. While the order of rearrangements is known and the cis-acting recombination signal sequences have been defined, little is known about the recombinational machinery, its regulation, or the factors governing its expression. Transfection of genomic DNA into NIH 3T3 cells, a fibroblastoid line that lacks V(D)J recombinase activity, can stably induce the expression of the V(D)J recombinase. Isolation of the genetic locus responsible for this induction led us to the identification of the Recombination Activating Genes, RAG-1 and RAG-2, lymphoid-specific genes whose co-expression correlates precisely with recombinase activity. RAG-1 and RAG-2 are unrelated genes that synergistically activate V(D)J recombination. Both genes are evolutionarily conserved between species that carry out V(D)J recombination. The RAG locus is unusual not only because the two convergently transcribed genes are located just 8 kb apart, but also because most, if not all, of the RAG-1 and RAG-2 coding and 3' untranslated regions are contained in single exons. RAG-1 and RAG-2 either serve as regulators of a pathway leading to the expression of the V(D)J recombinase or, more likely, directly encode lymphoid-specific components of the recombination machinery. We are attempting to distinguish between these possibilities through a combination of biochemical and in vivo functional studies. To date, it has not been possible to carry out the V(D)J recombination reaction in vitro, and we are interested in developing such a system with which to study the biochemical mechanism of the reaction. More recently, we have demonstrated that RAG-1 and RAG-2 are differentially expressed and may play important functions in processes other than classic V(D)J recombination. We have demonstrated that RAG-2, but not RAG-1, is expressed in the chicken bursa of fabricius. RAG-2 expression correlates exactly with the gene conversion process that diversifies chicken immunoglobulin genes, suggesting that RAG-2 plays a role in this process. Furthermore, we have demonstrated that RAG-1, but apparently not RAG-2, is expressed in neurons in the mouse central nervous system. We are currently attempting to understand the function, if any, of RAG-1 in neuronal development.

Transposition

F 021 MECHANISM AND REGULATION OF TN10 TRANSPOSITION.
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(1). Translational stabilization of IS10 transposase message. Translation of the IS10 transposase message occurs at an unusually low level. We find that translation stabilizes the transposase mRNA, apparently by protecting it from degradation from RNaseE. Thus, inefficient translation reduces the level of transposase both directly, by reducing the intrinsic rate of translation initiation, and indirectly and by reducing the functional halflife of the message.

(2) Preferential *cis* action depends upon message halflife and upon the quality of transposase. IS10 transposase acts preferentially in *cis*. We find that the degree of *cis* action can be altered by changes that affect the functional halflife of the transposase message: increased functional halflife is correlated with a decrease in *cis* action and *vice versa*. Two models that can explain this effect will be discussed. Variations in the level of translation initiation have particularly profound effects. Since *cis* action is likely to be important for decreasing the rate at which cells accumulate IS10 elements, these observations provide additional evidence that inefficient translation plays a critical role in the biology of IS10. Preferential *cis* action is also affected by changes in the nature of the transposase protein itself.

(3) Role of host factors. Transposition of Tn10 requires host functions. *In vitro*, transposition of both Tn10 and IS10 requires either IHF or HU at an early stage in the reaction. We have now found that, paradoxically, intermolecular transposition *in vivo* *increases* in an IHF$^-$ strain. We can reconcile these paradoxical observations as the sum of two different effects: an intrinsic requirement for a host factor for the initial stages of the reaction (provided by HU in an IHF$^-$ host) plus modulation of the reaction at later stages by general changes in chromosome structure (altered by the absence of IHF from the cell). Transposition is likely to require host functions at other stages of the reaction as well. Preliminary characterization of new host mutants will be presented.

(4) Functional domains of transposase and the transposon ends. The inverted repeats at the ends of Tn10 can be subdivided into two functional domains. Bp1-3 are not required for excision of the transposon from target DNA but are required for strand transfer; major groove interactions in bp 6-13 are required for excision and probably interfere with transposase binding.

Three groups of transposase mutations alter the transposition reaction in three specific ways: by blocking transposition after the excision step, by conferring relaxed insertion specificity, or by increasing the ability of transposase to work on ends that are defective for transposase binding. These mutations map to three different sets of codons within a small region of transposase protein.

F 022 MOLECULAR MECHANISMS IN RETROVIRAL DNA INTEGRATION, Richard Katz[1], Kathryn Jones[1], Joseph Kulkosky[1], Thomas Laue[2], Joseph P. G. Mack[3], George Merkel[1], and Anna Marie Skalka[1], [1]Fox Chase Cancer Center, Institute for Cancer Research, Philadelphia, PA 19111, [2]University of New Hampshire, Durham, NH 03824 and [3]NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, Frederick, MD 21701.

The 30-40 kDa retroviral integrase (IN) is the only protein required for specific cleavage of retroviral sequences and their joining to target DNA. Development of simple *in vitro* assays for either the viral DNA processing reaction or the joining reaction, together with the availability of large amounts of bacterially-expressed normal and mutant IN proteins, make it possible to use standard physical and kinetic analyses to study reaction mechanisms. Computer-assisted protein sequence alignments highlight residues and regions that are conserved in retroviral and retrotransposon integrases and certain bacterial transposes. Thus, insights gained through study of the retroviral enzymes should be applicable to a broad class of recombinases. Our site-directed mutagenesis of invariant or conserved residues in HIV and RSV integrases has identified several amino acids which are sensitive to even the most conservative changes and thus may be important catalytically. Since mutagenesis of all such residues analyzed to date affects the processing and joining activities similarly, we propose that both reactions occur at a single active site. Kinetic and physical studies of the Rous sarcoma virus enzyme indicates that IN is a dimer even in the absence of substrate, and that dimerization is required for the reaction. Models for roles of specific residues and the multimeric structure of integrase will be presented.

F 023 RETROVIRAL INTEGRATION, Harold E. Varmus, Peter Pryciak, Andrew Leavitt, Lily Shiue, Departments of Microbiology and Immunology, Biochemistry and Biophysics, University of California, San Francisco.

Retroviral integration is an efficient and precise eukaryotic recombination reaction that normally occurs during the early phase of a retrovirus life cycle: att sites at the ends of a newly synthesized linear DNA in a viral nucleoprotein complex are joined to host chromosomes by the viral integration protein, IN. To define the interactions among the central components (IN, att sites, and target DNA), we have tested wild type and mutant forms of HIV IN purified from a yeast expression system for their ability to act upon oligonucleotides representing normal and mutant att sites and a variety of targets. Findings to date emphasize the importance of the conserved CA dinucleotide near the 3' ends of viral att sites and of conserved amino acid residues in IN proteins. We are also attempting to define target site selection by using minichromosomes as targets for integration mediated in vitro by viral nucleoprotein complexes and by purified IN protein. The frequency of insertion can be accurately measured at many sites with single-base resolution, using a PCR-based method. We have found that retroviral integration occurs readily into DNA assembled into nucleosomes; moreover, integration occurs preferentially in the major groove facing out from the nucleosome, producing a 10 bp periodic pattern when nucleosomes are rotationally positioned on the DNA target. Control experiments in which naked DNA serves as a target do not display this periodic pattern, but the distribution of sites is affected by nucleotide sequences. Moreover, integration into regions of DNA recognized by a protein that binds in the major groove is blocked by addition of the protein. We are also able to map integration sites in SV40 minichromosomes during co-infection of cells with MLV and SV40; such assays may allow us to correlate use of integration sites with transcription and DNA replication.

Replication Fork

F 024 YEAST DNA REPLICATION AND ITS REGULATION, J. L. Campbell, Clay Brown, Martin Budd, Seng Loo, P. Rhode, Rati Verma, H-J Yoon, Division of Biology, California Institute of Technology, 147-75CH, Pasadena, CA 91125.

DNA Polymerases: (1) We have isolated temperature sensitive mutants of DNA polymerase ε - *pol2-ts*. They are defective in DNA synthesis at the non-permissive temperature. (2) We have also further studied the apparently polymerase ε specific stimulatory factor, SF1. SF1 is a single-stranded DNA binding protein with properties quite distinct from RP-A. We have cloned the SF1 gene and shown that it is essential for viability of yeast. (3) We have identified a new DNA polymerase during fractionation of DNA polymerase δ. The new polymerase is active on templates with low primer to template ratios, has a weak 3' to 5' exonuclease activity and is sensitive to aphidicolin and insensitive to ddTTP. It's molecular weight appears to be only 60 kDa, although, proteolysis cannot be ruled out. It is not the product of the *POL1, POL3 REV3* or *MIP1* genes. Thus, it is either a new polymerase or an altered form of DNA polymerase ε.

ARS Binding Proteins: ABF1 specifically recognizes the motif, RTCRYBNNNACG, at many sites in the yeast genome including promoter elements, mating-type silencers and ARSs. Since ABF1 is an essential gene, to better define the roles of ABF1, we have isolated conditional lethal mutations in the *ABF1* gene that confer temperature sensitive growth. Three of the ts-mutants isolated show rapid growth arrest at the nonpermissive temperature. The *abf1-ts* mutants show distinct phenotypic variations in ABF1-DNA binding activity. Marker rescue and DNA sequence analysis reveal that two of the missense mutations (*abf1-1 and abf1-4*) responsible for these ts-phenotypes fall within the bipartite DNA binding domain of ABF1 and the third mutation (*abf1-5*) changes a residue located between the binding domains.

Flow cytometric analysis of *abf1-5* mutants synchronized with α-factor and then released at the non-permissive temperature shows that cells fail to progress efficiently from G1 through S phase, indicating a cell cycle defect in this mutant. In addition, metabolic labeling reveals that DNA and RNA synthesis are inhibited 75% and 70% respectively, compared to cells released at the permissive temperature, while protein synthesis was normal for several hours. To examine the effects of ABF1 on DNA replication and transcription in more detail, we measured ARS activity and ABF1 binding site-specific UAS activity in the *abf1-ts* mutants. Loss of *ARS121-CEN3* plasmids in the *abf1-1* and *abf1-5* mutants grown at the semipermissive temperature is 15 fold greater than in an isogenic ABF1 strain, verifying the importance of ABF1 in ARS activity. Together with the cell cycle defects, these results provide evidence for a direct role for ABF1 in the initiation of DNA replication. Transcriptional defects were also detected as transcriptional activation of a heterologous promoter by the ABF1 binding site decreased 2 fold in the *abf1-1* mutant at the semipermissive temperature. ABF1 is multiply phosphorylated, the phosphorylation is regulated by carbon source and binding to specific genes is affected by phosphorylation state.

Regulation of DNA Replication: Two aspects of regulation of the G1/S transition involve induction of genes essential for DNA replication and post-translational modification of proteins essential for replication. We have characterized a site involved in coordinate regulation of the replication genes, purified a protein that binds to the site and characterized the periodic function of the *cis* and *transacting* elements. Second, we will describe purification of the Cdc7 protein kinase and the nature of the requirements for periodic activity in the cell cycle as well as physiological substrates.

F 025 CONTROL OF OKAZAKI FRAGMENT SYNTHESIS AT THE *E. COLI* DNA REPLICATION FORK,
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The response, to changes in the concentration of substrates, effectors, enzymes, and ionic strength during rolling-circle DNA replication supported by a tailed form II template, of replication forks reconstituted with the *E. coli* (ϕX-type) primosomal proteins, the SSB, and the DNA polymerase III holoenzyme (Pol III HE) has been used to develop a model describing the mechanisms governing replication fork function. Studies utilizing purified subunits of the Pol III HE were in collaboration with J. Reems, A. H. Hughes, Jr., M. Franden, and C. S. McHenry (University of Colorado Health Sciences Center, Denver, CO). All factors that affected Okazaki fragment size did so by perturbing an event during the cycle of Okazaki fragment synthesis that occurred prior to actual nascent chain elongation. Variation in the concentration of NTPs or the distributively acting primase affected the frequency of primer synthesis, while variation in the concentration of dNTPs or the β subunit of the Pol III HE affected the efficiency of utilization of primers for the initiation of Okazaki fragment synthesis.

Regulation of primer size (which, when uncoupled from DNA synthesis can reach 70 nt) at 10 nt, the same size observed *in vivo* could be attributed to an interaction between the Pol III core and primase. This interaction was independent of DNA synthesis and preceded preinitiation complex formation on the primer terminus. Active forks could respond to changes in the environment by altering the size of the fragments synthesized. Thus, a mechanism where Okazaki fragment size was regulated by templating the size of the penultimate fragment could be excluded. In addition, the dNTP effect could not be explained by variation in the rate of replication fork movement. It was therefore unlikely that variation in Okazaki fragment size could be accounted for solely by differential rates of polymerization of the leading- and lagging-strand DNA polymerases.

Replication forks assembled under conditions of limiting Pol III core could be induced to synthesize aberrantly short Okazaki fragments that were separated on the lagging-strand template by large gaps. Nevertheless, under the same conditions, fragment size could be increased by a factor of ten when the priming frequency was decreased (be decreasing the DnaG concentration). This indicated that the signal for synthesis of a new primer could not be the stalling of the lagging-strand polymerase when it encountered the penultimate Okazaki fragment, instead it most likely was the association of DnaG with the fork that acted to signal the lagging-strand polymerase to terminate Okazaki fragment synthesis, whether or not all the available template had been copied.

In this view, the cycle of Okazaki fragment synthesis is keyed by the association of DnaG (at a rate governed by its K_a) with DnaB at the fork (i). This occurs while Okazaki fragment synthesis is ongoing. Primer synthesis is initiated (ii) and a primase lagging-strand Pol III core interaction is established that serves to limit primer synthesis and most likely also exposes the 3'-end of the primer (iii). Okazaki fragment synthesis continues. A preinitiation complex then assembles on the primer-terminus (iv). During this time, under normal circumstances, the Okazaki fragment being synthesized is completed. An assembled preinitiation complex is likely to be the signal (perhaps mediated by some holoenzyme subunits) for the lagging-strand Pol III core to release the template and transit to the primer terminus (v).

Within this context, it can be seen that since lagging-strand template is generated continuously, increased priming frequency (step i) would result in a smaller fragment, whereas decreases in the efficiency of primer utilization at either step (iii), because of premature termination of primer synthesis, or step (iv), because of bungled preinitiation complex formation, would result in larger fragments. The appearance of small fragments separated by large single-stranded gaps could be attributed to an increase in the time required to execute steps (iv) and (v).

F 026 DNA HELICASES: A BIOCHEMICAL AND GENETIC CHARACTERIZATION,
Steven W. Matson, Dan W. Bean, James W. George, Kathleen A. Kaiser-Rogers, Veera Mendonca, Brad S. Morton, Bill Nelson, Janet E. Yancey, Department of Biology, University of North Carolina, Chapel Hill, NC 27599.

DNA helicases catalyze the NTP hydrolysis-dependent unwinding of duplex DNA. Nine distinct DNA helicases have been described in *E. coli*. It is likely that each helicase participates in a specific aspect of DNA metabolism. To begin to elucidate functional relationships between the different helicases found in *E. coli*, deletion mutants in helicases II, IV and Rep protein were constructed. The genes encoding helicases II (*uvrD*), IV (*helD*) and Rep protein (*rep*) can be deleted from the chromosome indicating that none of these genes encode essential gene products. In addition, we have constructed double and triple deletion strains using these null mutants. The *helD/uvrD* and *helD/rep* double mutants are viable. However, a *uvrD/rep* double deletion is not viable and appears to require a supressor mutation for viability. Thus, either helicase II or Rep protein is required in some essential cellular function. Moreover, one helicase can apparently substitute for the other in this role in the cell. The phenotype of *helD* mutants, in an appropriate genetic background, suggests this protein may be involved in a mutagenic repair pathway.

Biochemical studies with DNA helicase I (F plasmid *traI* gene product) have shown that, in addition to ATPase and helicase activities, this enzyme catalyzes a site- and strand-specific nicking reaction within the F plasmid origin of transfer (*oriT*). DNA strand transfer during bacterial conjugation is initiated from this nick. The nicking reaction catalyzed by helicase I required a supercoiled plasmid containing the F *oriT* and $MgCl_2$. The enzyme cleaves a single phosphodiester bond to produce a 3'-OH with helicase I covalently bound on the 5'-side of the nick site. Thus helicase I is positioned to unwind the F plasmid to provide the single strand of DNA for transfer into the recipient bacterium.

Presumably helicases encounter proteins bound on the DNA in the cell. To address the effect of site specific DNA binding proteins on unwinding reactions catalyzed by helicases we have constructed a partial duplex substrate with a Lac repressor binding site. Both helicase I and Rep protein are able to unwind this substrate when Lac repressor is bound. Helicase II is partially inhibited by bound Lac repressor and helicase IV is substantially inhibited. Additional studies using other DNA binding proteins are in progress.

F 027 PROTEIN INTERACTIONS AT THE REPLICATION FORK OF BACTEROPHAGE T7,
Charles C. Richardson, Benjamin B. Beauchamp, Yeon-Bo Chung, Jeff Himawan, Young Tae Kim, Quingyun Liu, Lynn V. Mendelman, Steven M. Notarnicola, Stanley Tabor, and Susannah M. Wurgler, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

Leading strand synthesis at the replication fork of phage T7 is accomplished by the T7 gene 5 protein, *E. coli* thioredoxin, and the T7 56-kDa gene 4 protein. Gene 5 protein, a distributive DNA polymerase, forms a 1:1 complex with thioredoxin, an interaction that increases the affinity of the polymerase for a primer-template 50-fold (K_d = 3 nM) and confers high processivity (>1000 nucleotides) on polymerization. The 56-kDa gene 4 protein is a helicase that translocates 5' to 3' on the lagging strand to catalyze the unwinding of duplex DNA, a reaction coupled to the hydrolysis of dTTP. The T7 DNA polymerase/thioredoxin complex interacts physically with the gene 4 protein and the complex of the three proteins catalyzes leadind strand DNA synthesis in a processive manner at a rate of 300 nucleotides/sec at 30 °C. T7 gene 2.5 protein, a single-stranded DNA binding protein (DNA binding constant of 1.2 x 10^6 M^{-1}), stimulates the activity and processivity of the T7 DNA polymerase/thioredoxin complex on single-stranded DNA. T7 DNA polymerase/thioredoxin forms a one to one complex with gene 2.5 protein (K_d = 1.1 μM) as measured by fluorescence emission anisotropy.

Single amino changes in thioredoxin can alter its binding to T7 gene 5 protein. Some mutant thioredoxins (i.e., gly-92 to asp-92) do not bind to gene 5 protein while others (i.e , gly-74 to asp-74) can restore nearly full polymerase activity, but only at concentrations that are several hundred-fold higher than wild-type. While neither of these two mutant thioredoxins can substitute for wild type thioredoxin for T7 growth *in vivo*, T7 mutants arise that grow on strains harboring the gly-74 to asp-74 mutant thioredoxin. All of the suppressor T7 mutations give rise to single amino acid changes in gene 5 protein. For example, the gly-74 to asp-74 mutation in the thioredoxin gene is suppressed by a replacement of glu-319 in the gene 5 protein to either a valine or lysine residue. Based on the homology of gene 5 protein to the large fragment of *E. coli* DNA polymerase I, the glu-319 residue of gene 5 protein is located at the edge of the DNA-binding groove within the polymerization domain.

Lagging strand synthesis requires, in addition to the proteins involved in leading strand synthesis, primase activity provided by the T7 63-kDa gene 4 protein. The 63-kDa protein consists of the 56-kDa gene 4 protein with an additional 63 amino acid residues at its N-terminus. In order to obtain 63-kDa protein free of 56-kDa protein, mutations were introduced to eliminate translation of the co-linear 56-kDa protein. The 63-kDa protein, like the 56-kDa protein, translocates on single-stranded DNA and has helicase activity. In contrast to the 56-kDa protein, the 63-kDa protein catalyzes the synthesis of the diribonucleotide pppAC at primase recognition sites having the sequence 3'-CTG-5'. However, only in the presence of 56-kDa protein does the 63-kDa protein efficiently synthesize the tetranucleotides pppACC(C/A) and pppACAC that can be used as primers by T7 DNA polymerase *in vitro* and *in vivo*. A putative zinc motif is located within the 63 amino acid N-terminal sequence unique to the 63-kDa protein; mutations in the cysteine residues of the motif abolish the ability of the protein to function *in vivo*. T7 gene 2.5 protein physically interacts with both forms of the gene 4 protein and increases the quantity of oligonucleotides synthesized by the 63-kDa gene 4 protein.

F 028 DNA REPLICATION IN HUMAN AND YEAST CELLS, Bruce Stillman, Stephen P., Bell, Steven J. Brill, Anindya Dutta, York Marahrens and Thomas Melendy, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

Eight cellular activities have been purified and shown to initiate DNA synthesis at the SV40 *ori*. These proteins include Replication Protein A (RPA), Replication factor C (RFC), Proliferating Cell Nuclear Antigen (PCNA), DNA polymerase α/primase complex (pol α-primase), DNA polymerase δ (pol δ) and topoisomerases I and II. To determine if these proteins play a role in cellular DNA replication, we have purified these proteins from *S. cerevisiae*, including the RPA and RFC replication factors (scRPA and scRFC). The genes encoding the three scRPA subunits have been cloned and genetic analysis demonstrates that they are essential. In addition, we have demonstrated that RPA from both human and yeast cells is phosphorylated in a cell cycle dependent manner. Human RPA is phosphorylated by multiple protein kinases *in vitro*, including the $p34^{cdc2}$ -cyclin kinase.

A major gap in these studies has been the identification of proteins that function at cellular origins of DNA replication. To begin addressing this problem, we have determined the nature of the *cis*-acting sequences within the cellular origin of DNA replication, ARS1. Four DNA elements are necessary and sufficient for efficient origin function; element A is essential whereas elements B1, B2 and B3 are required for maximal activity. Although elements B1, B2 and B3 are functionally distinct, any combination of two of these elements will support efficient DNA replication. Element B3 corresponds to the binding site for the previously identified ABF1 transcription factor. We have demonstrated that other transcription factors will substitute for ABF1, provided that they contain a transcriptional activating domain.

Finally, we have searched for other proteins that interact with yeast origins of DNA replication. A multiprotein complex has been purified that binds to element A, containing the ARS consensus sequence, but which does not bind to *ori* $^-$ derivatives. This protein binds to all ARS sequences tested and alters the structure of the DNA in element B1.

Fidelity in Genome Duplication

F 029 LOCAL SEQUENCE EFFECTS ON DNA SYNTHESIS FIDELITY, Myron F. Goodman[1], Linda B. Bloom[1], Steven Creighton[1], John Petruska[1], and Joseph M. Beechem[2], [1]University of Southern California, Department of Biological Sciences, Los Angeles, CA 90089-1340, [2]Vanderbilt University School of Medicine, Nashville.

Mutations are distributed nonrandomly in DNA. Their occurrence is determined by a series of complex interactions between DNA primer-template molecules, and proteins involved in replication and repair. The properties of DNA polymerase that determine insertion, extension, and excision rates for correct and incorrect nucleotides play a prominent role in the mutagenic process. Compared to the influence of homopolymer runs on the appearance of frameshift mutations, the influence of local DNA sequence on base substitution mutagenesis is poorly understood. To model nonrandom base substitution errors in DNA, it is necessary to analyze the effect of local DNA sequence on the dynamics of dNTP incorporation. The analysis requires rapid and convenient assays to measure polymerase kinetics and well-defined model systems to investigate normal and aberrant nucleotide insertion, excision and extension. We have developed a steady-state polymerase fidelity assay using polyacrylamide gel electrophoresis to measure elongation of ^{32}P-labeled primer molecules. In this assay, nucleotide incorporation fidelities are deduced by measuring the relative velocities of inserting right and wrong nucleotides as a function of [dNTP], in separate reactions, at template target sites. The target sites are situated either immediately adjacent to a primer 3'-terminus (standing start) or several nucleotides downstream from a primer end (running start). The fraction of misinserted nucleotides (f_{ins}) is given by the ratio of V_{max}/K_m values for wrong (w) and right (r) insertions, $f_{ins} = (V_{max}/K_m)_w/(V_{max}/K_m)_r$. We show that under "single hit" steady-state conditions, (i.e., primer-templates are extended at most once in a running start reaction), the nucleotide insertion velocity is given by the ratio of integrated band intensities measured at the target (T) and previous (T-1) template positions multiplied by the polymerase dissociation rate constant at the previous site; $V_{ins}=(I_T/I_{T-1})k_{off}$. A standard DNA trapping measurement can be used to determine k_{off}. When the running start assay employs a Phosphorimager to measure integrated gel band intensities, nucleotide insertion rates of several hundreds of nucleotides/sec can be measured. Our measurements for Klenow exo$^-$ polymerase yield kinetic parameters, k_{cat} = 50 nt/sec and $K_{m,dNTP}$ = 10 mM, similar to those obtained by analysis of presteady-state kinetics. We also show that DNA polymerase incorporation fidelity in the presence of proofreading is also obtained by measuring the band intensity ratios I_T/I_{T-1}, for correct and incorrect incorporations, provided that polymerase dissociation rates are approximately the same at the two adjacent template sites. Incorporation data using a model system in which the fluorescent mutagenic nucleotide analogue 2-aminopurine (2dAPTP) is used as a substrate for DNA polymerase are discussed from the viewpoint of analyzing the effects of base composition on 2AP incorporation opposite T and misincorporation opposite C. Data from three assays are compared: (i) running start steady-state kinetics; (ii) presteady-state kinetics; (iii) time-resolved fluorescence intensity and anisotropy measurements performed during the reaction.

F 030 THE FIDELITY OF SV40 ORIGIN-DEPENDENT DNA REPLICATION, Thomas A. Kunkel, John D. Roberts and David C. Thomas, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, NC, 27709

During DNA replication, several asymmetries provide an opportunity for non-random replication error rates. For example, current eukaryotic replication fork models suggest that two or more DNA polymerases are required for high fidelity semiconservative DNA replication and that the replication proteins that start or finish chains may be different than those that perform the bulk of chain elongation on the leading and lagging strands. Furthermore, the concentrations of the four dNTP precursors in a cell are not equal and they are not constant throughout the cell cycle. The eukaryotic genome consists of two subgenomes that replicate at different times and differ in nucleotide composition and gene content. We are attempting to determine the effects of these asymmetries on the fidelity of SV40 origin-dependent replication in HeLa cell extracts using an M13mp2 DNA substrate.

We have compared the fidelity of replication to that of DNA synthesis by DNA polymerases α, δ and ε. The data suggest that both replication and pol ε are highly accurate. Exonuclease-containing calf thymus DNA polymerase δ, which requires PCNA for efficient synthesis, is less accurate, while the DNA pol α-primase from HeLa cells or calf thymus is least accurate. Sequence analysis of mutants generated by each enzyme shows that they all produce base substitution and single-base frameshift errors and larger deletions. However, the three polymerases have distinctly different error rates and error specificities. This has implications for their roles in the various stages of DNA replication.

We have also asked whether exonucleolytic proofreading occurs during SV40-origin-dependent, bidirectional DNA replication in extracts of human HeLa cells, and we have compared the fidelity of leading and lagging strand DNA synthesis. Providing an excess of a single dNTP over the other three dNTP substrates in a replication reaction generates defined, strand-specific base-substitution errors that revert a TGA codon in the *lacZα* gene. Fidelity measurements with two vectors having the origin of replication on opposite sides of the opal codon demonstrate that error rates for two different A•dCTP and T•dGTP mispairs increase when deoxyguanosine monophosphate is added to replication reactions or when the concentration of deoxynucleoside triphosphates is increased. The data suggest that exonucleolytic proofreading occurs on both strands during bidirectional replication. Measurements using the two SV40 origin containing vectors suggest that base-substitution error rates are similar for replication of the leading and lagging strands. A similar analysis with a second set of vectors designed to score frameshift errors indicates that replication fidelity is high for one-nucleotide deletions, that exonucleolytic proofreading also occurs for these errors and that error rates are similar for replication of the leading and lagging strands. The analysis is being extended to examine replication error rates as a function of dNTP substrate pool imbalances, sequence context and the position of the origin relative to the mutational target. The results will be discussed in light of models for eukaryotic replication and the architecture of the eukaryotic genome.

F 031 MISMATCH DETECTION AND THE PRECISION OF DNA REPLICATION AND RECOMBINATION PROCESSES. Miroslav Radman, In
Jacques Monod, CNRS-Université Paris 7, Paris, France

The detection of mispaired and unpaired bases in DNA is a central element for mutation avoidance, genetic recombination and provides a genetic barrier between closely related species. Mismatches may arise as a result of DNA replication errors, strand exchange between homologous but non-identical sequences or deamination of 5-methylcytosine to thymine.
Mismatch repair systems can be divided into two broad classes on the basis of their mechanism of action. The long-patch mismatch repair (LPMR) systems act by removing a large number of nucleotides from one strand of a DNA double helix in the region of a mismatch.Very-short-patch mismatch repair (VSPMR) systems act by removing only the mismatched nucleotide and few if any surrounding nucleotides.
LPMR systems are "generalized" because they act to repair many of the different mismatches. These systems have been well characterized in bacteria and in vertebrates. In *E. coli*, in the LPMR system improves the precision of DNA replication by about 10^3-fold through removed of mispaired or u bases from the newly synthesized strands and by "melting" DNA se structures in the template thus preventing deletions arising through past such secondary structures (Tex phenotype).
The LPMR system prevents recombination between partially hom sequences, presumably by aborting mismatched heteroduplex interm Hence LPMR accounts for the stability of chromosomes and genomes repeated diverged sequences and for the genetic barrier between related species (e.g., the bacterial genera *Escherichia* and *Salmonella*) The multiple implications of mismatch-monitoring in the evolution and chromosome structure in eucaryotes, the roles of sequence div and polymorphism, and the origins and evolutionary value of "junk will be discussed.

General Recombination

F 032 HOMOLOGOUS PAIRING PROTEINS IN *SACCHAROMYCES CEREVISIAE*. R. Kolodner, E. Alani, A. Johnson, D. Norris and D. Tishkoff. Division of Cellular and Molecular Biology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA 02115

Our laboratory has described a protein, called Sep1, that promotes ATP-independent homologous pairing and strand exchange of circular ssDNA and homologous linear dsDNA with a 5' to 3' polarity with respect to the displaced strand. Overproduced Sep1 was a 175,000 Mr protein having two independent activities; homologous pairing activity and an intrinsic 5' to 3' exonuclease activity. The exonuclease activity degraded RNA, ssDNA and dsDNA and had turnover numbers of 50 and 20 bases/enzyme/min on ssDNA and dsDNA substrates, respectively. In 20 min strand exchange reactions where up to 90% of the substrates are converted to joint molecules with up to 7250 bases of heteroduplex per joint molecule, 400 bases were degraded per end of the linear duplex. When exonuclease activity was inhibited by Ca ions, joint molecule formation was inhibited. If the linear duplex was resected with exoIII (3' to 5') or T7 gene6 exo (5' to 3'), then Sep1 promoted homologous pairing and strand exchange when the nuclease was inhibited. Extensive regions of heteroduplex DNA were formed by a protein promoted strand displacement reaction yielding partially exchanged molecules and full length duplex circular products. These data are consistent with the exonuclease activity of Sep1 being required for initiation of pairing by producing a short single-stranded tail on the linear duplex substrate and this determines the polarity of the reaction observed when the linear dsDNA has blunt ends.

Insertion and deletion mutations in the *SEP1* gene caused mitotic and meiotic phenotypes. The mitotic phenotypes included slow growth, a slight sensitivity to UV-irradiation and a delay in return to growth after UV-irradiation. *sep1* mutants did not sporulate and arrested in meiosis after DNA replication but before the M1 cell division. In return to growth experiments, meiotic recombination between *his4* heteroalleles was increased 3 to 5 fold whereas recombination between *his7* heteroalleles, *lys2* heteroalleles, single-site conversion at *cyh2* and crossing over between *cyh2* and *trp5* were all reduced up to 15 fold. 1 to 2% of the cells sporulated; spore viability was 50 to 60% and the viable spores showed 1 to 2% aneuploidy. Crossing over occurred in these spores but was not associated with positive interference. These data suggest that Sep1 plays an important role in meiotic recombination.

Two proteins were identified that allowed Sep1 to function in strand exchange at lower levels. The first protein was RPA, a 3 subunit ssDNA binding protein. RPA was found to bind to ssDNA with a site size of 95 bases, high affinity ($K\omega = 5x10^9$) and cooperativity ($\omega = 10^{-4}-10^{-5}$), and organized the DNA into nucleosome like structures. Under conditions of saturating RPA, the amount of Sep1 required was reduced 2 to 4 fold and the renaturation activity of Sep1 was completely inhibited. The second protein was SF1, which decreased the amount of Sep1 required 300 fold. Overproduced Sf1 was a 55,000 Mr protein that bound weakly to ssDNA and promoted the renaturation of homologous ssDNA. Sf1 did not promote homologous pairing and strand exchange of single-stranded circular DNA and homologous linear duplex DNA when the linear substrate had blunt ends. However, when the ends were resected with either exoIII or T7 gene6 exo, then Sf1 would promote homologous pairing and strand exchange yielding partially exchanged molecules and full length duplex circular products. These data indicate that Sf1 is a second strand exchange protein and explains how Sf1 can stimulate Sep1 so markedly.

F 033 BIOCHEMISTRY OF RECOMBINATION HOTSPOT ACTIVITY AT CHI SITES, Stephen C. Kowalczykowski and Dan A. Dixon, Division of Biological Sciences, Section of Microbiology, University of California, Davis, CA 95616.

Homologous recombination in *E. coli via* the RecBCD pathway is elevated in the vicinity of Chi sites. These sites are cis-acting elements with the sequence 5'-GCTGGTGG-3'. *In vitro*, the recBCD enzyme was found to nick specifically 4 to 6 nucleotides to the 3'-side of Chi, leading to the proposal that the generation of an invasive 3'-end at the Chi site is a crucial step in the initiation of a recA protein-dependent DNA strand exchange event. Recently, our laboratory demonstrated the formation *in vitro* of homologously paired DNA molecules that requires the coordinated action of the recA, recBCD, and SSB proteins. In this reaction, the helicase activity of recBCD enzyme initiates events by unwinding linear dsDNA to produce the ssDNA needed for recA protein-dependent joint molecule formation; SSB protein facilitates this reaction by trapping the ssDNA produced. Furthermore, when a properly oriented Chi sequence is present in the linear donor dsDNA, a Chi-specific joint molecule is generated with homologous recipient supercoiled DNA. As predicted, the Chi-specific joint molecules formed result from strand invasion by the newly generated 3'-end formed by nicking at Chi. However, an unexpected observation is the finding that, on the Chi-sequence containing DNA strand, only ssDNA "downstream" of the Chi site is recovered, leading to the proposal that the dsDNA exonuclease activity of recBCD enzyme is both asymmetric and attenuated by interaction with Chi. Recent results clearly demonstrate that the nuclease activity of recBCD enzyme is asymmetric, with the strand ending 3' at the site of recBCD entry being degraded more extensively than the strand ending 5' at the entry site. Furthermore, the nuclease activity of recBCD enzyme is attenuated upon recognition of the Chi sequence, thereby preserving the strand ending 3' at the Chi site. This observation reconciles the seemingly disparate degradative and recombination function of the recBCD enzyme and suggest that the stimulation of recombination downstream of Chi sites *in vivo* is a consequence of the reduced nuclease activity of the recBCD enzyme that, in turn, results in an increased survival of the chromosome located downstream of Chi. The ssDNA generated by the attenuated recBCD enzyme is the appropriate substrate for subsequent recA protein-dependent strand invasion with a recipient chromosome. Thus, a Chi site stimulates genetic recombination as a result of both the recBCD enzyme-dependent generation of an invasive 3' end by unwinding and nicking at Chi, and its action as an effector site that attenuates the nuclease activity of recBCD enzyme.

F 034 TRIPLEX DNA IN RECOMBINATION MEDIATED BY REC A PROTEIN, B.J. Rao, Bruce Burnett, Biru Jwang, Sung Kay Chiu, U. Ezekiel, G. Reddy & Charles Radding, Yale University School of Medicine, New Haven, CT, 06510

E. coli RecA protein and similar proteins that are ubiquitous in prokaryotes polymerize on single-stranded DNA to form nucleoprotein filaments that recognize homology in naked duplex DNA and promote extensive strand exchange.Recent findings promise to reveal how homology is recognized in a duplex molecule. Rec A protein can form stable joints at either the proximal or distal end of duplex DNA in relation to the polarity of strand exchange between the linear duplex and circular single-stranded DNA. In such reactions, deproteinized, thermostable intermediates have previously been detected that contain all three strands in a nuclease resistant form (1-3). In natural DNA, triplex regions of several thousand nucleotide residues were detected when a large region of heterology blocked the progression of strand exchange that had started at the proximal end, or when heterology at the proximal end allowed the formation of a distal joint but blocked the initiation of strand exchange (2,3). To characterize triple-stranded joints further,we used a self-complementary,duplex deoxyoligonucleotide, 35 base pairs in length, in which a proximal hairpin connection of the two strands blocked the initiation of strand exchange for want of a free end of the required polarity.With ATP as cofactor, RecA protein formed stable joints between the hairpin substrate and either another oligonucleotide or circular single-stranded DNA, whereas, in the absence of the hairpin connection, strand exchange promptly ensued and stable joints were undetectable. In the joints formed by the hairpin oligonucleotide, the strand that would otherwise be displaced by strand exchange was resistant to diethylpyrocarbonate, indicating that it was not in a single-stranded state. Hairpin joints were hypersensitive to DNase I and to copper phenanthroline, both of which react with the minor groove of duplex DNA. Whereas triplex joints melted at the same temperature as a control duplex of the same sequence, the enzymatic methylation of several residues of the hairpin in the major groove, at N6 adenine and N4 cytosine, lowered the Tm of triplex joints by 6-9°C, but had no effect on the Tm of the control duplex. On the other hand, methylation at N7 guanine by dimethyl sulfate had no effect on the thermal stability of triplexes.Mismatches between bases in the single strand and base pairs in the hairpin duplex reduced the yield and thermal stability of triplex joints. These observations support the conclusion that RecA protein makes a triple-stranded intermediate in which the third strand is antiparallel to its complement and is paired to duplex DNA via additional hydrogen bonds in the major groove. Whereas deproteinized triplex joints are stable, the addition of Rec A protein to isolated triplex joints dissociates them by a reaction that appears to require the hydrolysis of ATP, observations which suggest a new and important rationale for the role of ATP in homologous pairing. (These experiments were supported by grants from the National Institutes of Health,GM-33504, and the American Cancer Society,NP90)

1) Hsieh, P., Camerini-Otero, C.S., & Camerini-Otero, R.D., Genes & Dev. 4, 1951-1963 (1990)
2) Rao, B.J., Jwang, B., & Radding, C.M., J. Mol. Biol. 213, 789-809 (1990)
3) Rao, B.J., Dutreix, M., & Radding, C.M., Proc.Natl.Acad. Sci. USA 88, 2984-2988 (1991)

F 035 HOMOLOGOUS RECOMBINATION IN *ESCHERICHIA COLI* AND *SCHIZOSACCHAROMYCES POMBE*, Gerald R. Smith, Susan K. Amundsen, Linda C. DeVeaux, Sam Ganesan, Susan L. Holbeck, Yukang Lin, Philippe Szankasi, Andrew F. Taylor, Jeffrey B. Virgin, and Wayne P. Wahls, Fred Hutchinson Cancer Research Center, Seattle, WA 98104.

Recombination involving dsDNA ends in *E. coli* proceeds by the RecBCD pathway, which depends upon RecBCD enzyme (and other proteins) and is stimulated by Chi sites, 5'G-C-T-G-G-T-G-G-3'. RecBCD enzyme binds with high affinity to dsDNA ends (K_D=0.5 nM) and protects from DNase I digestion 15 and 21 nucleotides at the 3' and 5' ends, respectively. The RecB and RecC subunits appear to contact the 3' and 5' ends, respectively. These strand-specific interactions presumably are the basis of the asymmetric loop-tail unwinding structures described previously (1). During its unwinding of DNA, RecBCD enzyme cuts the DNA strand containing the Chi sequence to generate a 3'-ended ssDNA tail, which is a substrate for RecA + SSB protein-stimulated formation of D-loops with an homologous duplex. As predicted from studies with purified components, heteroduplex DNA accumulation in λ-infected *E. coli* requires RecA protein and RecBCD enzyme and is stimulated ~6-fold by Chi. We have found that RecBCD enzyme detectably cuts at only one Chi site on a given DNA molecule; this feature assures exactly one exchange near each end of the dsDNA fragment in recombination. We propose that recombination by the RecBCD pathway is non-reciprocal, due to replication primed by the invading 3' tails. We present a unified mechanism for recombination by the RecBCD pathway during conjugation, transduction, transformation and ds break repair (2).

Meiotic recombination in *S. pombe* is locally stimulated by the *M26* site created by the *ade6-M26* mutation; the heptamer 5'A-T-G-A-C-G-T-3' is necessary for this hotspot (3). The heptamer is not, however, sufficient for hotspot activity, since moving it on DNA fragments up to 7 kb in length to other genomic locations fails to yield hotspot activity. We infer that a second site or proper chromosomal context is required to activate the *M26* hotspot. By a gel "band-shift" assay we have detected and partially purified a protein that binds to the *M26* sequence. We infer that this protein is required for *M26* hotspot activity, since single bp mutations within the heptamer co-ordinately reduce binding and hotspot activity, whereas mutations outside the heptamer affect neither. Using a screen for mutants deficient in *ade6* intragenic recombination, we have isolated 50 recessive recombination-deficient mutations, which define 16 *rec* genes (*rec6* to *rec21*). Based upon the severity of the recombination-deficiency (3- to 1000-fold reduction), these genes fall into three discrete classes (4). The mutations decrease meiotic but not (as far as tested) mitotic recombination. We have cloned and sequenced the *rec7* and *rec8* genes. Their mRNA's are strongly induced during meiosis; this induction presumably accounts for their meiotic specificity. We have purified two nucleases, exonucleases I and II, and cloned the genes encoding them. ExoI is induced about 5-fold during meiosis; it digests dsDNA to produce 5'NMP's and 3' ss tails, putative invasive substrates during meiotic recombination. ExoII, which is not detectably induced during meiosis, digests ssDNA from the 5' end. We anticipate that further analysis of these genes, enzymes and sites will allow formulation of a biochemical model for meiotic recombination in *S. pombe*.

(1) Taylor, A. and G.R. Smith (1980) Cell 22: 447-457; Braedt, G. and G.R. Smith (1989) PNAS 86: 871-875.
(2) Smith, G.R. (1991) Cell 64:19-27.
(3) Schuchert, P., M. Langsford, E. Käslin, and J. Kohli (1991) EMBO J. 10:2157-2163.
(4) DeVeaux, L.C., N.A. Hoagland, and G.R. Smith, Genetics (in press).

F 036 ON THE MECHANISM OF CHI ACTIVITY, Franklin W. Stahl[1] and Andrew V. Kuzminov[1,2], [1]Institute of Molecular Biology, University of Oregon, Eugene, OR 97403-1229, [2]Institute of Cytology and Genetics, Novosibirsk, USSR.

Previous work (1) has indicated that χ-stimulated recombination is similar to recombination effected by RecBCD enzyme whose D subunit has been inactivated by mutation. A salient phenotype of a *recD* null mutation is the loss of ExoV activity (2), suggesting that χ contributes to recombination in two (related) ways: (i) by preserving the substrate through its ability to inactivate ExoV activity, (ii) by converting the anti-recombinagenic RecBCD enzyme to the recombinagenic RecBC enzyme. When χ^+ is present in only one parent in a phage λ cross, it can promote the formation of both complementary recombinants (3) in apparent disagreement with the view that the χ^+ parent duplex has been degraded from *cos*, the RecBCD point of entry, to χ^+, where ExoV is presumed to be inactivated. However, the relative frequencies of the complementary recombinants are dependent on the relative frequencies of the infecting parental phage types. The nature of the dependence indicates that formation of the recombinant inheriting χ^+ requires the participation of two χ^+-containing chromosomes and one χ^0 chromosomes (4). It was proposed that one χ^+-containing chromosome initated the event, and was digested from *cos* to χ, while another contributed the segment so lost in a nonreciprocal recombination event initiated by the invading terminus left at or near χ. The implied triparental interactions can be seen in λ crosses conducted in cells carrying plasmids that have homology with part of λ. The pickup of a plasmid or a marker therefrom into λ appears to involve a conservative recombination event with a non-conservative λ x λ exchange occurring nearby to the right (on the λ map). These studies have an experimental counterpart in work from Steve Kowalczykowski's lab (pers com), which shows that χ protects "downstream" DNA against digestion by purified RecBCD *in vitro*. We have examined cosmid DNA linearized *in vivo* by terminase induced off a second plasmid. When the cosmid carries χ^+ and *cos* oriented "correctly" (as judged by Chi activity in λ crosses) , the DNA is digested at a reduced rate. Prolonged incubation prior to DNA extraction reveals a double-chained fragment whose size indicates that χ protects preferentially the DNA downstream from itself.

1. Thaler DS, Sampson E, Siddiqi I, Rosenberg SM, Thomason LC, Stahl FW, Stahl MM (1989) Genome 31: 53-67.
2. Amundsen SK, Taylor AF, Chaudhury AM, Smith GR (1986) Proc Natl Acad Sci USA 83: 5558-5562.
3. Kobayashi I, Stahl MM, Fairfield FR, Stahl FW (1984) Genetics 108: 773-794.
4. Stahl FW, Thomason LC, Siddiqi I, Stahl MM (1990) Genetics 126: 519-533.

Cell Cycle Regulation of Replication

F 037 STARTING DNA SYNTHESIS DURING THE EUKARYOTIC CELL CYCLE. James Roberts, Rati Fotedar, Robert Marraccino, Eduardo Firpo, Andy Koff, Gennaro D'Urso and Motoaki Ohtsubo. Department of Basic Sciences, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104.

Recent experiments from a number of laboratories have suggested that the p34 CDC2 kinase, or a closely related kinase, performs a necessary step in the start of cellular S phase in all eukaryotic cells. Our laboratory used cell free replication of SV40 DNA as a model for chromosome replication and showed that a p34 CDC2/cyclin complex can activate DNA replication upon addition to inactive extracts from human G1 phase cells. Our efforts are now focused upon understanding the mechanisms that activate the p34 CDC2 kinase during the G1 phase of the cell cycle and the pathway through which the CDC2 kinase starts DNA synthesis.

Using highly synchronous human cells we have established that the p34 CDC2 kinase becomes activated in late G1, just prior to the start of S phase. We have also found that cyclin A is one activator of p34 CDC2 at this point in the cell cycle. We have shown that cyclin A synthesis begins before S phase starts and that the cyclin A/p34 CDC2 complex assembles, and is activated at the G1 to S phase transition. We have also cloned a new human cyclin, called cyclin E, based upon its ability to functionally substitute for the S. cerevisiae CLN type cyclins. Cyclin E can bind and activate both the p34 CDC2 and the related p33 CDK2 kinase. The timing of the cyclin E/CDC2 and cyclin E/CDK2 associations are under investigation. We suggest that multiple kinases and cyclins are required to integrate the diverse array signals that regulate progression through G1 and entry into the S phase of the cell cycle.

We have studied the structure and composition of replication initiation complexes assembled in vitro at the SV40 origin in order to understand the mechanism by which the p34 kinase regulates the start of replication. We have shown the association of 4 proteins with the template DNA during the initiation of DNA replication - SV40 T antigen, a helix destabilizing protein called RP-A, p34 CDC2 and cyclin A. The interaction of all these proteins with DNA requires the SV40 replication origin. Moreover, this complex does not form in extracts from G1 cells indicating that the inability of G1 extracts to replicate DNA results from a failure to assemble the requisite proteins at the replication origin. The DNA associated p34 CDC2 protein has protein kinase activity and will phosphorylate RP-A, in cis, on the DNA as well as exogenously added histone H1. These studies have also revealed that second kinase, unrelated to CDC2, phosphorylates the 33 kd subunit of RP-A within the initiation complex, while the free RP-A that does not participate in replication remains unphosphorylated. We speculate that the origin dependent phosphorylation of RP-A signals the completion of the initiation reaction and is necessary for the subsequent formation of the elongation complex.

DNA Structure & Topology

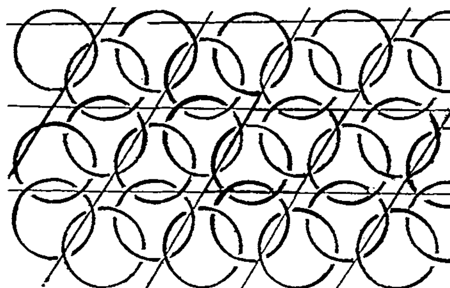
F 100 TOWARD A DYNAMICAL STRUCTURE OF DUPLEX B-DNA IN SOLUTION: COMPARISON OF THEORETICAL AND EXPERIMENTAL NOE INTENSITIES FOR d(CGCGAATTCGCG)$_2$, D. L. Beveridge, Jane M.Withka, S.Swaminathan, Jayashree Srinivasan and Philip H. Bolton, Chemistry Department, Wesleyan University, Middletown, CT 06459.

We have recently been made predictions of the solution structure for the DNA dodecamer d(CGCGAATTCGCG) on the basis of extensive molecular dynamics simulations including counterions and water explicitly. A full matrix relaxation method has also been devised to calculate theoretical NOE effects based on a dynamical model, including considerations of interproton motions obtained from MD simulation and anisotropic tumbling of the DNA. Measurements of experimental NOE intensities for duplex d(CGCGAATTCGCG) and calculations of the corresponding quantities based on several of our recent MD simulations and also the canonical A and B forms of the dodecamer as well as the crystal structure have been carried out and compared. The results obtained based on MD simulation agree significantly better with experiment than those obtained from either of the canonical forms or the crystal structure. The dynamical structure obtained from simulation is thus an improved prediction of the structure of the DNA dodecamer in solution. Including anisotropic motion of the helix and local interproton motions both improve the quality of agreement between the theoretical and experimental results. Consideration of various aspects of molecular motion is essential for obtaining an accurate theoretical account of observed NOE intensities and improved predictions of the structure of DNA in solution.

F 101 THE TOPOLOGY OF KINETOPLAST DNA, J. Chen, C.A. Rauch*, P. T. Englund*, J. H. White** and N. R. Cozzarelli, Department of Molecular and Cell Biology, University of California, Berkeley, *Department of Biological Chemistry, Johns Hopkins School of Medicine, and **Department of Mathematics, University of California, Los Angeles.

The kinetoplast DNA of Crithidia is a unique structure in nature. It is a massive network composed of thousands of topologically interlocked circles. Most of these circles are minicircles (2.5 kb) and about 50 are maxicircles (37kb). Each cell contains only one network, which resides in the matrix of its single mitochondria. By E.M., we found that the periphery of the network seems is composed of coalesced maxicircles and together with other maxicircles which run through the network interior may form a framework of the network.

The E.M. also shows that the network is a monolayer and we assume that all minicircles are linked identically to their neighbors. We have developed the following strategy to deduce the pattern of the kDNA network. (1) We model possible network structures graphically. (2) We use these model to make predictions about the the structure and relative frequencies of the products expected from a random restriction of networks. (3) we restrict kDNA networks enzymatically and analyze the products. (4) we compare our kDNA results from (3) with the prediction from our model. Using this strategy, we are able to show that, there are three other minicircles linked to each minicircle, i.e. the network has a valence of 3, which is the smallest number required to form a two dimensional network. We present a model of the network structure and a possible mechanism for its replication.



F 102 BIOLOGICAL FUNCTIONS OF TOPOISOMERASE I IN *S. CEREVISIAE*, Michael F. Christman, Dept. of Radiation Oncology, UCSF, San Francisco, CA 94143 and Gerald R. Fink, Whitehead Institute and Dept. of Biology, MIT, Cambridge, MA 02142.

The goal of this project is to identify the critical functions of topoisomerase I in *S. cerevisiae* through a molecular genetic analysis of mutations that are inviable in combination with *top1* mutations. Topoisomerase I is thought to be important to relieve torsional stress encountered during DNA replication and transcription. However, yeast mutants with a null mutation in the *TOP1* gene are viable. One explanation for the viability of *top1* mutants is that there are proteins providing overlapping functions. To identify genes encoding overlapping functions, we have isolated mutants that are inviable unless topoisomerase I is being expressed (*tpr* mutants for topo-I requiring). The *TPR* genes define at least five complementation groups. *TPR1* is allelic to *HPR1*, a gene that is homologous to *TOP1* over two short regions. *TPR2* is RNA polymerase II subunit *RPB4*, a subunit required for transcription initiation in vitro.

F 103 EFFECT OF CASEIN KINASE II-MEDIATED PHOSPHORYLATION ON THE CATALYTIC CYCLE OF TOPOISOMERASE II, Anita H. Corbett, Russell F. DeVore*, and Neil Osheroff, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146 and *Department of Medicine, West Virginia University School of Medicine, Morgantown, WV 26505

Topoisomerase II influences virtually every aspect of eukaryotic DNA metabolism. This enzyme is essential for cell viability and is required for chromosome segregation, structure, and condensation. In addition, it is involved in DNA replication and recombination. Topoisomerase II exists in the cell as a phosphoprotein. Both *in vivo* and *in vitro* the enzyme is a high affinity substrate for casein kinase II. Moreover, as determined by DNA relaxation, catenation, or decatenation assays, modification by this kinase stimulates the enzyme's overall catalytic activity 2.5- to 3.0-fold. In order to delineate the mechanism by which the enzyme's activity is enhanced, the effects of casein kinase II-mediated phosphorylation on the individual steps of the *Drosophila* topoisomerase II catalytic cycle were characterized. Phosphorylation had no effect on any of the reaction steps which preceded hydrolysis of the enzyme's high energy cofactor. This included the enzyme's binding to DNA and recognition of DNA topology, its pre-strand passage DNA cleavage/religation equilibrium, its double-stranded DNA passage event, and its post-strand passage DNA cleavage/religation equilibrium. In contrast, the rate of topoisomerase II-mediated ATP hydrolysis was stimulated ~3-fold following phosphorylation by casein kinase II. It is therefore concluded that phosphorylation modulates the overall catalytic activity of topoisomerase II by stimulating the enzyme's ability to hydrolyze ATP and subsequently reinitiate a new round of catalysis.

Supported by NIH Grants GM-33944, DK-43325, CA-09582 and ACS Faculty Research Award FRA-370.

F 104 IDENTIFICATION OF ESSENTIAL REGIONS OF THE *ESCHERICHIA COLI* DNA TOPOISOMERASE III POLYPEPTIDE REVEALED BY CHEMICAL AND SITE-SPECIFIC MUTAGENESIS OF THE *topB* GENE, Russell J. DiGate*+ and HongLiang Zhang+, *Department of Biomedicinal Chemistry, *Medical Biotechnology Center, and +Program in Molecular and Cellular Biology, University of Maryland at Baltimore, Baltimore, MD 21201

The gene encoding *E. coli* topoisomerase III (*topB*) has been placed under the control of a bacteriophage T7 RNA polymerase promoter and the *topB* gene product has been overexpressed in *E. coli* using a bacteriophage T7 transient expression system. The wild-type gene can only be expressed using a system where T7 RNA polymerase is supplied exogenously by infection of cells, harboring the *topB* plasmid DNA, with a bacteriophage λ containing T7 RNA polymerase under the control of the phage P_L promoter. Cells that contain the T7 RNA polymerase gene under the control of a cellular, inducible, *lac*UV5 promoter can not be transformed with the *topB* expression plasmid DNA. This presumably reflects the lethality of DNA topoisomerase III (Topo III) overproduction and is most likely due to the slight production of T7 RNA polymerase in the absence of induction.

The inability of the wild-type *topB* gene to transform cells containing the cellular, inducible, T7 RNA polymerase has been used as a selection to identify defective Topo III polypeptides. The wild-type gene, subjected to both chemical and site-specific mutagenesis, was used to transform cells containing an inducible T7 RNA polymerase gene. Colonies from the transformation, possessing potentially defective Topo III polypeptides, were induced and analyzed by SDS-PAGE. This selection procedure resulted in a collection of both missense and nonsense mutations, whose properties will be discussed in detail.

F 105 RECOMBINATION OF KNOTTED SUBSTRATES BY RESOLVASE : IMPLICATIONS FOR THE FLEXIBILITY OF DNA-MOVEMENT IN KNOTS, THE ENERGETICS OF KNOTTING, AND THE ROLE OF SUPERCOILING DURING DNA STRAND EXCHANGE, Peter Dröge and N.R. Cozzarelli*, Dept. of Biology, Nucleic Acids Research Group, Univ. of Konstanz, P.O. Box 5560, D-7750 Konstanz, Germany; * Dept. of Molecular and Cell Biology, Univ. of California, Berkeley, CA 94720, U.S.A.

We have extended our studies on recombination of knotted substrates and found that torus knots can be efficiently recombined by γδ resolvase under standard reaction conditions in the absence of (-) supercoiling. Kinetic experiments show that knotted supercoiled substrates are recombined about ten times faster than in the absence of torsional stress. Interestingly, the efficiency of recombination of nicked knots increases with knot-node density, as it increases with (-) supercoil density for standard substrates. This dependency on knot node density is lost when the substrates are under (-) torsional stress. In this case we also detect a new set of topologically distinct products, which are most likely the result of a 360° instead of a 180° rotation of the cross-over sites during strand exchange. By placing the two res-sites at different positions within the nicked knots, we show that the efficiency and the kinetics of recombination is not affected by the different positioning, which we take as a measurement for the flexibility of DNA segment movement within the knots.

F 106 CONDITIONAL GROWTH OF *Escherichia coli* DUE TO THE EXPRESSION OF VACCINIA DNA TOPOISOMERASE I, M. Elena Fernandez-Beros and Yuk-Ching Tse-Dinh, Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595

Vaccinia virus-encoded DNA topoisomerase I can relax both positively and negatively supercoiled DNA. The plasmid p1940 contains the entire gene cloned into pUC19 cloning vector (Shuman et al., 1988). It was found that vaccinia topoisomerase I was expressed in *E. coli* cells containing this plasmid. Moreover, the plasmid p1940 conferred an abnormal growth response of the host cells to shift in temperature. Several different strains carrrying the plasmid p1940 were grown at 30ºC for 3 hrs. Half of the culture was shifted to 42ºC. The growth curves showed a decline of the number of viable cells per ml at 42ºC. Depending on the strain, typically 2-3 logs of difference could be observed. The death of the cells was not due to plasmid loss. The same results were obtained when the experiment was repeated with no ampicillin in the media. No temperature-dependent growth difference was seen with the same strains carrying the cloning vector.
When the plasmid was introduced into *gyrA* and *gyrB* strains, the cells grew equally well at 42ºC and 30ºC. We are now trying to determine if the deleterious effect of vaccinia topoisomerase I at 42ºC is due to its relaxation of positive supercoils, or if negative supercoiling by gyrase is required for cell killing.

F 107 INTRINSIC INTERMOLECULAR DNA LIGATION ACTIVITY OF EUKARYOTIC TOPOISOMERASE II, Kevin C. Gale and Neil Osheroff, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146

Drosophila melanogaster topoisomerase II is capable of covalently joining cleaved *φX174* (+) strand DNA to duplex oligonucleotide acceptor molecules by an intermolecular ligation reaction (Gale, K.C. and Osheroff, N. (1990) *Biochemistry* 29, 9538-9545). This intermolecular DNA ligation reaction provides an efficient *in vitro* model in which to study the mechanism of potential topoisomerase II-mediated recombinational events. Therefore, further characterization of this activity was conducted. Intermolecular DNA ligation proceeded in a time dependent fashion and was concentration dependent with respect to oligonucleotide. Southern analysis and alkaline gel electrophoresis demonstrated that *φX174* DNA was covalently linked to oligonucleotide acceptor molecules following intermolecular ligation. Topoisomerase II (which becomes covalently linked to the 5'-phosphate terminus of *φX174* DNA following cleavage) required the 3'-OH, but not the 5'-phosphate termini of the oligonucleotide acceptor molecule for intermolecular ligation. Topoisomerase II-mediated intermolecular DNA ligation required a divalent cation, was inhibited by salt, and was not affected by the presence of ATP. The enzyme was capable of ligating the *φX174* (+) strand DNA to either single-stranded oligonucleotide acceptor molecules or double-stranded oligonucleotides that contained 5'-overhang, 3'-overhang, or blunt ends. As previously demonstrated for the enzyme's intramolecular religation of double-stranded DNA, the chemotherapeutic agent, etoposide, also inhibited intermolecular DNA ligation. These results demonstrate that topoisomerase II has an intrinsic ability to carry out illegitimate DNA recombination *in vitro* and suggests possible roles for the enzyme in the recombination of nucleic acids *in vivo*.

Supported by NIH grants GM-33944, DK-43325, and Faculty Research Award FRA-370.

F 108 HETEROLOGOUS BENDS CAN SUBSTITUTE FOR INTEGRATION HOST FACTOR (IHF) IN EXCISION OF BACTERIOPHAGE LAMBDA,
Steven D. Goodman and Howard A. Nash, Laboratory of Molecular Biology, National Institute of Mental Health, Bethesda, MD 20892
Bacteriophage lambda requires the *E. coli* protein IHF for efficient integration and excision. IHF is a heterodimeric protein that binds to and bends DNA sequences that contain the consensus sequence WATCAANNNNTTR. Three such sequences, H1, H2 and H' are located in *attP*, the attachment site of phage lambda. We have examined whether IHF function is mediated exclusively through its ability to bend DNA. Our strategy has been to construct chimeric attachment sites in which IHF binding sites are replaced by alternative sources of DNA deformation. Previously, we demonstrated that properly phased bends can substitute for the binding of IHF at the H2 site of *attP*. Although this result is highly suggestive of a critical role of IHF-promoted bending in lambda integration, its interpretation is obscured by the continued need for IHF binding to the H1 and H' sites of these constructs. In order to rule out adventitious binding of IHF to bend swap chimeras and to test for roles of IHF in addition to DNA bending, we have now developed two IHF-independent recombination systems.
To facilitate this approach, we focused on excisive recombination, which only requires the H2 and H' sites. In the first system we employed HU protein in place of IHF. HU protein is a homolog of IHF that compacts DNA but shows little or no sequence-specific binding. We find that HU can efficiently substitute for IHF *in vitro*, particularly during intramolecular recombination. The second IHF-independent system replaced the protein entirely. A population of sequence-directed bends were placed both in the vicinity of H2 and within the H' consensus. Even in the absence of IHF, attachment sites with properly phased bends are active for in vitro excision. Our success, although tempered by limited efficiency of these systems, completes the proof that IHF functions primarily as an architectural element.

F 109 PROTEIN-INDUCED ALTERATIONS IN DNA STRUCTURE AT THE DHFR OBR.
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The strand specificity of Okazaki fragment synthesis has been used to locate an origin of bidirectional DNA replication (OBR) to a 450 bp region 3' to the Chinese hamster *dhfr* gene (Burhans et al., Cell 62:955-965, 1990). Sequence and DNA structure analysis shows that the origin region contains a region of stably bent DNA that contains binding sites for the protein factors fos/jun and RIP60. Gel mobility assays with circularly permuted DNA fragments show that the center of DNA bending in the OBR in absence of protein is located near bend element B3. Binding of RIP60 to the bent DNA motif enhances DNA bending, and moves the center of bending approximately 25 bp 3' to bend element B3. Located 20 bp 5' to the RIP60 site between bend elements B3 and B4 is a consensus AP1 site. Using bacterially expressed polypeptides that encompass the dimerization and DNA binding domains of c-fos and c-jun, we have begun examining the combined effects that binding of fos/jun heterodimers, jun/jun homodimers, and RIP60 dimers have on the structure of the bent DNA motif. Binding of RIP60 and jun/jun homodimers induces DNase I hypersensitivity near a 12 bp palindrome that separates the AP1 and RIP60 sites, whereas binding of fos/jun heterodimers in the presence of RIP60 produces a different pattern of DNAse I hypersensitivity. Phasing of binding sites appears to be an important determinant in the degree and direction of protein-induced DNA bending in the origin region. These data suggest a model in which multiple transcription and replication factors participate along with bent DNA in the formation of a higher-order nucleoprotein structure at the dhfr OBR during initiation of DNA replication.

F 110 CHEMILUMINESCENT DETECTION OF DIGOXIGENIN-LABELED NUCLEIC ACIDS:OPTIMIZATION OF THE DETECTION PROTOCOL
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A fast and simple protocol for the chemiluminescent detection of digoxigenin (DIG) labeled nucleic acids with anti-digoxigenin antibody Fab-fragments coupled to alkaline phosphatase and 3-(4-methoxyspiro[1,2-dioxetane-3,2'-tricyclo-[3.3.1.1^{3,7}]decan]4-yl)phenyl phosphate (DPP) as substrate is described. The washing and blocking procedure was optimized to yield low background even on positively charged nylon membranes. The sensitivity of the system is equal or better than of radioactive methods. Exposure to X-ray or Polaroid film for up to 30 minutes is sufficient for the detection of 70 fg homologous DNA. Human single-copy genes are detected in Southern-blots of as low as 0.3 ug total placenta DNA. Blots can be reprobed multiple times very easily.
The advantages of the DIG-system, high sensitivity, absence of background and ease of reprobing, are illustrated by applications for single-copy gene detection in genomic blots of human DNA, Northern-hybridizations to rare mRNA, detection of E. coli genes on blots of genomic digests after pulse field gel electrophoresis as well as for nonradioactive DNA sequencing blots with DIG-labeled primers.

F 111 **THE RECOGNITION OF DNA STRUCTURE BY TOPOISOMERASE II**
Michael T. Howard and Jack D. Griffith, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599

The type II topoisomerases are a class of enzymes involved in altering the topological state of DNA. The factors that determine the interaction of topoisomerase II with it's substrate are not well understood. We have identified a strong topoisomerase II binding site located approximately 600 base pairs upstream of an integrated Human Immunodifficiency Virus (HIV). This region of DNA contains an unusual DNA structure as defined by abnormal electrophoretic migration and S1 nuclease sensitivity (Sosa et al.(1989), Biochemical and Biophysical Research Communications 161, 134-142). It was proposed that this structure could form a slipped pairing structure or an extensive cruciform. The interaction of topoisomerase II with other unusual DNA structures will be described and discussed. In addition, we will describe topoisomerase II binding and cleavage sites within the 5' Long Terminal Repeat of an integrated HIV.

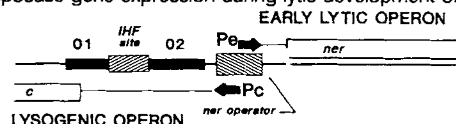
F 112 MECHANISM OF DNA TOPOISOMERIZATION BY THE ENZYMES FROM HYPERTHERMOPHILIC ARCHAEBACTERIA, Sergei A. Kozyavkin, Karl O. Stetter* and Alexei I. Slesarev*, Laboratory of Molecular Biology, NIDDK, National Institutes of Health, Bethesda, MD 20892-0002 and *Lehrstuhl für Mikrobiologie, Universität Regensburg, D-8400 Regensburg, FRG

DNA topoisomerases from hyperthermophilic archaebacteria act at temperatures where DNA may be partly or completely denatured. In contrast to the situation at moderate temperatures, the linking number of relaxed DNA, Lk_o, is different from that of relaxed completely duplexed DNA, Lk_o^d, since only ϑ_o part of a molecule is helical. So, $Lk_o = \vartheta_o \cdot Lk_o^d$, which decreases to 0 as the temperature increases[1]. It is also unusually sensitive to the ionic strength. This was shown in experiments with the relaxing ATP-independent thermophilic topoisomerase. We used this data in studies of the mechanism of action of another hyperthermophilic type I DNA topoisomerase - reverse gyrase. We found that its binding to DNA and formation of the cleavable complex does not require ATP or magnesium[2]. Our data show that the next step in the reaction - transfer of one DNA strand through the break in the other strand and religation of the break depends on the state of DNA. In the absence of ATP, reverse gyrase is able to go through this step only if it will increase Lk and decrease the free energy of DNA. The increase of Lk is limited by the ratio of reverse gyrase to DNA. Subsequent catalytic topoisomerization is restored upon addition of ATP. Thus when reverse gyrase relaxes DNA the binding of ATP to the enzyme can occur either before or after strand transfer reaction. Without ATP the enzyme fails to increase Lk against the DNA free energy or to decrease it at the expense of the DNA free energy. However the ATP concentration may be as low as 0.1 µM to supply free energy for positive supercoiling reaction at 95°C. Under these conditions we observed molar stoichiometry between the ATP molecules and the change of Lk. These data show very tight coupling between topological and ATPase cycles of reverse gyrase and imply that the binding of ATP to the enzyme should occur prior to the strand transfer in the course of the positive supercoiling reaction.

1. A.I.Slesarev, D.A.Zaitsev, V.M.Kopylov, K.O.Stetter and S.A.Kozyavkin (1990) *J. Biol. Chem.* **266**, 12321-12328.
2. O.I.Kovalsky, S.A.Kozyavkin and A.I.Slesarev (1990) *Nucleic Acids Res.* **18**, 2801-2805.

F 113 CONTROL OF D108 TRANSPOSASE GENE EXPRESSION: DNA STRUCTURAL ALTERATIONS IN THE LEFT-END REGULATORY REGION, George Kukolj and Michael S. DuBow, Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3A 2B4

Regulation of temperate Mu-like bacteriophage D108 transposition occurs at multiple levels. During lysogeny, the randomly integrated copy of the phage genome remains quiescent through the expression and action of the c repressor. DNase I footprinting reveals that oligomers of c repressor bind two operators (O1 and O2), one of which (O2) overlaps the early promoter such that bound c repressor prevents RNA polymerase binding and transcription of the early operon. During lytic growth, expression of the early (transposase) operon is controlled by the phage encoded *ner* repressor in conjunction with the host protein IHF. The *ner*-operator, which spans five turns of the DNA helix, is composed of two 11 bp inverted repeats separated by an 8 bp AT-rich region and overlaps the transcriptional initiation sites for both the repressor (Pc) and early (Pc) promoters. Gel-shift analysis of DNA fragments of identical length in which the *ner*-binding site is located in circular permutations, show that the intrinsically bent *ner*-operator is conformationally altered upon protein binding. Moreover, potassium permanganate hypersensitivity reveals that the apex of operator bending localizes to the AT-rich spacer. In the absence of IHF, bound Ner prevents RNA polymerase from binding to either Pc or Pe. However, in the presence of IHF, which binds and bends the DNA upstream of Pe, RNA polymerase is capable of associating with Pe irrespective of the Ner-DNA complex. Thus, the IHF-induced DNA structural change may, in part, partially relieve the Pe (but not Pc) promoter from Ner repression to ensure stable transposase gene expression during lytic development of the phage.



F 114 **DNA Topological Requirements for the Initiation of Bacteriophage λ DNA Replication.** Brian A. Learn and Roger McMacken. Department of Biochemistry, The Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205

The DNA topological requirements for the initiation of λ DNA replication were determined *in vitro* using template topoisomers of a 6.1 kb oriλ plasmid and a purified-protein DNA replication assay. In the absence of DNA gyrase, no DNA synthesis was initiated on topoisomers containing an average of less than 23 negative (-) superhelical turns (superhelical density, σ, = -0.037), but as the number of (-) supercoils of the template plasmid exceeds 23, the efficiency of initiation increases to a maximum when the DNA templates contain 38 (-) supercoils (σ = -0.062). In addition, we have examined the topological requirements for DNA structural changes at the λ origin induced by the λ DNA replication initiator protein, λ O. The specific binding of λ O to four 19 bp repeats at the λ origin induces a supercoiling-dependent destabilization of an A/T-rich sequence adjacent to the λ O binding repeats. We determined the effect of template superhelical density on the ability of λ O to induce DNA structural changes within the A/T-rich region using OsO_4 as a probe. In the absence of λ O, the A/T-rich region of oriλ is insensitive to OsO_4 at all superhelical densities tested; however, as the number of (-) supercoils increases above 28, thymidine runs within inverted repeats in the λ origin become OsO_4-sensitive, consistent with the extrusion of cruciform structures under superhelical stress. In contrast, when λ O binds to templates with > 28 (-) supercoils, it induces a cooperative structural change throughout the 40 bp A/T-rich region. Moreover, the thymidine runs within the origin inverted repeats are inert to OsO_4 in the presence of λ O, even at very negative superhelical densities (-0.088), suggesting that the binding of λ O destabilizes cruciform structures elicited by superhelical tension. These results demonstrate that the initiation of λ DNA replication *in vitro* and λ O-induced destabilization of the A/T-rich sequence require a free superhelical density < -0.04.

F 115 QUANTITATIVE STUDIES OF SITE JUXTAPOSITION IN SUPERCOILED DNA, S.D. Levene,[1] P.M. Abola,[1] A.V. Vologodskii,[2] and N.R. Cozzarelli[1], [1]Department of Molecular and Cell Biology, University of California, Berkeley and [2]Institute of Molecular Genetics, USSR Academy of Sciences, Moscow, USSR.

Site-specific recombination of DNA is an important event in many biological. The proteins that mediate these events interact with at least two distinct sites on the DNA contour to form a nucleoprotein structure called a synaptic complex. The DNA between the recombination sites is looped out, an unfavorable DNA conformation that is strongly influenced by DNA tertiary structure. Many replication and site-specific recombination systems require DNA supercoiling.

We used Monte Carlo simulation to investigate the structure of DNA containing physiological levels of supercoiling. Structurally, the simulated molecules have the same conformations of supercoiled DNA as observed by electron microscopy. We find that supercoiling strongly promotes juxtaposition of DNA sites located distantly along the contour of the molecule. In circular molecules containing native levels of supercoiling, the effective concentration of one site around another site is two to three orders of magnitude greater than in a relaxed molecule.

Data are presented for the effective DNA concentration, or loop closure probability, as a function of contour and through-space separation, superhelical density and DNA length. We analyze separately the radial (through-space) and site-orientation contributions to this quantity. These results are related to the measured effects of supercoiling on site-specific recombination systems.

F 116 A NOVEL YEAST TOPOISOMERASE I MUTANT, Nikki Levin and Gerald R. Fink, Department of Biology, MIT, Whitehead Institute, 9 Cambridge Center, Cambridge, MA 02142

DNA topoisomerases have been proposed to play a role in such critical cell functions as transcription, DNA replication, and recombination. Despite the evidence suggesting that topoisomerases are required for these functions, the yeast topoisomerase I gene, TOP1, is not required for growth. While deletion of TOP1 causes only a slight growth defect, it greatly increases recombination, specifically in the ribosomal DNA, which is an array of approximately 200 tandemly repeated genes. We have isolated an altered function allele of TOP1 (designated top1-103) which not only causes higher levels of recombination in the rDNA arrays than a deletion of TOP1, but also elevates recombination at other loci. Unlike simple deletion mutants of TOP1, this allele also causes a severe growth defect, and is lethal to wild type cells when overexpressed. The mutation is lethal in combination with several DNA repair mutations and elevates expression of a DNA damage- inducible gene, suggesting that top1-103 damages DNA.

To account for the novel recombination and toxicity phenotypes of the top1-103 mutant, we propose the following model: top1-103 is able to initiate the topoisomerization reaction, nicking DNA and becoming covalently bound to one end of the broken DNA strand. It is defective, however, in the resealing step and therefore leaves nicks in the DNA. These nicks could be converted to double-stranded breaks during replication, resulting in DNA damage which is recombinogenic and toxic to the cell. Assays of partially purified top1-103 suggest that this enzyme is in fact able to nick a supercoiled substrate, but completes the reaction only inefficiently.

Since many of the phenotypes of the top1-103 mutant are suppressed by overexpressing wild type TOP1, we believe that topoisomerase I normally acts as part of a multiprotein complex, and that the wild type and mutant proteins compete for spaces in this complex. We are currently isolating high copy suppressors in order to identify other proteins in this complex.

F 117 INTERACTION OF THE BACTERIAL HISTONE-LIKE PROTEIN H1a AND THE GENE 5.5 PROTEIN OF BACTERIOPHAGE T7

Qingyun Liu and Charles C. Richardson, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

The H1a protein is found tightly bound to the gene 5.5 protein of bacteriophage T7. Gene 5.5 of T7 is located within the DNA metabolism gene cluster and encodes a protein with a MW of about 11kD. T7(5.5$^-$) is able to form normal but somewhat smaller plaques on *E. coli*. Packaging extracts made from T7(3$^-$5$^-$5.5$^-$6$^-$)-infected cells package exogenous T7 DNA at an efficiency that is 10- to 100-fold lower than extracts of T7(3$^-$5$^-$6$^-$)-infected cells do in several *E.coli* strains tested.

During the process of purifying gp5.5, a protein with a MW of about 17 kD was noticed to consistently co-purify with gp5.5. Microsequencing of this 17kD protein revealed that it is the *E. coli* H1a (also called H-NS) protein. The binding of H1a to gp5.5 is tight enough to sustain any non-denaturing fractionation methods attempted to separate these two proteins. The coding sequence of gene 5.5 was also fused to the maltose-binding protein (MBP). When the MBP-gp5.5 fusion protein was eluted from amylose resin, H1a protein is also found in the eluate, which further supports the interaction between gp5.5 and H1a. Furthermore, when the MBP-gp5.5 fusion protein is overexpressed in *E. coli*, it induces one of the phenotypes caused by H1a mutation: derepression of the *proU* promoter. The physiological significance of the H1a-gp5.5 interaction is under investigation and will be further discussed.

F 118 ISOLATION OF THE MITOCHONDRIAL GENOME COMPLEXED WITH ITS PHYSIOLOGIC DNA BINDING PROTEIN, Robert L. Low, Mariana Gerschenson and Kathryn L. Houmiel, Department of Pathology, University of Colorado Health Sciences Center, Denver, CO 80262

The mitochondrial (mt) genome of the mammalian mitochondria can be gently isolated without removing proteins that naturally bind and stabilize the DNA. The isolation procedure developed uses bovine-heart mitochondria. It requires that the mitochondria first be treated with pancreatic DNaseI, extensively washed and then sedimented through a linear sucrose gradient, in order to eliminate contaminants of nuclear DNA. The organelles are then disrupted with 1% (w/v) Triton X100 and the mtDNA isolated through cycles of differential centrifugation. In this state, the mtDNA is intact and coated by DNA binding proteins, and maintain a tight, yet noncovalent association with an insoluble matrix-like complex that contains less than 2% of the total mt protein. Remarkably, the mtDNA:protein complex supports the rapid synthesis of the heavy (H)-strand in the displacement (D)-loop region *in vitro*, when incubated in a standard DNA replication assay. This reaction is resistant to N-ethylmaleimide and aphidicolin, and occurs in the absence of additional proteins. These results suggest a D-loop replicase activity resides stably bound to the mtDNA *in vivo* and possibly functions to preserve the D-loop structure that is seen in a large proportion of mtDNA circles.

F 119 GYRASE BINDING SITES DO NOT SUBSTITUTE FOR THE pSC101 *par* LOCUS IN PRODUCING PLASMID STABILITY OR INCREASED SUPERHELICITY, Christine Miller and Stanley N. Cohen, Department of Genetics, Stanford University School of Medicine, Stanford, California 94305.

To investigate the role of DNA gyrase binding in the *par* locus mediated supercoiling and stabilization of the pSC101 plasmid, other gyrase binding sites were substituted for the *par* locus. Whale and Kornberg (EMBO J. 1988) have shown that the *par* locus contains a good gyrase binding site; another sequence, the REP (repetitive extragenic palindrome) sequence characterized by Yang and Ames (PNAS, 1988) as a good gyrase binding site was substitited for *par*. Our results show that the REP sequence does not either stabilize *par* deleted plasmids or increase their overall superhelicity. Because the presence of a strong gyrase binding site is not sufficient to impart an observable increase in overall superhelicity to plasmid DNA and concominant stabilization of the plasmid; we conclude that the *par* locus has other functions in addition to its gyrase binding ability. In contrast to promoter-induced stabilization which is position dependant, the *par* locus can stabilize a *par* deleted plasmid and increase its negative superhelicity at loci distant from its natural location.

F 120 INTRAMOLECULAR DNA TRIPLEXES: NOVEL SEQUENCE REQUIREMENTS AND INFLUENCE ON DNA POLYMERIZATION, Andrey Dayn and Sergei M. Mirkin, Department of Genetics, University of Illinois at Chicago, Chicago, IL 60612.

Intramolecular DNA triplexes are known to be formed by homopurine-homopyrimidine mirror repeats *in vitro*. These structures may be involved in DNA replication, transcription and recombination. Two different types of such triplexes are known. Under mildly acidic conditions, H DNA is formed by half of the purine strand and the hairpin formed by the pyrimidine strand. It consists of CGC+ and TAT base triads. In the presence of bivalent cations, a related H* DNA is formed by half of the pyrimidine strand and a hairpin formed by the purine strand. It is stabilized by GGC and AAT base triads. By probing with chemical agents specific for unusual DNA conformations, we have now demonstrated the formation of intramolecular triplexes consisting of GGC and TAT base triplets by DNA sequences that are neither homopurine-homopyrimidine, nor mirror repeats. This finding significantly enlarges the number of sequences that could form DNA triplexes. Such triplexes cause specific termination of DNA polymerization in vitro, and may have implications for the regulation of DNA replication in vivo.

F 121 DNA topology in the thermophilic Archaebacterium *Sulfolobus shibatae* B12.

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Sulfolobus shibatae B12 is an interesting model in terms of DNA topology at high temperature. This organism is a thermoacidophilic Archaebacterium (growth conditions: pH 3 and 81°C). It contains an inducible viral particle named SSV1. The viral DNA has been shown to be positively supercoiled before encapsidation[1]. On another hand, we have previously purified an archaebacterial topoisomerase likely responsible for this enzymatic reaction. This enzyme has been called Reverse Gyrase: it is a type I topoisomerase which catalyzes positive supercoiling of the DNA by an ATP-dependent process[2]. The positively supercoiled state of SSV1 DNA suggests that this DNA is a preferential substrate for Reverse Gyrase. We used this property to study the SSV1 DNA cleavage due to Reverse Gyrase *in vitro*: the enzyme induced a specific cleavage pattern. Cleavable complexes were obtained in the absence of ATP. Their detection was increased when the reaction was stopped by alkali instead of SDS or LDS.

In the course of the purification of *Sulfolobus shibatae* B12 Reverse Gyrase, we detected another topoisomerase activity. This new enzyme performs an ATP-independent relaxation of DNA. We defined a new procedure for the purification of both these enzymes. They were separated by using a phenyl-Sepharose chromatography. The purified Reverse Gyrase from *S. shibatae* B12 presents the same molecular mass and similar enzymatic characteristics as that of *S. acidocaldarius* DSM 639. Moreover, it is recognized by antibodies raised against DSM 639 protein. The other topoisomerase is a Mg-dependent enzyme able to relax negatively supercoiled DNA exclusively.

In conclusion, we have defined a new system we called "B12 system": it is an homogenous system containing purified topoisomerases and one of their preferential substrates, which could be easily used in order to study the role of these enzymes in the archaebacterial world.

[1] Nadal, M., Mirambeau, G., Forterre, P., Reiter, W.-D. et Duguet, M. Nature (1986), *321*, 256-258.
[2] Nadal, M., Jaxel, C., Portemer, C., Forterre, P., Mirambeau, G. et Duguet, M. Biochemistry (1988), *27*, 9102-9108.

F 122 REGULATION OF YEAST DNA TOPOISOMERASES, J.L. Nitiss[1,2], M. Jannatipour[1], D.A. Adams[1], and V. King[2]
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Eukaryotic DNA topoisomerases play essential roles in DNA metabolic events such as transcription, replication and recombination. In prokaryotes, the DNA topoisomerases are regulated in a homeostatic fashion in order to maintain an optimal level of DNA supercoiling. Since the bulk of eukaryotic DNA is relaxed, it is unlikely that a similar mode of regulation is utilized. However, it is possible that topoisomerase gene promoters may respond to transient supercoiling produced by processes such as transcription. Little information is currently available in any eukaryotic system on the regulation of these enzymes. We have therefore begun to analyze the regulation of the topoisomerase I (TOP1) and topoisomerase II (TOP2) genes of yeast.

Yeast TOP2 is expressed preferentially in proliferating cells and is cell cycle regulated. TOP2 is induced by DNA damaging agents such as MMS, and is also induced in meiosis. Unlike TOP2, uniformly high levels of TOP1 activity are seen in both log phase and stationary phase cells, and the TOP1 gene is not induced by DNA damaging agents. We have found that the expression of both the TOP1 and TOP2 genes are affected by topoisomerase mutations. While little difference is seen in cells carrying either Δtop1 or $top2^{ts}$ single mutations, topoisomerase expression is increased in cells carrying both mutations. The increase in expression is particularly striking in cells with a plasmid carrying β-galactosidase under the control of the TOP1 promoter; Δtop1 $top2^{ts}$ strains show up to a 20 fold increase in expression compared to isogenic TOP+ strains. We are presently testing the hypothesis that the double mutant produces DNA that is under superhelical tension, and that this superhelicity is responsible for the high level of expression of topoisomerase genes in the top1 $top2^{ts}$ double mutant.

F 123 A ROLE FOR THE PASSAGE HELIX IN TOPOISOMERASE II-MEDIATED DNA CLEAVAGE: A TWO-SITE MODEL FOR ENZYME ACTION, Neil Osheroff, Anita H. Corbett, E. Lynn Zechiedrich, and Kevin C. Gale, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146

Fundamental to the physiological activities of topoisomerase II is the enzyme's double-stranded DNA passage reaction. At the time of the DNA strand passage event, the enzyme must interact with two separate helices of DNA, the helix in which it creates a double-stranded break (i.e., the *cleavage helix*) and the helix which it passes through this break (i.e. the *passage helix*). Electron microscopy studies indicate that topoisomerase II is capable of binding both helices prior to its DNA cleavage event. This raises the possibility that the presence of the passage helix is a prerequisite for efficient enzyme-mediated DNA cleavage. To determine whether the passage helix plays an active role in the enzyme·DNA complex, the ability of *Drosophila* topoisomerase II to cleave a short double-stranded oligonucleotide was assessed. A 40-mer that contained a specific enzyme recognition/cleavage sequence was employed for these studies. A sigmoidal rather than a linear oligonucleotide concentration dependence for DNA cleavage was obtained. This indicates that the enzyme must interact with more than a single 40-mer in order for cleavage to take place. Topoisomerase II·DNA binding experiments were carried out to further characterize interactions between the oligonucleotide and the enzyme. No binding cooperativity for the oligonucleotide was observed. These findings strongly suggest a *two-site model* for topoisomerase II action. In this model, the passage and the cleavage helices bind to the enzyme independently, but the passage helix must be present for efficient topoisomerase II-mediated DNA cleavage to occur.

Supported by NIH Grants GM-33944 and CA-09582 and ACS Faculty Research Award FRA-370.

F 124 TRANSCRIPTION -DEPENDENT DELETIONS IN AN E. COLI TOPOISOMERASE I MUTANT, Gail J. Pruss, Biology Dept, University of South Carolina, Columbia, SC 29208
DNA topoisomerases appear to act as suppressors of some types of recombination, possibly by relaxing negatively supercoiled DNA (see Wang et al., Cell **62**, 403, 1990, for a review). To determine the effect of negative supercoiling on deletion formation, I looked at the generation of deletions in plasmids under conditions in which plasmid DNA supercoiling is determined by transcription on the plasmid. This system is based on the observation that transcription of the *tet* gene on pBR322 in topoisomerase I (*top*) mutants of E. coli causes very high negative supercoiling of the plasmid DNA (Pruss & Drlica, PNAS **83**, 8952, 1986), and it uses derivatives of pBR322 that have the *tet* gene under control of a *tac*-related promoter. The promoter is embedded within a region of palindromic DNA that contains direct repeats. Deletions occurred within this region when the plasmids were in *top*$^-$ cells, but no deletions were detected in *top*$^+$ cells. Repressing expression from the *tac*-related promoter (with *laci*^{Q} on a compatible plasmid) eliminated the deletions in *top*$^-$ cells. Thus, transcription-driven negative supercoiling appears to be required for deletion formation in this system.

F 125 MOLECULAR ANALYSIS OF MCRBC, A GTP-DEPENDENT RESTRICTION ENDONUCLEASE FROM *E. COLI* K-12. Elisabeth A. Raleigh, Ellen Sutherland, Deborah Dila, Pamela Briggs, Julia Kelleher, Linda Coe, Barton Slatko and Laurie Moran, New England Biolabs, 32 Tozer Rd, Beverly, Ma. 01915.
The McrBC system is one of three modification-dependent restriction systems used by *E. coli* K-12 to monitor the origin of invading DNA and determine its fate. The system is encoded by two genes of low GC composition flanked by two similar dyad symmetries, suggesting that the system may have been imported from elsewhere. Both genes are required for restriction *in vivo* of a variety of modified targets, including those with 5-methylcytosine, 5-hydroxymethylcytosine and N^4-methylcytosine.
Three proteins are expressed from the two genes, two of which are required for *in vitro* activity. For each of these proteins ($McrB_L$ and McrC), constructs that forced translation initiation at either of two potential start codons yielded enzymatically active product. One of each was purified to >90% purity. The *in vitro* cleavage activity reflected the *in vivo* properties of the system in its requirement for a modified substrate and in the spectrum of site-specific modifications that were sensitive to cleavage. GTP was required for cleavage. Non-hydrolysable analogues of GTP inhibited the reaction, as did ATP. We are unaware of any other nuclease with an absolute requirement for a guanosine nucleotide.
The nature of the cleavage site was examined further by mapping sites on natural substrates, delimiting the cleavage site by primer extension, and cleaving synthetic oligonucleotide model substrates. Different sites are cleaved with differing efficiencies. Our current model is that cleavage requires the sequence $R^{m}C(N_{40-70})R^{m}C$, with multiple cleavage positions on both strands distributed within the spacer region.

F 126 EFFECTS OF NOVEL QUINOLONE DERIVATIVES ON EUKARYOTIC TOPOISOMERASE II, Megan J. Robinson, Sarah H. Elsea, Barbara A. Martin*, Thomas D. Gootz*, Paul R. McGuirk*, and Neil Osheroff, Department of Biochemistry, Vanderbilt University, Nashville, TN 37232-0146 and *Pfizer Central Research, Groton, CT 06340
Although quinolone-based drugs are widely used as antimicrobial agents, they have been shown to affect eukaryotic topoisomerase II. Therefore, the effects of novel quinolone derivatives on the activities of eukaryotic topoisomerase II were examined. The most effective derivative, CP-115,953, was twice as potent at enhancing levels of DNA cleavage mediated by the *Drosophila* enzyme as was the antineoplastic drug etoposide. Quinolones in the CP-115,953 series were considerably more potent against calf thymus topoisomerase II. Religation assays indicated that quinolone derivatives enhanced DNA cleavage without impairing the ability of topoisomerase II to religate cleaved DNA. In addition, these agents were cytotoxic to mammalian cells, and a drug-resistant cell line displayed crossresistance to quinolones, albeit to a lesser extent than for other topoisomerase II-targeted drugs. Structure-activity studies indicate that the C_8 fluorine (X) and the N_1 cyclopropyl (Y) substituents contribute to quinolone potency. Furthermore, the hydroxyphenyl ring at position C_7 appears to be important for eukaryotic specificity. These results strongly suggest that novel quinolones constitute a novel mechanistic class of topoisomerase II-targeted drugs which have potential as antineoplastic agents.
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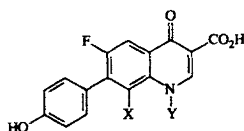
Compound	X	Y
CP-115,953	F	Cyclopropyl
CP-115,955	H	Cyclopropyl
CP- 67,804	F	Ethyl



F 127 STRUCTURAL PERTURBATIONS INDUCED BY THE BINDING OF THE λO PROTEIN TO THE VIRAL REPLICATION ORIGIN, Devanur S. Sampath and Roger McMacken, Department of Biochemistry, The Johns Hopkins University, Baltimore, MD 21205.
The binding of 4 dimers of the λO replication initiator protein to the viral origin(oriλ) induces a structural change in the adjacent(rightward) 40 base pair A/T-rich region. The prevailing view is that this alteration represents the localized melting of the A/T-rich region into its component A-rich and T-rich single strands. Using various chemical and enzymatic probes of DNA structure and primer extension analysis, we are examining in more detail the nature of the perturbations in origin structure that are induced by O binding.

Binding of O to supercoiled oriλ DNA under optimal replication conditions, renders two cytosine residues markedly susceptible to oxidation by $KMnO_4$, a reagent which modifies pyrimidine bases that are present in a non-B DNA configuration. These residues are near the 5'-ends of the two O recognition site iterons located closest to the A/T-rich region on the T-rich strand. Moreover, these residues are paired with guanine residues that experience a pronounced hypersensitivity to dimethyl sulphate under identical conditions. Surprisingly, only one thymine residue inside the A/T-rich region, just two residues away from the nearest iteron experiences $KMnO_4$ oxidation. Under these same conditions, P1 nuclease cleaves both strands of the A/T-rich region throughout the first 20 bases to the right of the origin repeats. All of these reactivities exhibit a marked sensitivity towards (i)the superhelical density of the DNA;(ii)temperature and(iii)magnesium concentration. We tentatively conclude that the binding of the λO initiator to the origin repeats on a supercoiled template induces the neighbouring A/T-rich DNA segment to adopt a 'non-B' DNA configuration that is <u>not</u> equivalent to unwound, single stranded DNA.

F 128 BIOLOGICAL EFFECTS OF TOPOISOMERASE IV IN S. TYPHIMURIUM M.B. Schmid, D.J. Sekula, A.L. Luttinger and A.L. Springer; Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014
The parCEF genes encode Topoisomerase IV, and were identified by conditional lethal mutants that fail to partition daughter nucleoids at the nonpermissive temperature. Ts mutants in parF, parC and parE have identical cytological phenotypes after a nonpermissive temperature shift. We are exploring the in vivo role of Topoisomerase IV by looking at the effect of parCEF mutants on DNA replication, transcription from supercoiling sensitive promoters, and illegitimate recombination. The parCEF genes are clustered at 65' (near metC). The DNA sequences of parC and parE show extensive homology to gyrA and gyrB, respectively, and preserve amino acids common among TypeII topoisomerases. The parF gene encodes a 27Kd protein with a strongly hydrophobic N-terminus. ParC and GyrA cannot substitute for one another, despite the strong structural homology. A plasmid that expresses the wild-type GyrA protein does not suppress parC(ts) mutations, nor does a plasmid that expresses the wild-type ParC protein suppress gyrA(ts) mutations. Ts mutants in parCEF show altered transcription from a set of "supercoiling-sensitive" promoters (including the gyrA promoter), as measured by lacZ transcript fusions. We have speculated that ParCEF (Topisomerase IV) forms a specialized, membrane-bound topoisomerase that plays an essential role in the partitioning process. These results suggest that Topoisomerase IV also has the more general ability to alter the DNA's superhelicity.

F 129 THE ROLE OF CONSERVED NUCLEOTIDE SEQUENCES IN THE REPLICATION OF KINETOPLAST DNA. Joseph Shlomai, Merav Zucker, Irit Kapeller, Vera Schindler, and Dale Frank. Department of Parasitology, The Hebrew University, Hadassah Medical School, Jerusalem 91010, Israel.
Kinetoplast DNA (kDNA) is a unique DNA network found in the single mitochondrion of trypanosomatids. Its major constituents are several thousand duplex DNA minicircles, which are interlocked topologically to form a catenane DNA network. Minicircles replicate as unlinked monomeric circles, through a process which includes their prereplication release from the network and their post replication reattachment to it. Our *in vitro* studies, using purified enzymes, suggest that this "release and reattachment" process is controlled through the action of a type II DNA topoisomerase, which catalyzes the topological interconversions of monomeric kDNA minicircles and catenane networks, and a unique endonuclease, which enables the discrimination between parental minicircles and newly replicated ones. The DNA topoisomerase II was recently purified to apparent homogeneity from *Crithidia fasciculata* cell extracts and was located to the kinetoplast organell using electron microscopy. Preliminary results suggest a possible structural relationship between the topoisomerase and the unique endonuclease. The involvement of a conserved bent DNA structure in the control of the topological reaction during kDNA minicircle replication and the specific recognition of the origin of kDNA minicircle replication by a protein present in the trypanosomatid cell extracts are discussed.

F 130 ACCELERATED DNA RENATURATION: COMPLEMENTARY RECOGNITION IN CONDENSED DNA, Jean-Louis Sikorav and George M. Church, Unité de Génétique et Biochimie du Développement, LA CNRS 361, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15 (JLS), and Department of Genetics, Harvard Medical School, and Howard Hughes Medical Institute, 25 Shattuck Street, Boston, MA 02115 (GMC).

The functional consequences of DNA condensation are investigated. The recognition of complementary strands is profoundly modified by this critical phenomenon. 1) Condensation of denatured DNA greatly accelerates the kinetics of DNA renaturation. We propose a unifying explanation for the effects of several accelerating solvents studied here including polymers, di- and multivalent cations, as well as effects seen with the phenol emulsions and single-stranded nucleic acid binding proteins. Optimal conditions for renaturation at or above the calculated three dimensional diffusion limit are theoretically consistent with a limited search space in the condensed phases. 2) In addition to these effects on association of two single strands, similar condensation acceleration effects can be seen in strand exchange experiments with double stranded DNA without proteins. These may model a mechanism of recombinational protein function.

F 131 MOLECULAR CLONING, EXPRESSION AND PURIFICATION OF HIV NUCLEOCAPSID PROTEIN. J. C. YOU and C. McHENRY. Department of Biochemistry, University of Colorado HSC, Denver, CO 80262.

HIV nucleocapsid protein (MW 7,000), a nucleic acid binding protein encoded near the carboxyl terminus of the *gag* gene polyprotein precursor and processed by HIV viral protease, is found in a ribonucleoprotein complex with its RNA genome in the viron core. The protein can be recognized by its basic amino acid composition and two small zinc finger sequences highly conserved in a group of retroviruses.

However, the functional role of nucleocapsid protein in viral replication and life cycle has not been elucidated because of the difficulty in obtaining sufficient amount of the protein for a rigorous study. Therefore, we have undertaken the present studies to produce a large quantity of HIV nucleocapsid protein.

We amplified the nucleocapsid protein coding sequence (165 bp) from viral cDNA by the polymerase chain reaction (PCR) using specifically designed synthetic oligonucleotide primers and cloned it downstream of the Ptac promoter in an expression vector. By developing a nitrocellulose filter binding assay as the functional assay using homopoly [8-^{3}H] adenylic acid as a substrate, HIV nucleocapsid protein was purified 30-fold to apparent homogeneity by employing Q-sepharose, S-sepharose and G-50 chromatography. Crude cell extracts were prepared by mechanical cell lysis method, polymin P, and ammonium sulfate precipitation. From this procedure, we have obtained 24 mg of the nucleocapsid protein from 60 g of cells.

The results from DNA sequencing, partial protein sequencing and amino acid analysis have confirmed that the authentic HIV nucleocapsid protein has been produced without any mutations, or proteolytic degradation. The extinction coefficient of the purified HIV nucleocapsid protein was determined to be 8,300 $cm^{-1}M^{-1}$ (280 nm). The intrinsic association constant (K) to poly (rA) was determined to be 2.0×10^6 M^{-1}.

F 132 INHIBITION OF TOPOISOMERASE I ACTIVITY IN NUCLEAR EXTRACTS OF MOUSE C127 CELLS BY 2',5'-OLIGOADENYLATES.

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The activity of topoisomerase I (topo I), a key enzyme in DNA metabolism, is inhibited with 5'-monophosphate trimer of 2',5'-oligoadenylate (2-5A). The activity of topo I in crude nuclear extracts of mouse C127 cells was assayed by the relaxation of supercoiled pBR322 plasmid DNA. By this method, we have found two different interactions of 2',5'-oligoadenylates with topo I. The first one is direct, as recently reported for pure topo I by F.J.Castora et al., [J.Interferon Res., 11:143-149 (1991)], with a low affinity binding of 2-5A. The second type is possibly indirect and 1,000 times more effective. It is not found with purified calf thymus topo I (BRL), and is labile in crude extract. The inhibition of topo I in this case strongly depends on Mn^{2+}. The level of inhibition is dependent upon the number of residues and degree of phosphorylation of 2-5A. When the activity of mono-, di-, tri-, tetra-, and pentanucleotides is compared, the most effective is the trimer form, for all degrees of phosphorylation. Amongst the 5'-mono, tri- and nonphosphorylated forms of 2-5A, the most effective are monophosphorylated oligomers. 3'-monophosphate forms of 2-5A, which are less sensitive to degradation by phosphodiesterase, exhibit high inhibition efficiency, comparable to that observed with the naturally occurring 5'-monophosphate forms. The average minimal concentrations of different 2-5A forms which still inhibit topo I are presented in the table below.

pA 50 µM		Ap 100 µM	
pA_2 20 µM		A_2p 2 µM	A_2 100 µM
pA_3 100 nM	$pppA_3$ 3 µM	A_3p 50 nM	A_3 10 µM
pA_4 5 µM	$pppA_4$ 5 µM	A_4p 10 µM	
pA_5 3 µM		A_5p 3 µM	

The trimer form of 5'-monophosphorylated 2',5'-oligoadenylate is the natural second messenger in normal cell metabolism, as well as during the interferon response. The concentration of this form of 2-5A needed for inhibition is close to the levels found intracellularly . This effect of 2-5A may play a role in regulating topo I activity in the cell during transcription and replication.

Polymerase Mechanism

F 200 CHARACTERIZATION OF THE 5'→3' EXONUCLEASE ACTIVITY OF *THERMUS AQUATICUS* DNA POLYMERASE, Richard D. Abramson, Pamela M. Holland, Robert Watson and David H. Gelfand, PCR Division, Cetus Corporation, Emeryville, CA 94608

Thermus aquaticus (*Taq*) DNA polymerase is a thermostable enzyme which has greatly simplified the polymerase chain reaction (PCR) method of DNA amplification. In addition to its polymerization activity, *Taq* DNA polymerase contains a 5'→3' exonucleolytic activity. The exonuclease cleaves 5'-terminal nucleotides of double-stranded DNA. The preferred substrate is displaced single-stranded DNA, with hydrolysis occurring at the phosphodiester bond joining the displaced region with the base paired portion of the strand. We designed various substrates to resemble nicks, gaps, and displaced single-stranded DNA. In the absence of polymerization, the rate of exonuclease cleavage at a nick with a displaced single strand is 15–20 fold greater than cleavage at a completely duplexed nick. Similarly, cleavage is 15–20 fold greater at a nick than a gap, and diminishes as gap size increases. In the presence of polymerization, a gap is filled to resemble a nick with a displaced single strand which is then cleaved. This cleavage occurs at a rate 2–3 times greater than cleavage at a nick in the absence of polymerization. Based on the size of the cleavage products generated, it appears that the enzyme prefers to perform limited strand displacement synthesis prior to exonucleolytic cleavage. We also describe a deletion mutant of *Taq* DNA polymerase. This mutant enzyme lacks the amino terminal one third of the protein containing the 5'→3' exonuclease domain, and is analogous to the Klenow fragment of *E. coli* DNA polymerase I. A thermostable DNA polymerase lacking 5'→3' exonuclease activity may be desirable in some instances of PCR, such as in the amplification of certain GC-rich templates, where the secondary structure of the DNA may resemble a preferred exonuclease substrate. In addition, it may minimize the "plateau effect" observed in late cycles of PCR, where forked structures are predicted to occur.

F 201 A ROLE FOR DNA POLYMERASE β IN DNA REPLICATION AND REPAIR IN *XENOPUS* OOCYTES AND EXTRACTS, Eric J. Ackerman, Timothy M. Jenkins, Joshua D. Levin, Jitendra K. Saxena, and Samuel H. Wilson* GBB, NIDDK, NIH, Bethesda, MD 20892; *Laboratory of Biochemistry, NCI, NIH, Bethesda, MD 20892

We are investigating the roles of various DNA polymerases and accessory proteins involved in repair of damaged DNA in both injected *Xenopus* oocytes and oocyte nuclear extracts [Saxena *et al.*, 1990 *Nucleic Acids Res.* **18**, 7425-7432]. We have also examined roles for these proteins in replication of single strand (ss) M13 to double strand (ds) M13 DNA. DNA replication in injected oocytes resulted in fully replicated molecules of form I and form II DNA, whereas a mixture of full-length and partial replication products was found in the *in vitro* system. We made use of various inhibitors and specific antibodies to DNA polymerases α and β. DNA replication in oocytes or the extract was blocked by aphidicolin or monoclonal antibodies to α-polymerase. Surprisingly, antibodies to DNA polymerase β blocked replication in both systems, as did the β-polymerase inhibitors, dideoxythymidine triphosphate (ddTTP) and dideoxycytidine triphosphate (ddCTP). The inhibitory effect of the antibody to DNA polymerase β could be reversed by purified polymerase β, thereby indicating an essential role for polymerase β in this DNA replication system. This finding may have particular significance towards emerging evidence for a role of ss DNA replication in eukaryotic cells. The role of β-polymerase in repair-type replication will also be discussed.

F 202 YEAST DNA POLYMERASE II REPLICATES CHROMOSOMAL DNA,

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In the yeast *Saccharomyces cerevisiae*, three distinct DNA polymerases (I, II, and III) have been purified and characterized. Conditionally lethal mutants of both DNA polymerases I (α) and III (δ) exhibit reduced chromosomal DNA synthesis at the restrictive temperature, showing that those DNA polymerases are involved in chromosome replication. DNA polymerase II (a probable homolog of mammalian DNA polymerase ϵ) consists of 5 polypeptides (>200, 80, 34, 30 and 29 kDa). The genes encoding the catalytic (>200 kDa) and two other subunits (80 and 34 kDa) have been isolated and sequenced. Gene disruption experiments revealed that the genes encoding the catalytic (*POL2*) and second largest subunits (*DPB2*), but not the third subunit gene (*DPB3*), are essential for cell growth. To elucidate the *in vivo* function(s) of DNA polymerase II, we have isolated thermosensitive mutants of *POL2* and *DPB2* by the plasmid shuffling technique. Temperature-sensitive *pol2* mutants, *pol2-9* and *pol2-18*, exhibited reduced chromosomal DNA synthesis at the restrictive temperature. Furthermore, DNA polymerase II activities partially purified from *pol2-9* and *pol2-18* cells were temperature-sensitive. *pol2-9* and *pol2-18* mutations each has a single base pair change leading to an amino acid substitution in region II and between regions II and VI, respectively, conserved among the aphidicolin-sensitive DNA polymerase family. Moreover, a thermosensitive mutant of the second subunit gene, *dpb2-1*, also showed reduced DNA synthesis at the restrictive temperature and DNA polymerase II holoenzyme was not obtained from the mutant cells, suggesting that DNA polymerase II holoenzyme participates in chromosomal DNA replication. These results indicate that the polymerase activity of DNA polymerase II is required for chromosomal DNA replication and support our current proposal that three DNA polymerases participate in chromosomal DNA replication in yeast. The similarities in structure and activities between the DNA polymerases of yeast and mammals make it likely that mammalian DNA polymerase ϵ too is required for chromosomal replication.

F 203 ROLE OF HUMAN CTF/NF-I INTERACTION WITH ADPOL IN ADENOVIRUS TYPE 2 DNA REPLICATION,

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Initiation of Adenovirus (Ad) DNA replication in vitro requires in addition to the Ad genome protein complex, two proteins encoded by the virus, the Ad DNA polymerase (AdPol) and the Ad preterminal protein (pTP), as well as CTF-1, a member of the CTF/NF-I human DNA binding proteins family. Results from our and other labs have suggested a direct interaction between CTF/NF-I and AdPol. Moreover, our co-immunoprecipitation studies have suggested that AdPol interacts with a CTF-1 domain comprised between amino acids 68-150, which overlaps the DNA binding and replication activation domain of the human factor (Chen, M, Mermod, N, and Horwitz, M. S., (1990) J. Biol. Chem. 265, 18634- 18642). These studies suggest that specific interactions between CTF-1 and AdPol may serve to direct the AdPol-pTP heterodimer into the replication preinitiation complex. To assess this possibility directly, we have generated site specific and deletion mutations within the putative region of CTF-1 that mediates interaction with AdPol (amino acids 68-150). 11 mutant proteins, expressed in rabbit reticulocyte lysate, have been tested for their ability to form dimers in solution and to bind the viral origin, as well as for their ability to activate initiation of Ad DNA replication. We show that dimerization is required for the binding activity of CTF-1, which in turn is necessary for activation of initiation of Ad DNA replication. Mutant proteins which show reduced dimerization ability also display reduced binding activity. One mutation (mut 6) affecting a positively charged amino acid prevents the binding of the protein on its cognate sequence but has little effect on dimerization. Indeed, a homodimer of this mutant is not able to recognize the CTF/NF-I binding site, but can do so when co-translated with the wild type protein, as detected by the presence of a heterodimer in the gel retardation assay. We suggest that this CTF-1 mutant has lost its DNA binding site specificity but not its unspecific DNA binding affinity. The interaction of the various mutants with AdPol and the role of this interaction in the initiation of Ad DNA replication will be discussed.

F 204 CO-CRYSTAL STRUCTURE OF THE KLENOW FRAGMENT OF DNA POLYMERASE I FROM *E. COLI* AND DUPLEX DNA.

Lorena S. Beese, Victoria Derbyshire, Marlon Cowart*, Jonathan M. Friedman, and Thomas A. Steitz, Department of Molecular Biophysics and Biochemistry and the Howard Hughes Medical Institute. Yale University, New Haven, CT 06511.*Abbott Laboratories, Abbott Park, IL 60064.

A tightly bound complex of duplex DNA (7+ 12 mer DNA) with a mutant of the Klenow fragment deficient in 3' to 5' exonuclease activity (D355A) [1] was formed by incorporating the suicide inhibitor 2',3' - riboepoxy ATP into the primer strand[2]. Co-crystals of this complex were obtained under both low and high ionic strength crystallization conditions. The structure of the crystal form grown from high ionic strength was determined by molecular replacement to 3.3 A resolution and refined to an R-factor of 0.25. A portion of the protein structure in contact with the DNA undergoes a significant conformational change compared with the apo enzyme. The region referred to as the "disordered" domain[3] is now visible and interacts with the duplex DNA. The 3' end of the DNA is single-stranded and bound in the exonuclease active site similar to the single stranded DNA complex described previously [4]. The complex may correspond to DNA bound in an editing conformation showing both single stranded and duplex DNA regions. The structure determination of the second crystal form is in progress.

1 Derbyshire et al. (1991) *EMBO J.* **10,** 17-24.
2 Catalano and Benkovic (1989) *Biochemistry* **28,** 4374-4382.
3 Ollis et al. (1985) *Nature* **313**, 762-766.
4 Beese and Steitz (1991) *EMBO J.* **10**, 25-33.

F 205 DNA POLYMERASES OF THE ARCHAEBACTERIUM HALOBACTERIUM HALOBIUM,

Kamel Ben-Mahrez[1], Irene Sorokine, Masachi Nakayama, Omrane Bel Hadj[2] and Masamichi Kohiyama, Institut Jacques Monod, Université Paris VII, 75251 Paris Cedex 05, 1 : Present adress : Laboratoire de Biochimie, Faculte de Médecine Dentaire, 5000 Monastir, Tunisia, 2 : Laboratoire de Biochimie, Faculté des Sciences de Tunis, 1060 Tunis, Tunisia .

Three DNA polymerases were purified from extracts of the halophile archaebacterium Halobacterium halobium . The first enzyme designated as DNA polymerase α may be considered as the equivalent of both eukaryotic DNA polymerases α and δ on the basis of its high molecular weight, its sensitivity to aphidicolin and N-ethylmaleimide, its indifference to dideoxyribonucleotides and its associated DNA primase and 3' to 5'-exonuclease activities .
The second DNA polymerase purified from H.halobium is distinguished from the DNA ploymerase α by its resistance to both aphidicolin and N-ethylmaleimide and its sensitivity to dideoxyribonucleotides . This enzyme called DNA polymerase β contains 3' to 5'- and 5' to 3'-exonuclease activities . Therefore the DNA polymerase β of H.halobium differs fundamentally from eukaryotic DNA polymerases β by its exonuclease activities .The third enzyme we purified from H.halobium is a multifunctional enzyme containing DNA polymerase, reverse transcriptase and RNase H activities . Unlike retroviral reverse transcriptases, the archaebacterial enzyme does not require a preformed primer to initiate DNA synthesis . This demonstrates that this enzyme is a primase-reverse transcriptase complex .

F 206 BIOCHEMICAL CHARACTERIZATION OF HIGHLY PURIFIED *ESCHERICHIA COLI* DNA POLYMERASE II, Cynthia A. Bonner[1], Sharon Hays[1], Hong Yu[1], Steve Creighton[1], Todd Stukenberg[2], Mike O'Donnell[2], Kevin McEntee[3], and Myron F. Goodman[1], [1]University of Southern California,Department of Biological Sciences, Los Angeles, CA 90089-1340, [2]Cornell University Medical College, Howard Hughes Medical Institute Department of Microbiology, New York, NY 10021, [3]UCLA School of Medicine, Department of Biological Chemistry and Molecular Biology Institute, Los Angeles, CA 90024.

Escherichia coli contains three DNA polymerases: pol I, II, and III. The roles of pol I and pol III in replication and repair have been extensively studied; little, however, is known about pol II. Recently, the gene for pol II has been cloned and sequenced . We have shown that pol II is encoded by the *dinA* "damage-inducible" gene under control of the SOS regulon; also, we have shown that Pol II belongs to the family of "α" polymerases. We have constructed a strain which overproduces *E. coli* DNA polymerase II 100-200 fold, allowing us to purify large quantities of polymerase to greater than 99% homogeneity. This has allowed us to biochemically characterize the pure enzyme. In particular, we have looked at the effect of the pol III accessory proteins β and γ-complex on the processivity of pol II and find that in the presence of SSB protein, these accessory proteins increase the processivity of pol II 20-200 fold. We have also looked at pol II fidelity, turnover, exonuclease activity, and the effect of ara-CTP on pol II activity. Finally, we have constructed both pol II deletion strains and pol I/pol II double deletion strains. Currently, we are using these strains to investigate possible phenotypes for pol II.

F 207 DNA POLYMERASE III OF *Bacillus subtilis*: LOCALIZATION OF THE EXONUCLEASE AND POLYMERASE DOMAINS ON THE PRIMARY STRUCTURAL MAP, Neal C. Brown, Marjorie H. Barnes, Russell A. Hammond, Christopher C. Kennedy, and Susan L. Mack, Dept. of Pharmacology, U. Mass. Med. Sch., Worcester, MA 01655

Structural gene mutants have been cloned and exploited to identify the major catalytic domains of *Bacillus subtilis* DNA polymerase III (BsPol III), a 162.4 kilodalton (1437 amino acid residues) polymerase:3'-5' exonuclease (pol-exo) required for replicative DNA synthesis.

Analysis of the sequence, mutagenicity, and catalytic behavior of natural and site-directed point mutants of BsPol III have unequivocally located the domain responsible for exonuclease catalysis within a 155-residue segment displaying homology with the exo domain of *Escherichia coli* DNA polymerase I and other pol-exos.

Sequence analysis of four structural gene mutations which specifically alter the enzyme's reactivity to the inhibitory dGTP analog, 6-(*p*-hydroxyphenylhydrazino)uracil, and the inhibitory arabinonucleotide, araCTP, have identified a provisional dNTP binding (pol) domain in the carboxy-terminal fourth of the enzyme, within a 98-residue segment spanning amino acids 1175-1273.

The primary structure of the pol domain and the rest of the enzyme not included in the exo domain is unique; it displays little or no conservation in any other DNA polymerase, including the distantly related pol IIIs of the Gram-negative organisms, *E. coli* and *Salmonella typhimurium*. (Supported by N.I.H. grant GM45330 to N.C.B.)

F 208 MOLECULAR CLONING AND EXPRESSION OF GENES CODING FOR SUBUNITS OF DNA POLYMERASE III HOLOENZYME, Jeffrey R. Carter, Mary Ann Franden, Ruedi Aebersold and Charles S. McHenry, Department of Biophysics, Biochemistry and Genetics, University of Colorado Health Sciences Center, Denver, CO 80262.

DNA polymerase III holoenzyme is the replicative complex of *E. coli*. Of the 10 holoenzyme subunits, α, ε, β, γ and τ have been cloned and overproduced. A thorough biochemical analysis of holoenzyme has been frustrated by a lack of large quantities of purified δ, δ', θ, χ, and ψ subunits. Our aim has been to use a reverse-genetics approach to clone the structural genes for the five remaining subunits into expression vectors and purify large quantities of each subunit.

Peptide sequencing of tryptic fragments of each subunit was performed; this sequence information was used to design primers to amplify a region of the corresponding structural gene using PCR. This segment of the structural gene was then cloned and used as an unambiguous probe to isolate the entire structural gene. For example, PCR reactions using primers for the δ' gene produced a 350-bp fragment of this gene. This fragment was cloned and used to probe a Southern blot of restriction-digested chromosomal DNA and determine that the entire gene for δ' was on an 18-kb *Eco*RI fragment. This restriction fragment was isolated and cloned into an expression vector. The 350-bp fragment was also used to probe the Kohara mini-set of chromosomal clones to determine that the gene maps at 24 minutes.

For two of the subunits, database searches revealed identities between our peptide sequences and amino-termini of translated open reading frames. From DNA sequences of these known open reading frames, we designed oligonucleotide probes and used these to clone the genes for δ and ψ. These genes map at 15 and 96 minutes, respectively. The gene encoding δ is part of an operon with two lipoprotein genes (Takase *et al.* 1987. J. Bacteriol. **169**:5692-5699). The gene for ψ appears to be part of an operon including genes coding for aminopeptidase A and valyl tRNA synthetase (Stirling *et al.* 1989. The EMBO J. **8**:1623-1627).

F 209 HeLa DNA POLYMERASE EPSILON, Gloria S.J. Chui, Hitomi Asahara, Jeff S. Goldsmith, Ed Lee, Stuart Linn, Division of Biochemistry and Molecular Biology, University of California, Berkeley, CA 94720

We have separated, purified, and characterized polymerases α, δ, and ε from the same HeLa cell extract in order to determine their relationship by comparing them for the first time from the same cell type (1). Catalytic and physical properties, coupled with previous immunologic comparisons, support the premise that HeLa DNA pols α, δ, and ε are distinct enzymes and analogous to yeast pols I, III, and II, respectively. However, the apparent HeLa pol ε subunits (>200 and 55 kDa) differ from those reported for yeast pol II (>200, 80, 34, 30, 29 kDa (2)). We have found no significant effect of PCNA (prepared in our lab), RF-A and/or RF-C (prepared in the Stillman or Hurwitz labs) on the processivity, activity, or substrate specificity of large HeLa pol ε (i.e., catalytic subunit > 200 kDa). The HeLa enzyme was able to copy an entire M13 DNA circle without exogenous factors. Yeast pol II has been cloned and sequenced (2); it has the six regions of sequence homology of the α-class of DNA polymerases. Comparing the sequences of the six homologous regions (ordered IV-II-VI-III-I-V) for yeast pol II to those of HeLa (and yeast) pol α, and yeast pol δ, we found sequences in regions IV, II, and III which were most unique to the yeast pol II and which had minimal codon degeneracy. Using PCR with primer sequences made from regions IV (left-to-right) and regions II and III (right-to-left), we obtained a PCR fragment from a human cDNA library with remarkable homology in sequence, spacing, and size to yeast pol II. (Homology extends well beyond the conserved regions.) We are now obtaining fragments and sequences of the cDNA and continue to find strong homology. Moreover, when the PCR fragment was used to probe HeLa mRNA, a 7.7 Kb transcript was detected (yeast pol II cDNA is 7.5 Kb (2)). Preliminary results have indicated that the level of the pol ε mRNA is approximately 5- to 6-fold higher in proliferating cells than in quiescent cells.

1. Syvaoja, J. et al. PNAS **87**, 6664 (1990)
2. Morrison, A. et al. Cell **62**, 1143 (1990)

F 210 CRYSTALLOGRAPHIC STUDIES OF A $P3_221$ COCRYSTAL OF THE KLENOW FRAGMENT OF DNA POLYMERASE I WITH DNA AND NUCLEOSIDE TRIPHOSPHATE ANALOGUES

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The molecular replacement method has been used to solve the structure of the Klenow fragment at 3.0/3.5 Å for a crystal form that may be grown with several different DNA duplexes and α,β-methylene dNTP's. The crystal is of a different space group and has different protein-protein contacts than the original form [Ollis, *et.al.* Nature **313**, 762 (1985)]. The enzyme has been assayed and shown to be active under conditions similar to those used for crystallization. One observes a conformational change similar to that observed by [Freemont *et.al.* PNAS **85**, 8924 (1988)] in cocrystals grown at low pH and high ionic strength, yet there is no electron density for the DNA at the exonuclease site. In addition, the stretch of amino acids that had been described as disordered in the original structure has a higher degree of relative order in the trigonal crystal form due to an increased number of intermolecular contacts. The position of the iodine atom in an iodinated dNTP analogue was determined by difference Fourier and is consistent with the position of the triphosphate observed in the tetragonal form [Beese, Friedman, and Steitz *in preparation*].

F 211 NEW METHODS FOR OBTAINING DNA SEQUENCE USING VERY SMALL AMOUNTS OF TEMPLATE: OPTIMIZING REACTION CONDITIONS FOR AN EXONUCLEASE DEFICIENT, THERMOSTABLE POLYMERASE

C. W. Fuller, U. S. Biochemical Corp., Cleveland, OH

A new method for sequencing very small amounts of template DNA will be presented. Dideoxy DNA sequencing reactions are performed in two steps (labeling and termination) both of which are thermally cycled. Cycling the labeling step using a polymerase that lacks exonuclease activity results in the accumulation of extended primer molecules which can be readily and efficiently labeled using α-^{35}S dATP or α-^{35}S dCTP. These labeled, extended molecules can then serve as primers for repeated cycles of synthesis in the presence of chain-terminating dideoxy-nucleotides. This method works equally well for single- and double-stranded templates without taking special measures to denature double-stranded templates. Sequences have been obtained from single M13 plaques and single plasmid-containing colonies without prior purification of the template DNA. Thus the steps of growing clones and purifying DNA can be bypassed for some sequencing projects.

F 212 DNA POLYMERASE III HOLOENZYME: A) DISTANCE BETWEEN THE β SUBUNIT AND THE PRIMER AND B) CONTINUOUS ASSAY FOR ITS ACTIVITY,

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Within a complex of holoenzyme bound to primed DNA we have measured the distance between a unique position on the β subunit and a unique position on the primer. The technique used was fluorescence energy transfer. The β subunit was labeled at cys333 with a fluorescence energy acceptor and the primer was labeled at the third nucleotide from its 3'-end with a fluorescence energy donor. The distance between these two sites was measured as 65 Å. Coupling this distance with other information a model was proposed for the positioning of the β subunit within the initiation complex.

A continuous assay for the polymerase activity of holoenzyme was developed. The assay monitored DNA polymerase activity by measuring the displacement of SSB from the template as the DNA chain was elongated. SSB bound to ssDNA has quenched tryptophan fluorescence and SSB fluorescence was recovered as it was displaced from the template during DNA replication. Comparison with the [^{3}H]dNTP incorporation assay showed that the two assays measured the same parameters. However, the SSB fluorescence recovery assay allowed for the continuous collection of data points at the expense of only a small sample. All of the usual steps involved in priming and DNA replication can be monitored with this assay. Primase binding, primer synthesis and DNA polymerase III holoenzyme binding did not displace SSB from the template and, as a result, these steps generated a lag in the time-dependence. Saturating primase sufficiently synchronized primer synthesis that the first round of replication was recorded as a linear increase in fluorescence recovery. Following the burst was a slower enhancement phase that corresponded to circles replicated after cycling of holoenzyme from completed templates.

F 213 STIMULATION OF HSV-1 DNA POLYMERASE ACTIVITY BY THE HSV-1 UL42 GENE PRODUCT IN VITRO,

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The DNA-dependent DNA Polymerase (UL30, Pol) of herpes simplex virus type-1 exists in infected cells as a stable complex with an accessory protein (UL42). This protein has previously been shown to be essential for viral replication in vivo, and to stimulate Pol activity in vitro. We have utilized purified HSV Pol and UL42 from recombinant baculovirus-infected cells to characterize UL42-mediated stimulation of HSV Pol activity in vitro. The stimulation was observed on a variety of primer-template combinations including singly-primed heteropolymeric templates and synthetic homopolymeric templates. The effect of UL42 on the biochemical parameters of Pol processivity and primer utilization were examined. The effects of antibodies raised against Pol or UL42 on Pol activity were also examined.

F 214 LASER CROSS-LINKING OF β-POLYMERASE TO NUCLEIC ACIDS, Joel W. Hockensmith*, Amalendra Kumar†, Samuel H. Wilson†, and Gordon S. Rule*, *Department of Biochemistry, University of Virginia Health Sciences Center, Charlottesville, VA 22908 and †Laboratory of Biochemistry, NCI, NIH, Bethesda, MD 20892.

β-polymerase is the smallest DNA polymerase available in sufficient quantity for biophysical studies. In order to pursue NMR studies of β-polymerase interactions with oligonucleotides, we have used a laser cross-linking methodology to establish that β-polymerase binds with similar affinities to both oligonucleotides and their spin-labelled derivatives. Our titrations of oligonucleotides have established that β-polymerase exhibits significant cooperativity upon binding to the nucleic acid. Furthermore, the cooperative binding of β-polymerase is dependent on the salt concentration. β-polymerase binding to DNA is stimulated by added salt at β-polymerase concentrations typical of enzymological assays (10^{-8} M) but is inhibited at higher β-polymerase concentrations (10^{-6} M) generally used for biophysical type studies.

F 215 FUNCTIONAL INTERACTION BETWEEN DNA POLYMERASE AND RNASE H DOMAINS IN THE RECONSTITUTED COMPLEX OF HIV-1 REVERSE TRANSCRIPTASE

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The constituent domains of HIV-1 reverse transcriptase (RT) were expressed separately and purified to homogeneity. Although the isolated RNase H (p15) domain has no detectable catalytic activity, its combination *in vitro* with the DNA polymerase (p51) domain leads to restoration of the RNase H activity. We were able to follow simultaneously products of RNA hydrolysis and DNA synthesis catalysed by the reconstituted complex of HIV-1 RT on a defined RNA/DNA hybrid substrate, derived from the *gag* region of HIV-1. Using a series of overlapping DNA primers extended by increments of 1 nucleotide, we systematically examined distance between the RNase H cleavage site in the RNA template and the 3' end of the complementary DNA primer. This distance was typically 15 nucleotides, less often 16 and in one case 17 nucleotides, indicating that some phosphodiester bonds are less readily cleaved by RNase H. The reconstituted complex produced a very similar pattern of RNA degradation/DNA synthesis products as the wt RT, suggesting the same topological orientation with respect to the RNA/DNA hybrid substrate. The observed RNase H activity will be discussed in the context of the three-dimensional structure of the RNase H domain of HIV-1 RT.

F 216 ISOLATION AND CHARACTERIZATION OF AN EXTRAGENIC SUPPRESSOR OF TEMPERATURE-SENSITIVE DNA POLYMERASES IN SACCHAROMYCES CEREVISIAE, Peter Hovland and Robert A. Sclafani, Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver CO 80262 (Supported by PHS grant GM35078 to R.A.S.).

In an effort to identify novel components of the eukaryotic DNA replication apparatus we employed a genetic approach. Double mutant haploid strains were created which bear temperature sensitive alleles of the two genes encoding the yeast DNA polymerase catalytic subunits, **POL1** and **POL 3.** Presence of either or both of these alleles causes the arrest of cell division at the restrictive temperature of 36° C. These strains were transformed with a wild-type, high-copy number library, and then screened for colony formation at the restrictive temperature. Six transformants of a **pol 1-1, pol 3-2** strain passed through this screen and all were found to bear an identical 3.8kB inserts. This purified plasmid suppresses temperature-sensitivity in double and single mutant strains in an allele-specific manner.

DNA restriction mapping of the putative wild-type insert indicate that it does not bear any of the known yeast genes which encode DNA polymerases, nor is it **POL30.** Southern hybridization to a chromosome separation blot indicates that the gene is on chromosome V. Preliminary nucleotide sequence information will be presented.

Several hypotheses are possible at this point, including the possibility that the insert encodes a protein which interacts directly with the catalytic subunits of the polymerases.

F 217 Structural and Funtional Studies of HIV Reverse Transcriptase: Subunit structure, Conformational Similarity to Klenow Fragment, and use of tRNA as a Primer L. A. Kohlstaedt, J. Wang, J. Friedman, P. Rice, and T.A. Steitz Dept. of Molecular Biophysics and Biochemistry and Howard Hughes Medical Institute, Yale University, New Haven, CT 06511

We have determined the crystal structure of HIV reverse transcriptase (RT). RT is a heterodimer of a 66kD subunit containing a polymerase domain and an RNase H domain and a 51kD subunit containing only a polymerase domain. RT crystalized in the space group C2 with a=224.3, b=70.2, c=106.2Å, ß=106.1°. The structure has been solved to 3.5Å resolution by MIR using data collected on a Xoung-Hamlin multiwire area detector system. Although the two polymerase domains of the heterodimer have the same sequence, they take on very different conformations in this crystal form. The two subunits of the enzyme are arranged asymmetrically with no simple rotational relation. HIV reverse transcriptase bears a striking conformational similarity to Klenow fragment of DNA polymerase I in some regions but is rather different in others.

In related work, we have explored the ability of HIV reverse transcriptase to use tRNAs as primers *in vitro*. RT can use either "cognate" $tRNA_3^{lys}$ or "noncognate" *E. Coli* $tRNA_2^{gln}$ as a primer. Primer utilization specificity appears to be dependent on sequences flanking the primer binding site.

F 218 INTERACTIONS BETWEEN TETRAHYMENA TELOMERASE AND DNA PRIMERS, Margaret S. Lee and Elizabeth H. Blackburn, Department of Microbiology and Immunology, Univ. of Calif., San Francisco, CA 94143

We are studying the mechanism by which the ribonucleoprotein enzyme telomerase from the ciliate Tetrahymena thermophila synthesizes the G-rich strand of the telomere.

Previous work has shown that the telomerase RNA serves as a template in the synthesis of T_2G_4 repeats, base-pairing with the 3' end of the primer and determining the next correct nucleotides to be added. We have analyzed in detail both the primer dependence of the reaction and the kinetics of the reaction itself. Oligonucleotides which represent the G-rich strand of the telomere or are antisense to the RNA 3' of the templating region are efficiently utilized (as judged by standard telomerase assays); however, at least one long (18 base) oligonucleotide lacking either of these characteristics also primes telomere addition unexpectedly well. These and other results suggest that there are other RNP interactions with the primer beyond pairing of the template with the 3' end of the primer that may be important in the mechanism of telomere synthesis. To address this hypothesis, sets of primers of fixed length and 3' end sequence but with varied 5' end sequences were compared.

We have observed that different steps and aspects of the telomerase reaction are differentially affected by the input primer. Therefore, assessing primer-telomerase interaction requires careful qualitative and quantitative determination of short and long product formation. Our results lead us to propose a kinetic scheme for the telomerase reaction which, while comparable to that for other nucleic acid polymerases, in which dissociation is favored for relatively short products and is dependent on primer sequence, is likely to have additional unique features.

F 219 ABORTIVE DNA SYNTHESIS IN dnaQ NULL MUTANTS OF S. typhimurium, Miriam R. Lifsics, Edward D. Lancy and Russell A. Maurer, Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106-4960.

DnaQ encodes the ε editing subunit of DNA polymerase III. Null mutations of this gene (ΔdnaQ) in S. typhimurium confer a severe growth defect in a wild type genetic background. Suppression of the growth defect depends on both a mutation affecting the α (polymerase) subunit of pol III and on the presence of adequate DNA polymerase I activity (e.g., the wild type level). Here, we report additional characterization of the feeble growth of unsuppressed ΔdnaQ strains. Feeble growth was associated with induction of the SOS response, suggesting that DNA synthesis tended to terminate prematurely, leading to regions of single-stranded DNA in the newly replicated chromosome. Satisfaction of the pol I requirement for suppression required the polymerase domain of pol I. It thus appears that the cell recruits pol I to fill gaps left by the defective pol III. The events leading to abortive DNA synthesis by pol III, whether wild type in the α subunit or mutant, are unknown. Premature termination of DNA synthesis could not be attributed to a lack of polymerase activity in the altered α subunit since the purified, altered α subunit exhibited polymerase activity comparable to or greater than that of wild type α. Sequestration of either pol I or pol III in repair functions did not appear to contribute to the growth defect of ΔdnaQ strains. These results clarify the nature of the physiological defect imposed by the loss of ε and the mechanisms which restore normal growth to such mutants.

F 220 Cloning large subunit of DNA Polymerase α from *D. melanogaster*, Gordon Lindberg and I. Robert Lehman, Stanford Biochemistry Department, Stanford University, Stanford CA 94305

We have isolated several cDNA clones that code for parts of the large subunit of DNA polymerase α from *D. melanogaster*. These were isolated by screening a λgt11 ovary cDNA expression library (generously provided by Tula Hazelrig) with monoclonal antibodies, generated and purified in our lab, that are specific for the large subunit of *D. melanogaster* polymerase α. Analysis of the clones revealed sequence motifs previously identified in all alpha DNA polymerases.

Northern analysis of total RNA isolated at different times during *Drosophila* development revealed a transcript the expected size for a protein of 160 kdaltons, even though the protein migrates at 180 kdaltons on SDS-PAGE gels. The steady state level of this mRNA is highest during embryonic and decreases during larval and adult stages of *Drosophila* development.

Using one of the cDNA clones, we have isolated several genomic clones from an EMBL3 λ phage library (provided by Matt Scott) and are using them to map the genomic location of the DNA polymerase gene.

F 221 CHARACTERIZATION OF THE ENZYMATIC ACTIVITIES OF THE *STREPTOCOCCUS PNEUMONIAE* DNA POLYMERASE I. Paloma López, Asunción Díaz, M. Elena Pons and Sanford A. Lacks[+], Centro de Investigaciones Biológicas, CSIC, Velazquez, 144, 28006-Madrid, Spain and [+]Brookhaven National Laboratory, Upton, NY 11973, USA.

Derivatives of plasmid pSM22, which contains the cloned *S. pneumoniae polA* gene, were constructed with various mutations in the *polA* gene. The altered gene products were produced, purified and characterized. Comparison of the enzymatic activities of the mutant *S. pneumoniae* PolI proteins show that the 5'-->3' exonuclease and polymerase domains present in the pneumococcal PolI are arranged in the same order as in the *Escherichia coli* PolI enzyme. However, the *S. pneumoniae* polymerase, like the *Thermus aquaticus* polymerase, was shown to lack 3'-->5' exonuclease activity. The 5'-->3' exonuclease domain was localized in the amino-terminal region of the wild type pneumococcal protein within the first 351 amino acids residues. The polymerase domain is included in the carboxyl-terminal two-thirds of pneumococcal PolI. Enzymatic properties of the 5'-->3' exonuclease and polymerase activities of *S. pneumoniae* PolI were characterized.

F 222 CHARACTERIZATION OF A HIGH FIDELITY DNA POLYMERASE ISOLATED FROM THE EXTREMELY THERMOPHILIC ARCHAEBACTERIUM, *Pyrococcus furiosus*, Eric J. Mathur, Kelly S. Lundberg, Dan D. Shoemaker, Kirk B. Nielson, Sandra A. Mathur, Brad R. Scott, Warren Schoettlin, Jane Rombouts, Janice Cline and Joseph A. Sorge, Bioseparations R&D, Stratagene Inc, 11099 North Torrey Pines Road, La Jolla, CA 92037.

We have isolated a novel DNA polymerase from the hyperthermophilic, anaerobic, marine archaebacterium, *Pyrococcus furiosus* (*Pfu*). The 92 kD, monomeric, multifunctional enzyme possesses both 5' to 3' DNA polymerase and 3' to 5' exonuclease activities. We have characterized *Pfu* DNA polymerase with respect to thermostability, DNA polymerase, 3' to 5' and 5' to 3' exonuclease, processivity, strand displacement, terminal transferase and proofreading activities. In order to determine the fidelity of DNA synthesis during the polymerase chain reaction (PCR), we amplified the entire lac I gene and the first 500 bases of the lac Z gene sequence using either *Pfu* or *Taq* DNA polymerase. The amplified products were subsequently cloned into a λ phage vector; following packaging and plating, the resulting plaques were analyzed for errors incurred during the amplification process. Mutations within the lac I gene which inactivate the lac repressor protein will allow the expression of β-galactosidase which, in the presence of X-gal, result in blue phage plaques. Therefore, the observed mutation frequency can be defined as the proportion of blue (mutant) plaques to the total number of plaques scored. Our results indicate that amplification performed with *Pfu* DNA polymerase resulted in 12 fold lower observed mutation frequencies than similar amplifications performed with *Taq* DNA polymerase. *Pfu* DNA polymerase may thus have important applications in amplification protocols which require high fidelity DNA synthesis, including the direct cloning of PCR products, PCR-based procedures for high efficiency double-stranded mutagenesis and PCR techniques designed to detect specific point mutations.

F 223 DNA DUPLEXES CONTAINING SUGAR SUBSTITUTIONS: STABILITY STUDIES & EFFECTS ON DNA POLYMERASE BINDING AND CATALYSIS. Thomas Mikita* and G. Peter Beardsley#. Departments of Molecular Biophysics & Biochemistry* and Pharmacology# and Pediatrics#, Yale University, Ct. 06510.

While much attention in the experimental literature has been given to the integrity of DNA base pairing, with its components of intra-strand stacking and inter-strand hydrogen bonding, much less information exists concerning the role of the sugar-phosphate backbone for polymerase function. In addition to proper nucleotide selection, the polymerase must helical track around the DNA to keep its catalytic site positioned at the primer terminus. It is likely that the sugar-phosphate backbone has a role in directing this motion. In addition, the 3'-OH of the primer sugar is the all important site of nucleotide addition. We would like to know the range of structural and configurational plasticity which can be tolerated in the DNA backbone for nucleotide incorporation and polymerase translocation to continue. In an attempt to gather information of this kind, we have carried out sugar substitutions, via phosphoramidite chemistry, to compare deoxyribose with ribose and arabinose. The order of base pair stability within a DNA duplex is dC-dG > rC-dG >araC-dG >> dT-dG(the most stable mispair). For polymerase catalysed incorporation of dG opposite template sites of dC, araC, and rC and dT, the rate is dC ≅ rC > araC>> dT, in keeping with the stability trend of these base pairs. However, for polymerase catalysed addition to primer terminal dC, araC, rC and dT in the above base pairs, the rate of next nucleotide addition is dC > rC >> dT >> araC, which is not in keeping with the stability trend of these base pairs. Quantitative footprinting experiments with an exo^{-} form of the Klenow Fragment and the dC and araC primed substrates show no difference in footprint size nor any difference in Kd, suggesting that binding is not what limits addition to araC. These and other data will be discussed in terms of the conformations these sugars prefer to adopt and how this effects the positional range of the 3'-OH, as well as the neighboring phosphates. The importance of the C2' position will also be discussed.

F 224 ENZYMATIC PROPERTIES OF A DNA POLYMERASE FROM *THERMUS THERMOPHILUS* ON RNA AND DNA TEMPLATES, Thomas W. Myers and David H. Gelfand, PCR Division, Cetus Corporation, Emeryville, CA 94608.

A recombinant DNA polymerase derived from the thermophilic eubacterium *Thermus thermophilus* (*Tth* pol) was found to possess very efficient reverse transcriptase (RT) activity at 70 °C in the presence of $MnCl_2$. The efficiency of this RT activity and the thermostability of *Tth* pol facilitated the coupling of a two step, high sensitivity target detection RT/polymerase chain reaction (PCR) assay [Myers and Gelfand (1991) *Biochemistry* 30, 7661-7666]. We have found that by modifying reaction parameters, *Tth* pol is capable of performing an efficient homogeneous reverse transcription/DNA amplification reaction, thus eliminating the requirement to alter reaction buffers for the two enzymatic steps. The emerging importance of this enzyme in diagnostic and molecular biology applications has prompted us to characterize further the enzymatic properties of the *Tth* pol on both RNA and DNA templates. Characterization was performed with a synthetic RNA transcript for reverse transcription, while a DNA template containing the same nucleotide sequence was utilized for DNA-dependent synthesis. Factors such as monovalent and divalent cations, cosolvents, enzyme and template concentration, reaction temperature, and reaction time were evaluated. Extension rates and processivity values were also determined for the enzyme. In addition to providing biochemically important information comparing two similar biological activities, these data should be useful in the diverse applications that utilize *Tth* pol.

F 225 CLONING, OVEREXPRESSION AND PURIFICATION OF THE δ' SUBUNIT OF THE DNA POLYMERASE III HOLOENZYME. Rene Onrust, and Mike O'Donnell. Howard Hughes Medical Institute, Microbiology Department, Cornell University Medical College, 1300 York Avenue, NYC, NY10021.

DNA polymerase III holoenzyme (holoenzyme), the main replicase of the *E. coli* chromosome, is composed of at least 10 subunits. The γ complex (γδδ'χψ) subassembly of the holoenzyme uses ATP to transfer the β subunit onto primed DNA. Once locked on to DNA, the β subunit confers rapid and processive synthesis onto the core polymerase. To obtain a better understanding of the role of the individual subunits of the γ complex we have identified the genes that had not previously been determined. This poster describes the gene encoding the δ' subunit. Analysis of δ' of the γ complex by SDS PAGE revealed a doublet of approximately 37kDa. Sequence analysis of tryptic digests revealed that the two proteins had peptides in common. Two synthetic DNA oligonucleotides were used to probe southern blots of restricted *E.coli* DNA. The pattern revealed by the probe was compared to the Kohara restriction map of the *E. coli* chromosome. A unique site was found on the *E. coli* chromosome which matched the pattern of the southern. A λ clone containing an insert that corresponded to this map position was obtained from Yuji Kohara. The gene for δ' revealed a 334 amino acid open reading frame and predicts a 36.9kDa polypeptide.The δ' gene was overexpressed to >30% of total cell protein in *E. coli*. The overexpressed protein was purified to apparent homogeneity using an in vitro assay to reconstitute processive DNA synthesis. Analysis of the overexpressed protein shows it contains two polypeptides just like the δ' purified from the holoenzyme. Mass spectroscopy indicates the lower band is the full length protein. Gel filtration analysis indicated a γδδ' complex could be reconstituted from γ, δ and δ' to form an active complex in an vitro replication assay.

F 226 IDENTIFICATION OF PEPTIDE DOMAINS IN THE HERPES SIMPLEX VIRUS UL42 PROTEIN REQUIRED FOR PHYSICAL AND FUNCTIONAL INTERACTION WITH THE VIRAL DNA POLYMERASE.
Deborah S. Parris and Steven J. Monahan, Department of Medical Microbiology and Immunology and Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210.

Herpes simplex virus type 1 (HSV-1) encodes 7 proteins which are required for origin specific amplification of plasmids. Two of these, a 65 kilodalton double-stranded DNA binding protein (encoded by UL42) and the DNA polymerase (pol) are physically associated and form a 1:1 stoichiometric complex. We have previously shown that the UL42 protein is a pol accessory protein which stimulates the activity of the pol on an activated DNA template. To identify the functional domains of UL42 required for interaction with the pol, we constructed a series of insertion and deletion mutations in the UL42 gene. Functional interaction with pol was measured by the ability of in vitro transcription/translation products of the UL42 gene to stimulate pol activity in co-translation reactions. The wild-type (488 AA) and C-terminal truncations of the UL42 gene up to AA 339 stimulated pol activity 6 to 9-fold. Deletion of UL42 product further to AA 248 or deletion of AA 37-282 yielded basal pol activity in co-translations with pol transcripts, despite the production of stable products. Several insertion mutations in the UL42 gene resulted in wild-type pol stimulation activity, although a 4 AA insertion after residue 140 abolished stimulation activity. Thus two regions of the UL42 (one around AA 140 and one between 248 and 339) contain domains critical for stimulation of pol activity and these are being further defined by site-directed mutagenesis. These regions are in excellent agreement with the domains defined by Coen and co-workers required for co-immunoprecipitation with pol or UL42-specific antisera. Synthetic peptides corresponding to domains on the UL42 and pol gene have been tested for their ability to interfere with complex formation and pol stimulation. The results of these experiments will be presented.

F 227 STRUCTURAL ANALYSIS OF *E. coli* DNA POLYMERASE III HOLOENZYME INITIATION COMPLEXES, Jo Anna Reems, and Charles S. McHenry, Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, CO 80262.

E. coli DNA polymerase III holoenzyme, in the presence of ATP and *E. coli* single-stranded DNA binding protein, forms an initiation complex that is capable of rapid and highly processive DNA replication. DNA polymerase III holoenzyme contains the core DNA polymerase III (α, ε, and θ subunits) and associated auxiliary subunits (β, τ, γ, δ, δ', ψ and χ). Two different approaches were taken to determine DNA polymerase III holoenzyme-primer interactions.

DNase I footprint analysis was used to determine the length of primer protected by holoenzyme. DNase I digestion of holoenzyme-primer-template complexes revealed that 30 ± 2 nucleotides of the primer were protected by the enzyme. Protection of the primer followed the same requirements as formation of initiation complexes. Generation of the footprint absolutely required ATP. Reconstituted initiation complexes assembled with core pol III, $\gamma\delta$ complex and β subunit produced a footprint identical to that of intact holoenzyme. This result indicated that reconstituted initiation complexes were structurally equivalent to complexes formed with intact holoenzyme. The addition of the τ subunit, which presumably contains a DNA binding domain, did not alter the footprint.

Secondly, site-specific photo cross-linkage was used to identify the position of the subunits within the footprinted region. Multiple primers were synthesized to contain a single photo-reactive probe at defined intervals within the 30 nucleotide footprint. Holoenzyme was site-specifically cross-linked to these primers at the position of the photo-reactive group. These results allowed us to map the linear arrangement of the subunits within the initiation complex.

F 228 A DISSECTION OF THE MULTIPLE FUNCTIONS OF DNA POLYMERASE I WITH MONOCLONAL ANTIBODIES, Theresa Ruscitti, Deborah A. Polayes and Stuart Linn, Division of Biochemistry and Molecular Biology, Univ. of Calif., Berkeley, CA 94720

DNA Polymerase I (Pol I) from E. coli is a multi-functional enzyme containing a polymerase, a 3' to 5' exonuclease and a 5' to 3' exonuclease. The enzyme can coordinate these activities to synthesize DNA with high fidelity and take part in the repair processes of the cell. Despite the concerted action of the polymerase and exonuclease activities, substantial evidence indicates that the three catalytic activities reside on separate domains of the protein (reviewed by A. Kornberg and T.A. Baker, DNA Replication, second ed., 1991, W.H. Freeman, New York). As all of these studies have used proteolytic fragments of the enzyme, we have asked whether the separation of sites exists for the full length Pol I. Our approach has been to raise antibodies to Pol I which selectively neutralize each one of the three activities while leaving the other two unaffected. We obtained antibodies which preferentially inhibit each activity, consistent with the model of separate sites. Antibodies from any two classes are able to bind to the same molecule of Pol I at the same time, further indicating that the enzyme is not folded such that the catalytic domains are in close proximity. This supports the crystal structure of the Klenow fragment demonstrating separation of the pclymerase and the editting exonuclease and is the first indication that the 5'to3' exonuclease is discrete from the rest of the enzyme in tertiary as well as primary structure.

The various modes of action of the 5' to 3' exonuclease have been further examined with several of the neutralizing antibodies. One preferentially inhibits the RNaseH activity, but not the DNase activity, of the exonuclease. Two other antibodies can neutralize the exonuclease when the ionic strength in the reaction is high but stimulate the activity when the ionic strength is low. We believe these antibodies will be useful probes for examining the roles of the RNaseH and DNase activities in physiological processes requiring Pol I and for further elucidating the mechanism of the exonuclease.

F 229 Mechanism of the Cycling Reaction of E. coli DNA Polymerase III Holoenzyme: Insights into Lagging Strand Replication. **P. Todd Stukenberg and Mike O'Donnell. Howard Hughes Medical Institute, Microbiology Department Cornell University Medical Center NY, NY 10021.**

Due to the scarcity of DNA polymerase III holoenzyme (HE) within the E. coli cell a single HE is presumed to continually recycle itself during the thousands of reinitiation events that are required in the discontinous replication of the lagging strand. We have developed a simple in vitro assay of HE recycling. In this assay, the HE is first "locked" onto a primed M13mp18 ssDNA circle using ATP. Addition of all 4 dNTPs results in processive replication of the 7.2 kb circular DNA within 15 seconds. However, after replication the HE does not dissociate off the first template and replicate new primed templates. The key to rapid recycling of HE to new templates is its accessory proteins, β and the γ complex. The γ complex uses ATP to clamp a β dimer onto a primed ϕx174 ssDNA. We call the β-DNA complex a preinitiation complex. If one now replicates a primed template in the presence of a second template endowed with a preintiation complex, the HE rapidly cycles from the initial replicated template to the new preinitiated complex.

We have undertaken a detailed mechanistic study of this cycling reaction. We find the cycling reaction can be separated into two half reactions. Hence, the HE is allowed to fully replicate a circular M13mp18 DNA, then is gel filtered. Using $\alpha^3[H]\varepsilon$ (polymerase) and $^3[H]\beta$ subunits we find both are retained on the replicated duplex through gel filtration. Upon mixing the gel filtered "termination complex" with primed ϕx174 ssDNA containing a preinitiation complex, the $\alpha^3[H]\varepsilon$ cycles to the ϕx174 preinitiation complex and leaves the $^3[H]\beta$ behind on the replicated M13mp18 DNA. Since the $\alpha\varepsilon$ polymerase remained adhered to the replicated DNA, it would appear that the preinitiation complex on the ϕx174 DNA somehow induced the HE to split apart; the $\alpha\varepsilon$ cycles to the ϕx174 and β stays on the replicated M13mp18. A model discussing the importance of this bisecting of HE on lagging strand replication will be presented.

F 230 IN VITRO SV40 DNA REPLICATION ACTIVITY OF THE HUMAN CELL 21S DNA REPLICATION COMPLEX. R.F. Swaby, R.J. Hickey, U. Kammula and L.H. Malkas, University of Maryland School of Medicine, Dept. of Pharmacology, Molecular and Cellular Biology Program, and Program in Oncology, Baltimore, MD 21201.

The large T-antigen dependent, semi-conservative replication of simian virus 40 (SV40) DNA in vitro from HeLa cell homogenates has been recovered as a 21S complex of enzymes and proteins essential for DNA replication.[1] The DNA synthetic activity of the human cell 21S DNA replication complex has been demonstrated to be ribo- and deoxynucleotide triphosphate dependent and fueled by ATP. In addition, E.coli single-strand binding protein stimulates the DNA synthetic activity of the 21S complex. Kinetic analyses of the 21S DNA replication complex indicate that the initiation of the DNA synthetic process occurs within the first fifteen minutes of the reaction. These and other results regarding the biochemical characterization of the 21S DNA replication complex will be presented.

1-Malkas et al, (1990) Biochemistry 29: 6362.

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F 231 MAMMALIAN DNA POLYMERASE β CAN SUBSTITUTE FOR DNA POLYMERASE I IN *E. COLI*, Joann B. Sweasy and Lawrence A. Loeb, Joseph Gottstein Cancer Research Laboratory, Department of Pathology, University of WA., Seattle, WA., 98195
We have shown that expression of mammalian DNA polymerase β in *Escherichia coli* restores growth in a polymerase I defective bacterial mutant. Sucrose density gradient analysis revealed that DNA polymerase β complements the replication defect in the mutant by increasing the rate of joining of Okazaki fragments. Our findings demonstrate that DNA polymerase β, believed to function in DNA repair in mammalian cells, can also function in DNA replication. Moreover, the complementation system will permit study of the in vivo function of altered species of DNA polymerase β, an analysis currently precluded by the difficulty in isolating mutants in mammalian cells.

F 232 STRUCTURE / FUNCTION STUDIES OF MOLONEY MURINE LEUKEMIA VIRUS REVERSE TRANSCRIPTASE, Alice Telesnitsky and Stephen P. Goff, Department of Biochemistry and Molecular Biophysics, Columbia University College of Physicians and Surgeons, New York, NY 10032

We are studying the effects of RT mutations on the DNA polymerase activity of retroviral reverse transcriptase, and are using these mutants to probe the interaction between the enzyme and its template. Reverse transcriptase catalyzes two distinct enzymatic activities essential in retroviral replication: a DNA polymerase activity which can use either RNA or DNA as a template, and a nuclease activity, called ribonuclease H, which degrades the RNA strand of RNA:DNA duplexes. In the case of M-MuLV RT, these two activities reside in physically separable domains of a single monomeric polypeptide. Some mutations in the RNase H domain which do not decrease DNA polymerase activity in the standard oligo dT: poly rA primer: template assay are nonetheless altered in elongation properties of DNA polymerization when assayed on heteropolymeric templates. A truncated form of reverse transcriptase which is missing the entire RNase H domain is one such mutant. Complexes formed between this enzyme and defined templates can be studied in gel retardation assays. A comparison of these complexes and those formed with the wild-type enzyme should allow us to probe the physical interactions between the domains of reverse transcriptase and its template.

F 233 MOLECULAR INTERACTIONS BETWEEN THE HSV-1 DNA POLYMERASE AND ITS ACCESSORY PROTEIN UL42, Daniel J. Tenney, Robert K. Hamatake, Pamela A. Micheletti, Warren W. Hurlburt, Marc Bifano, John T. Stevens, James T. Matthews, Anthony R. Sanchez and Michael G. Cordingley, Virology, Bristol-Myers Squibb PRI, Princeton, NJ08543-4000.
The herpes simplex virus type 1 DNA-dependent DNA polymerase (Pol) and UL42 protein (UL42) associate to form a stable complex during viral infection and in vitro. The interaction of UL42 with Pol results in increased processivity of the enzyme. The carboxyl-terminal region of the Pol protein is necessary and sufficient for complex formation with UL42 in vitro (Digard and Coen, 1990. JBC, 265:17393-17396). Variant HSV Pol proteins, with defined mutations in this domain, have been expressed in yeast cells. The ability of these catalytically active mutant polymerase molecules to bind and be stimulated by UL42 has been addressed. Co-immunoprecipitation of radiolabeled Pol/UL42 complexes has been used to further elucidate specific protein sequences involved in the HSV polymerase-UL42 interaction.

F 234 HIGH PURIFICATION OF A PLANT DNA POLYMERASE AND ITS POSSIBLE ROLE.

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Three different DNA polymerase activities can be resolved by passing a protein extract from 24h imbibed maize axes through DEAE-cellulose. One of them, DNA polymerase B, elutes at 100-120 mM phosphate. This enzyme was purified further through Heparin-Sepharose, Sephacryl S-300 and DNA cellulose. Purification was nearly 5000 fold. The enzyme was characterized: absolutely needs Mg, is stimulated by K, has an optimum pH at 7.0-7.7, optimum temperature 30-37°C and do not use synthetic polyribonu cleotide templates. A molecular weight around 500Kd can be calculated through Sephacryl, and SDS-PAGE gives evidence of several bands of molecular weights 86,64,34-42. The same bands can be visualized if proteins from crude extracts are analyzed by western using an antibody against human DNA polymerase α. Finally, specific activity of this enzyme increases 100 fold during maize germination - whereas polymerases C and nuclear virtually do not increase. The possibility that polymerase B is a replicative enzyme is discussed.

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F 235 The Homologous DNA Polymerase α from Human and Yeast Displays Species Specificity In Vivo

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Human DNA polymerase α and S. cerevisiae polymerase α (formerly named polymerase I) are homologous replicative DNA polymerases in the cell. The primary sequence of human DNA polymerase α contains six regions of striking similarity to yeast polymerase α and to several other prokaryotic and eukaryotic replicative DNA polymerases. In addition, conservation between human and yeast DNA polymerase α are present throughout the entire sequence of these two proteins. Here we report that, despite the overall sequence conservation between these two replicative polymerases, functionally active recombinant human DNA polymerase α expressed in S. cerevisiae fails to substitute several different temperature sensitive pol1 alleles for growth at non-permissive temperature, and is also unable to complement a pol1 null allele in germinating spores or in vegetatively growing cells. Furthermore, the functionally active recombinant human polymerase α catalytic polypeptide is unable to associate with the yeast primase and the yeast p86 subunit. Thus, stringent species specificity between DNA polymerase α catalytic polypeptide and other replicative proteins is required in cellular DNA replication.
These results support the conclusion that the stringent species specific demand between polymerase α and other replicative proteins observed in viral DNA replication is also required in cellular DNA replication.

F 236 ALLOSTERIC REGULATION OF THE HIV-1 REVERSE TRANSCRIPTASE BY SUBSTRATE.

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The HIV-1 reverse transcriptase is a holoenzyme containing a 1:1 ratio of two subunits of 51 kDa and 64 kDa. These two subunits are derived from the pol ORF and are the products of the modification of the gag/pol polyprotein by the viral protease. They share the same amino terminus and differ in that the 51 kDa subunit is missing the C terminal RNaseH domain. While both subunits possess the catalytic site for polymerization only the 64 kDa subunit appears to be active. Purified reverse transcriptase of the human immunodeficiency virus type I (HIV-1) has been extensively examined with respect to the steady state kinetics of polymerization of dNTPs into product DNA. When dNTPs were used as the variable substrate, the kinetics of the enzyme deviated from standard Michaelis-Menten kinetics. The plot of increase in initial velocity of incorporation as a function of substrate concentration displayed substrate inhibition at high substrate concentrations and curvature consistent with negative cooperativity at lower substrate concentrations. Further examination of the incorporation of substrate dNMP in the presence of nucleotides not complementing the template, demonstrated that dNTPs may act as noncompetitive inhibitors as well as substrate. This allowed for the evaluation of the K_i of the enzyme for dNTPs by the application of the rate equation for noncompetitive inhibition. A working model is presented that accounts for the substrate inhibition. In this model, the reverse transcriptase is a multisubunit holoenzyme, possibly a dimer, with a one to one ratio of the 51 and 64 kDa subunits. Noncompetitive inhibition is allosterically mediated by one subunit, possibly the 51 kDa subunit, by binding nucleotide and down regulating the activity of the polymerizing 64 kDa subunit. With additional assumptions, this model can accommodate the negative cooperativity observed.

F 237 PRIMARY STRUCTURE OF THE CATALYTIC SUBUNITS OF HUMAN AND BOVINE DNA POLYMERASE DELTA: SEQUENCE SIMILARITIES WITH OTHER DNA POLYMERASES,

Jian Zhang, Dominic Chung, Cheng-Keat Tan, Earl W. Davie, Kathleen M. Downey and Antero G. So, Departments of Biochemistry/Molecular Biology and Medicine, University of Miami, Miami, FL 33101 and the Department of Biochemistry, University of Washington, Seattle, WA 98195.

The cDNAs encoding the catalytic subunits of both bovine and human DNA polymerase delta have been cloned by polymerase chain reaction methodology and the chromosomal location of the human pol delta gene has been determined. The nucleotide sequences of the cDNAs for human and bovine pol delta predict polypeptides of 1107 amino acids for human pol δ and 1106 amino acids for the bovine enzyme, with a calculated molecular weight of 124,000 for both. The human polypeptide is 94% identical to bovine pol δ and 45% identical to its counterpart from *Saccharomyces cerevisiae*. The catalytic subunits of both bovine and human pol δ contain the seven conserved regions found in a number of bacterial, viral and eukaryotic DNA polymerases. They also contain five additional regions that are highly conserved among human, bovine and yeast pol δ but share little or no homology with the α polymerases. Four of these regions are also homologous to corresponding regions in the herpes virus family of DNA polymerases, whereas one region is apparently unique to the δ polymerases. The human, bovine and yeast enzymes also contain two putative zinc finger domains in the carboxyl terminal region of the protein.

F 238 MOLECULAR CLONING AND CHROMOSOMAL LOCALIZATION OF THE cDNA FOR THE CATALYTIC SUBUNIT OF HUMAN DNA POLYMERASE DELTA, Peng Zhang, Robert Kemper, Chun-Li Yang, Huiling Hao, N. Lan Toomey, Long Sheng Chang‡, Mark Rabin and Marietta Y.W.T. Lee, Departments of Medicine and Pediatrics, University of Miami School of Medicine, Miami, FL 33101 and ‡Department of Pediatrics, Childrens' Hospital, Ohio State Univ., Columbus, OH 43205.
DNA polymerase delta is now recognized to have a central role in eukaryotic DNA replication, and the determination of its primary sequence is important in understanding its structural relationships with other polymerases. We have used a PCR amplification strategy, exploiting sequence information on the yeast pol III, the yeast analog of mammalian delta, to isolate partial clones for human delta. These were then used to isolate cDNA clones from human cDNA libraries. The cDNA consists of 3.4 kb with an open reading frame encoding a protein of 1106 residues with a calculated molecular mass of 125 kDa, in good agreement with the reported molecular weight of the catalytic polypeptide of DNA polymerase delta. C-terminal peptide polyclonal and monoclonal antibodies were produced and characterized. These are specific for DNA polymerase delta and blot only DNA polymerase delta enzyme. Northern blots of poly A+ mRNA from IMR 90, 293 and HeLa cells revealed a single mRNA species of 3.4 kb. Comparison of the human delta primary sequence with those of other eukaryotic polymerases show that it is most closely related to yeast pol delta (46% identity), followed by the polymerases from the human herpes virus family (EBV, CMV, VZV and HSV). Examination of delta mRNA and activity in serum restimulated IMR 90 cells show that both are induced in a similar pattern which is consistent with its function as a nuclear DNA replication enzyme. We have localized the delta gene to human chromosome 19Q13.3. Current efforts are directed to the expression of pol delta in prokaryotic and eukaryotic expression vector systems.

F 239 THE HIGHLY CONSERVED LYSINE RESIDUE IN FAMILY B DNA POLYMERASES IS REQUIRED FOR PROTEIN-PRIMED INITIATION AND POLYMERIZATION BY PRD1 DNA POLYMERASE, Weiguo Zhu and Junetsu Ito, Department of Microbiology and Immunology, College of Medicine, University of Arizona, Tucson, AZ 85724
The PRD1 DNA polymerase is a small multifunctional DNA polymerase which catalyzes protein-primed initiation and strand-displacement DNA synthesis. This DNA polymerase has been classified into the family B (α-type) DNA polymerases based on its amino acid sequence similarity with that of *E. coli* DNA polymerase II. There are several highly conserved regions among the family B DNA polymerases. The lysine residue at position 340 of the PRD1 DNA polymerase is highly conserved. To evaluate the functional and structural roles of this lysine residue in the DNA polymerase, we have constructed three mutants (K340H, K340N, and K340E) by means of site-specific mutagenesis. Both wild type and mutant PRD1 DNA polymerases have been overexpressed and purified to near homogeneity. All these DNA polymerases have been characterized in various *in vitro* assays. *In vitro* DNA replication experiments indicated that all three mutants are inactive in protein-primed initiation (PRD1 terminal protein-dGMP formation) and DNA chain elongation. Furthermore, in contrast to wild type PRD1 DNA polymerase, all three mutant polymerase were incapable of performing the filling-in reaction. Our results have shown that all the mutant DNA polymerases retain normal levels of 3' to 5' exonuclease activity. Based on our results, together with the observation that lysine-340 of the PRD1 DNA polymerase is similar to lysine-758 in alpha helix O of the *E. coli* DNA polymerase I Klenow fragment (a family A DNA polymerase), we propose that lysine-340 of the PRD1 DNA polymerase is involved in the binding of dNTPs. Our results further support the notion that family A and family B DNA polymerases share a common ancestral gene.

Multiprotein Complexes

F 240 STRUCTURE OF THE SYNAPTIC COMPLEX MADE IN THE DNA STRAND-EXCHANGE REACTION BY RECA PROTEIN, Kenji Adzuma, Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

One of the least understood aspects of the DNA strand-exchange reaction catalyzed by the recA protein is the mechanism by which recA mediates homologous pairing of two DNA molecules (usually ssDNA and dsDNA). In particular, the issue of whether recognition of homology is mechanistically coupled with disruption of Watson/Crick base-pairing in the participating dsDNA is crucial to understanding the mechanism of this DNA synapsis step. An intriguing possibility is that, by forming some kind of triple helix, the synapsis might be accomplished without disrupting the base-pairing in the dsDNA.

To gain an insight into the molecular mechanism of synapsis, the status of the three DNA strands in the synaptic complex made in the presence of ATPγS was probed by chemical footprinting using potassium permanganate ($KMnO_4$). $KMnO_4$ oxidizes unstacked Ts in ssDNAs, but not in dsDNAs, and has been widely used as a structural probe for "single-strandedness".

The results indicated that, regardless of the presence or absence of homologous ends, all three strands in the synaptic complex are susceptible to $KMnO_4$-attack. No Ts in naked duplex DNA are sensitive to $KMnO_4$ under these conditions. However, the footprint-patterns are different among the strands in the complex; Ts in the strand to be displaced show a stronger and uniform susceptibility to $KMnO_4$, whereas Ts in the other two strands exhibit variability in their reactivity, depending on the location of the base. A similar experiment using dimethyl sulfate (DMS), which primarily methylates N^7 of G that is thought to be involved in known triple-helices, is now in progress. Preliminary results, together with the results of $KMnO_4$-footprinting, suggest that the DNA structure in the synaptic complex may resemble that of the end-products of strand-exchange.

F 241 RECA PROTEIN-FACILITATED DNA STRAND BREAKS: A MECHANISM FOR BYPASSING DNA STRUCTURAL BARRIERS DURING STRAND EXCHANGE, Wendy A. Bedale, Ross B. Inman and Michael M. Cox, Department of Biochemistry, University of Wisconsin, Madison, WI 53706
RecA protein promotes an unexpectedly efficient DNA strand exchange between circular ssDNA and duplex DNAs containing short (100-400 bp) heterologous sequences at the 5' (initiating) end. The major mechanism by which this topological barrier is bypassed involves DNA strand breakage. Breakage is both strand- and position-specific, occurring almost exclusively in the displaced (+) strand of the duplex within a 15 base pair region at the heterology/homology junction. Breakage also requires recA protein, ATP hydrolysis, and homologous sequences 3' to the heterology. In addition, breakage requires the presence of a single-stranded DNA exonuclease, which acts on the recA-ssDNA-dsDNA complex to facilitate strand exchange past the heterologous barrier. The role of the exonuclease in this unusual reaction is currently under investigation.

F 242 SPECIFICITY OF RuvC FOR FOUR-STRANDED RECOMBINATION INTERMEDIATES.

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Studies in this laboratory have shown that the *E.coli* RuvC protein resolves Holliday junctions made by RecA protein *in vitro*. However, whilst *ruvC* single mutants are repair deficient, they are only deficient in conjugational recombination in *recBCsbcA*, *recBCsbcBC* or *recG* genetic backgrounds, which may indicate the presence of an alternative resolvase in *E. coli*, perhaps with a different substrate specificity. To investigate the cleavage specificity of the RuvC protein, its action on three-stranded recombination intermediates was examined. Intermediates of RecA mediated reactions between single-stranded circular DNA and linear duplex DNA were not cleaved by the RuvC protein. The substrate specificity of RuvC on a variety of substrates is currently under investigation.

F 243 *E. COLI* RECA PROTEIN REQUIRES ATP HYDROLYSIS TO INCORPORATE MISMATCHES INTO HETERODUPLEX DNA,

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RecA protein hydrolyzes ATP vigorously when bound to DNA. However, it has been shown that recA requires ATP hydrolysis neither for the search of homology, nor for heteroduplex formation between homologous DNA molecules. Eukaryotic recombinases are similar in this respect, since they do not consume ATP. From the thermodynamic point of view, strand transfer between perfectly homologous DNA molecules is a $\Delta G=0$ reaction, requiring no free energy input.

So why are bacterial recombinases ATPases? We have tested the requirement for ATP in the formation of heteroduplex DNA containing mismatched bases, a reaction that is *not* thermodynamically isoenergetic. If ATP hydrolysis is allowed, recA will produce heteroduplex DNA containing a significant fraction of mismatched bases, or even large insertions and deletions (Bianchi and Radding, 1983, Cell 35:511-520). We have now shown that if the non-hydrolyzable analog ATPγS is used, strand transfer between perfectly homologous single-stranded and duplex DNA proceeds to completion, whereas strand transfer between molecules containing insertions and deletions does not proceed past the non-homologous site. Therefore, recA needs the free energy of ATP hydrolysis to separate the strands of duplex DNA when concerted reannealing is not possible. This fits nicely with the observation that recA has a helicase activity on duplex DNA that is supported by ATP but not by ATPγS (Bianchi et al., EMBO J. 4:3025-3030).

In conclusion, the free energy requirement for mismatched DNA formation can be met by recA thanks to its associated ATPase and helicase activities; we predict it will not be met by eukaryotic recombinases.

F 244 DISSOCIATION OF TRIPLEX DNA BY RECA PROTEIN

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When a circular single strand of DNA that has been coated with recA protein reacts with homologous linear duplex DNA, strand exchange proceeds from the proximal to the distal end of the duplex, the proximal end corresponding to the 5' end of the non-complementary strand. When the proximal end was blocked by a long region of the heterology, stable joints formed although strand exchange could not take place. When recA protein was removed by treatment with EDTA, SDS, and proteinase K, a stable triplex DNA intermediate was found (Rao et al 1991). In the present studies, we have found that in the continued presence of recA protein, these distal triplex joints are further processed although strand exchange can not occur. To examine the ability of recA protein to process triplex intermediates, we formed joint molecules with chimeric linear duplexes which had homology either at the proximal or distal ends. Reactions with these substrates showed that the accumulation of ADP specifically dissociated distal joints. ATPγS prevented this dissociation. In addition, distal joints underwent cycles of dissociation and reassociation, as revealed by the effect of added unlabeled excess homologous duplex DNA. Recycling was specific to distal joints. Finally, when recA protein was added back to protein-free distal triplexes, they dissociated. This reaction, as well, was inhibited by ATPγS. These observations suggest that recA protein catalyzes both the formation and dissociation of triplex intermediates, and that dissociation requires the hydrolysis of ATP.

F 245 OPPOSITE EFFECTS OF UvrD IN RecA-PROMOTED HOMOLOGOUS PAIRING AND STRAND TRANSFER,

Era Cassuto, Sandrine Séguret, James Hejna and Patrice Morel, Génétique Microbienne, Institut des Biotechnologies, INRA, Jouy-en-Josas, 78352 Cedex, France.

The T_4 dda helicase has been shown to inhibit homologous pairing (1) and to stimulate strand transfer promoted by UvsX (2). Because UvrD (helicase II) has no effect in the UvsX system whereas Dda also inhibits RecA dependant homologous pairing, these properties were thought to be specific to the T_4 enzyme.

We have found that UvrD displays analogous activities in the RecA system, although it translocates in a direction opposite to that of Dda. We propose a model that reconciles these observations at the mechanistic level.

Given the known effects of uvrD mutations in repair and recombination, the stimulation of strand transfer is a surprising finding. We suggest a mechanism that renders both the "recombinase" and "antirecombinase" activities of UvrD consistent with its postulated role in the suppression of interspecies recombination (3).

(1) Kodadek, T. *J.Biol.Chem.* 1991, 266:9712-9718
(2) Kodadek, T. and Alberts, B.M. *Nature* 1987, 326:312-314
(3) Rayssignier, C., Thaler, D.S. and Radman, M. *Nature* 1989, 342:396-401

F 246 RESOLUTION OF RECOMBINATION INTERMEDIATES BY *E. COLI* RuvC PROTEIN.

Hazel J. Dunderdale, Fiona E. Benson, Carol A. Parsons and Stephen C. West, Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts. EN6 3LD UK.

The RuvC protein has been over-expressed and purified. The 19 KDa protein resolves recombination intermediates (Holliday junctions) produced *in vitro* by RecA protein. The action of RuvC protein on Holliday junctions has been studied using both natural (RecA-made) and synthetic stuctures. In both cases, resolution occurs to produce nicked linear duplex products. Resolution is dependant upon homology at the site of the junction, and can occur in either orientation to give rise to genetically sensible products. The orientation of cleavage is affected by the presence of the RecA protein.

F 247 PROBING THE ACTIVITIES OF RecA PROTEIN WITH CHROMIUM(III) ADENINE NUCLEOTIDE COMPLEXES.

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The recA protein of *Escherichia coli* utilizes MgATP to perform a number of diverse functions in the cell, including homologous recombination and induction of the SOS response for DNA repair. The coordination of MgATP at the binding site of recA was examined using the exchange-inert Cr(III) adenine nucleotide complexes, β,γ-bidentate $Cr(H_2O)_4ATP$, γ-monodentate $Cr(H_2O)_5ATP$, α,β-bidentate $Cr(H_2O)_4ADP$ and β-monodentate $Cr(H_2O)_5ADP$. All of these compounds served as competitive inhibitors of the recA ATPase activity; the monodentate complexes were more effective inhibitors than the corresponding bidentate complexes. Furthermore, β,γ-bidentate $Cr(H_2O)_4ATP$ (β,γ-CrATP) functioned as an active site directed irreversible inhibitor of recA. Kinetic analysis of the inactivation reaction suggests the formation of a dissociable enzyme-CrATP complex prior to the inactivation step, which probably involves insertion of an amino acid side chain into the coordination sphere of the Cr(III). ATP protected against inactivation, implying that β,γ-CrATP was incorporated at the active site. Approximately one mole of β,γ-CrATP was incorporated per recA monomer. The ability of the Cr(III) nucleotide complexes to serve as cofactors for recA-catalyzed cleavage of the lexA repressor, a critical step in the induction of the SOS response, was also investigated. None of the chromium nucleotides stimulated lexA cleavage in the manner of MgATP. Rather, the chromium compounds inhibited the MgATP induced lexA cleavage. These results imply that MgATP and MgADP form monodentate complexes at the nucleotide binding site of recA. However, the lack of induction of lexA cleavage by recA-CrATP complexes suggests that CrATP is unable to induce the conformational change required for interaction with lexA. This conformational change may be triggered by insertion of an amino acid side chain of recA into the coordination sphere of the metal ion at the nucleotide binding site. Such an insertion may not easily occur with the exchange-inert Cr(III) analogues. This study provides a molecular description of the ATP binding site of recA.

F 248 **THE ANALYSIS OF THE DNA BINDING SITE OF THE RecA PROTEIN OF *Escherichia coli.***

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The RecA protein of *E. coli* promotes a strand transfer reaction through binding both to single- and double-stranded DNA. To understand the DNA binding mechanism of the RecA protein, we introduced mutational changes at the regions so far proposed as a DNA binding region by sequential homology to other DNA binding proteins. These includes N-terminal region and central region. Among the 15 constructed mutant *recA* genes, *recA-R243A, recA-K248A and recA-Y264A* showed highly defective on the resistance to UV irradiation. We purified these three mutant RecA proteins and compared the biochemical characteristics with the wild-type protein. The RecA-K248A protein did not show any activity of the protein. The other two mutant RecA proteins showed lower binding affinities to single- and double-stranded DNA than the wild-type protein, although their Km values for ATP in the DNA dependent ATPase reaction were similar to that of the wild-type protein. Since the apparent affinity to DNA would be influenced by the alteration of RecA-RecA interaction property, we also examined the self-oligomerization property of these mutant proteins in the absence of DNA by ATPase activity and light scattering in a high salt condition. The results obtained were that the RecA-R243A protein showed similar oligomerization profile to the wild-type, but the RecA-Y264A protein did not. These results suggest that the region including Arg^{243} participates in the binding to single- and double- stranded DNA, in contrast, the region including Tyr^{264} participates in the RecA-RecA interaction. With the other results obtained, we will discuss the binding mechanism of the RecA protein to DNA in the strand transfer reaction.

F 249 THE EFFECT OF MISPAIRED BASES ON DNA BRANCH MIGRATION,

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Upon the completion of strand exchange in homologous recombination, Holliday structures containing regions of heteroduplex DNA are formed. These heteroduplex regions can undergo branch migration which affects the amount of genetic information exchanged between homologous chromosomes. We are examining the effect of mispaired bases on DNA branch migration. Using synthetic, three-stranded DNA substrates having defined mismatches, deletions or insertions, we are studying the effect of perturbations in DNA structure on branch migration. The assay consists of a slow annealing step between an oligonucleotide and a partial duplex molecule made by annealing a ^{32}P-labeled oligonucleotide to complementary sequences in M13mp18 single-strand DNA, followed by a rapid branch migration step leading to displacement of the ^{32}P-labeled oligonucleotide. We observe that nonenzymatic branch migration does not proceed to any significant extent through as few as two consecutive mispaired bases (mismatches or deletions). This barrier to branch migration posed by mispaired or unpaired bases appears to be relatively insensitive to sequence context. We are currently studying branch migration using four-stranded DNA substrates.

F 250 THE PUTATIVE THREE-STRANDED DNA PAIRING INTERMEDIATE IN RECA PROTEIN-MEDIATED DNA STRAND EXCHANGE: NO ROLE FOR GUANINE N-7, Sarita K. Jain, Michael M. Cox and Ross B. Inman, Department of Biochemistry, University of Wisconsin, Madison, WI 53706
As an early step in DNA strand exchange reactions, the recA protein aligns homologous sequences within two DNA molecules to form a putative triple-stranded intermediate. In virtually all models for three-stranded DNA proposed to date, hydrogen bonds involving the N-7 position of guanine have played a prominent structural role. To determine whether the N-7 position of guanine is required for triple helix and heteroduplex formation in the recA protein-mediated DNA pairing reaction, guanine was completely replaced by the base analog 7-deazaguanine in both strands of the duplex DNA substrate using PCR. This modified dsDNA was reacted with unmodified ssDNA *in vitro*. The 7-deazaguanine-substituted DNA functioned as well as the unsubstituted DNA in recA protein-mediated DNA three-strand exchange reactions. Strand exchange reactions involving four strands also proceeded normally when three of the four strands contained 7-deazaguanine rather than guanine. In fact, the rate of strand exchange improved somewhat when the modified DNA substrates were used. This indicates either that the N-7 position of guanine is not essential for the formation of the putative triple-stranded DNA pairing intermediate, or that a three-stranded (or four-stranded) structure is not an obligate intermediate in recA protein-mediated DNA strand exchange.

F 251 FUNCTIONAL INTERACTION BETWEEN SINGLE-STRADED DNA AND THE PHAGE T4 UVSX, UVSY AND GENE 32 PROTEINS, Hua Jiang and Tomas Kodadek, Department of Chemistry and Biochemistry, University of Texas at Austin, TX 78712
Presynapsis is out of the early steps in genetic recombination. In the bacteriophage T4 system, three proteins are involved in this process. The UvsX protein, a strand transferase, is analogous to the *E. coli* recA protein and catalyzes homologous pairing and branch migration. The UvsY and gene 32 proteins strongly stimulate these activities and are an integral part of the presynaptic filament. The organization of these three interacting proteins in the filament is currently unknown. To address this question we have carried out hydroxyl radical protection studies of a 60 base oligonucleiotide in the presence of various combinations of the T4 proteins. These experiments have led to a new model for the T4 presynaptic filament.

F 252 ENZYMATIC EFFECTS OF MUTATIONS IN THE CONSENSUS ATP-BINDING MOTIFS OF THE RecB AND RecD PROTEINS, Douglas A. Julin, Firouzeh Korangy, and Susie S.-H. Hsieh, Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742
Both the RecB and RecD subunits of the RecBCD enzyme from *Escherichia coli* contain a consensus ATP-binding sequence. We have changed the conserved lysine residue in this sequence to glutamine in each subunit by site-directed mutagenesis. Extracts of cells expressing the mutant RecB subunit and unaltered RecC and RecD have no ATP-dependent nuclease activity with double-stranded DNA at ATP concentrations up to 1 mM. We are in the process of purifying this enzyme. The RecD mutant enzyme (RecBCD-K177Q) retains the ATP hydrolysis, ATP-dependent nuclease, and DNA unwinding activities of the wild-type enzyme. All are reduced compared to the wild-type enzyme. The K_m for ATP in the ATP hydrolysis reaction is less than that of the wild-type enzyme, which we attribute to hydrolysis by the unaltered RecB subunit. The ATP hydrolysis reaction catalyzed by the RecBCD-K177Q enzyme is also less processive than the wild-type, as shown by its sensitivity to heparin, to the single-stranded DNA binding protein, and by direct examination of the DNA substrate on agarose gels.

F 253 RECOMBINATION PROTEIN MEDIATED TRIPLEX DNA: R-FORM DNA. M.G. Kim[1], P. Hsieh[1], C.S. Camerini-Otero[1], G. Raghunathan[2], R.L. Jernigan[2], V.B. Zhurkin[2], and R.D. Camerini-Otero[1], [1]Genetics and Biochemistry Branch, NIDDK, [2]Laboratory of Mathematical Biology, NCI, NIH, Bethesda, MD 20892

Our previous data showed that the deproteinized joint molecules generated by several general recombination proteins have unexpectedly high melting temperatures, indicating that these joint molecules are comprised of three stranded DNA. We propose a model with the structural features that are a direct consequence of the overriding biological constraint that the third strand must be identical and parallel to one of the duplex strands. This molecularly defined model structure is testable and we have designated this novel DNA triplex as R-form DNA. It is plausible to expect that recombination proteins, particularly recA, can lower the activation energy involved in the formation of R-form DNA. We show that R-form DNA may be a family of related structures all distinguished from previously described triplexes by the parallel orientation of the third strand with respect to its identical strand in the Watson-Crick duplex. Chemical footprinting and isosteric base substitution data confirm some of the molecular interactions in the proposed triplets. The data demonstrate that the third strand is in contact with purines in the major groove of the Watson-Crick duplex.

F 254 ANALYSIS OF MUTATIONS IN THE ATP BINDING DOMAINS OF THE RECBCD NUCLEASE OF ESCHERICHIA COLI, Sidney R. Kushner, Miroslawa Wlodarczyk, Stephen Vigo and Thomas Nemetz, Department of Genetics, University of Georgia, Athens, Georgia 30602
The RecBCD enzyme of Escherichia coli carries out a number of ATP catalyzed reactions that play a role in genetic recombination, DNA repair and cell viability. These include the ATP-dependent degradation of both single- and double-stranded DNA as well as an ATP-dependent DNA helicase activity. In addition, the enzyme endonucleolytically cleaves covalently closed single-stranded DNA in an ATP-stimulated fashion. Both the RecB and RecD subunits have been shown by DNA sequence analysis to contain putative ATP binding domains. Using site-directed mutagenesis, we have altered the proposed ATP binding domain in the RecB subunit. While such recB mutants have dramatically altered phenotypic properties, surprisingly, only a few of the catalytic activities of the modified RecBCD enzymes have been affected. These results suggest a more sophisticated role for the RecBCD enzyme in both genetic recombination and DNA repair. (This work was supported in part by a grant from NIHGMS (GM27997) to S.R.K.).

F 255 **Mutagenesis of the "P-loop" Region of the *E. coli* RecA Protein ATP Binding Site**
Karen M. Logan and Kendall L. Knight, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA. 01655.
We have performed oligonucleotide cassette mutagenesis in order to investigate the amino acid requirements in the "P-loop motif" of the RecA protein. This motif was originally defined as a consensus sequence that exists in a large number of otherwise unrelated NTP binding proteins [G-X-X-X-X-G-K-T(S)]. The recently solved X-ray crystal structure confirms that residues within this motif contact the phosphate groups of bound NTP (R. Story/T. Steitz, personal communication). We have introduced a variety of single, double, and multiple amino acid substitutions within the following sequence: ^{66}Gly-Pro-Glu-Ser-Ser-Gly-Lys-Thr-Thr-Leu^{75}. Mutant RecA proteins have been divided into 3 categories (functional, partially functional, and non-functional) based on the results of several screens: **1.** survival in the presence of 4-nitroquinoline-1-oxide (NQO), **2.** survival following U.V. irradiation, **3.** formation of plasmid oligomers, and **4.** the ability to support plaque formation by a *Chi*$^+$ *red*$^-$ *gam*$^-$ λ phage. Western blot analysis was performed in order to measure the expression and *in vivo* stability of each of the mutant RecA proteins. Non-functional protein resulted when any kind of substitution occurred at those residues identified as important by the consensus sequence (Gly^{66}, Gly^{71}, Lys^{72}, and Thr^{73}). However, despite being very highly conserved among the RecA proteins from 23 different bacterial species we found that a number of different amino acids are allowed at Pro^{67}, Glu^{68}, Ser^{69}, Ser^{70}, and Leu^{75}. Substitutions at Thr^{74} are limited and may reflect a requirement for a side chain capable of forming a hydrogen bond to the ribose oxygen of bound ATP. In several cases mutations that resulted in a partial loss of function were suppressed by the simultaneous occurrence of other substitutions in this region. We have attempted to correlate the effects of specific amino acid substitutions and their position in the 3-dimensional structure of RecA.

F 256 **BIOCHEMICAL STUDIES WITH RecF PROTEIN;** Murty Madiraju, V.V. S. and Alvin J. Clark; Department of Molecular and Cell Biology, University of California, Berkeley, CA-94720.

Homologous recombination occurs in *Escherichia coli* by RecBCD and RecF pathways. RecF is one of the important proteins involved in the RecF pathway of recombination and mutations in *recF* cause a wide variety of phenotypes. We have been interested in understanding the role of RecF protein in genetic recombination and repair. We previously hypothesized that *in vivo* RecF protein aids RecA protein to carry out recombination and repair reactions effectively. In this work we report that, in apparent contradiction of our hypothesis, our purified RecF protein preparation interferes with the joint molecule forming ability of RecA. We also found that RecF protein interferes with the ssDNA dependent ATPase activity catalyzed by RecA protein. To further understand these reactions we are presently characterizing the DNA binding properties of RecF protein. Also, we are seeking to remove the contaminating proteins present in RecF protein preparation to see whether the level interference caused by RecF protein changes. RecF protein has a consensus sequence associated with binding of the phosphate moiety of nucleotide (Sandler and Clark, Submitted; Gorbalenya and Koonin, J. Mol. Biol. 1990). We have been able to cross-link 8-Azido ATP, a photoaffinity analogue to RecF. Presently we are attempting to determine whether the Azido-ATP cross linking to RecF protein is due to a specific ATP binding site or due to a DNA binding site. The implications of these experiments and DNA binding experiments described above, in context to how RecF protein works *in vivo* will be discussed.

F 257 INTERACTION OF HEAT-SHOCK PROTEIN GROEL WITH THE SINGLE-STRANDED DNA-BINDING PROTEIN OF *E. COLI*: SUPPRESSION OF REPLICATION, REPAIR AND RECOMBINATION DEFECTS OF *ssb-113*, Ralph R. Meyer and Phyllis S. Laine, Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221
The *E. coli* heat-shock protein GroEL plays essential roles in the folding, unfolding, association, dissociation, and transport of many cellular proteins. It is inducible by heat or other stress conditions and serves an important function in protecting against heat stress. We have previously identified an allele, *groEL411*, which specifically suppresses the temperature-sensitive DNA synthesis of the single-stranded binding protein mutation *ssb-1*. We have now identified a second *groEL* mutation that specifically and completely suppresses the defects in DNA replication, repair and recombination of the *ssb-113* mutation. There is, however, no reciprocal suppression of the λ^r defect of these *groEL* mutant cells. The biochemical defects of SSB-1 and SSB-113 proteins are markedly different. While SSB-1 is unable to form or maintain active tetramers at the restrictive temperature, we believe that SSB-113 fails to interact with other proteins involved in replication, repair and recombination. The *ssb-113* mutation (a.a. 176, pro → ser) lies in a highly conserved C-terminal domain, and this substitution could easily lead to abnormal folding. It is proposed that the *groEL* suppressor, by virtue of its "foldase" activity, may fold SSB-113 into a functional conformation. This occurs at all temperatures, and, unlike the GroEL411 suppression of SSB-1, heat-shock is not necessary for the suppression.

F 258 THE UvsX RECOMBINASE OF BACTERIOPHAGE T4 PROMOTES TRANSLESION DNA SYNTHESIS BY A COPY-CHOICE MECHANISM IN VITRO

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During late stages of bacteriophage T4 infection in *E. coli*, the initiation of phage DNA replication is dependent on the recombination activity of the T4 uvsX protein (a recA protein-like homologous recombination enzyme). The central steps of the T4 recombination-dependent DNA replication pathway have been reconstituted in an in vitro system containing eleven cloned and highly purified T4 recombination and replication proteins. We have used this in vitro system to investigate the role of uvsX protein in T4 recombination-dependent DNA repair pathways. Our results indicate that during replication of damaged templates, the uvsX protein promotes translesion DNA synthesis by a copy-choice type of mechanism, in which DNA synthesis switches from a damaged template to a homologous but undamaged template via recombination. The translesion DNA synthesis in vitro system serves as a model for recombinational DNA repair processes in bacteriophage T4, and may provide clues as to how these processes work in higher organisms as well.

F 259 IDENTIFICATION OF A PROTEIN WITH SPECIFICITY FOR HEMIMETHYLATED *oriC* DNA, Piotr J.Polaczek, James W.Fontaine, Rene Gallegos and Moselio Schaechter, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111

The control and timing of initiation of DNA replication in *E.coli* is not well understood. The initiation event is precisely timed and occurs once per cell cycle. Immediately following initiation, newly replicated DNA is in a hemimethylated state which persists in the *oriC* region approximately 5 times longer than at other regions of the chromosome. Mutations in the *dam* methylase gene have been found to cause asynchrony of replication. It was found in this laboratory that hemimethylated origin DNA specifically binds to membrane fractions of *E. coli*. This suggests that the hemimethylated state of the *oriC* region is involved in replication synchrony and possibly, chromosomal segregation. We are investigating the specific binding of a protein to hemimethylated *oriC* DNA. We have identified a hemimethylated origin binding (Hob) protein, using the Kohara phage library lysates. We have subcloned the candidate gene on a pUC19 plasmid for *in vivo* genetic studies. Currently, we are purifying the Hob protein for *in vitro* studies.

F 260 INTERACTION OF DnaA AND FIS PROTEINS WITH THE *E.COLI* REGULATION ORIGIN,

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FIS protein (Factor for inversion stimulation) binds to a specific site in the *E.coli* regulation origin, oriC. This FIS binding site is directly adjacent to a binding site for the initiation protein DnaA, DnaA box R3, which may play a pivotal role in the initiation of replication. Binding of FIS to its site and of DnaA to box R3 are mutually exclusive.

We show that binding of FIS protein bends oriC DNA. FIS protein is required for the efficient replication of minichromosomes. Presumably, FIS protein (as well as IHF and HU proteins) assists in the formation of a nucleoprotein complex for the initiation of DNA replication.

F 261 RESOLUTION OF HOLLIDAY JUNCTIONS IN *E. coli*: MOLECULAR AND GENETIC ANALYSIS OF WILD-TYPE AND MUTANT *ruvC* GENES, Gary J. Sharples and Robert G. Lloyd, Department of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK.

The *ruv* region of *Escherichia coli* specifies activities that are involved with DNA repair and recombination. Three genes have been identified at this locus (1). *ruvA* and *ruvB* form a single operon which is regulated by LexA as part of the SOS response (2,3). *ruvA* specifies a 22 kDa DNA binding protein, *ruvB* a 37kDa ATPase (4,5) and *ruvC* a nuclease that resolves Holliday junction intermediates in genetic recombination into recombinant products (6).

ruvC was located between *aspS* and the *ruvAB* operon by DNA sequencing and deletion analysis of *ruvC* plasmids. Examination of the DNA flanking *ruvC* indicated that the gene is transcribed independently of *ruvAB* operon and is not under SOS control. It encodes a protein of 18747 Da which has been over-produced and purified (7). A comparison of the predicted amino acid sequence of RuvC with those of phage T4 endonuclease VII and T7 endonuclease I, both of which are also able to resolve Holliday junctions, revealed no major homologies.

Three mutant alleles of *ruvC* have been sequenced. *ruvC51* and *ruvC53* specify single amino acid substitutions, while *ruvC55* is a nonsense mutation. Over-expression of RuvC51 protein in a ruv^{+} background confers sensitivity to UV light. The mutant protein is stable and may be able to form non-functional heterodimers with the wild-type protein.

1. Sharples, G.J., *et al. Mol. Gen. Genet.* **221**, 219-226 (1990).
2. Benson, F.E., *et al. Nucl. Acids Res.* **16**, 1541-1549 (1988).
3. Shinagawa, H., *et al. J. Bacteriol.* **170**, 4322-4329 (1988).
4. Iwasaki, H., *et al. J. Bacteriol.* **171**, 5276-5280 (1989).
5. Shinagawa, H., *et al. Biochimie* **73**, 505-508 (1991).
6. Connolly, B., *et al. Proc. Natl. Acad. Sci.* **88**, 6063-6067 (1991).
7. Dunderdale, H.J., *et al. Nature* - submitted, (1991).

F 262 CHARACTERIZATION OF THE RecA-MEDIATED STRAND EXCHANGE BETWEEN DNA MOLECULES CONTAINING NON BASE-PAIRED REGIONS, Andrzej Stasiak and Walter Rosselli, Laboratoire d'Analyse Ultrastructurale, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne-Dorigny, Switzerland.

The main function of the RecA-mediated recombination reaction in bacterial cells is the participation in DNA repair. DNA damages frequently manifest themselves by the presence of non base-paired regions. Therefore, we decided to test if RecA would be able to perform a strand exchange reaction when one of the DNA partners of the reaction contains non base-paired regions. Our experiments show that the presence of a non base-paired region in the substrate duplex has several interesting implications for the mechanism and outcome of the RecA-mediated recombination reaction. In the case of the reaction between two double-stranded DNA molecules, non-paired regions can serve as nucleation sites for the formation of the presynaptic RecA-DNA complexes that are active in the recombination process. In the case of the nonreciprocal strand exchange between double-stranded linear and single-stranded DNA, non base-paired regions do not block the reaction but can shift the equilibrium between the products and the substrates of the reaction. When the strand exchange reaction leads to conversion of non base-paired region into a fully base-paired one, the equilibrium of the reaction is strongly shifted toward the products.

The fact that RecA can recognize non base-paired regions as initiation sites for the formation of presynaptic complexes and the fact that RecA has a tendency to convert non-base paired regions into fully base-paired regions show new aspects of the involvement of RecA in the DNA repair.

F 264 THE ROLE OF THE SMALL *ESCHERICHIA COLI* HEAT SHOCK PROTEINS, DnaJ AND GrpE, IN THE INITIATION OF λ DNA REPLICATION, M. Zylicz[1], J. Osipiuk[1], J. Marszalek[1], K. Liberek[1,2], D. Skowyra[1], A. Maddock[2], D. Ang[2] and C. Georgopoulos[2], [1]Division of Biophysics, Dept. of Molecular Biology, University of Gdansk, Kladki 24, 80-822 Gdansk, Poland, [2]Dept. of Cellular, Viral and Molecular Biology, University of Utah School of Medicine, Salt Lake City, UT 84132

Genetic analyses have shown that bacteriophage λ growth is dependent on several *E. coli* proteins, including the DnaK, DnaJ and GrpE heat shock proteins. Based on these analyses, we have developed an *in vitro* λ DNA replication system composed entirely of purified *E. coli* and λ proteins. Initiation of λ DNA replication requires the assembly of the proper nucleoprotein complex: *ori*λ - λO - λP - DnaB. Subsequently, DnaK, DnaJ and GrpE act upon this complex, resulting in initiation of λ DNA replication. The detection of specific protein-protein interactions, isolation of active nucleoprotein intermediates, and modulation of DnaK's enzymatic activities suggest that both DnaJ and GrpE are involved in the following two reactions:

1. Binding of DnaK protein to λP protein. In the presence of GrpE, DnaK has a higher affinity for the λP - DnaJ protein complex than in the absence of GrpE.
2. Hydrolysis of ATP catalyzed by DnaK protein. DnaJ and GrpE stimulate the ATPase activity of DnaK, thus accelerating release of λP protein from the preprimosomal complex.

The molecular mechanism of GrpE and DnaJ action in the initiation of λ DNA replication will be compared with the mechanism by which these two small heat shock proteins modulate DnaK's "chaperoning" function, specifically in the reactivation of heat-inactivated RNA polymerase holoenzyme.

F 263 DNA BINDING AND NUCLEASE ACTIVITY ASSOCIATED WITH THE *REC1* GENE PRODUCT OF *USTILAGO MAYDIS*.

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The *REC1* gene, which restores normal recombination and DNA repair to *rec1* mutants of *Ustilago maydis*, encodes a protein with regional sequence similarities to several DNA binding proteins and also to the 3'→5' exonuclease portion of α-type DNA polymerases. The 58kDa *REC1* gene product, obtained after overexpression of the *REC1* ORF in bacteria, bound to DNA, and in the presence of Mg^{2+} hydrolyzed nucleotides from the 3'- (but not 5'-) termini of both duplex and single-stranded DNA. In order to assess the effect of individual mutations on protein function, products of *rec1* alleles were overproduced and tested for DNA binding and 3'→5' exonuclease. The native protein possessing these activities has been purified from *U. maydis*. It was recognized by antibody to the overexpressed *REC1* gene product, and appeared to be associated in equimolar amount with two other proteins of M_r 20k and 70k. We are investigating whether the three proteins together may form a recombinational repair machine.

Replication Initiation & Termination - Prok

F 300 ANALYSIS OF DNA DAMAGE-INDUCIBLE ORIGINS OF DNA REPLICATION IN *E. coli*. Tsuneaki Asai, Thomas R. Magee and Tokio Kogoma, Departments of Cell Biology and Microbiology, University of New Mexico Medical Center, Albuquerque, NM 87131

Upon induction of the SOS response in *E. coli*, DNA replication can occur in the absence of normally required protein or RNA synthesis. This type of DNA replication has been termed inducible stable DNA replication (iSDR). Initiation of iSDR occurs at two origins, one (termed *oriM1*) located in the *oriC* region and the other (*oriM2*) in the *terC* region. Neither origin requires DnaA protein for initiation. Initiation from *oriM1* was inactivated by a 6-kilobase deletion in the *oriC* region of the chromosome. To further delimit the *oriM1* region, we inserted various *oriC* fragments into pBR322 and measured the copy number of these chimera plasmids after induction of the SOS response in the presence of rifampin, which normally inhibits the initiation of DNA replication from the origin of pBR322. The result suggested that *oriM1* exists in the *oriC* region between -178 and +425. In the course of the experiment, however, we noticed that in cells induced for the SOS response, certain derivatives of pBR322 can replicate in the presence of rifampin [Rifampin-Resistant Replication (RRR): T. Magee and T. Kogoma, J. Bacteriol. 173, 4736, 1991]. RRR requires the presence of an insert DNA of a certain sequence in pBR322, and one such sequence was found in the *oriC* region. Thus, in order to discriminate iSDR from RRR, in this study, we employed minichromosomes for the analysis and confirmed the previous result, i.e. *oriM1* lies between -178 and +489. Precise mapping of *oriM1* is in progress and will be discussed.

F 301 STRINGENT CONTROL OF P1 PLASMID REPLICATION, Stuart J. Austin, Ann L. Abeles and Therese G. Brendler, Laboratory of Chromosome Biology, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

The stringently controlled plasmid replicon (repR) that maintains the plasmid prophage of bacteriophage P1 consists of an origin of replication, a gene for the synthesis of the RepA initiator protein, and a copy-control locus, incA. Both the origin and incA loci contain multiple 19-bp binding sites for RepA protein. Genetic studies carried out in vivo, and in an in vitro replication system, provide evidence for the following:

Control of initiation does not primarily involve limitation of RepA supply. Rather, it is carried out by DNA replication itself. Once two plasmids are present, RepA promotes plasmid pairing, with the incA control locus of each daughter in contact with the origin of the other. This inactivates the origins of both daughter plasmids. Reinitiation does not occur until a partitioning event parts the copies and puts them into separate daughter cells. This automatically synchronizes replication control to the cell cycle. This type of mechanism may have application to the control of replication of eukaryotic chromosomes.

A second level of control involves monitoring of the methylation state of the origin DNA sequences. The origin core contains essential 7-bp repeat sequences encompassing dam methylation sites. These must be methylated for efficient origin function. We present evidence that a novel host protein binds to this region only when the DNA is methylated. Presumably, dam methylation is needed because this protein plays some essential role in initiation.
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F 302 F PLASMID PARTITIONING FUNCTIONS AND DNA SUPERCOILING. Donald Biek and Joquetta Strings. Department of Microbiology & Immunology, University of Kentucky Medical Center, Lexington, KY 40536.

We are studying the process of segregation of low copy number plasmids F and P1 with the goal of identifying and characterizing *E. coli* functions that participate in the process. The partitioning regions of these plasmids each encode two proteins and a cis-acting centromere-like site that are required for partitioning. Several of our current studies relate to a report (Miller, Beaucage and Cohen, Cell 62:127) that partition defective (*sop*) mini-F plasmids are stably maintained in *topA* mutant strains in which negative supercoiling of the DNA is increased. We are testing the hypothesis that the *sop* genes of F may act to influence the supercoiling state of the plasmid and that this may play a role in the partitioning process.

Mutations in the mini-F plasmid *sop* genes have been found to alter plasmid supercoiling. Mutation of the *sopB* gene, or the cis-acting *sopC* region to which the SopB protein binds, is reflected in increased negative superhelicity of the isolated plasmid DNA; while a mutation in the autoregulatory *sopA* gene leads to decreased plasmid supercoiling. In addition, the SopAB proteins encoded by one plasmid are able to decrease the superhelicity of an unrelated plasmid carrying the *sopC* region. Experiments are in progress to test whether this is due to a relaxing topoisomerase activity associated with SopB, or more likely, whether the effect reflects the formation of a wrapped nucleoprotein structure in which SopB binding of *sopC* in a right-handed wrap retains positive supercoils. We have used this supercoiling assay to explore *sopC*-mediated incompatibility and the implications it has for the mechanism of mini-F partitioning.

F 303 COMPLETE ALIGNMENT OF THE 3´-5´ EXONUCLEASE DOMAIN OF PROKARYOTIC AND EUKARYOTIC DNA POLYMERASES. MUTAGENESIS AT THE EXO III REGION OF Ø29 DNA POLYMERASE. Luis Blanco, María S. Soengas, Antonio Bernad, José Antonio Esteban, José M. Lázaro and Margarita Salas. Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain.

The 3´-5´ exonuclease active site of *E. coli* DNA polymerase I (Pol I) was predicted to be conserved for both prokaryotic and eukaryotic DNA polymerases based on amino acid sequence similarity (1, 2). Thus, three regions (ExoI, Exo II and Exo III), containing the critical residues in Pol I involved in metal binding and catalysis, were identified in the N-terminal portion of prokaryotic and eukaryotic DNA polymerases. Selective inactivation of the 3´-5´ exonuclease activity has been demonstrated by site-directed mutagenesis at the predicted exonuclease active site in Pol I (regions ExoI, Exo II and Exo III; 3), Ø29 DNA polymerase (Exo I and Exo II; 1), T7 DNA polymerase (Exo I; 4) and DNA pol δ of *S. cerevisiae* (Exo II; 5). However, similar studies demonstrated that the predicted Exo I region of T4 DNA polymerase (6) and DNA pol δ of *S. cerevisiae* (5) is not critical for the 3´-5´ exonuclease activity.

In this report we present the complete alignment of the 3´-5´ exonuclease domain of 30 DNA-dependent DNA polymerases belonging to the main two subfamilies (Pol I-like and α-like). This alignment allowed to identify, in addition to the most conserved regions (ExoI, II and III), other segments of amino acid similarity which contain residues recently involved in single-stranded DNA binding at the exonuclease domain of Pol I (7). Analysis of Ø29 DNA polymerase mutants in the amino acid residues corresponding to Pol I residues Tyr497 and Asp501 (region Exo III), thought to be involved in exonucleolytic catalysis and metal binding, respectively, support the critical role of the conserved region Exo III in the 3´-5´exonuclease activity of DNA-dependent DNA polymerases.

References: (1) Bernad et al.(1989) Cell 59,219-228; (2) Blanco et al. (1991) Gene 100, 27-38; (3)Derbyshire et al. (1991) EMBO J. 10, 17-24; (4) Patel et al. (1991) Biochemistry 30, 511-525; (5) Simon et al. (1991) EMBO J. 10, 2165-2170; (6) Reha-Krantz et al. (1991) PNAS 88, 2417-2421; (7) Beese and Steitz (1991) EMBO J. 10, 25-33.

F 304 THE INITIATION MASS OF Escherichia coli K-12 VARIES WITH GROWTH RATE AND DOES NOT REFLECT INTRACELLULAR DNAA CONTENT.
Erik Boye, Brigitte M.R. Voss, and Sture Wold, Department of Biophysics, Institute for Cancer Research, Montebello, 0310 Oslo, Norway.

The nature of the coupling between DNA replication and general cell growth is not known in any organism. In *Escherichia coli*, regulation of the rate of DNA replication occurs at the level of initiation. It is a long standing observation that the ratio between cell mass and the number of replication origins at initiation (termed the initiation mass) is approximately constant and independent of growth rate (Donachie, Nature 219, 1077, 1968). The constancy of the initiation mass is central for the understanding of the regulation of the cell cycle in *E. coli*.

In the present work we have employed flow cytometry to measure the initiation mass at different growth rates. Strain AB1157 was grown in the presence of different carbon sources and amino acid supplements, fixed, stained, and subjected to flow cytometry. The size of individual cells was measured as scattered light, protein content was measured as fluorescence from FITC-labelled bacteria, and DNA content was quantitated after staining with Mithramycin/ethidium bromide. Initiation mass was shown to increase monotonously and by a factor of 2 when the doubling time increased from 30 min to 100 min. Immunoblotting revealed that the contents of DnaA protein per unit cell mass increased by about a factor of 2 in the same interval. In contrast, we have earlier shown that increasing the DnaA concentration by inducing transcription from a *plac-dnaA* construct results in premature initiations and decreased initiation mass (Boye and Løbner-Olesen, Cell 62, 981, 1990). The present data demonstrate that when the growth rate is varied the DnaA concentration is not determining initiation mass.

F 305 THE DELIVERY OF THE DNAB HELICASE TO SSDNA CATALYZED BY DNAC PROTEIN,
David Bramhill, Dorina Trusca, Chris Thompson, Julia Hsu and Katherine Young, Building 80Y-325, MSDRL, PO Box 2000, Rahway, NJ 07065.

The *E. coli* DnaB helicase is essential for replication. It unwinds the duplex DNA at a replication fork to expose the template, and also guides primase to lay down RNA primers on the discontinuous strand. In vivo, a single hexamer of DnaB may be responsible for processive unwinding of up to half of the *E. coli* chromosome. Paradoxically, DnaB exhibits a low affinity for its ssDNA substrate (K_M of 80 μM). Another essential replication protein, DnaC, co-ordinates efficient delivery of DnaB to ssDNA. Six DnaC monomers form a complex with a DnaB hexamer, stabilized by six molecules of ATP bound by DnaC. This DnaB-DnaC complex can interact readily with ssDNA in a series of steps which we have analyzed. By using substrate analogs, and by using purified mutant proteins in place of wild type proteins, intermediates in the delivery pathway have been trapped and isolated. Based on the properties of these protein-DNA complexes, the following sequence of events has been deduced: First, a loose complex is formed with ssDNA which can readily dissociate. Subsequently, a conformational change occurs which leads to a tightly bound DnaB-DnaC-ssDNA complex. Next, the bound ATP molecules are hydrolysed, allowing the release of the DnaC subunits and leaving DnaB tightly associated with the ssDNA ready to act as a helicase and as a guide for primase. With a much reduced efficiency, DnaB can find its own way on to ssDNA. The resulting DnaB-ssDNA complexes are indistinguishable from those formed with the aid of DnaC, being equally stable and fully capable of guiding primase. We propose a model to account for these observations which can also explain why replication initiated at *oriC* is bidirectional.

F 306 DNA REPLICATION PROTEINS IN HIGHER PLANT CHLOROPLASTS AND IN CYANOBACTERIA,
Gordon C. Cannon, Sabine Heinhorst and Lisa A. Hedrick, Department of Chemistry and Biochemistry, University of Southern Mississippi, Hattiesburg, MS 39406-5043.

Chloroplasts of eukaryotic algae and higher plants contain genetically active DNA that consists of double stranded, covalently closed circles within a size range of 120-160 kilobase pairs (kbp). Although the entire nucleotide sequence of the plastid genome from several higher plants is known, the mechanism by which the plastome is replicated, its origin of replication and many replication proteins have as yet remained elusive. Likewise, DNA replication in cyanobacteria, the presumed evolutionary ancestors of chloroplasts, is largely unexamined.

We have begun to purify, characterize and compare DNA replication proteins from soybean chloroplasts and from the cyanobacterium *Anacystis nidulans*. To date, we have highly purified and characterized two forms of γ-like DNA polymerase from the chloroplast and one DNA polymerase from the cyanobacterium which resembles *E. coli* Pol I. No obvious biochemical similarities exist between the organellar and the cyanobacterial enzymes, and it remains to be seen if these differences also extend to the primary sequence level. We are examining the role of the cyanobacterial enzyme in DNA replication while attempting to ascertain the presence of Pol II and Pol III homologs. In order to determine which form of the chloroplast DNA polymerase functions in replication we have begun to purify and characterize replication proteins present in a chloroplast extract capable of site specific initiation on cloned chloroplast DNA *in vitro*. We have so far identified DNA helicase, topoisomerase I and primase activities as well as DNA binding protein in this extract.

F 307 MEMBRANE FLUIDITY AND ACIDIC HEAD GROUPS ACTIVATE DnaA, THE INITIATION PROTEIN OF REPLICATION IN *ESCHERICHIA COLI*,
Celina E. Castuma and Arthur Kornberg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305-5307.

Acidic phospholipids in a fluid phase dissociate the adenine nucleotide bound to DnaA protein and, in the presence of ATP and DNA, restore an inactive ADP-form to full activity (Yung, Y. M. and Kornberg, A. (1988) Proc. Natl. Acad. Sci. USA 85, 7202). Further studies of the interactions between DnaA protein and lipids have used two functional assays: (1) release of nucleotides from dnaA, and (2) DNA synthesis upon rejuvenation of inactive DnaA protein-ADP complex. Tested were a wide variety of pure phospholipids, as well as phospholipid mixtures from *E. coli* auxotrophs unable to synthesize unsaturated fatty acids (*fab a*) and supplemented with different fatty acids. Fatty acid composition was determined by gas liquid chromatography and membrane fludity by fluorescence spectroscopy using 1,6-diphenyl-1,3,5-hexatriene as a probe. Lipid requirements of DnaA proteins were shown to be: (1) phospholipids in a fluid phase (i.e., above the transition temperature), (2) a charged polar head group, (3) a lamellar phase (i.e., hexagonal II structures were inactive), and (4) an unsaturated fatty acid esterified in the *sn2* position of the glycerol backbone. That an unsaturated fatty acid is required because of the fluid state it promotes in the bilayer instead of the particular conformation of the *cis* double bond directly in contact with the DnaA was proved by: (1) cholesterol, known to decrease the fluidity of lipids without interacting with protein surfaces, and (2) a branched fatty acyl derivative. Both agents demonstrated that membrane fluidity accounts for the unsaturated fatty acid requirement for DnaA protein function *in vitro* consistent with studies of chromosome initiation in growing cells.

F 308 *E. coli* HEAT SHOCK PROTEINS DnaJ AND DnaK HELP P1 PLASMID REPLICATION BY STIMULATING INITIATOR BINDING TO THE ORIGIN, Dhruba Chattoraj, Shanmuga Sozhamannan, Gauranga Mukhopadhyay and Peter Papp, Lab of Biochemistry, NCI, NIH, Bethesda, MD 20892

Replication of plasmid P1 requires the plasmid encoded initiator protein RepA. Binding of RepA to the origin is defective in *dnaJ*, *dnaK* and *grpE* mutant cells and this defect probably accounts for the inefficiency of plasmid replication in these cells[1]. The replication and binding defects are overcome by overproduction of RepA in *dnaJ* mutant cells; *dnaK* and *grpE* cells have not been tested. Thus, facilitation of RepA binding to the origin in wild type cells may be through increasing the concentration of active RepA.

DnaJ and DnaK (but not GrpE) proteins also stimulate RepA binding to the P1 origin in vitro[2]. Binding measurements under equilibrium conditions revealed that the reaction follows 2nd-order binding isotherms. k_{on} at 1×10^{-9} M RepA at ionic strength of 0.1 M was 1×10^{9} $M^{-1}sec^{-1}$, whereas k_{off} was 2×10^{-4} sec^{-1}, corresponding to K_D of 2×10^{-13} M. Measurements at different DnaJ and DnaK protein concentrations indicated that the stimulation does not result from an alteration in the intrinsic binding affinity of RepA, but rather from an increase in the active form of RepA. These results are consistent with the idea that these heat shock proteins exert a conformational change on RepA.

1) K.Tilly, S.Sozhamannan & M.Yarmolinsky (1990) The New Biologist 2:812-817. 2) S.Wickner, J.Hoskins & K.McKenney (1991) Nature 350:165-167.

F 309 ACTIVATION OF *dnaX* EXPRESSION BY AN INTERNAL SEQUENCE, Ken-Shiung Chen and James R. Walker, Department of Microbiology, The University of Texas, Austin, TX 78712-1095

The 6820 bp *dnaX* region of the *E. coli* K-12 chromosome contains 6 genes, all or most of which are involved in nucleic acid metabolism. *apt*, *dnaX*, *orf12-recR*, *htpG* and *adk* are adjacent and transcribed in the same direction. The *dnaX* gene encodes both the τ and γ subunits of DNA polymerase III. τ is the full-length product of the *dnaX* reading frame, but the shorter γ protein is produced from within the *dnaX* reading frame by a programmed ribosomal frameshift followed by a nonsense codon in the new reading frame. The *dnaX* promoter has been localized to a 168 bp fragment, and the 5' end of the mRNA has been mapped. Expression of the *dnaX* gene is activated by a sequence within the *dnaX* coding region. A 620 bp, *NarI-AflII* internal fragment that had no promoter activity alone stimulated the upstream *dnaX* promoter 4-10 fold. No stimulation of transcription was observed when this fragment was inserted upstream of *dnaX* in both orientations or when its orientation downstream of *dnaX* was reversed. The sequence necessary for stimulation has been further localized on a 36 bp segment; 16 of which share extensive homology with the operator site of the purine regulon. The possibility that the purine repressor (or a similar protein), which normally represses purine biosynthesis, acts as a positive regulator is under study.

F 310 INITIATION OF BACTERIOPHAGE T7 DNA REPLICATION, Yeon-Bo Chung, and Charles C. Richardson, Department of Biological Chemistry and Molecular Pharmacology, Boston, MA 02115

Bacteriophage T7 has at least five potential replication origins. When replicating molecules were isolated from an isopycnic gradient after density-labelling, the primary origin, located 15% of the distance from the genetic left end of the chromosome was predominantly utilized. To identify the mechanism of initiation of DNA replication, we investigated the 5'-ends of leading strands generated during phage-infection of *Escherichia coli* by primer extension. The 5'-ends of the newly synthesized DNA were protected from further processing by inactivation of the exonuclease activities responsible for the digestion of RNA primers. We found that the newly synthesized DNAs arising from the primary origin are all associated with the T7 gene 4 protein (primase/helicase) recognition sequence. While the gene 4 primase site downstream of the AT-rich region of the primary origin was seldom used, those within and upstream of the AT-rich region was utilized efficiently suggesting the initial binding of gene 4 protein at AT-rich region. It also suggested that the generation of leading strand is not made at a specific site but at any site upstream of the primary origin, although the proximal ones have higher possibility of utilization. The simultaneous activity of the secondary origins with the primary origin are inferred from the same rate of radioactive thymidine incorporation on different regions of T7 DNA.

F 311 PHYSICAL AND BIOCHEMICAL PROPERTIES OF THE *E.COLI* TUS PROTEIN, Filiz F. Coskun-Ari, Aikaterini Skokotas and Thomas Hill, Department of Bioscience and Biotechnology, Drexel University, Philadelphia, PA 19104

The Tus protein of *E. coli* arrests DNA replication when bound to specific sequences in the chromosome called *Ter* sites. We have examined the physical properties of purified Tus protein as part of our analysis of the Tus-*Ter* binding interaction. The combination of gel filtration chromatography and sucrose density gradient centrifugation allowed us to determine the Stokes radius (23.2 Å), the sedimentation coefficient (2.8×10^{-13} sec) and frictional ratio (1.06) of native Tus protein, which exists as a monomer in solution. These results suggest that the shape of Tus protein is roughly spherical with an axial ratio of 2. The isoelectric point (7.5) and extinction coefficient ($\varepsilon_{280\ nm}$ = 39000) of purified Tus protein have also been determined. In addition, we have performed partial proteolysis of unbound Tus protein to determine if a minimal DNA-binding domain could be identified. Our results demonstrated that a single cut by trypsin or chymotrypsin resulted in loss of DNA-binding activity of Tus. In contrast, Tus complexed with DNA containing a *Ter* site was completely protected from tryptic or chymotryptic digestion.

F 312 CLONING, OVEREXPRESSION AND PURIFICATION OF THE Ψ SUBUNIT OF E.COLI DNA POLYMERASE III HOLOENZYME,

René Crombie and Mike O'Donnell, Howard Hughes Medical Institute, Hearst Microbiology Department, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

DNA polymerase III holoenzyme, the chromosomal replicase of Escherichia coli, is composed of at least 10 subunits. The second smallest of these is the ψ subunit, with an apparent molecular weight of 12kDa on SDS-PAGE. The ψ subunit is a component of the five-protein γ complex (γδδ'χψ) which utilizes ATP to transfer the β accessory protein onto a primed template to form the preinitiation subassembly. The ATP-activated β clamp confers rapid and processive synthesis onto the core polymerase. The ψ subunit is not essential to reconstitute holoenzyme, although the χ and ψ subunits stabilize the reconstituted holoenzyme to added salt. A homology search of the N-terminal sequence of the naturally isolated ψ protein revealed a GenBank match to an open reading frame overlapping the RimI gene. The ψ gene is located at 99.3 minutes on the E.coli chromosomal map, near the deo operon. Analysis of DNA and protein sequence indicates a hydrophobic domain and putative helix-turn-helix concensus sequence. The ψ gene was cloned and expressed from a T7 promoter in E.coli cells containing an inducible T7 RNA polymerase gene (Studier). The overexpressed ψ protein represents greater than 10% of total cell protein, and has been purified to apparent homogeneity. The interaction of ψ with the other subunits of holoenzyme and its function in the reconstituted holoenzyme system is currently under investigation.

F 313 *OriC* DNA STABILIZES *E. coli* DnaA PROTEIN DURING REJUVENATION BY PHOSPHOLIPIDS,

Elliott Crooke, Celina Castuma, and Arthur Kornberg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305.

DnaA protein (the initiator protein) binds and oligermizes at the four dnaA boxes of the *E. coli* chromosomal origin (*oriC*) and promotes the strand opening required for subsequent DNA replication. The activity of DnaA protein is profoundly influenced by the tight binding of ATP and ADP. Replication events following binding to the dnaA boxes require DnaA protein to be in the ATP-form; the ADP-form, generated by hydrolysis of the bound ATP, is inactive. Rejuvenation of ADP-DnaA protein, by replacement with ATP, is catalyzed by acidic phospholipids in a fluid bilayer. We find that interaction of DnaA protein with *oriC* DNA is necessary to stabilize DnaA protein during this phospholipid-mediated rejuvenation process: (1) DnaA protein bound to *oriC* DNA responds to phospholipids, but free DnaA protein is inactivated by phospholipids and fails to bind *oriC*. (2) *OriC* DNA facilitates the high affinity binding of ATP to DnaA protein during treatment with phospholipids. The role of *oriC* DNA sequence motifs in the stabilization of DnaA protein during phospholipid treatment will be presented.

F 314 COOPERATIVE BINDING OF IHF PROTEIN TO THE REPLICATION ORIGIN OF PLASMID R6K.

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The minimal replication origin of plasmid R6K (γ ori) contains a set of seven 22-bp direct repeats (iterons) which is flanked by two Integration Host Factor (IHF) binding sites. Site ihf1 contains two adjacent IHF consensus sequences and IHF protein binds regardless of sequence context, bending the DNA. Site ihf2 contains two overlapping and antiparallel IHF consensus sequences. One sequence is perfect and the other contains one mismatch from the IHF consensus. However, we demonstrate that only one consensus sequence of either binding site is recognized by IHF. Furthermore, IHF does not bind DNA fragments containing ihf2 when ihf1 is mutated or physically removed, or if six of the seven iterons spanning both sites are deleted. When IHF is bound to both ihf sites, specific phosphodiester bonds within the iterons become more sensitive to DNase I. The data we present here support a model in which IHF binds initially to site ihf1, then a long-range cooperative interaction allows binding to site ihf2, looping out the intervening iteron segment.

F 315 ENRICHED SOURCES OF *E. coli* REPLICATION PROTEINS

Nicholas E. Dixon, Mary E. Argall, Jennifer L. Beck, Jeffrey A. Crowther, Christopher M. Elvin, Philip Hendry, Penelope E. Lilley, N. Patrick J. Stamford, Brian P. Surin, Phillip R. Thompson and Subhash G. Vasudevan, Centre for Molecular Structure and Function, Research School of Chemistry, Australian National University, Canberra 2601, Australia.

Large quantities of purified *E. coli* replication proteins were required for chemical, spectroscopic and crystallographic studies. A modified series of λ promoter vectors (Elvin *et al.* [1990] *Gene* **87**, 123-126) was used to generate plasmids that separately directed overproduction of the products of the *dnaA, dnaB, dnaC, dnaE, dnaG, dnaN, dnaQ, dnaT, dnaX, gyrA, gyrB, rnhA* and *ssb* genes to levels such that each comprised between 10 and 50 % of cellular protein.

In the general case, the gene was trimmed to remove its natural promoter(s) and ribosome-binding site, then inserted into one of the vectors so that its transcription could be controlled by tandem λ P_R and P_L promoters, and its ATG start codon was placed just downstream of a ribosome-binding site *perfectly complementary to the 3' end of 16-S rRNA*

Most of the gene products were readily isolated in a soluble fraction. In some cases where proteins normally associate with others *in vivo* (*e.g.*, dnaB and dnaC, gyrase A and B), insoluble products were obtained. Use of plasmids that directed simultaneous overproduction of both proteins gave soluble complexes.

F 316 GENE IDENTIFICATION, OVERPRODUCTION-PURIFICATION OF THE δ SUBUNIT OF DNA POLYMERASE III HOLOENZYME.

Ziming Dong, Rene Onrust, Maija Skangalis and Mike O'Donnell, Howard Hughes Medical Institute, Microbiology Department, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

DNA polymerase III holoenzyme (holoenzyme) is the polymerase which replicates the E. coli chromosome. The holoenzyme is composed of ten subunits. holoenzyme hydrolyzes ATP to lock onto a primed template and thereafter is rapid and highly processive in DNA synthesis. The holoenzyme can be reassembled from the following components: the core subassembly ($\alpha\epsilon\theta$) which contains the polymerase activity, the γ complex subassembly ($\gamma\delta\delta'\chi\Psi$), and the β subunit. In the reassembly process, the γ complex recognizes primed DNA and couples ATP to clamp β onto the DNA. The β clamp converts the core to a processive polymerase by holding the core polymerase tightly to the primed DNA.

Toward our goal of understanding the individual function of each holoenzyme subunit we have identified the genes encoding the subunits for which the gene assignment had not been previously determined. This poster describes the gene encoding the δ subunit. We obtained the amino terminal and some internal amino acid sequence information using δ purified from the holoenzyme which led to discovery of the δ gene. The gene maps to approximately 15 minutes on the E. coli chromosome. The δ gene sequence reveals a 344 amino acid open reading frame and predicts a 38.7 kda polypeptide consistent with δ purified from wild type cells. The gene sequence contains a putative ATP binding motif. The δ protein was overexpressed to 20% of total cell protein in E. coli using a bacteriophage T7 expression system. The overexpressed protein was purified to homogeneity using an assay in which δ is combined with other purified overproduced subunits of the holoenzyme to reconstitute rapid and processive DNA synthesis.

F 317 The Temporal Expression of Proteins Involved in the Initiation or Repression of DNA Replication of *Bacillus subtilis*

Bethanie Eident-Wilkinson, José Tori, and William Firshein, Molecular Biology and Biochemistry, Wesleyan University, Middletown, CT 06459

A *Bacillus subtilis* membrane-associated protein that binds specifically to the origin region of DNA replication may act as an inhibitor of initiation of DNA replication (J. Laffan and W. Firshein .1988.Proc. Natl. Acad. Sci. USA. 85:7452-7456). This protein, originally estimated to be 64 kilodaltons(kda), had a slightly lower molecular weight (57kda) as determined by SDS PAGE gels, and is now referred to as the putative repressor protein (PR). A complex pattern of expression was revealed in which significant levels were detected in spores; levels decreased dramatically during germination and increased after the first round of DNA replication. The decrease during germination was due to protease activity as demonstrated by the addition of protease inhibitors, and radioactive labeling-chase experiments. During vegetative growth, the PR protein levels increased until stationary phase, after which there was another decrease during sporulation. The decrease during sporulation may be partially due to sequestering of the protein into forespores, since as the PR protein decreased in the mother cell, it increased in the forespores. However, protease activity was also involved in the decrease in the mother cell. The changes in expression of this protein are consistent with its role as a repressor of initiation of DNA replication. Similar studies were performed using antibodies prepared against dnaA and dnaB proteins of *B. subtilis*,which have been demonstrated to be involved in the initiation of DNA replication (T. Fukuoka, S. Moriya, H. Yoshikawa, and N. Ogasawara.1990. J. Biochem.107:732-739. and W. Firshein and U.N. Streips. (1991) *In* Modern Microbial Genetics. U. N. Streips and R.E. Yasbin, ed., Wiley-Liss, Inc., New York, pp55-75). The results show a different pattern of temporal expression during *B. subtilis* stages of development as compared to the PR protein. DnaA protein is very stable and levels remain fairly constant throughout germination and vegetative stages; but there is a decrease during late sporulation stages. The dnaB protein is very unstable and is susceptible to proteolytic cleavage throughout the entire *B. subtilis* cycle. Only the smaller cleaved form of dnaB protein (48 kda) is seen in spores. the native form of dnaB protein (55 kda) is not observed until initiation of DNA replication during germination. After germination, the native form is seen throughout the vegetative and sporulation stages as well as the cleaved form. However, levels of the native form appear to be slightly less than the cleaved form during the sporulation stage.

F 318 STUDIES OF IN VITRO SYNTHESIS OF A BACILLUS SUBTILIS OriC PLASMID IN SOLUBLE AND MEMBRANE ASSOCIATED SYSTEMS

William Firshein and Shigeki Moriya, Dept. of Molecular Biology & Biochemistry, Wesleyan University, Middletown, CT 06457

An autonomously replicating sequence (ars) from the origin region of Bacillus subtilis was recently cloned by Moriya et al (submitted). The minimum replication fragment (termed the OriC plasmid) contained two regions of repeated sequences identical to those in Escherichia coli which bind the dnaA protein separated by a region coding for the dnaA gene. However, the dnaA gene could be deleted or mutated without effecting ars activity whereas removal of one of the repeat regions did eliminate such activity.

Studies were initiated to construct an in vitro replicating system using the OriC plasmid in two ways. The first involved a soluble system slightly modified from one first described by Fuller et al (1) for an E. coli OriC plasmid. The second involved a membrane associated system developed in this laboratory by Mele and Firshein (unpublished results). Initial experiments with the soluble system revealed that when the OriC plasmid was added, replication occurred for 60 min and was completed after 90 min. In contrast, vector synthesis without the cloned origin increased only slightly over the 90 min incubation period. A cloned origin fragment with a specific deletion at one end which did not replicate in vivo did, however, replicate in vitro. Antibodies prepared against the dnaA and dnaB proteins inhibited replication of the OriC plasmid severely. Very precise conditions were required in order for replication to occur including the elimination of chloride ions from the assay solution and replacement of KC1 by potassium glutamate during preparation of the extract.

Initial experiments with the membrane associated system have not been as clear cut since significant synthesis (probably repair) still occurs with the vector although addition of the OriC plasmid does often yield higher rates of synthesis. However, we are encouraged by the observations that both DNA ligase and DNA gyrase activity are present in the membrane system as well as the dnaA and dnaB proteins. In addition, the same requirements for potassium glutamate and lack of chloride ions during the preparation of the active membrane fraction are evident. Significant synthesis also occurs with the endogenous template present in the membrane system in the absence or presence of the OriC plasmid. The molecular weight of the endogenous chromosomal DNA is high suggesting that fragmentation of the template is minimal. This latter probability stems from the fact that both lysozyme and minimal pressures (1500 to 2000 psi) are used to break the cells in the French Press. Further work is underway to improve both replicating systems. 1). Fuller, R. S., Kaguni, J. M. and Kornberg, A (1981) Proc. Natl. Acad. Sci. USA, 78, 7370-7374

F 319 **AN EARLY STEP IN PARTITION: ASSEMBLY OF THE P1 PLASMID PARTITION COMPLEX.**

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Stable maintenance of the P1 plasmid prophage in *Escherichia coli* requires (a) faithful DNA replication, and (b) proper segregation, or partition, of daughter plasmid copies at cell division. The P1 partition system encodes two proteins, ParA and ParB, which act upon a plasmid DNA site called *parS*. Partition models predict that P1 uses these components in conjunction with host *par* functions; that is, P1 "hitch-hikes" along with the host partition machinery. One of the earliest steps in partition is the assembly of a protein complex at *parS*, containing ParB and the *E. coli* integration host factor, IHF. The ParB binding sites flank the IHF binding site. We are probing the nature of these protein-DNA interactions on supercoiled plasmids since complex assembly strongly prefers DNA superhelicity. The formation of this complex significantly increases the affinity of both proteins for *parS*. For example, ParB binding affinity is over 10,000-fold greater to *parS* in the presence of IHF than in its absence. IHF induces a large bend in the middle of its binding site. This bend is particularly sensitive to chemical modification reagents such as dimethylsulfate. Stoichiometry measurements suggest that two dimers of ParB, and one molecule of IHF are contained within the complex. These findings indicate that *parS* DNA is specifically wrapped around a core of ParB and IHF. We have generated a variety of low-affinity *parS* variants by mutation, and have measured the ability of high- and low-affinity complexes to promote (or interfere with) plasmid stability *in vivo*. Further characteristics of the assembly of the partition complex will be presented.

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F 320 LONG-RANGE INTERACTIONS IN ENHANCEMENT OF PLASMID pT181 DNA REPLICATION, Maria Laura Gennaro, Public Health Research Institute, 455 First Avenue, New York, NY 10016.

The multicopy plasmid pT181 carries a DNA sequence, called *cmp*, that enhances the activity of the replication origin (*ori*) from a distance. Deletion of *cmp*, which maps about 1 kilobase away from *ori*, results in the inability of the *cmp*⁻ plasmid to coexist with another plasmid having the same *ori*. Besides impaired competitivity, no other phenotype is associated with the *cmp* deletion during steady-state plasmid replication. However, exponential replication (which follows conditional shut-off of the normal plasmid copy control) occurs at a reduced rate in the *cmp*⁻ plasmid as compared to that in the wild-type plasmid.

cmp works in either orientation and at several locations in the plasmid genome with decreasing efficiency as it is moved away from *ori*. The *cmp* sequence is approximately 100 bp in length; it contains a loosely repeated sequence motif and a periodic pattern of oligo(dT) tracts, consistent with the presence of a locus of static DNA bending.

The effects of *cmp* on both plasmid competition and exponential replication require the presence in *cis* of only two components of the pT181 genome, *ori* and *cmp*. Moreover, *cmp* does not alter the level of synthesis of RepC, the plasmid-encoded initiator protein, which is the rate-limiting factor for replication. On the basis of these observations, our current interpretation is that *cmp* affects utilization of the RepC protein by *ori*. Consistent with this interpretation, we have found that second site mutations in pT181 that partially suppress the *cmp* defect map in the *repC* gene.

The *cmp* element specifically binds to a ~70-kDa protein (CBF, *cmp* binding *f*actor), which is encoded by *Staphylococcus aureus*. Thus, the regulatory interaction between *ori* and *cmp* may involve two distantly located sets of site-specific protein-DNA complexes; one is originated by RepC binding at *ori*, the other by CBF binding at *cmp*.

F 321 EFFECT OF DELETION OF THE CARBOXY-TERMINUS OF T4 DNA POLYMERASE UPON ITS INTERACTION WITH ITS ACCESSORY PROTEINS. L.D. Goodrich, T.-C. Lin, E.K. Spicer, and W.H. Konigsberg, Dept. of Molec. Biophys. and Biochem., New Haven, CT. The T4 DNA polymerase accessory proteins (44P/62P and 45P) stimulate the polymerase (pol) activity of T4 DNA polymerase, and also stimulate the 3'→5' exonuclease (exo) activity of the polymerase on a double-stranded DNA substrate (reviewed by Nossal and Alberts [1983] in *Bacteriophage T4* [Mathews *et al.*, eds.], pp. 71-81, Amer. Soc. Micro., Wash. D.C.). The accessory proteins are thought to interact with the polymerase through its carboxy-terminal domain based on the finding that the exo activity of a polymerase that lacked the carboxy-terminal 19% of the protein was not stimulated on double-stranded DNA by the addition of the accessory proteins (Venkatessan and Nossal [1982], J. Biol. Chem. *257*: 12435). An overexpression vector which contains the polymerase gene inserted downstream of an inducible promoter was used to engineer stop codons at various positions in the region of the polymerase gene that encodes the carboxy-terminal portion of the protein. The mutant constructs subsequently were used to overexpress the truncated polymerases, and the proteins were purified and assayed for pol and exo activity. In general, mutant polymerases with larger deletions of the carboxy-terminus retain less pol activity than those with smaller deletions, and the pol activities of the mutant enzymes are much more reduced than their exo activities. Detailed studies of pol activity using a synthetic primer-template indicate that a polymerase mutant (N881) which lacks the carboxy-terminal seventeen amino acids of wild-type polymerase has a two-fold lower k_{cat} and four-fold higher K_M(DNA) than wild-type polymerase. This mutant polymerase was tested for stimulation of its exo activity on double-stranded DNA by addition of the polymerase accessory proteins. Whereas the exo activity of the wild-type polymerase was approximately six-fold higher in the presence of the accessory proteins, no stimulation of the N881 mutant polymerase exo activity could be detected. Therefore, removal of seventeen amino acids from the carboxy-terminus of polymerase in some way disrupts its interaction with its accessory proteins.

F 322 **PHAGE ø29 DNA REPLICATION INTERMEDIATES PRODUCED IN VITRO.** Crisanto Gutiérrez[1], José M. Sogo[2] and Margarita Salas[1]. [1] *Centro de Biología Molecular, Universidad Autónoma, Canto Blanco 28049 Madrid, Spain* and [2] *ETH-Hönggerberg, Zürich, CH-8093, Switzerland.*

Bacteriophage ø29 DNA replication is initiated at either end of its double-stranded DNA (dsDNA) molecule. After initiation, ø29 DNA replication proceeds by strand-displacement. Electron microscopic analysis of the replicative intermediates (RIs) produced in the *in vitro* ø29 DNA replication system showed that they were similar to those observed *in vivo*: type I (full-length dsDNA with single-stranded DNA (ssDNA) branches) and type II (full-length DNA with a portion of dsDNA and the rest of ssDNA).

We have observed that: (i) initiation occurs asynchronously at both ends of the same DNA template (≥80%), (ii) type II RIs appear well-before a full-length ssDNA molecule is displaced and (iii) the replication of a mutant ø29 DNA template lacking terminal protein at one end led to both an accumulation of full-length ssDNA molecules and a lack of type II RIs. These studies strongly suggest that type II RIs are formed when two growing DNA chains, running from opposite ends, merge rather than by initiation on a panhandle RI.

The ø29 SSB protein p5 binds specifically to the ssDNA branches of RIs producing, under replication conditions, a two-fold reduction in the ssDNA length. SSB protein p5 does not change the DNA elongation rate. However, it increases the proportion of type I RI, probably by preventing non-productive binding of ø29 DNA polymerase to ssDNA.

F 323 THE KINETICS OF DNA POLYMERASE HOLOENZYME DISSOCIATION AFTER COMPLETION OF AN OKAZAKI FRAGMENT, K. Hacker and B. Alberts, Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143-0448.

We are studying the molecular mechanism that enables the T4 bacteriophage DNA polymerase holoenzyme to synthesize DNA processively on the leading strand of the replication fork for many minutes, while allowing the same holoenzyme on the lagging strand to recycle from Okazaki to Okazaki fragment in < 4 sec. Like the mammalian delta DNA polymerase and *E. coli* DNA polymerase III holoenzyme, the T4 DNA polymerase holoenzyme contains accessory proteins with DNA-dependent ATPase activity (T4 44/62 protein) that are stimulated by an additional accessory protein (T4 45 protein). These proteins form a sliding clamp for the polymerase, whose assembly and disassembly are regulated to control DNA polymerase action. We have developed a system to measure the dissociation rate of the holoenzyme under conditions that mimic those at the lagging strand of the replication fork. In this system, the holoenzyme is assembled at a primer and then pauses by dCTP omission, after synthesizing 8 nucleotides. All non-engaged DNA polymerase molecules are disabled by the addition of excess competitor DNA and dCTP is added. The polymerase holoenzyme immediately encounters a perfect hairpin helix of 15 base pairs and stalls. Polymerase dissociation is monitored during the stall by the delayed addition at intervals of excess 32 protein, which rapidly melts out the hairpin and allows the polymerase molecules that have not dissociated to continue synthesis to the end of the template. We find that the holoenzyme dissociates with a half-life of < 2 sec after hitting the helix, as required for polymerase recycling during Okazaki fragment synthesis *in vivo*. Further characterization provides details of how the clamp is disassembled.

F 324 DNA SEQUENCE OF THE *CIS*-ACTING GENE *A* AND *ORI* REGION OF BACTERIOPHAGE P2,
Elisabeth Haggård-Ljungquist, Shamol Saha and Yuanfang Liu, Department of Microbial Genetics, Karolinska Institutet, Box 60400, S-10401 Stockholm, Sweden
A P2 replicon has been constructed. It contains the P2 region from 76.7 to 91.5 % (about 4.800 nt) from the left end of the P2 genetic map, including genes *B* and *A*, known to be required for phage DNA replication.
The DNA sequence of the 80.3 to 91.8 % region of phage P2 has been determined. This region contains the early gene *A*, which codes for a *cis* acting protein known to induce a single strand cut at or near *ori*, and the P2 *ori*, which has been located by electron microscopy at 89 % from the left end of the genome. The 80.3 - 91.8 % region has the capacity to code for at least 6 previously unknown polypeptides (*orf1-6*). They are all preceded by a good ribosome binding site and they are expressed in an *in vitro* transcription translation system. *orf1*, *orf2*, *orf3*, *orf4* and *orf5* codes for polypeptides 74, 100, 74, 91 and 109 amino acids long respectively. The *A* gene, located between *orf4* and *orf5*, codes for a 760 amino acids long polypeptide.
The *A* gene has been cloned under the control of a T7 promoter, and after induction the A protein is expressed in large amounts.
There are no mutations known in the possible polypeptides corresponding to *orf1-5*, therefore their functions in the phage cycle are unknown.
However, two of the polypeptides, *orf3* and *orf4*, are very homologous to two polypeptides located in the region required for DNA replication of phage 186.
The DNA sequence of the region supposed to contain P2 *ori*, shows no homology to other known *E. coli ori* sequences and it shows very few secondary structures. However, at 89.? % there is a GC rich inverted repeat, which might be part of *ori*.

F 325 ENZYMATIC SYNTHESIS AND EXONUCLEOLYTIC DEGRADATION OF FLUORESCENT DNA CONTAINING RHODAMINE AND FLUORESCEIN NUCLEOTIDES
Mark L. Hammond, Babetta L. Marrone, Alberto Haces*, and Roger S. Lasken*, Los Alamos National Laboratory, Life Sciences Division and Center for Human Genome Studies, Los Alamos, NM, and * Life Technologies, Inc. (GIBCO BRL), Gaithersburg, MD
A rapid DNA sequencing technique, based on single molecule detection, has been proposed by R. A. Keller et al. (GATA 8(1):1-7, 1991). This technique requires: 1) selection and manipulation of single DNA duplexes 2) fluorescent labeling of the nucleotides in a base specific manner, 3) exonucleolytic digestion of the labeled DNA strand, and 4) detection and identification of the released fluorescent nucleotides in a flowing sample stream. Using Rhodamine-dCTP (Rhod-dCTP) and Fluorescein-dUTP (Fluor-dUTP) analogs, we are synthesizing enzymatically fluorescent DNA molecules and characterizing their exonucleolytic degradation. Although the rate of incorporation of these fluorescent nucleotides by modified T7 or T5 polymerases is 5-10 times slower than that of unmodified nucleotides, Rhod-dCTP or Fluor-dUTP could be incorporated into products greater than 500 nucleotides in length. Competition studies suggest that the fluorescent nucleotides are rapidly inserted, but attenuate subsequent primer elongation. Consistent with this interpretation, pauses in replication correlate well with sites of contiguous fluorescent nucleotide insertion. Six different exonucleases (or polymerases with exonuclease activities) were examined for their abilities to degrade fluorescent DNA substrates. All six exonucleases tested (snake venom phosphodiesterase, Exo III, and the 3'-exonuclease activities of Klenow fragment, native T7 polymerase, T4 polymerase, and E. coli pol III holoenzyme) degraded DNA substrates containing either Rhod-dCTP or Fluor-dUTP. Additional experiments using native T7 polymerase and defined 300-mer DNA substrates containing Rhod-dCTP or Fluor-dUTP indicated a cleavage rate of 5-20 nucleotides/sec (2-5 times slower than unmodified DNA). Ongoing experiments are aimed at 1) synthesizing longer, labeled DNA strands and 2)increasing the rate and determining the processivity of the exonucleases under investigation. This work was conducted under CRADA No. LANL-C-91-001.

F 326 INITIATION OF Ø29 DNA REPLICATION: ACTIVATION BY PROTEIN p6.
José M. Hermoso, Manuel Serrano, Raimundo Freire, Crisanto Gutiérrez and Margarita Salas. *Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain.*

B. subtilis phage Ø29 DNA replication is initiated at either end of its double-stranded DNA molecule by a mechanism in which the viral DNA polymerase catalyzes the formation of the initiation complex, terminal protein-dAMP.
Protein p6 is required *in vivo* for viral DNA replication and activates the initiation reaction in an *in vitro* system with purified proteins. This activation has been related to the formation of a nucleoprotein complex at the replication origins that extends 200-300 bp. Footprinting analysis, as well as restraining of positive supercoiling led us to propose a model in which a dimer of protein p6 binds to 24 bp interacting with the minor groove of DNA and generating a right-handed superhelix in which DNA wraps a multimeric p6 core (1). By deletion analysis, we have located the main p6 recognition regions, that contain predicted bendable sequences with a periodicity of 12 bp (2).
We have constructed head-to-tail concatemers of a p6 binding sequences of 24 bp (dimer binding unit). Protein p6 binds to these sequences in the same position and with higher affinity than to the replication origin. Series of pUC19 derived plasmids containing different number of p6 dimer binding units were used to measure changes in linking number (ΔLk) induced by protein p6. The observed ΔLk/p6 dimer was 0.25 ± 0.09. Formation of p6-DNA complexes in the plasmid at the region containing p6 binding units was confirmed by electron microscopy. In addition, p6-DNA complexes were detected along the Ø29 genome. The minimal size of those complexes was measured by electron microscopy (90 bp) and by resistance to micrococcal nuclease digestion (84 ± 9 bp). This could correspond to the maximun value for one turn of the superhelix stabilized by protein-protein interaction. With these results a refined model of protein p6-DNA complex will be discussed.
The activation of initiation increases as the temperature is lowered, suggesting DNA unwinding is involved, furthermore it requires a precise positioning of protein p6 at the replication origin and the presence of a region containing a polypyrimidine tract.

1.Serrano, M., Salas, M. and Hermoso, J.M. (1990). *Science* **248**, 1012.
2.Serrano, M., Gutierrez, J., Prieto, I., Hermoso, J.M. and Salas M. (1989). *EMBO J.* **8**, 1879.

F 327 DIFFERENTIAL INHIBITION OF DNA TRANSLOCATION AND DNA HELICASE ACTIVITY BY THE *E. COLI* TUS PROTEIN.
Hiroshi Hiasa and Kenneth J. Marians, Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021
Escherichia coli Tus protein bound specifically to a *ter* site is sufficient to arrest the progression of a replication fork. However, little is known about the molecular mechanism of this reaction. We have studied the effect of Tus protein on various DNA helicase activities using a strand displacement assay. When the substrate was a short oligomer hybridized to a circular ssDNA, strand displacement by DnaB, PriA, and the preprimosome, but not UvrD, was blocked by Tus. However, no inhibition of DnaB helicase activity was observed when the substrate was an elongated (250 nt) duplex region hybridized to the ssDNA. With this elongated substrate, both PriA helicase activity, as well as that of the preprimosome in the 3'→5' direction, was partially inhibited. The 5'→3' helicase activity of the preprimosome was almost completely inhibited by Tus with either type of substrate. This likely accounts for Tus-induced arrest of replication fork progression in the pBR322 replication system *in vitro*. These results suggest that while Tus may, in general, be capable of inhibiting in a polar fashion translocation of some proteins along single-stranded DNA, inhibition of *bona fide* DNA helicase activity is more likely to involve specific protein-protein interactions.

F 328 EQUILIBRIUM, KINETIC, AND FOOTPRINTING STUDIES OF THE TUS-*TER* PROTEIN-DNA INTERACTION,
Thomas Hill, Su Wu, Xiaolin Zhang, and Philip Gottlieb, Department of Bioscience and Biotechnology, Drexel University, Philadelphia, PA 19104 and Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716
Arrest of DNA replication in the terminus region of the *E. coli* chromosome is mediated by protein-DNA complexes composed of the Tus protein and nucleotide sequences called *Ter* sites. Using a filter-binding assay, we examined the *in vitro* binding of purified Tus protein to a 37 basepair oligomer containing the chromosomal *TerB* sequence. The measured equilibrium binding constant (K_D) for the *TerB* site was 3.4×10^{-13} M. Kinetic studies revealed that the Tus-*TerB* complex was very stable, with a half-life of 550 minutes, a dissociation rate constant of 2.1×10^{-5} sec^{-1}, and an association rate constant of 1.4×10^{8} $M^{-1}sec^{-1}$. Tus binding to the *TerR2* site of the plasmid R6K showed a 30-fold lower affinity compared to the Tus-*TerB* interaction. We also examined the DNA-protein contacts of Tus bound to the *TerB* sequence. Extensive DNA-protein contacts between the Tus protein and the *TerB* sequence were observed in the highly conserved 11 base-pair "core" sequence common to all identified *Ter* sites. Projection of the footprinting data onto B-form DNA indicated that the majority of the alkylation interference and hydroxyl radical protected sites were arranged on one face of the DNA helix. We also observed DMS protection of two guanine residues on the opposite side of the helix, suggesting that part of the Tus protein extended around the double helix. The distribution of contacts on the *TerB* sequence was asymmetric, consistent with the functional polarity of the Tus-*Ter* complex.

F 329 INTERACTION OF *E. coli* HEAT SHOCK PROTEINS WITH λ P PROTEIN,
Heidi J. Hoffmann, Susan K. Lyman, and Harrison Echols, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

The DnaJ and DnaK heat shock proteins act to release λ P protein from the bacteriophage λ preinitiation complex. This activity may be an example of the general role of heat shock proteins in the assembly and disassembly of multi-protein structures. We have studied the *in vitro* interactions of purified DnaJ and DnaK with purified P protein using glycerol gradient centrifugation. Denatured, but not native P protein showed an interaction with DnaJ. This appears to be a specific interaction, as DnaJ did not interact with denatured λ O protein. These data may indicate that unfolding or partial unfolding of P is a component of the mechanism of release of P from the preinitiation complex.

Excess DnaK showed an interaction with native P, which was abolished in the presence of ATP. This interaction was also seen in crosslinking reactions; the P•K association was enhanced by the presence of ATPγS. When P, DnaK, and DnaJ were mixed in the presence of ATP, all three proteins sedimented more rapidly than when mixed in pairwise combinations. These data indicate that DnaJ and DnaK may interact in the presence of ATP in order to release P from the preinitiation complex, possibly through a P•DnaJ intermediate.

F 330 CHARACTERIZATION OF AN ORIGIN OF DNA REPLICATION (oriK2) THAT FUNCTIONS IN E. coli rnhA MUTANTS LACKING RNASE HI ACTIVITY.
Xiankang Hong, Gregory Cadwell, Tsuneaki Asai, and Tokio Kogoma, Departments of Cell Biology and Microbiolgy, University of New Mexico Medical Center, Albuquerque, NM 87131.

In E. coli rnh mutants, chromosome replication is initiated by a mechanism that involves neither the oriC site or DnaA protein. Thus, rnh mutants can dispense with these two factors essential for the normal initiation of DNA replication. The alternative initiation mechanism requires instead RecA protein, a preprimosome protein (protein i, the product of the dnaT gene) and an event of transcription which is under the stringent control. Initiation can occur in the presence of chloramphenicol (CM) (i.e. stable DNA replication). It begins at several sites (collectively termed oriK) and is random with respect to time in the cell cycle as well as choice of origins. Based on our earlier observation that such a site (R-loop) stimulates homologous recombination, we have isolated several sequences that stimulate integration of a mini-F into the chromosome when replication of the plasmid is inhibited. One of the sequences (oriK2) which maps at 44.3 min on the chromosome has been shown to allow a mini-pSC101 plasmid to replicate in the presence of CM. The recombination-stimulation activity has been localized within a 945-bp region. The determination of the nucleotide sequence has revealed that the region contains an ORF which potentially encodes a hydrophilic protein of 224 amino acid residues and contains a sequence that closely resembles the n′-pasA(ColE1), an n′ protein (PriA) binding site.

F 331 RECOMBINATIONAL HOTSPOT ACTIVITY NEAR DNA REPLICATION TERMINUS SITES IN RNASE H-DEFECTIVE E. COLI MUTANT; TERMINATION EVENT DEPENDENCY.
Takashi Horiuchi, *Hideo Nishitani, Masumi Hidaka and Takehiko Kobayashi *National Institute for Basic Biology*, Okazaki and *Graduate School of Medical Science, Kyushu Univ. Fukuoka, JAPAN
To clone new replication origin(s) activated in an RNase H-defective (rnh^-) *E. coli* mutant, whole chromosomal DNA digested with the *Eco*RI enzyme was ligated with the Km^r DNA fragment, transformed to rnh^- derivative host and from the Km^r transformants, we obtained 8 kinds of plasmid-like DNA, each of which contains a specific DNA fragment, termed "Hot", derived from *E. coli* genome.

Because Hot DNA was unable to be transformed into a mutant strain in which the Hot corresponding region on the chromosome was deleted, the Hot DNA, though obtained as ccc DNA, formed through excision from the host chromosome into which Hot DNA was once integrated, rather than through autonomous replication, thereby suggesting that the Hot fragments are hyper-recombinogenic.

All but one of the Hot DNA fragments locates at DNA replication termination region on the *E. coli* genome. All Hot fragments so far tested have Chi sequence. In 3 Hot groups, introduction of tau^- (tus^-; termination-less) into rnh^- host genome reduces the recovery of Hot plasmid-like DNA to less than 1/10, that is the Hot activity is dependent on termination event *per se.*

From these data and our previous observations that under rnh^- conditions, the DNA replication fork blocked at terminus are accumulated as Y-shaped molecule, we propose a tentative model in which the RecBCD enzyme enters the DNA molecule through the Y-shaped portion at the terminus site, travels to the replicated, branched portion of the Y-shaped molecule, encounters and activates the Chi sequence and homologous recombination is triggered.

F 332 KEY REGULATORY PROTEINS, DnaA AND IciA, INTERACT WITH THE 13-MER ITERONS IN THE REPLICATION ORIGIN OF *ESCHERICHIA COLI*, Deog Su Hwang and Arthur Kornberg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305-5307

Opening of the three tandem repeats (iterons) of a 13-mer in the replication origin (*oriC*) of *E. coli* is a prime event in the replication of minichromosomes [Bramhill, D. and Kornberg, A., Cell 54, 915-918 (1988)]. DnaA protein, the initiator protein, requires proteins HU or IHF, along with a millimolar level of ATP to open the 13-mer region. Negative superhelical density in the plasmid is essential. The extent of opening, as judged by cleavage by a single-strand-specific endonuclease (i.e., P1 nuclease) is closely correlated with replication of the *oriC* plasmid.

We now show by mutational alterations of the 13-mer region that *oriC* function, both *in vivo* and *in vitro*, requires AT-richness in the left 13-mer (L) and sequence specificity in the middle (M) and right (R) 13-mers. Interactions of DnaA protein with the M and R 13-mers are crucial to opening of the region. Binding of the protein to the top strand of the 13-mers appears to maintain the single-strandedness in the bottom strand.

IciA protein, the inhibitor of initiation, binds the three 13-mers and blocks the opening of the region (Hwang, D. S. and Kornberg, A. (1990) Cell 63, 325-331). The degrees of inhibition by IciA protein of 13-mer opening and *oriC* plasmid replication of various mutant forms of the 13-mers are correlated with its binding affinity. Binding of IciA protein to the 13-mers does not affect the binding of DnaA protein to its four 9-mer boxes, but blocks its interaction with the 13-mers. The selective interactions of DnaA and IciA proteins with the 13-mer region appear to be components of the on/off switch of the initiation of *E. coli* chromosomal replication.

F 333 **The pSC101 par locus affects RepA/DnaA protein interactions within the plasmid replication origin.** Hanne Ingmer and Stanley N. Cohen. Department of Genetics, Stanford School of Medicine, Stanford University, Stanford, CA 94305.

The distribution of post-replicative pSC101 molecules to daughter cells at the time of division normally is dependent on the plasmid's *par* locus. However, *repA7* (previously known as *repA**) a mutation in a pSC101 gene that encodes a protein required for the plasmid replication, can overcome the requirement for *par*. Because this mutation leads to overproduction of the *repA* gene product, we tested whether overexpression of wild type RepA, either made in *trans* under the control of an adventitious promoter or made in *cis* by an operator mutant defective in autoregulation by RepA, could stabilize the inheritance of *par* deleted plasmids. The results showed not only a lack of stabilization of par^- pSC101 by overproduced wild type RepA, but inhibition of replication was observed; in contrast, par^+ plasmids replicated normally. The inhibition was reversed by concomitant overproduction of the *E. coli* DnaA protein, implying that RepA and DnaA can compete *in vivo* for sites within the pSC101 origin region. As deletions in the *par* locus affect the *in vivo* footprint at RepA and DnaA binding sites within the origin (H. Ingmer and S.N. Cohen, in preparation), our current results suggest that the *par* locus, which is known to alter DNA superhelicity, may help maintain a DNA conformation that reduces sensitivity of the pSC101 replication origin to fluctuations in cellular concentrations of RepA and DnaA.

F 334 DNAJ AND GRPE HEAT-SHOCK PROTEINS MODULATE THE ACTIVITIES OF DNAK IN THE INTITIATION OF BACTERIOPHAGE λ DNA REPLICATION

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Initiation of bacteriophage lambda DNA replication requires the precise assembly and partial disassembly of a highly ordered multi-protein complex at a specific site on the phage chromosome. The initiation pathway has been well characterized *in vitro* with the development of a reconstituted system consisting of ten highly purified proteins that specifically replicates supercoiled plasmid templates containing the λ-origin of DNA replication (Ori λ). DNA replication, as reconstituted, requires only two phage-encoded proteins, λO and λP, and five host-encoded proteins, DnaB, primase, single stranded binding protein, and DNA polymerase III holoenzyme. Three *E. coli* heat-shock proteins, DnaJ, DnaK, and GrpE also play an essential role in the initiation process. The heat-shock proteins act to partially disassemble a pre-initiation ori λO-P-DnaB nucleoprotein complex which is formed at the origin of DNA replication. This rearrangement results in the liberation of the DnaB helicase from the inhibitory effects of λP. Once freed, DnaB unwinds the DNA and interacts with the rest of the replication proteins required for propagation of the replication fork.

Our goal is to understand how the *E. coli* heat-shock proteins facilitate the activation of DnaB during the initiation of λ DNA replication. We have been attempting to elucidate how the DnaJ and GrpE proteins assist DnaK hsp70 protein function in the activation process. We have discovered that certain small peptides stimulate the ATPase activity of DnaK and that these same peptides effectively inhibit λ DNA replication *in vitro*. The presence of GrpE both (i) partially relieves the inhibitory effects of peptides on DNA replication *in vitro* and (ii) stimulates the peptide dependent ATPase activity of DnaK. In contrast, DnaJ lowers the concentration of peptide required to half maximally saturate the peptide-dependent ATPase reaction. We suggest that DnaJ improves the specificity of DnaK action whereas GrpE stimulates the turnover of DnaK-reaction complexes during initiation of λ DNA replication.

F 335 CHROMOSOME INITIATION IS ACTIVATED IN A *dnaAcos* MUTANT BOTH *IN VIVO* AND *IN VITRO*, Tsutomu Katayama and Arthur Kornberg, Department of Biochemistry, Stanford University, Stanford, CA 94305

DnaA protein plays an essential role in initiation of replication at the chromosomal origin (*oriC*). A *dnaA46* mutant, in which GroELS chaperonins are overproduced, shows cold sensitivity - a failure to grow at 30°C. Chromosomal replication overinitiates at the *oriC* region at 30°C, resulting in an over 6-fold increase of copy number of the region, despite no concomitant protein synthesis[1]. Rather than an increased dosage of DnaA protein, a conformational activation of the mutant DnaA protein by the GroELS proteins is suggested.

In a similar vein, initiation is excessive in a cold-sensitive *dnaAcos* mutant which also fails to grow at 30°C[2]. (The *dnaAcos* gene is an intragenic suppressor-allele of the *dnaA46* mutation[3].) Although the amount of DnaA protein was not elevated relative to the wild-type strain both *in vivo* and in crude extracts, replication of an *oriC* plasmid, dependent on DnaA protein, was at least ten times as great in extracts of the mutant compared to the wild-type. Thus, initiation by the DnaAcos protein appears to be highly activated *in vivo* and *in vitro*. The nature of this activation is under study.

1) Katayama,T and Nagata,T (1991) MGG 226: 491-502
2) Kellenberger-Gujer,G et al. (1978) MGG 162: 9-16
3) Braun,R et al. (1987) J. Bacteriol 169: 3898-3903

F 336 THE E. *coli* HEAT SHOCK PROTEIN DnaK IS INVOLVED IN PSC101 PLASMID REPLICATION,

France Keppel, Danièle Rifat and Lucien Caro, Department of Molecular Biology, University of Geneva, 1211 Geneva 4, Switzerland

The plasmid pSC101 encodes an autoregulated protein, RepA, which is essential for its replication. In *E. coli*, it also requires DnaA, DnaB, IHF and DNA gyrase. To identify new bacterial functions involved in pSC101 replication, we investigated the heat shock proteins DnaK, DnaJ and grpE which are necessary for phage lambda, P1 plasmid and miniF duplication. Transformation experiments in strains defective in dnaK, dnaJ or grpE showed that pSC101 can replicate in these mutants. However, the stability of pSC101 was reduced in the mutant strain DnaK 103 whereas no segregation was detected in dnaJ- and grpE- strains. Bandshift experiments were performed with total proteins extracted either from wild type or DnaK mutant strains transformed with a plasmid expressing RepA under its own promoter. The results indicate that DnaK is involved in the binding of RepA to the repeated sequences located at the replication origin but not in the binding of RepA to its promoter. Thus, DnaK may play a role in the initiation of pSC101 replication without affecting the autoregulation of the initiator protein.

F 337 MAPPING OF THE *IN VIVO* START SITE FOR LEADING STRAND DNA SYNTHESIS IN THE PLASMID R1 REPLICON,

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We have previously constructed *intR1* strains, in which an R1 plasmid is integrated into the origin of chromosome replication, *oriC*, of the *Escherichia coli* chromosome. In such strains, *oriC* is inactive and chromosome replication is instead controlled by the unidirectional R1 replicon. Due to the large size of the chromosome, replication intermediates generated at the R1 origin in these strains are considerably more long-lived than those of unintegrated R1 plasmids. Here, we have used this property of the system to map the free 5' DNA ends of the replication intermediates that are formed during R1 replication *in vivo*. The mapping was performed by primer extension analysis on total DNA isolated from exponentially growing and stationary phase *intR1* strains. The sensitivity of the mapping was considerably increased when cycles of repeated primer extensions (RPE) were performed, using a polymerase chain reaction (PCR) protocol.

The mapped free 5' DNA ends were assumed to represent normal *in vivo* start sites for leading strand DNA synthesis in plasmid R1, and the positions of the sites are in good agreement with *in vitro* results from other laboratories. The same start positions were utilized also in the absence of the DnaA protein, indicating that DnaA does not participate in the determination of the exact position where DNA synthesis starts during initiation of replication at the R1 origin.

F 338 ACTIVITY OF THE *BACILLUS SUBTILIS dnaB* OPERON AND SOME PROPERTIES OF THE *dnaB* PROTEIN.

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The *dnaB* gene of *Bacillus subtilis* is essential for initiation of DNA replication and salt-resistant membrane binding of the chromosome and some plasmids like pUB110. Northern blot analysis shows that the *dnaB* gene is transcribed as an operon with two other genes, ORF-Y and *dnaZ*. Using an *in vitro* DNA replication system which is derived from an extracted DNA/membrane complex that demonstrates DNA replication (initiation, elongation and repair) without adding exogenous enzymes or template DNA (only nucleotides and cofactors are added), we can demonstrate that the *dnaZ* and the *dnaB* proteins are involved in initiation. While the function of ORF-Y is unknown, amino acid sequence comparison reveals that the ORF-Y protein may function like the *E. coli dnaC* protein - assisting in DNA-protein binding.

The *dnaB* gene has been cloned separately into the inducible expression vector pGEX-2T (Pharmacia). We are studying the purified over-expressed protein. *DnaB* seems to be a very strong non-specific single-stranded DNA binding protein. The binding is very salt resistant. This is consistent with earlier observations that the *dnaB* product is required for salt resistant binding of DNA to the membrane. The protein *in vivo* appears to be translated as an inactive 55 kD protein then processed into an active form of approximately 37 kD. The expressed protein also under goes this cleavage. The Bacillus *dnaB* protein appears to be unrelated to the *E. coli dnaB* helicase.

F 339 THE PHAGE P4 GENE α PRODUCT IS A MULTI-FUNCTIONAL INITIATOR PROTEIN,

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The α gene product (M_r = 84,841 Da) of the satellite phage P4 of *E.coli* known as the major phage-specified replication protein (Krevolin, M. D. & R. Calendar [1985] *J. Mol. Biol.* **182**, 509-517) was overproduced using a *Ptac/lacI*^{Q} expression vector and purified. P4α-associated activities include: I) sequence specific binding ability of *ori* and *crr* DNA, II) initiation of complementary strand synthesis on ssDNA of small phages and III) strand separation of duplex DNA. Analyses of P4α-DNA complexes by gel electrophoresis and electron microscopy indicated that *ori* type I repeats (TGTTCACC, ref.: Flensburg, J. & R. Calendar [1987] *J. Mol. Biol.* **195**, 439-445) are recognized which occur in P4 DNA six times in the *ori* and ten times in the *crr* region as direct and inverted repetitions. In addition, the α-protein possesses a DNA primase activity resembling the activity of primases encoded by conjugative plasmids. Purified α-protein catalyzes in a rifampicin resistant reaction uncoupled from DNA synthesis ssDNA dependent incorporation of NTPs and dNTPs into short oligonucleotide primers. This finding is paralleled by a significant sequence similarity between primases encoded by conjugative plasmids RP4(IncPα), R751(IncPβ) and ColIb-P9 (IncI1) and the P4 α protein. Site-specific mutagenesis of the sequence motif -EGYATA-, common to three conjugative primases and P4α, demonstrated the functional relevance of the motif for the priming activity. Two amino acid replacements (Y $\rightarrow$ F, T $\rightarrow$ S) abolish or strongly reduce the specific activity, whereas an E $\rightarrow$ Q change increases or leaves the activity unaltered. Another sequence motif found in P4α, a type A NTP binding site, is probably essential to a strand separation activity on duplex DNA. The helicase activity is fueled by nucleoside triphosphate hydrolysis accepting all NTPs and dNTPs as substrates except UTP and dTTP. The enzyme unwinds preferentially tailed DNA duplex molecules in a 3' $\rightarrow$ 5' direction and also separates strands of blunt end DNA fragments but less efficiently. Thus, the P4α protein combines in a single polypeptide chain at least three activities which are needed for initiation of DNA replication. These functions are likely to account for P4 replicating independently of *E.coli* genes *dnaA*, *dnaB*, *dnaC* and *dnaG*.

F 340 REPLICATION DEFECTS in *priA* MUTANTS of *E. COLI* LACKING or DEFICIENT in the PRIMOSOMAL REPLICATION PROTEIN, Eui Hum Lee and Arthur Kornberg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305-5307.
The *priA* gene, which encodes PriA protein essential for the replication of phage ϕX174 and ColE1-like plasmids *in vitro*, has been cloned and overexpressed (E. H. Lee *et al.* (1990) Proc. Natl. Acad. Sci. USA 87, 4620-4624). The null *priA* mutant shows filamentous morphology, failure to support ϕX phage growth, and inability to maintain plasmids with ColE1, *oriC*, or R1 replication origins (E. H. Lee and A. Kornberg (1991) Proc. Natl Acad. Sci. USA 88, 3029-3032). We have confirmed the role of PriA protein in maintenance of ColE1 and *oriC* plasmids by employing a *priA*-encoding plasmid (pSC101ts-*priA*) with a temperature-sensitive replicon. The copy numbers of ColE1 and *oriC* plasmids were decreased by 10^3 to 10^5 when this pSC101ts-*priA* plasmid was cotransformed into a *priA*-null-mutant strain at a restrictive temperature. Availability of *priA* mutants, temperature-sensitive in the structural gene, should further clarify the role of PriA protein in chromosomal as well as plasmid DNA replication.

F 341 APPARENT TERMINATOR DEGENERACY IN REPLICATION FORK ARREST IN *BACILLUS SUBTILIS*, Peter Lewis, Mark Smith, Carmel Carrigan, Robert Pack and Gerry Wake, Department of Biochemistry, The University of Sydney, Sydney, N.S.W., 2006, Australia.
Termination of chromosome replication in *Bacillus subtilis* occurs at a fixed site known as *terC* which is situated approximately opposite *oriC* on the circular chromosome. The *terC* region is characterised by a large imperfect inverted repeat region (IRR) comprising IR I (47 bp) and IR II (48 bp) just upstream of a gene, *rtp*, which encodes the IRR-binding replication terminator protein. A model has been proposed in which RTP bound to specific sites within the IRR is able to effect replication fork arrest through the polar inhibition of the activity of the major replicative helicase situated at the apex of a replication fork. In support of this model, preliminary experiments using DnaB, the major replicative helicase from *Escherichia coli*, indicate that RTP can inhibit DnaB activity when approached from one direction, but not the other. Work is currently under way to identify and isolate the major replicative helicase from *B. subtilis* for more extensive experiments.

The proposed model involves RTP binding to both IR I and IR II and implies that replication fork arrest would be observed no matter what the orientation, or position, of *terC* in the chromosome. This was found not to be so. On relocation of *terC* to two chromsomal sites, the *pyr* gene cluster (139°) and *metD* (100°), fork arrest was only observed when *terC* was oriented such that IR I was operative. *terC*-containing plasmids, derived from the unidirectionally replicating plasmid pIL253 have also been used to study the activity of IR I and IR II *in vivo*. Assays of *terC* activity of these plasmids in cells in which *rtp* expression was constitutive, indicated that both IR I and IR II were active (fork arrest was observed for both orientations of the *terC* region), but the level of fork arrest was much less for IR II than for IR I. This would suggest that IR II is a weak terminator which may normally be inactive in cells where the level of RTP may be too low for binding to this region. It is interesting to note that the *terC* region is slightly offset in the chromosome to a position that would require only IR I to function *in vivo*.

F 342 INITIATION OF PHAGE λ REPLICATION: ADDITION OF DnaJ TO THE OPB STRUCTURE. Susan K. Lyman, Heidi J. Hoffmann, and Harrison Echols, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

We are studying the initiation of replication of phage λ in its host *E. coli*. Initiation at oriλ occurs through the formation of a specialized nucleoprotein structure which arises by the ordered addition of several phage and host proteins. First, the initiation protein λO binds to oriλ to generate the looped O-some structure. The λP protein binds the host dnaB helicase, and the λP•DnaB complex associates with the O-some, resulting in the very stable OPB•oriλ structure. However, the helicase is unable to function in the grip of λP, so the next step in initiation utilizes the host heat shock proteins DnaJ, DnaK, and GrpE to partially disassemble the OPB structure. This disassembly step results in the freeing of the helicase to allow for productive initiation.

We have been examining the protein-protein interactions involved in the action of the heat shock proteins using both physical and biochemical methods. DnaJ associates with the OPB•oriλ structure. In a two protein system, DnaJ stimulates the DNA-dependent ATPase activity of DnaB, suggesting a functional interaction between the two proteins. Analysis by glycerol gradient sedimentation indicates an interaction between DnaJ and the λP•DnaB complex that is not found for λP and DnaJ alone. Thus, the ordered addition of DnaJ to the origin nucleoprotein complex is likely to involve an interaction with DnaB. Similar conclusions have been derived from other approaches used by Georgopoulos, Zylicz, *et al.*

F 343 THE INTERACTION OF THE INITIATOR PROTEIN REPA1 WITH THE ORIGIN OF REPLICATION OF THE INCFII-TYPE REPLICON REPFIC. Renata Maas, Peter L. Bergquist and W.K. Maas, Department of Microbiology, New York University School of Medicine, New York, NY 10016.
RepFIC is a basic replicon about 3 kb long which is widely distributed among IncF plasmids. It codes for the RepA1 protein which is required for initiation, for regulatory genes that are involved in the regulation of both the transcription and translation of the *repA1* gene and for an origin of replication on which the RepA1 protein presumably acts. The initiator gene has been cloned separately and overexpressed. Its product is a soluble protein of MW 40,000. It appears to act more efficiently on a *cis*- than on a *trans*-origin. Furthermore, when the continuity of the replicon is interrupted with a 2 kb insertion between the initiator gene and the origin, the efficiency of replication as measured by transformation into a *polA* recipient (RepFIC is *polA* independent) is very much diminished. Thus the initiator functions efficiently only when the continuity of the RepFIC replicon is maintained.

F 344 STUDY OF FUNCTIONAL DOMAINS IN THE REPA PROTEIN OF PLASMID pSC101.

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The origin of replication of plasmid pSC101 contains multiple binding sites for essential proteins such as plasmid encoded RepA protein, and the host encoded proteins DnaA and IHF. The pSC101 replication protein RepA is auto-regulated by binding to a palindromic sequence, IR, in the promoter region, thus regulating its own transcription. RepA also binds, upstream of its promoter, in the *ori* region, to three directly repeated sequences, RS1, RS2, RS3, which are partially homologous to its self regulation binding site. The simultaneous binding of RepA, IHF, and DnaA to their respective site may lead to the formation of a replisome. The entire coding sequence of the protein is required for overall activity. The aim of the project is to study functional domains in the RepA protein. To this end, we have constructed series of 3' end and 5' deletions. We monitored their ability to bind to the inverted repeat and to the direct repeats by bandshift assay. We find that there is a difference between the binding to the repeated sequences and that to the IR site. The last 3' end 90 aa are not needed for binding to any the sites. The 5' end deletion of only 8 aa destroys binding to the direct repeated sequences while a 5' end deletion of 12 aa still binds to the inverted repeat (i.e. the site of autoregulation of RepA).

F 345 THE C. crescentus ORIGIN OF DNA REPLICATION AND dnaA GENE, Gregory T. Marczynski, Gary Zweig, and Lucy Shapiro, Dept. of Developmental Biology, Stanford University Medical Center, Stanford, CA 94305.
C. crescentus cell division is asymmetric and yields swarmer and stalked cell progeny. Only the stalked cell initiates chromosomal replication. To understand this developmental control of replication, we localized, cloned, and sequenced the C. crescentus origin of DNA replication and its homologue of the dnaA gene. A plasmid that employs only the C. crescentus origin of DNA replication has a cell cycle DNA synthesis pattern that resembles that of the bona fide chromosome suggesting that cis-acting control elements are linked to the origin. Deletion analysis determined that the autonomous replication sequences are contained within a 450 to 700 bp length of DNA. This DNA contains sequences that are common to other bacterial origins, such as DnaA boxes and an A+T-rich region. Point mutations in the DnaA box or deletions inside the A+T-rich region abolish replication. However, a "13-mer" replication motif is dispensable in C. crescentus. This origin also requires unique motifs: Six (AAGCCCGG) motifs and five (GTTAA-N7-TTAA) motifs are also required for replication function. C. crescentus homologues of the E. coli dnaK, dnaJ, hemE, and dnaA genes are adjacent to the origin. S1 nuclease protection assays mapped the start of the dnaA mRNA and showed that its transcription peaks during DNA replication. Future studies will address the biochemical basis of asymmetric replication control.

F 346 THE EFFECT OF GrpE HEAT SHOCK PROTEIN ON THE ATPASE ACTIVITY OF DnaK AND ITS CATALYTIC FUNCTION IN BACTERIOPHAGE λ DNA REPLICATION, Andrew F. Mehl and Roger McMacken, Department of Biochemistry, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD 21205
GrpE, DnaK, and DnaJ, three *E. coli* heat shock proteins, are required for replication of bacteriophage λ DNA *in vivo*. *In vitro* DnaK and DnaJ are absolutely essential, whereas GrpE functions to lower the amount of DnaK needed for replication of plasmids containing a λ replication origin (oriλ). Our goal is to understand the effect of GrpE on the intrinsic ATPase activity of the DnaK hsp70 protein and, subsequently, to define its role in λ replication. The grpE gene was amplified by PCR from the *E. coli* genome and cloned under the control of a heat-inducible promoter and then transformed into a DnaK mutant strain. We have developed a simple, high-yield purification protocol for GrpE. The purified GrpE protein acts in a catalytic manner in the *in vitro* DNA replication assay, and the active form is a dimer as determined by crosslinking studies. Investigation of the energetics of the ATPase reaction of DnaK indicate that GrpE increases the off rate of ADP by 3-fold and that of ATP by 10-fold. In contrast, DnaJ increases the rate of the chemistry step (DnaK·ATP ⇒ DnaK·ADP) by as much as 50-fold. GrpE and DnaJ, by increasing the overall rate of ATP hydrolysis, presumably enhance the rate at which DnaK can carry out its function of disassembling the preinitiation nucleoprotein complex formed at oriλ.

F 347 PROTEIN, DNA, AND NUCLEOTIDE REQUIREMENTS OF BACTERIOPHAGE T7 GENE 4 PRIMASE. Lynn V. Mendelman, Stephen M. Notarnicola, and Charles C. Richardson. Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.

Gene 4 of bacteriophage T7 encodes two co-linear polypeptides of 56- and 63-kDa. The 56-kDa gene 4 protein catalyzes unidirectional translocation on single-stranded DNA and has helicase activity. Both of the 56-kDa protein activities are coupled to the hydrolysis of dTTP. Purified 63-kDa gene 4 protein has both helicase and primase activities. Optimal primase activity, however, requires the presence of the 56-kDa gene 4 protein. Primase activity of the 63-kDa gene 4 protein has been examined using synthetic oligodeoxynucleotide templates containing single primase recognition sequences. Point mutations in the primase recognition sequence show that a three base sequence, 3'-CTG-5', is necessary and sufficient to support synthesis of pppAC dimers. Hydrolysis of dTTP is also required for oligoribonucleotide synthesis. The 56-kDa gene 4 protein has two effects on primer synthesis by 63-kDa gene 4 protein. It increases the total number of oligoribonucleotides synthesized by 63-kDa gene 4 protein and it increases the ratio of tetraribonucleotides to diribonucleotides; only the former are functional as primers for T7 DNA polymerase. Results from *in vivo* experiments further define the roles of the 63-kDa and 56-kDa proteins during T7 DNA replication. *E. coli* strains that express either 63-kDa gene 4 protein, 56-kDa gene 4 protein, or site-directed mutants of the two gene 4 proteins were tested for their ability to complement and support the growth of a T7 phage lacking gene 4. It appears that the 63-kDa gene 4 protein can catalyze both primase and helicase activities during T7 growth, but the rate of DNA synthesis is increased by the presence of 56-kDa gene 4 protein.

F 348 DNA-DEPENDENT ATPASE A IS THE EUKARYOTIC ANALOG OF THE BACTERIOPHAGE T4 GENE 44 PROTEIN.

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Monoclonal Antibodies (MAbs) have been developed against calf thymus DNA-dependent ATPase A. The MAbs react with antigens from bacteriophage T4, *E. coli*, *S. cerevisiae*, *Xenopus laevis*, two Chinese hamster cell lines and HeLa cells. The bacteriophage T4 antigen has been identified as the gene 44 protein, which is a DNA-dependent ATPase involved in T4 DNA replication. Both the gene 44 protein and ATPase A exhibit preferential use of primer-template junctions as effectors for ATP hydrolysis, suggesting common structural elements for nucleotide recognition. At least 12 of our MAbs recognize unique epitopes that exist in both ATPase A and the gene 44 protein. The identification of epitopes common to both ATPase A and the gene 44 protein supports our earlier proposal that ATPase A is the eukaryotic analogue of this bacteriophage T4 protein.

F 349 STRAND-DISPLACEMENT SYNTHESIS BY THE BACTERIOPHAGE T4 *ts*L141 ANTIMUTATOR DNA POLYMERASE IS DEPENDENT ON THE T4 GENE 59 PROTEIN,

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The *ts*L141 and *ts*CB120 T4 polymerase mutants were originally characterized as temperature sensitive for DNA replication and as antimutators at the permissive temperature. Both of these mutants are changed at a position (A737V) which is C-terminal to the region where T4 shares homology with the polymerase α family, and may be in a domain important for polymerase interactions with other replication proteins. Reha-Krantz and her co-workers have identified second site mutations within the polymerase that suppress the antimutator phenotype of *ts*L141, one of which is L771F (J. Cell. Biochem. **13D** 140 (1989)). We have purified the *ts*L141, L771F , and double mutant polymerases from plasmids constructed by site directed mutagenesis of the cloned gene.

The *ts*L141 polymerase is stimulated by the 44/62 and 45 accessory proteins and 32 DNA binding protein, but is arrested at pause sites much more frequently than the wild type on single-stranded DNA templates. The L771F mutant resembles the wild type, and the double mutant is arrested much less than the *ts*L141 polymerase. The *ts*L141 polymerase is almost completely unable to carry out strand displacement synthesis on a forked DNA template with the accessory proteins, 32 protein, and the 41 protein helicase, even at the permissive temperature. Hacker, Barry, and Alberts (J. Cell. Biochem. **13D** 87 (1989)) have shown that the T4 gene 59 protein stimulates the primase and helicase activities of the 61 and 41 proteins. We find that addition of the 59 protein is absolutely required for strand displacement synthesis with the L141 polymerase.

F 350 REQUIREMENTS FOR PROPER INTERACTION OF THE RK2 INITIATION REPLICATION PROTEIN WITH THE PLASMID ORIGIN REPEATS;

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The plasmid-encoded TrfA proteins are required for replication of the broad host range plasmid RK2. Using gel mobility shift and DNaseI footprinting we have previously shown that highly purified TrfA proteins bind specifically to the cluster of five repeats (iterons) contained in the minimal origin of replication. These repeats are 17 bp long and are spaced by 5 or 6 nonconserved base pairs. Earlier studies of the affinity of TrfA proteins for the monomer and the dimer forms of an origin repeat and of stability of the corresponding complexes revealed that the TrfA protein had a lower affinity for the monomer 17 bp form than for the dimer containing a spacer sequence. These results could be due to interactions between proteins or iterons in the formation of stable complexes and/or to requirements for specific sequences adjacent to the iterons. The present work analyzes these possibilities *in vivo* and *in vitro*. For this purpose a series of dimer forms of the origin repeats have been constructed in which the spacer region and the relative orientation of the iterons were varied. Furthermore, using oligo-directed site-specific mutagenesis the adjacent and not highly conserved bases have been mutated. The effect of all changes has been analyzed *in vitro* with techniques such as gel mobility shift and footprinting, and *in vivo* by incompatibility studies using an active RK2 origin. Our findings indicate a requirement for the nonconserved bases adjacent to the 17 bp repeat for proper TrfA binding to an origin iteron.

F 351 DNA METABOLISM IN *E. coli* CELLS DEPENDENT ON THE F FACTOR SINGLE-STANDED-DNA-BINDING PROTEIN,

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A variety of large, self-transmissible plasmids carry genes encoding single-stranded-DNA-binding proteins (SSBs). Portions of these plasmid-encoded SSBs show strong amino acid sequence homology with the SSB encoded by the essential *E. coli ssb* gene while other portions are quite different. There are also both differences and similarities in their *in vitro* ssDNA binding properties as compared to the chromosome-encoded SSB. In earlier work, it was demonstrated that the plasmid-borne *ssf* gene of the *E. coli* F factor could support the viability of an *E. coli* strain with a deletion of its chromosomal *ssb* gene. This *ssf*-dependent strain appears to have altered DNA replication properties in that the cells grow more slowly and show a tendency to filament.

Although *ssf*-dependent cells appear to have altered DNA replication properties, both the frequency and the linkage relationships of recombination occurring subsequent to Hfr conjugation are unaffected by *ssf* dependence. At the same time, *ssf* dependence results in a greatly increased sensitivity of the cells to UV irradiation. The effect of *lexA3* on filamentation and UV sensitivity will be reported as well as other investigations of the effects of *ssf*-dependence on DNA metabolism.

F 352 MAPPING dnaB HELICASE ACTIVE SITES USING ts dnaB MUTANTS. Daman Saluja and G. Nigel Godson, Department of Biochemistry, New York University Medical Center, New York, NY 10016

dnaB protein, a DNA dependent helicase, is essential for the replication of the bacterial chromosome. A hexamer of dnaB subunits interacts with a number of other proteins required for DNA replication. Using PCR the dnaB gene from the classical ts dnaB mutants PC8 (dnaB8), RS162 (dnaB252), CR34/454 (dnaB454) and CR34/43 (dnaB43) have been isolated and cloned into the T7 expression vector pET-11c. The mutant proteins have been overexpressed, purified and compared for activities in a) the general priming pRNA synthesis reaction using primase and ssDNA fragments, b) ATPase activity and c) subunit assembly at 30°C and 42°C. The mutants fall into three groups. All except dnaB252 synthesise negligible pRNA at 42° C and do not have ATPase activity at 42°C. dnaB252 protein is as active as wt dnaB protein in pRNA synthesis and ATPase activity at 42°C. The other three mutant proteins could be further subdivided into two groups, (dnaB8 and dnaB454) and (dnaB43) respectively, based on relative temperature sensitivity. The mutations are being mapped by DNA sequencing. The pET-vectors even in the absence of T7 polymerase transcribe sufficient cloned insert RNA from cryptic promoter in the tet gene, to allow genetic complementation (Godson, GENE (1991) 100:59-64). This system has been used to check genetic complementation of the dnaB alleles. dnaB252 complements dnaB43 indicating that the two mutations are in different domains of the protein and suggests that the active dnaB hexamer must be a mixed hexamer. The ts dnaB genes from PC6 (dnaB6), CR34/500 (dnaB500), E107 (dnaB107), CR34/313 (dnaB313), FA22 (dnaB22) and FA2104-1 (dnaB2) are also being isolated, overexpressed and tested for functional activity.

F 353 *ESCHERICHIA COLI* BACTERIOPHAGE PRD1 PROTEIN-PRIMED DNA REPLICATION WITH PURIFIED PROTEINS, Harri Savilahti, Javier Caldentey, Tiina M. Pakula and Dennis H. Bamford, Department of Genetics, Arkadiankatu 7, University of Helsinki, 00100 Finland

Bacteriophage PRD1 is a member of a close group of bacteriophages infecting gram-negative bacteria harboring P, N or W incompatibility group plasmids. The host spectrum includes *E. coli* and *S. typhimurium*. The phage linear dsDNA genome is 14925 bp long and contains 110 bp inverted terminal repeats as well as a 5'-covalently linked terminal protein at each end. The replication of the genome is protein-primed similarly to that of adenovirus and bacteriophage φ29. During initiation of replication the phage-specific DNA-polymerase (P1) catalyses the linking of dGMP into a Tyr-residue in the terminal protein (P8). Chain elongation proceeds from this initiation complex and simultaneously the parental strand of the same polarity is displaced.

Four early genes putatively involved in PRD1 replication have been identified. We have overexpressed these genes in *E. coli* and purified the corresponding proteins (P1, P8, P12 and P19). An *in vitro* minimal replication system has been constructed using P1 and P8. The role in DNA replication of the other two PRD1 early proteins having DNA binding properties has also been studied. This is the first protein-primed DNA replication system originally operating in *E. coli* that is available *in vitro*. Since the genetic background of *E. coli* is well established and, in addition, many DNA replication mutants as well as purified replication proteins are available, the PRD1 system offers a good opportunity to study the protein-primed DNA replication.

F 354 IN SEARCH OF REGULATORY PROTEINS AT oriC: THE 35 kD AND FIS PROTEINS, Kirsten Skarstad, Beat Thöny, Deog Su Hwang and Arthur Kornberg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305, USA and Department of Biophysics, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway.

The initiation of chromosomal replication is a key control point in the cell cycle and is likely influenced by many regulatory proteins and DNA elements. Thus, the origin region of E. coli was probed for binding proteins by the aid of gelshift assays. Two proteins of molecular weights 35 kD and 12 kD were identified and purified. The 35 kD protein bound specifically to the right border of oriC, next to one of the binding sites of the initiator protein, DnaA (Fig. 1). Around 5000 monomers of the 35 kD protein were present per cell. The 12 kD protein bound nearer the center of oriC and appears to be identical to FIS (factor for inversion stimulation).

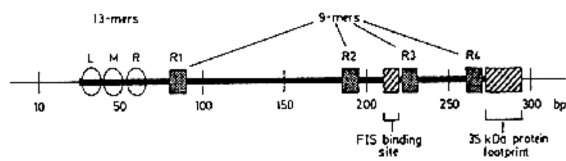

Figure 1: The E. coli chromosomal origin of replication, oriC, with the four DnaA binding sites (R1 to R4) and the AT-rich region with three 13-mers (L, M and R) which are melted by the DnaA protein. The binding sites of the 35 kD and the FIS proteins are indicated.

F 355 MUTATIONS IN THE SUPPOSED ATP-BINDING REGION OF THE *E. coli* DNAA PROTEIN. **Ole Skovgaard,** Institute of Life Science and Chemistry, University of Roskilde, Denmark and **Andrew Wright,** Department of Molecular Biology and Microbiology, Tufts Medical School, Boston, MA, USA.

The DnaA protein is essential for and has a regulatory role in *oriC* specific initiation of DNA replication in *E. coli*. The DnaA protein recognizes a specific DNA sequence, a DnaA-box. This box is found four times in the minimally *oriC*. Binding of DnaA protein to these boxes probably melts an AT rich region close to *oriC*, what allows the DnaB helicase to enter and hereafter are the replication complexes assembled. Some "small" replicons, like P1 and mini-F, and some transposons also depend on the DnaA protein for replication and transposition, respectively.

A consensus ATP binding site was found in the DnaA protein by a database search [1]. Purified DnaA protein binds ATP and ADP with a high affinity [2]. DnaA protein free of nucleotide binds to DnaA-boxes, whereas the ATP form is required for complete initiation [3].

Three mutants of the *E. coli dnaA* gene has been constructed by "oligo-directed" mutagenesis in this consensus sequence. These mutant genes were tested for their ability to complement a chromosomal *dnaA46*(Ts) mutation, to support *in vivo* replication of P1, mini-F and *oriC* and to perform auto-repression.

Even though the three mutants behaves different it is concluded that the requirements to the DnaA protein for replication of P1 are less than for replication of mini-F and *oriC*. Whether this difference is due to ATP binding or other changes caused by the mutations must await characterization of purified mutant proteins. Meanwhile it has been observed that the ADP form of the DnaA protein supports *in vitro* replication of P1, but not of *oriC* [4].

1. D.C. Fry, S.A. Kuby & A.S. Mildvan (1986) Procl. Natl Acad. Sci. USA 83:907-911.
2. K. Sekimuzu, D. Bramhill & A. Kornberg (1987) Cell 50:259-265.
3. D. Bramhill & A. Kornberg (1988) Cell 52:743-755.
4. S. Wickner, J. Hoskins, D. Chattoraj & K McKenney (1990) J. Biol. Chem. 265:11622-11627.

F 356 CLONING, OVERPRODUCTION, AND PURIFICATION OF THE θ SUBUNIT OF E. COLI DNA POLYMERASE III HOLOENZYME. Patricia S. Studwell-Vaughan, Ziming Dong and Mike O'Donnell. Howard Hughes Medical Institute, Department of Microbiology, Cornell University Medical College, New York, NY 10021

DNA polymerase III holoenzyme (holoenzyme) is the ten subunit replicase of E. coli. The function of the the 10 kilodalton θ subunit of holoenzyme is not known but θ can be isolated in a core polymerase complex with the essential α (polymerase) and ε (3'-5' exonuclease) subunits. θ from this core subassembly was used for N-terminal sequence analysis and 40 amino acids were identified. This sequence allowed synthesis of synthetic oligonucleotide probes and subsequent cloning of the θ gene. Using the Kohara ordered library of E. coli[1], we were able to map the θ gene on the E. coli chromosome.

The θ gene was subjected to site directed mutagenesis in order to put it into a pET3C expression vector. The θ protein was overproduced in E. coli to approximately 5% of total soluble protein and subsequently purified to apparent homogeneity using FPLC and conventional chromotography. Using the purified protein we are examining the subunit contacts of θ within holoenzyme and the role of the θ subunit in reconstituted holoenzyme activity.

[1] Kohara, Y., Akiyama, K., and Isono, K. (1987) Cell 50:495-508.

F 357 INTERACTION OF E. Coli SSB WITH PRIMASE DURING pRNA SYNTHESIS ON G4ori_c AND ssDNA. Wu-liang Sun and G. Nigel Godson, Biochemistry Department, NYU Medical Center, New York, NY 10016

Using small ssDNA fragments ranging in size from 21 to 383 nucleotides, the interaction of SSB with the specific primase origin G4ori_c and with random control ssDNA under conditions of pRNA synthesis has been investigated. Using gel shift technology with either [^{32}P] labelled ssDNA or [^{35}S] labelled SSB followed by DNAseI digestion of the intermediate gel shift species, it has been shown that SSB interacts with random ssDNA in steps, sequentially assembling SSB tetramers that bind to approximately 65 nt stretches of DNA and leaving 34 nt linkers between SSB octomers exactly as the model proposed by Chrysogelos and Griffith (PSNAS 69:5803, 1982). The 278 nt G4ori_c ssDNA fragment binds SSB in gel shift steps exactly as a random 285 nt fragment of ssDNA. However, DNAseI digestion of each gel shift step indicates that a core of about 100 nt probably containing the hairpin loops is protected by one SSB tetramer, not 65 nt as in the control. Using a smaller nt core G4ori_c fragment consisting of mainly hairpin looped DNA, similar results aree obtained. Adding primase to the SSB coated G4ori_c results in a further gel shift and pRNA synthesis. The G4ori_c has to be completely coated with SSB before pRNA synthesis can take place. Primase, however, appears to interact with SSB not DNA as it also gives a second gel shift in the control SSB coated random ss DNA (no pRNA synthesis). Preincubation of primase with free SSB also inhibits its activity to bind SSB coated G4ori_c. T4 gene32 protein or other control proteins do not interact with primase. A model is proposed to suggest that the double stranded sequences of G4ori_c function to define the phasing of SSB octomers in order to leave CTG pRNA priming site uncoated . Deletion mutants of primase are being studied for their effect on this interaction.

F 358 STABILITY OF THE INITIATOR PROTEIN AND INHERITANCE OF THE REPLICATION COMPLEX IN λ PLASMIDS, Karol Taylor, Grzegorz Wegrzyn, Alicja Pawlowicz and Elzbieta Kwasnik, Department of Molecular Biology, Universitiy of Gdansk, Poland.

Amino acid starvation should inhibit λ plasmid replication due to the inhibition of protein synthesis. We found, however, that λ plasmid replication proceeds for hours in an aa-starved relaxed mutant, whereas it is inhibited in the stringent partner. λ plasmid replication in aa-starved relaxed cells reveals an absolute λO initiator dependence, although there is no λO synthesis in these conditions. Detecting ^{35}S-λO by immunoprecipitation we confirmed the rapid decay of λO, but we found a fraction resistant to proteolysis; we presume that it represents λO enclosed in the replication complex. Stable λO was present in λ phage-infected as well as in λ plasmid-harboring cells; but it was absent from cells that were UV-irradiated before infection. Kinetic studies of plasmid DNA synthesis and amplification reveal that the number of replication complexes remains constant during starvation. The results of DNA density shift experiments are compatible with a model assuming that only one of two plasmid copies synthesized before the onset of starvation operates in the first round of replication. λ plasmid replication perpetuated by the once assembled replication complex is rifampicin sensitive. Since *cro* repression does not function in this system, transcriptional activation of ori λ seems to be the only initiation controlling event.

F 359 A NOVEL ORIGIN BINDING PROTEIN (35 kDa) POTENTIALLY INVOLVED IN REGULATING THE *E. coli oriC* FUNCTION, Beat Thöny, Kirsten Skarstad and Arthur Kornberg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305-5307.

Initiation of chromosomal replication in *E. coli* is localized at a 245-bp sequence (*oriC*) (Fig. 1). Within this sequence, several highly conserved and biologically essential regions have been identified: (1) four 9-mer binding sites for the DnaA protein (R1 to R4), and (2) an AT-rich region, containing three 13-mers (L, M and R), which are melted by the DnaA protein when bound to its sites. DnaA protein action can be specifically inhibited by binding of the IciA protein (inhibitor of chromosome initiation) to the unopened 13-mer region.

In order to identify other proteins potentially involved in regulating replication initiation events at *oriC*, gel-shift assays have been performed to isolate a novel 35 kDa protein (see abstract, Skarstad *et al.*), which binds to the right border of *oriC* next to R4. Based on its N-terminal amino acid sequence, the corresponding gene was cloned, sequenced and overexpressed. The gene is at 99.8 min on the chromosomal map, between the *trpR* gene and the operon containing *phoM*. Properties of the analyzed DNA sequence and of the purified 35 kDa protein in replication *in vitro* will be presented.

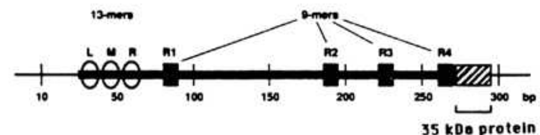

Fig. 1. *E. coli* chromosomal origin of replication (*oriC*). See text for details.

F 360 TRFA INITIATOR PROTEIN MUTANTS ALTERED FOR BINDING TO PLASMID RK2 ORIGIN SEQUENCES. Aresa Toukdarian, Joan Lin, and Donald R. Helinski, Center for Molecular Genetics and Department of Biology, University of California, San Diego, La Jolla, CA 92093-0634.

We have been studying interactions between the RK2 replication initiation protein, TrfA, and the plasmid's origin sequences. An *in vivo* antibiotic selection system for isolating genes encoding sequence specific DNA binding proteins was adapted for use with TrfA. A binding site consisting of two 17-bp iterons separated by a 6-bp spacer region was cloned into the vector pNN388 (1) such that an increased level of spectinomycin resistance was dependent on TrfA specific binding to the iteron sequences. The results with the *in vivo* system were found to correlate well with observations of *in vitro* DNA binding activity of several previously characterized TrfA mutants. The system was then used to isolate mutants which were either defective or more effective in DNA binding. These latter mutants were found to fall into three classes: those active with only the complete (eight-iteron) RK2 origin, with only the minimal (five-iteron) RK2 origin, or with both types of origin. Additional properties of these different classes of mutant TrfA proteins will be described.

1. Elledge, S. et al. (1989) Proc. Natl. Acad. Sci. USA *86*:3689-3693.

F 361 INTERACTION OF BACTERIOPHAGE λ O PROTEIN WITH THE λ REPLICATION ORIGIN Soo-jong Um and Roger McMacken, Department of Biochemistry, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD 21205

The binding stoichiometry of bacteriophage λ O protein to the 4 recognition site iterons in the λ replication origin (ori λ) was determined with a gel mobility shift assay, using O protein labeled with ^{3}H-leucine and an ori λ DNA fragment labeled with ^{32}P. At subsaturating levels of O, 4 retarded ori λ-O complex bands were observed. Each shift was shown to result from the binding of an additional dimer of O protein. At saturation 4 dimers of O protein form the nucleosome-like O-some observed by electron microscopy. Characterization of free O protein by cross-linking and equilibrium sedimentation confirm that it is a dimer in solution, as is an amino-terminal, 162 amino acid DNA-binding fragment of O (O^N). Further evidence that the bound species is an O dimer was obtained from gel mobility shift assays. A heterodimeric species was formed when O and O^N were mixed with a DNA fragment containing a single recognition site. Equilibrium binding measurements were performed to determine the affinity of λ O protein for a single recognition site. From Scatchard analysis, the equilibrium dissociation constants were 3×10^{-9} M for the O-iteron interaction and 2×10^{-9} M for the O^N-iteron interaction. The binding constants calculated from the measured kinetic association and dissociation rate constants agree well with the observed equilibrium constants.

F 362 MECHANISM OF DNA-UNWINDING BY *E. coli* REP HELICASE. I. Wong, K.L. Chao, and T.M. Lohman. Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110.

DNA replication, recombination, and repair require DNA helicases to catalyze the transient melting of ds-DNA in a reaction driven energetically by the hydrolysis of ATP. The *E. coli* Rep helicase is essential for the replication of several ss-DNA phages, *e.g.* ϕX174, M13, f1, and P2 (Lane, H.E.D. and Denhardt, D.T. (1975) *J. Mol. Biol.* **97**, 99; Colasanti, J. and Denhardt, D.T. (1987) *Mol. Gen. Genet.* **209**, 382), and *E. coli rep*$^-$ mutants exhibit reduced rates of replication-fork movement (Lane, H.E.D. and Denhardt, D.T. (1974) *J. Bacteriol.* **120**, 843). Thus, it seems likely that the Rep helicase plays a functional role during replication *in vivo*. Rep, a 72 kD monomer when free in solution undergoes a DNA-binding induced dimerization. Both subunits of a dimer bind DNA and chemically cross-linked Rep-dimers retain both ATPase and ATP-dependant helicase activities (Chao, K.L. and Lohman, T.M. (1991) *J. Mol. Biol. in press*). Using short synthetic oligonucleotides that are long enough to bind only one Rep monomer, we have determined quantitatively the dimerization constant and the DNA-binding constants at both subunits for ss- and ds-DNA by nitrocellulose filter-binding. We observe DNA-induced allosteric interactions between the two subunits: the binding affinity for ss- versus ds-DNA to the second subunit depends on whether ss- or ds-DNA is bound to the first subunit. We also observe modulation of DNA-binding by nucleotide cofactors. In the presence of the nonhydrolyzable ATP analog, AMPPNP, Rep-dimers favor binding of ss-DNA to one subunit and ds-DNA to the other. However, in the presence of the hydrolysis product, ADP, binding of ss-DNA to both subunits is favored. These results suggest a model for ATP-dependent unwinding in which ATP-binding is coupled to translocation while ATP-hydrolysis drives unwinding.

F 363 ELECTRON MICROSCOPY OF REPLICATING NUCLEOPROTEIN COMPLEXES, Claire Wyman and Harrison Echols, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

The polymerase III holoenzyme (pol III) is the replicative polymerase of *Escherichia coli* and is the likely polymerase involved in SOS induced mutagenic bypass of replication blocking lesions. We have been developing methods to study replication complexes by electron microscopy. As a first step in this study an immuno-electron microscopy assay was developed to identify pol III associated with DNA in replicating complexes. Replication complexes formed *in vitro* with purified proteins and oligonucleotide primed ΦX174 circular DNA were fixed, stained with antibodies to pol III subunits and protein A conjugated to colloidal gold and spread for electron microscopy. Pol III remains associated with a completely replicated circle during the time course of these reactions. IgG from polyclonal sera raised against either the α, β, or τγ subunits stained a majority of the replicated circles with associated proteins. IgG from serum raised against the ε subunit did not stain pol III in these complexes. Conditions that result in pol III stalling were then established in order to study the interactions of proteins with stopped replication complexes, such as those involved in SOS rescue of polymerases. By omitting two nucleotides from the *in vitro* replication reactions, pol III will initiate replication but stall at the first position where one of the missing nucleotides should be inserted. Replication complexes formed in such reactions were fixed and stained for electron microscopy using IgG against the α subunit. 44% of the primed DNA molecules were stained by this antibody.

F 364 Gene Identification and Protein Overproduction-Purification of the χ Subunit of DNA Polymerase III Holoenzyme,

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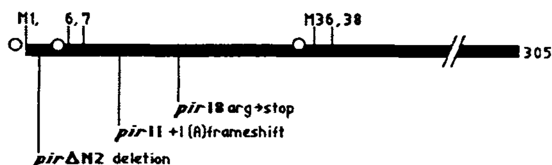
DNA polymerase III holoenzyme, the chromosomal replicase of Escherichia coli, is composed of at least 10 subunits. The γ complex subassembly ($\gamma\delta\delta'\chi\psi$) initates processive replication by coupling ATP hydrolysis to clamp the β subunit onto primed DNA. The β clamp tethers the core polymerase ($\alpha\epsilon\theta$) of the holoenzyme to the template for processive replication. Using χ protein purified from the holoenzyme, we obtained the N-terminal amino acid sequence. Analysis of tryptic peptides yielded further amino acid sequence information. The sequence led to identification of the gene encoding the χ protein. The gene sequence predicts that χ is a 147 amino acid protein (16.6 kda). We have cloned the χ gene into an expression vector which produces χ to a level of ~5-10% of total cell protein and have purified χ to apparent homogeneity. Function of χ and its interaction with other holoenzyme subunits is under investigation.

F 365 EXPRESSION OF DNA REPLICATION PROTEINS INVOLVED IN LAGGING STRAND SYNTHESIS MAY BE COORDINATED AT THE LEVEL OF mRNA STABILITY. Vijay Yajnik and G. Nigel Godson, Dept of Biochemistry, NYU Medical Center, 550 First Avenue, New York, NY 10016

The rpsU-dnaG-rpoD operon messenger RNA that encodes primase and sigma-70 is cleaved under normal physiological conditions and the dnaG coding portion of the mRNA is then rapidly degraded. An endonuclease activity has been isolated that cleaves dnaG mRNA in vitro. This activity has been identified as RNase E, and the identity confirmed by the accumulation of the unprocessed operon polycistronic mRNA in RNase E mutants. Also, extracts prepared from RNase E mutant strains fail to cleave dnaG mRNA in vitro. The dnaG mRNA RNase E cleavage site is only 50% homologous to the ribosomal RNA RNase E cleavage site. A computer search of E.coli data banks with dnaG mRNA RNase E cleavage site reveals a sequence of complete identity in the coding region of the dnaB gene, near the 3' end as in dnaG. Although little is known about the dnaB gene transcription, it is likely that RNase E may regulate the stability of its mRNA. Sequences with one or two mismatches were found in genes encoding different subunits of holoenzyme III. Fowler and McHenry (J. Mol. Bio. 220 649, 1991) demonstrate that the strength of the dnaZX operon promoter does not correlate with the poor synthesis of its gene products. Preliminary data from our laboratory suggests that the dnaZX mRNA is stabilized in RNase E mutants. Primase, dnaB helicase and holoenzyme III are expressed at low amounts in a cell. We propose that the presence of RNase E cleavage sites could be a mechanism that enables stochiometric expression of the components of the complex DNA replication machinery.

F 366 MULTIPLE TRANSLATIONAL OPTIONS FOR THE *pir* GENE, WHICH ENCODES π, THE REPLICATION INITIATOR PROTEIN OF PLASMID R6K. Dona York, John Hoffman, Jacek Gan, and Marcin Filutowicz, Department of Bacteriology, University of Wisconsin, Madison, WI 53706

The *pir* gene of *E. coli* plasmid R6K encodes the replication initiator protein π. In addition to being required for replication, π also inhibits replication and autoregulates its own synthesis at the transcriptional level. We have considered the possibility that the various functions of π may be performed by different products made from the *pir* gene. These products may results from translation initiating at different locations within the *pir* mRNA. There are three putative ribosomal binding sites (indicated by circles below) followed by met codons 1, 6, 7, 36, and 38 (also indicated below). Mutational analysis of the 5' end of the gene revealed that 3 in-frame polypeptides can be made from the *pir* gene. We will discuss their possible functions in R6K biology.



F 367 ATPASE DEFICIENT MUTANTS OF THE *ESCHERICHIA COLI* DNA REPLICATION PROTEIN PriA ARE CAPABLE OF CATALYZING THE ASSEMBLY OF ACTIVE PRIMOSOMES. Kenton H. Zavitz and Kenneth J. Marians, Program in Molecular Biology, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021 and Graduate Program in Molecular Biology, Cornell University Graduate School of Medical Sciences, New York, NY 10021

The PriA replication protein of *Escherichia coli* (formerly replication factor Y or protein n') is multifunctional. It is a site specific single-stranded DNA dependent ATPase (dATPase), a potent 3' → 5' DNA helicase and serves to guide the ordered assembly of the primosome, a mobile multiprotein DNA replication priming/helicase complex. Although not absolutely required for viability, *priA* null mutant cells grow very slowly, have poor viability, and form extensive filaments. In order to assess which of the multiple activities of PriA are required for normal replication and growth, site directed mutagenesis was employed to make single amino acid substitutions for the invariant lysine within the consensus nucleotide binding motif of PriA. Biochemical characterization of the representative purified mutant PriA proteins reveals them to be completely deficient in nucleotide hydrolysis, incapable of translocation along an SSB-coated single-stranded DNA template and unable to manifest the 3' → 5' DNA helicase activity of the wild-type PriA. These mutant proteins are, however, capable of catalyzing assembly of an active primosome *in vitro*. Furthermore, when supplied in trans to insertionally inactivated *priA* cells , plasmids containing a copy of these mutant *priA* genes restore the wild-type growth rate, viability, and cell morphology. Thus, the (d)ATPase/helicase activities of PriA can be uncoupled from its ability to catalyze the assembly of an active primosome and it is the latter function that is required for normal cellular growth and replication.

Replication Initiation & Termination - Euk

F 368 CHROMATIN STRUCTURE OF REPLICATION ORIGINS, GENE PROMOTER AND MATRIX ATTACHMENT REGION IN THE AMPLIFIED DIHYDROFOLATE REDUCTASE DOMAIN.
Sergei G.Bavykin, Alexander J.Pemov and Joyce L. Hamlin. Engelhardt Inst. of Molec.Biol., Moscow, USSR, and Univ. of Virginia Sch. of Medicine, USA
The Chinese hamster ovary cell line, CHOC 400, contains 1,000 copies of the dihydrofolate reductase (DHFR) domain, each of which is 240 kb in length. We have previously shown that replication initiates at many random sites within a 30-40 kb zone, possibly more frequently at two loci within this zone(ori-β and ori-γ) that are separated by a matrix attachment region (MAR)[reviewed in BBA 1087: 107 (1990)]. We have examined the chromatin structure of selected regions of the DHFR amplicon by limited micrococcal nuclease cleavage and by protein-DNA cross-linking experiments. Our results suggest that, in exponentially growing cells, the chromatin structure varies within this large domain. The chromatin organization of ori-γ is similar to that of the DHFR promoter region (but not to ori-β). Both ori-γ and the promoter are decondensed and display broad zones at least 1 kb in length in which the amount of histones is dramatically decreased relative to bulk chromatin (10-fold and 3-5-fold for the promoter and ori-γ, respectively). In the promoter region, the remaining histones organize "nucleosomal islands" containing 2 or 3 nucleosomal particles. The similar "islands" were founded in ori-γ, in which the usual chromatin structure also exists, but the nucleosomal repeat is 10 bp shorter than in bulk chromatin. In contrast, the chromatin in the MAR region has a more condensed structure, with the nucleosomal repeat being longer by 5 bp than in bulk chromatin. To our surprise, no unusual features were found in the chromatin structure of the ori-β region, in which a well-defined bidirectional origin of replication has been suggested to exist [Cell 62: 955 (1990)].

F 369 STUDIES OF THE INITIATION OF SV40 DNA SYNTHESIS IN VITRO.
Peter Bullock, Department of Biochemistry, Tufts University, Health Sciences Campus, Boston, MA. 02111

Initiation of SV40 DNA synthesis in crude extracts of HeLa cells has been examined using pulse-chase techniques[1]. It has been observed that in the absence of NTPs other than ATP, the preinitiation complex formed in crude extracts remains within, or close to, the SV40 origin. Upon introduction of dNTPs and rNTPs during pulse-labeling, SV40 initiates DNA synthesis on an unwound topoisomer termed Form U_R. Furthermore, using restriction endonucleases, it was demonstrated that SV40 initiates DNA synthesis in the vicinity of the SV40 origin. These studies indicate that in this system, DNA synthesis and unwinding events are coupled.

To further characterize initiation events, an analyses of the parental strands serving as templates during initiation of SV40 DNA synthesis was performed. These studies indicated that DNA synthesis initiated on strands, that relative to the replication forks, are templates for lagging strand DNA synthesis.

Pulse labeling is eliminated by BuPdGTP, a selective inhibitor of pol α at low concentrations. This is one of several observations that suggest that SV40 DNA synthesis events are initiated by the pol α-primase complex. Antibodies which neutralize proliferating cell nuclear antigen (PCNA) inhibit, but do not abolish, synthesis of DNA during a 5 s pulse. The length of the DNA products formed in the absence and presence of anti-PCNA serum averaged ~200 and ~34 nucleotides, respectively. The small-DNA product formed after a 5 s pulse in the presence of anti-PCNA serum (primer-DNA) were covalently linked to oligoribonucleotides ~10 nt long. Primer-DNA is also detected in standard pulse reactions and its formation is independent of the presence of anti-PCNA serum: the available evidence indicates that this species is the product of the pol α- primase complex.

Primer-DNA can be detected at both initiation and elongation stages of SV40 DNA synthesis in vitro. Moreover, primer-DNA formed in the presence or absence of anti-PCNA serum arose from replication of lagging strands. These results suggest that in addition to priming leading strand DNA synthesis, primer-DNA also has a role in the synthesis of lagging strands.

1) Bullock, P.A., Y.S. Seo and J. Hurwitz (1991) Mol. Cell. Biol. 11, 2350-2361.

F 370 CHARACTERIZATION OF REPLICATION FACTOR C FROM HUMAN AND YEAST CELLS
Fred Bunz, Karen Fien and Bruce Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724

Fractionation of cell extracts that will support the replication of SV40 origin-containing DNA *in vitro* has resulted in the purification of a number of essential factors. One of these is Replication Factor C (RFC), a multisubunit enzyme composed of polypeptides of 140, 41 and 37kDa. Reconstitution of SV40 DNA replication with purified proteins has shown that RFC is involved in the switching of polymerases following synthesis of the first nascent strand and in the subsequent elongation of the leading strand by DNA polmerase delta. Biochemical characterization revealed that RFC binds to heteroduplex DNA in a structure specific manner, and has an intrinsic ATPase activity that is stimulated by DNA and by Proliferating Cell Nuclear Antigen (PCNA), another essential replication factor. Further enzymatic studies of the RFC ATPase allowed the determination of the K_m and V_{max} for ATP. In addition, the stimulation by various cofactors and ligands have been investigated. The results suggest a heterotropic allosteric interaction between the DNA binding and ATPase moities and a model for the assembly and disassembly of the leading strand complex at the replication fork will be presented. Currently, we are using monoclonal antibodies to study the role of protein further.

RFC has been purified from the yeast *S. cerevesiae*. Like its human homologue, scRFC exhibits structure specific DNA binding and a DNA dependent ATPase. In addition, like its human counterpart, scRFC cooperates with the RPA and PCNA replication factors to stimulate DNA polymerase delta activity. These similarities further demostrate the striking conservation of the DNA replication apparatus between higher eucaryotes.

F 371 DNA REPLICATION ORIGINS AND NUCLEOSOME SEGREGATION IN MAMMALIAN CELLS.
William Burhans[1] Lyubomir Vassilev[1], Jiarui Wu[2], Jose Sogo[2], Ferez Nallaseth[1] and Mel DePamphilis[1], [1]Roche Institute of Molecular Biology, Nutley, NJ 07110; [2]Institute of Cell Biology, ETH-Honggerberg, 8093 Zurich, Switzerland

Hybridization of CHO and CHOC 400 cell Okazaki fragments to cloned template DNA demonstrates that Okazaki fragments are synthesized predominantly on the *retrograde* arms of mammalian replication forks, and that a specific origin of bidirectional DNA replication (OBR-1) occurs downstream of the DHFR gene in hamster cells (Burhans et al., *Cell* **62**, 955-965 (1990)). To confirm and extend these observations, we analyzed products of DNA synthesis on the *forward* arms of replication forks by applying an assay described by Handeli et al. (*Cell* **57**, 909-920 (1989)) to the DHFR initiation locus in these cells.

In agreement with the data of Handeli and coworkers, our results demonstrated that emetine, an inhibitor of protein synthesis, induced a 5 to 7-fold accumulation of nascent DNA on forward arms of replication forks relative to retrograde arms, revealing that at least 85% of replication forks in this region emanated from OBR-1 (Burhans et al., *EMBO J.* (1991) *in press*). However, three lines of evidence argue that this bias did not result from conservative nucleosome segregation to forward arms, as previously assumed, but from preferential inhibition of Okazaki fragment synthesis on retrograde arms (imbalanced DNA synthesis). First, the bias existed in long nascent DNA strands prior to nuclease digestion of non-nucleosomal DNA. Second, the fraction of RNA-primed Okazaki fragments was rapidly diminished. Third, electron microscopic analysis of SV40 DNA replicating in the presence of emetine revealed large numbers of replication forks with single-stranded DNA on one arm, and nucleosomes randomly distributed to both arms.

Analysis of imbalanced DNA replication in cell lines that contain integrated copies of OBR-1 reveals that OBR-1 requires cis-acting sequence elements. We are delineating these elements by analyzing the function of integrated OBR-1 sequences in various configurations.

F 372 SEQUENCE-SPECIFIC SINGLE STRANDED DNA BINDING FACTORS, INCLUDING RPA, RECOGNIZE THE SV40 CORE ORIGIN IR DOMAIN IN A CELL CYCLE DEPENDENT MANNER,

Ellen P. Carmichael, Janet M. Roome and Alan F. Wahl. Department of Cellular and Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT 06495

To understand S-phase control it has become increasingly important to study replication associated proteins that bring about the onset of DNA synthesis. The inverted repeat domain (IR domain) within the SV40 origin of replication is the site of DNA melting prior to the onset of DNA synthesis. We have shown that two distinct protein complexes bind opposite strands of the IR domain. RPA, a previously described non-specific single stranded DNA binding protein, preferentially binds the pyrimidine rich strand. We characterized the second protein complex, IRBF-B, which specifically recognizes the complementary strand of the IR domain. The activity of IRBF-B varies significantly with cell proliferation and the cell cycle. Binding of IRBF-B to the IR domain is negatively correlated with the onset of DNA synthesis, and is displaced by the viral T-antigen, whereas binding of RPA is not perturbed by T-antigen.

As cells transit from quiescence to proliferation these DNA protein complexes are diminished, then reestablished, coincident with the peak of DNA synthesis. Throughout the proliferative cell cycle IR domain binding of these complexes is minimal just prior to entry into S-phase and maximal at the peak of DNA synthesis. Extracts of cells staged by metabolic block in G1, G1/S and S-phase of cell cycle also show a buildup of one of these factors, IRBF-B, in G1 and its subsequent decrease upon onset of DNA synthesis. Biochemical fractionation indicates that the two complexes co-purify with DNA polymerase δ through initial chromatographic steps, and each can be resolved by subsequent AMP Sepharose chromatography and rate sedimentation. We propose that RPA and IRBF-B bind opposite strands of the IR domain to function in tandem to regulate origin activation.

F 373 DISSECTION AND RECONSTRUCTION OF A NUCLEAR ORIGIN OF REPLICATION: THE ARS121 OF SACCHAROMYCES CEREVISIAE,

Shlomo Eisenberg, Scott. S. Walker and Stephen. C. Francesconi, Ajay Malik and Brett Robinson. Dept. of Microbiology, The University of Connecticut Medical School, Farmington, CT 06030 USA.

The ARS*121* origin was mapped to chromosome X of *S. cerevisiae*. Analysis of chromosomal replication intermediates by the Brewer and Fangman 2-dimensional electrophoresis procedure suggests that ARS*121* is an efficient origin of replication in its natural chromosomal location. We have delineated this origin by a combination of site-directed *in vitro* mutagenesis and deletion analysis. The results of this analysis indicate that ARS*121* is composed of three distinct functional units: (1), an essential core element (~35bp), which includes an ARS consensus-like (2 bp. mismatch) sequence ; (2), an ATR (AT-rich) domain (72 bp) located 3' to the T-rich strand of the consensus; (3), an enhancer of DNA replication (22 bp), recognized by the OBF1 protein. We have now reconstructed origin activity by fusing isolated functional units. These studies have shown that the enhancer and the ATR domains act synergistically to enhance the activity of the essential core. The enhancer can work even when situated at long distances from the essential element in an orientation independent manner, while the ATR works in either orientation but only when present 3' to the essential domain. Furthermore both domains, the enhancer and ATR, have stimulated the activity of the essential core sequences of heterologous ARSs as well, including ARS*1* and ARS*307*, suggesting a common initiation mechanism for all origins of replication. The possible function of these units in initiation of DNA replication will be discussed.

The gene encoding the OBF1 protein,which binds to the enhancer, was isolated. These studies revealed that OBF1 is identical to BAF1 and ABF1, believed to function as transcriptional regulators. Thus we suggest that OBF1 fulfils a function in both replication and transcription. Recently we demonstrated that the OBF1 protein exists *in vivo* as a phosphoprotein phosphorylated at serine and threonine residues, suggesting that phosphorylation may regulate the different OBF1 activities. Mapping of the phosphorylation sites will also be presented.

F 374 EBNA1 MEDIATES A DNA LOOP WITHIN THE EBV LATENT ORIGIN OF REPLICATION, ORIP.

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Epstein-Barr virus nuclear antigen 1 (EBNA1) activates the viral latent origin, oriP, by a mechanism which involves its interaction with multiple binding sites in the two functional elements of oriP, the dyad symmetry element and the family of repeats. We have used electron microscopy to examine the assembly of EBNA1 onto oriP. A titration of homogeneous EBNA1 overproduced using the baculovirus system (bEBNA1) with oriP revealed an order to the assembly of bEBNA1 onto oriP. At low concentrations, bEBNA1 bound only to the family of repeats of oriP. As the amount of bEBNA1 was increased to stoichiometric levels, a looped structure in which the family of repeats and the dyad symmetry element were joined through bEBNA1 became the most prevalent DNA-protein complex. bEBNA1-mediated DNA looping was also observed when the distance between the two oriP elements was increased to 3 kb. bEBNA1 bound to multiple binding sites in the family of repeats or the dyad symmetry element did not appear as a linear array of protein, but rather appeared roughly spherical with the DNA arms usually entering and exiting the same side of the complex. These observations, together with DNA length measurements which indicate that bEBNA1 may condense the DNA, are suggestive of DNA wrapping around the bEBNA1 complex. The data suggest that bEBNA1-mediated DNA looping may play a role in activating latent phase replication of the Epstein-Barr virus.

F 375 MOLECULAR INTERACTIONS AT THE ADENOVIRUS TYPE 2 ORIGIN OF DNA REPLICATION,

Ronald T. Hay and Simon M. Temperley, Department of Biochemistry and Microbiology, University of St. Andrews, Fife, Scotland. KY169 AL

Initiation of adenovirus type 2 (Ad2) DNA replication is preceded by formation of a large nucleoprotein complex containing three viral proteins: DNA binding protein (DBP), DNA polymerase (pol), preterminal protein (pTP) and two cellular proteins: nuclear factor I (NFI) and nuclear factor III (NFIII). NFI makes a direct contact with pol and it is thought that this interaction contributes to the correct positioning of the pTP-pol heterodimer at the replication origin. However this interaction alone is not sufficient to direct specific initiation of replication and we have therefore investigated origin recognition by the pTP-pol heterodimer. Using pTP and pol purified from recombinant baculovirus infected insect cells we have demonstrated that the two viral proteins independently recognise DNA sequences in the 1-18 core of the replication origin. DNA binding properties of the individual proteins and the pTP/pol heterodimer were examined by HPLC gel filtration and gel electrophoresis DNA binding assays. Specificity of the observed interaction was further analysed by competition and and DNase I footprinting experiments. It therefore appears that pTP and pol contribute multiple DNA-protein and protein-protein interactions that stabilise a preinitiation complex at the adenovirus type 2 origin of DNA replication. The roles of NFI and DBP in the stabilisation of this complex will be discussed.

F 376 A REPLICATION ORIGIN AND TERMINUS ON CHROMOSOME III OF THE YEAST, *SACCHAROMYCES CEREVISIAE*, Jiguang Zhu[1], Leslie R. Davis[1], Carol S. Newlon[2] and Joel A. Huberman[1], [1]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY 14263 and [2]Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, NJ 07103

Two dimensional gel electrophoretic techniques were used to locate all functional DNA replication origins and termini in a 27.2 kbp stretch of yeast (*Saccharomyces cerevisiae*) chromosome III. Only one origin was detected (the C1G origin), and it colocalized with an *ARS* element (*ARS306*) as have all previously mapped yeast origins.

A terminus was found in between the C1G origin and the neighboring, previously mapped A6C origin. This terminus is located about 15 kbp towards the centromere from the A6C origin and about 16 kbp towards the telomere from C1G origin. The mechanism of termination is not sequence specific. Instead, termination appears to be the consequence of replication forks converging at random sites within a stretch of DNA of about 4 kbp.

F 377 SEQUENCE AND PROPERTIES OF PUR, A MAMMALIAN SINGLE-STRAND DNA-BINDING PROTEIN WITH SPECIFIC AFFINITY FOR AN ELEMENT PRESENT AT SEVERAL EUKARYOTIC ORIGINS OF REPLICATION, Edward M. Johnson and Andrew D. Bergemann, Department of Pathology and Brookdale Center for Molecular Biology, Mount Sinai School of Medicine, New York, NY 10029

A protein has been identified in HeLa nuclear extracts with specific affinity for a sequence element present near prominent sites of DNA bending in two initiation zones for chromosomal DNA replication, one in the human *c-myc* locus and one in the hamster *dhfr* locus. This protein has specific affinity for the purine-rich single strand of the element, termed *PUR*. Methylation interference maps a pattern of contact points with guanosine bases in a 24-mer oligonucleotide containing the element. UV cross-linking reveals that contact is made by a polypeptide of <30 kD. The *PUR* element is present in gene flanking regions and reported origins of replication in several eukaryotes, and a consensus sequence has been derived. A 24-mer oligonucleotide representing the hamster *dhfr* version of the *PUR* element effectively competes with the human *c-myc* version for binding to the HeLa nuclear Pur protein. We have isolated a clone from a human fetal liver cDNA library based on specific affinity of the expressed protein for the single-strand *c-myc PUR* element. Sequencing of the clone reveals a polyglycine motif, common to other single-strand polynucleotide binding proteins, as well as motifs present in known transcription factors. The Pur protein could be involved in opening or maintaining open the DNA duplex at a specific site. We discuss implications of this regarding regulation of initiation of replication in mammalian cells. (Supported by NIH CA55219.)

F 378 DNA UNWINDING ELEMENTS AT YEAST ORIGINS OF REPLICATION, D. Kowalski, C.A. Miller, R.-Y. Huang, R.M. Umek, & D.A. Natale, Molec. & Cell. Biology Dept., Roswell Park Cancer Institute, Buffalo, New York 14263

Autonomously replicating sequences (*ARS*) function as origins of DNA replication on plasmids in *S. cerevisiae* and in some cases serve as chromosomal origins. Efficient plasmid replication requires an 11 bp consensus element as well as an ca. 100 bp 3′-flanking sequence which exhibits little sequence similarity among *ARS* elements. Autonomous replication of *ARSs* with mutations in the 3′-flank correlates with hypersensitivity to single-strand-specific nucleases in negatively supercoiled plasmids and a low free energy cost for localized unwinding. Certain origin defective mutations that raise the energy cost can be suppressed at elevated growth temperature. We call the nuclease hypersensitive sequence a DNA unwinding element (DUE) and have proposed that the DUE facilitates the initial unwinding of parental strands, permitting entry of the replication enzymes into the DNA helix.

A DUE appears to be a general component of yeast replication origins and its orientation, 3′ to the T-rich consensus strand, is conserved. A broad 3′-flanking sequence is nuclease hypersensitive in the H4 *ARS*, the 2μ plasmid origin and the following *ARS* elements that serve as chromosomal origins: ribosomal DNA (rDNA), C2G1 (*ARS307*) and *ARS305*. The helical instability of the 3′-flank is quantitatively related to the replication efficiency for a large collection of C2G1 *ARS* mutants. The DUE in the rDNA *ARS* has an elevated energy cost for unwinding compared to the H4 *ARS* DUE. Plasmid replication activity of the rDNA *ARS*, but not the H4 *ARS*, is cold sensitive between 34°C and 20°C, consistent with the role proposed for the DUE.

Although not all *ARS* elements are chromosomal origins, the activity of certain *ARS* elements on plasmids does reflect chromosomal origin activity. At the tandemly-repeated rDNA locus, chromosomal origin usage is cold sensitive as predicted plasmid studies. For *ARS305*, deletions that abolish *ARS* activity, either by raising the energy cost of the DUE or by removing the 11bp consensus, also abolish chromosomal origin activity.

F 379 SIMIAN VIRUS 40 SMALL t ANTIGEN INHIBITS SV40 DNA REPLICATION *IN VITRO*, A. S. Levine#, J. Hauser#, M. Carty#, K. Rundell*, K. Dixon#, M. Carbone#, #Section on Viruses and Cellular Biology, National Institute of Child Health and Human Development, Bethesda, MD 20892 and *Department of Microbiology, Northwestern University, Chicago, IL 60611

The SV40 large T (Tumor) antigen is a multifunctional protein essential for the expression and replication of the viral genome. To initiate DNA replication, T must be phosphorylated at threonine-124, but the protein has to be dephosphorylated at serine sites. Phosphatase 2A (PP2A) dephosphorylates T at these serine sites and enhances the ability of T to support DNA replication. SV40 small t antigen interacts with PP2A by binding to the regulatory subunit. This observation suggested to us that the interaction of small t with PP2A might modulate T-mediated DNA replication. To test this hypothesis, we used an *in vitro* DNA replication system which included an HeLa cell extract, and as substrate the plasmid pZ189 (which contains the SV40 replication origin). Plasmid replication in this *in vitro* system is completely dependent on SV40 T-antigen. When purified small t (400 ng) was added to this system, replication was inhibited ~ 70%. The inhibitory effect was observed under various experimental conditions and using different T and t antigen preparations. The level of inhibition is dependent on the amount of purified small t added to the T antigen-dependent DNA replication system and increases as a function of time of incubation of the system. We demonstrated by immunoblotting that t-antigen coprecipitated with PP2A in this *in vitro* system. Moreover, addition of the free catalytic subunit of PP2A to *in vitro* replication reactions containing small t restored DNA replication activity, and the phosphorylation state of large T is modified in replication mixtures containing small t. Our results indicate that small t inhibits DNA replication *in vitro*, and that this inhibitory effect may be mediated by the interaction of small t with PP2A. Preliminary results in an *in vivo* system suggest that an increased level of small t inhibits wild-type SV40 DNA replication in permissive cells.

F 380 GENOMIC MAPPING OF ORS12, A MAMMALIAN AUTONOMOUSLY REPLICATING SEQUENCE, BY IN SITU HYBRIDIZATION. David C.W. Mah, Awatef Shihab-El-Deen, Gerald B. Price and Maria Zannis-Hadjopoulos, McGill Cancer Centre, McGill University, Montréal, Québec, CANADA H3G 1Y7.
ORS12, isolated from CV-1 cell nascent DNA by extrusion from replication bubbles active at the onset of S phase, has been demonstrated to function as an origin of DNA replication in autonomously replicating plasmids and in a cell-free system. The leftward portion of the 812-bp ORS12 sequence consists of alpha-satellite sequences, with an estimated copy number per haploid CV-1 genome of $>10^6$; the non-alpha satellite portion of the sequence has been estimated at 5-7 copies per haploid genome. Using the non-alpha satellite sequence as a probe we have determined a genomic restriction map of ORS12. In situ hybridization of CV-1 metaphase chromosomes using a biotinylated probe of the entire ORS12 sequence, positively identifies highly repetitive sequences on the centromeric region of all chromosomes. When only the non-alpha satellite region of ORS12 is used as a probe, however, it clearly identifies a low, or possibly single, copy sequence on the centromeric regions of 6-8 chromosomes. The significance of these findings will be discussed.

(Research supported by grant MT-7965 from MRC Canada).

F 381 ROLE OF TRANSCRIPTIONAL ACTIVATOR PROTEINS IN INITIATION OF POLYOMA VIRUS DNA REPLICATION, Haim Manor, Moshe Baru and Meir Shlissel, Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel.
We have replaced the polyomavirus (Py) enhancer, which is an essential component of the Py origin of DNA replication, with the yeast GAL4 upstream activating sequences (UAS). Plasmids containing this modified Py origin (test plasmids) and plasmids encoding the GAL4 transcriptional activator protein, or hybrid derivatives of this protein, were cotransfected into mouse cells which constitutively synthesize a temperature-sensitive Py large T-antigen. Replication of the test plasmids was monitored by DpnI assays. These studies showed that in the presence of a functional Py large T-antigen, the GAL4 protein and hybrid proteins including the GAL4 DNA binding domain and activating domains of the adenovirus Ela or Herpesvirus VP16 proteins transactivated the modified Py origin. A truncated protein including just the GAL4 DNA binding domain was inactive in these assays. The authentic GAL4 protein was found to be a more efficient replication transactivator than the hybrid proteins, although the latter proteins were more efficient activators of transcription. Deletion of the Py promoter from the test plasmids, or transfer of the GAL4 UAS to sites located >1200 bp from the Py origin core, reduced but did not abolish the replicational transactivation by the GAL4 protein. These results indicate that transcriptional activator proteins do not activate the Py origin by virtue of their interactions with general transcription factors, or by disruption of chromatin structure in the vicinity of the origin core. Instead, these data favor a model in which the DNA forms a loop such that the activator proteins interact with and stabilize the replication initiation complex formed at the origin core.

F 382 AUTONOMOUS REPLICATION OF PLASMIDS CARRYING HUMAN CHROMOSOMAL DNA, Hisao Masukata, Chikashi Obuse, Hiroyasu Satoh and Tuneko Okazaki, Department of Molecular Biology, School of Science, Nagoya University, Nagoya 464-01, Japan

We have developed a method to isolate human genomic DNA sequences that replicate autonomously in a human cultured cell. pUC119 library carrying approximately 10 kb long human genomic DNA was introduced into human 293 cell and the replicated molecules were labeled with 5-bromodeoxyuridine (BrdU). Heavy density molecules were separated by centrifugation in a CsCl gradient and used to transform E. coli. After two cycles of selection procedures, certain clones were found to be enriched more than twenty-fold compared with their population in the original library. From 400 clones we have so far isolated eight different ARS candidates that carry 8-15 kb inserts.

Replication of ARS candidates in human cultured cell was examined by introducing each ARS candidate as well as a control plasmid, which was not enriched by the selection procedures, into 293 cell. The BrdU-labeled plasmid DNA was subjected to CsCl centrifugation followed by gel electrophoresis and Southern hybridization. For ARS candidates, 15-20% of molecules replicate during a generation, while the corresponding values of vector alone and the control plasmid are 1% and 5%, respectively. Analysis by methylation sensitive restriction enzymes MboI and DpnI confirmed the replication of ARS candidates in 293 cell.

Following evidences strongly suggest that candidate plasmids carry ARS segment. Firstly, analysis of half-heavy density molecules by a subsequent centrifugation in an alkali CsCl gradient showed that ARS candidates replicate semiconservatively. Secondly, replication of ARS candidates are not mediated by excision of chromosomally integrated plasmid copies. Thirdly, ARS candidates replicate in the S-phase after synchronization by aphidicolin. And finally, long term replication assay by using an EB virus vector which has a nuclear retention activity showed that the segment from the ARS candidates support maintenance of the plasmid.

F 383 Herpes Simplex Virus UL9 Nucleoprotein Complexes, Samuel D. Rabkin, Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.
Herpes simplex virus (HSV) UL9 is an essential replication gene which encodes a sequence-specific origin-DNA binding protein. UL9 protein assembles a nucleoprotein complex at the origin of DNA replication (ori_S). Ori_S contains two UL9 protein binding sites, I and II, bracketing an AT rich region. Recombinant UL9 protein, purified from baculovirus vector-infected insect cells, forms a specific complex at ori_S in the absence of fixative that is visible in the electron microscope. UL9 protein covers about 120 basepairs of DNA at the origin. Upon formation of the nucleoprotein complex, the apparent contour length of the DNA is shortened, suggesting that the DNA is wrapped or condensed by the protein. Intermolecular complexes form between two bound DNA molecules with the junction at the position of protein binding. We are currently defining the structure of these complexes in more detail.

F 384 ANALYSIS OF T ANTIGEN MUTANTS LACKING PHOSPHATE ACCEPTOR SITES,

Donald Small[1], Alicia Russo[2], Kathy Collins[2], David Virshup[3], Ismael Moarefi[4], Ellen Fanning[4], and Thomas J. Kelly[2], Departments of Oncology and Pediatrics[1], Molecular Biology and Genetics[2], Johns Hopkins University School of Medicine, Baltimore, MD 21205; Program in Human Molecular Biology and Genetics, Univ. of Utah, Salt Lake City, Utah 841123; Institut fur Biochemie der Universitat Munchen, 8000 Munich 2, Germany[4]

Recent studies have shown that the phosphorylation state of SV40 T antigen (Tag) affects its replication activity. Previous studies revealed that phosphorylation of Tag threonine residue 124 is required for Tag activity whereas dephosphorylation of serine residues 120 and 123 are stimulatory. We have used in vitro assays to compare wild type Tag to mutant Tag's that lack PO_4 acceptor residues to assess the effect of phosphorylation on a number of defined activities including origin binding, origin unwinding, non-specific helicase, initiation, and replication.

Previous studies utilizing a modified McKay assay indicated the lack of phosphorylation at threonine 124 resulted in defective binding to the origin. We performed DNA mobility shift assays that revealed no defects in DNA binding. In addition, the mutant Tag was able to unwind a DNA fragment containing the origin in an origin dependent manner. However, the threonine 124 mutant Tag was partially defective in initiating DNA replication in a purified protein system and was completely unable to replicate DNA in vitro utilizing crude extracts. This suggests the defect in DNA replication lies beyond the initial steps of binding and unwinding.

Mixing experiments in which an increasing amount of the mutant Tag was added to replication reactions containing wild-type Tag demonstrated inhibition of DNA replication.

F 385 STUDIES OF THE HERPES SIMPLEX VIRUS ORIGIN OF DNA REPLICATION PROTEIN USING THE SINDBIS EXPRESSION SYSTEM,

Erik C. Stabell and Paul D. Olivo, Department of Medicine, Division of Infectious Diseases, Washington University School of Medicine, St. Louis, MO 63110

We are studying the DNA binding properties of the HSV origin binding protein (OBP). UL9 is one of seven Herpes simplex virus (HSV) proteins required for viral DNA replication and it is thought to play an essential role in the initiation of DNA replication. As part of our studies of UL9 we have constructed a recombinant Sindbis virus that expresses a 35 kdal polypeptide which contains the site-specific DNA binding domain of UL9. The Sindbis expression system has many features which facilitate a combined genetic and biochemical analysis which we are performing on the origin binding domain of UL9. Full length UL9 has been shown to bind specifically to both HSV origins of DNA replication (oriS and oriL) and there are at least two UL9 binding sites on oriS which occur on either side of the oriS palindrome which contains an AT-rich center. Characterization of the UL9 binding site by one group suggests that the the UL9 binding site is an octamer consisting of two inverted pentamers with a two base pair overlap (Koff and Tegtmeyer J. Virol. 62:4096, 1988). This structure led to the hypothesis that UL9 binds as a dimer to each binding site. Consistent with this notion UL9 sediments in solution as a homodimer. It is not known whether dimerization is required for DNA binding. Other studies of UL9 binding have shown that it binds cooperatively to the origin (Elias et al. JBC 265:17167, 1990) and more recently, that UL9 binding to the origin leads to a conformational change in the DNA (Koff et al. J. Virol 65:3284, 1991). These observations suggest that other UL9-UL9 interactions besides homodimerization may occur following binding to the origin. The Sindbis virus-expressed 35kdal polypeptide is a non-fusion protein which is soluble and localizes to the cytoplasm as shown by immunofluorescence using antibody to the carboxy terminal portion of UL9. It exhibits origin binding activity in a gel retardation assay. Velocity sedimentation analysis reveals that the 35 kdal OBP sediments as a monomer. Therefore origin binding activity does not require a preformed dimer.

F 386 CIS AND TRANS REQUIREMENTS FOR BPV DNA REPLICATION.

Arne Stenlund, Mart Ustav, Ene Ustav, and Paul Szymanski Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 117 24.

To study BPV DNA replication we have developed a transient replication assay based on a highly efficient electroporation procedure. Two viral polypeptides are necessary and sufficient for replication of BPV DNA in mouse C127 cells. One of these polypeptides is a 72 kD phosphoprotein encoded from the entire E1 open reading frame, and the other is the 48 kD E2 transcriptional trans-activator. The sequences required in *cis* for replication have been mapped using short term replication assays. A minimal *ori*-fragment, that we have defined as containing all the sequences required in *cis* for replication, is limited to a 60 nucleotide long fragment overlapping the unique Hpa I site of BPV. Thus, replication of BPV does not require the previously characterized PMS sequences. The *ori*-sequence is also required for stable replication, since mutants in the minimal *ori* fail to establish as replicating plasmids.

We have expressed the E1 polypeptide in E. coli and have determined that the full length protein has sequence specific DNA binding activity. E1 serves as the *ori* recognition factor and binds specifically to a palindromic sequence in the minimal *ori*. Point mutations in this palindromic sequence destroy E1 binding and inactivates the ori. Binding sites for the viral transactivator E2 are not required in the *ori* fragment. However, several point mutations in E2 that affect DNA binding but not dimerization of the protein are incapable of supporting replication. These results indicate that the binding specificity of E2 may be altered or relaxed for its role in replication.

F 387 INTERACTION OF EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN 1 (EBNA1) WITH THE LATENT VIRAL ORIGIN OF REPLICATION (oriP),

Dan Zhang, Lori Frappier, Vytautas Naktinis, and Mike O'Donnell, Howard Hughes Medical Institute, Microbiology Department, Cornell University Medical College, New York, NY 10021

The Epstein-Barr virus (EBV), the causative agent of infectious mononucleosis, persists latent in human B lymphocytes as a multicopy episome. Extrachromosomal maintenance of the EBV plasmid requires only two viral elements: an origin of replication, oriP, and EBV nuclear antigen 1 (EBNA1). oriP consists of two components: the family of repeats (FR), which contains 20 direct repeats, and the dyad symmetry element (DS), which contains 4 copies of the repeat. The FR and DS are separated by 980 bp of spacer DNA. EBNA1 binds to both FR and DS within each of the repeats. There is increasing evidence suggesting that DNA replication initiates at or near the DS element, while FR acts to activate initiation at the DS.

We would like to know how EBNA1 activates oriP for replication. In this study we examine the ability of EBNA1 (overproduced in baculovirus) to distort the structure of oriP by probing with $KMnO_4$, a reagent known to oxidize the pyrimidine residues within duplex DNA only at regions that are melted or otherwise distorted. EBNA1 induces two $KMnO_4$ hypersensitive sites in oriP, both located exclusively in the DS element. The two $KMnO_4$ sites lie in the first and fourth repeats (EBNA1 binding sites) of DS. Correlation of the $KMnO_4$ reactive sites with replication function of oriP, and whether the two $KMnO_4$ sites are independent or separable is being studied using mutant DS sequences.

F 388 IDENTIFICATION AND CHARACTERIZATION OF A CHROMOSOMAL REPLICATION ORIGIN IN *SCHIZOSACCHAROMYCES POMBE*, Jiguang Zhu[1], Hisanori Kurooka[2], Mitsuhiro Yanagida[2], David Kowalski[1] and Joel A. Huberman[1], [1]Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, NY 14263, [2]Department of Biophysics, Kyoto University, Faculty of Science, Sakyo-ku, Kyoto 606, Japan

Two dimensional (2D) gel electrophoretic techniques were used to find functional chromosomal replication origin(s) on chromosome III of *S. pombe*. Cosmid clones covering ~250 kb of genomic DNA, including the *ura4* gene, were used as starting material for restriction mapping and subcloning. After walking a few kilobases upstream of the *ura4* gene, a replication origin region was identified (we call it the *ura4* origin) with both the neutral-alkaline (N/A) and neutral-neutral (N/N) 2D replicon mapping techniques. The *ura4* origin appears to be more complex than the chromosomal replication origins found in *S. cerevisiae*, and in some respects it resembles higher eukaryotic replication origins.

Unidirectional deletion plasmids were constructed covering 2.7 kb of the *ura4* origin region for *ARS* mapping and also for sequencing. A strong *ARS* element was localized to a stretch of about 200 bp, and it colocalizes with the region of lowest free energy for DNA unwinding. A single 11/11 and three 10/11 matches to the *S. pombe ARS* consensus are found in the same region.

Fine mapping of the *ura4* origin by N/N and N/A 2D techniques suggests that replication initiates in a zone of about 3 kb, flanking but not overlapping the *ARS* and easily unwound region. Thus, the *S. pombe* origin resembles origins of *Drosophila* and CHO cells in the sense that, in all three cases, 2D gel electrophoretic data suggest that replication can initiate some distance away from cis-acting elements which are, probably, the primary determinants of origin location.

Site-specific Recombination

F 400 KINETICS OF LAMBDA SITE-SPECIFIC RECOMBINATION, Michael F. Bruist and Rebeca Leggiere, Department of Chemistry, Vassar College, Poughkeepsie, NY 12601

The site-specific recombination reaction between bacteriophage λ DNA and the *E. coli* chromosome is believed to proceed as follows: The intasome [a complex of phage DNA (*att*P), the phage protein Int, and the *E. coli* protein integration host factor (IHF)] binds the bacterial integration site (*att*B). No additional proteins bind to *att*B. The synapsed intermediate then exchanges the top strands of the DNAs forming a branched Holliday intermediate. The branch migrates through the overlap sequence and is then resolved by exchange of the bottom strands of the DNAs.

This scheme provides the basis for kinetics experiments which measure the affinity of the intasome for *att*B and which will try to determine the relative rates of the various steps in the reaction. Computer modeling indicates value ranges that can be assigned to the rate constants to fit the experimental data.

The weak interaction between the intasome and *att*B has been measured. The rate of recombination, at a fixed concentration of the intasome, shows saturation as the concentration of *att*B is increased. The saturation curve is described by a dissociation constant of about 200 nM. We interpret this as a saturation of the intasome by the *att*B during the initial binding step. This interpretation is supported by inhibition experiments. Recombination is inhibited by the presence of a second *att*B containing an altered overlap sequence, a *saf* mutation. Inhibition constants were measured for safT*att*B inhibiting wt*att*P x wt*att*B and for wt*att*B inhibiting safT*att*P x safT*att*B. The same value of about 200 nM was obtained in each case. The similarity of the saturation constant and the inhibition constants support the idea that initial binding *att*B is rapid relative to subsequent steps.

We are now comparing the ability of other altered *att*Bs to interfere with recombination. These experiments should reveal the minimum sequence recognized by the intasome.

F 401 FUNCTIONAL ANALYSES OF MUTANTS OF FLP AND R RECOMBINASE FROM YEAST, Jing-Wen Chen, Jehee Lee, Barbara Evans, Marie-Claude Serre, Hiroyuki Araki, Yasuji Oshima and Makkuni Jayaram. Department of Microbiology, University of Texas at Austin, Austin, TX 78712, and Department of Fermentation Technology, University of Osaka, Osaka 565, Japan

The yeast family site-specific recombinases show a significant degree of homology among them and share a number of invariant residues. We have obtained multiple mutations at selected invariant positions in the Flp recombinase from *Saccharomyces* and the R recombinase from *Zygosaccharomyces rouxii*. These mutations are localized in two highly conserved regions called box 1 and box 2. In many cases the mutations at a particular residue were identical in both recombinases. The activity of these mutant proteins were tested *in vitro* in substrate cleavage and strand exchange assays using full and half recombination sites. The results of these assays indicate that, at least for a subset of the residues altered, identical changes result in similar defects in the activity of the two recombinases.

F 402 COMPLEMENTATION OF X RAY-SENSITIVE SCID FIBROBLASTS BY HUMAN GENOMIC DNA TRANSFECTION,

Kathryn Hall, Eric Hendrickson and David Weaver, Dana Farber Cancer Inst. and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston MA 02115

Severe combined immune deficient (scid) mice are unable to execute normal V(D)J recombination. Such recombination events give rise to aberrant deletions in antigen receptor coding sequences undergoing rearrangement. We have recently shown that scid cells are also unable to repair the double-strand breaks (DSBs) wrought by DNA damaging agents (i.e., X rays). These and other experiments predict that the scid mutation exerts its effect during or after the formation of the site specific or random breaks which are generated as recombination or DNA damage intermediates.

To further study the common role for scid in gene rearrangement and general double-strand break repair, we are attempting to clone the scid gene by functional complementation.

We have previously shown that scid fibroblasts are up to 4 orders of magnitude more sensitive to X rays than wild-type NIH3T3 cells and up to 2 orders of magnitude more sensitive than wild-type BALB3T3, over a dose range of 0-900 rads.

The scid X ray defect was complemented by transfection of human genomic DNA into X ray sensitive scid fibroblasts to generate primary transfectants. Genomic DNA from these primary resistant cell lines was transfected into scid fibroblasts and one X ray resistant secondary transfectant was isolated. The primary and secondary complemented transfectants demonstrated up to 2 orders of magnitude increase in X ray resistance which was equal to that of BALB3T3 cells. We have also complemented the X ray sensitivity of scid cells by fusing scid fibroblasts to human fibroblasts. These hybrids have similar levels of X ray resistance to that of BALB 3T3.

We will report the X ray DSB repair and V(D)J recombination characteristics of the transfected and fusion cell lines. We are in the process of cloning human DNA sequences from these cell lines.

F 403 CLONING AND DNA SEQUENCE ANALYSIS OF A cDNA ENCODING NONAMER BINDING PROTEIN (NBP), A COMPONENT OF THE V(D)J RECOMBINASE SYSTEM WITH DNA STRAND TRANSFER ACTIVITY.

Brian D. Halligan, Ming Teng, Tom Guilliams, J. Brian Nauert, and Nadine L. N. Halligan, Department of Microbiology, Medical College of Wisconsin, Milwaukee, WI 53226

Immunoglobulin genes are assembled by a series of recombination events that join gene segments from the Variable (V), Diversity (D), and Joining (J) families to form the mature, expressed Ig genes. These DNA joining events are due to V(D)J recombination, a conservative, site-specific recombination process. V(D)J recombination is mediated by a motif of recombinational signal sequences (RSS) that includes a semi-palindromic heptamer sequence, a conserved length spacer region, and a nonamer sequence. Previously, a lymphoid protein that specifically recognizes the nonamer sequence portion of the Ig RSS, Nonamer Binding Protein (NBP) had been identified. We have cloned and sequenced an apparent full length cDNA encoding NBP and have analyzed its DNA and predicted protein sequence. Amino acids sequences at the carboxyl terminus of NBP were found to be homologues to sequences at the carboxyl terminus of some DNA ligases. Bacterially expressed NBP fusion proteins have been found to be able to covalently transfer DNA strands between molecules in a RSS dependent manner. A similar RSS-dependent DNA strand transfer activity has been identified in extracts of lymphoid cells and has been shown to co-extract and co-chromatograph with nonamer sequence binding activity. We are currently investigating the nature of the NBP DNA strand transfer reaction and its role in V(D)J recombination.

F 404 SITE-SPECIFIC RECOMBINATION IN *SACCHAROMYCES CEREVISIAE* AND *ESCHERCHIA COLI*,

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Yeast 2 μm circle and bacteriophage P1 encode site-specific recombination systems called Flp-FRT and Cre-*lox*, respectively. Both systems carry out a variety of reactions *in vitro* . FLP and Cre will recombine sites that are intermolecular or intramolecular, in direct or inverted orientation, and supercoiling of the substrate is not required. This promiscuous activity *in vitro* makes these systems promising for investigating recombination in eukaryotic and prokaryotic cells. Additionally, it raises the question of whether conditions *in vivo* are permissive for such a wide range of activities.

These systems have been used as recombination probes in *Escherchia coli* and *Saccharomyces cerevisiae*. In yeast, recombination of circular plasmid substrates was achieved utilizing GAL- based expression systems. The rate of plasmid recombination was strongly influenced by the rate of DNA replication and topoisomerase II inhibition. The topology of recombination products *in vivo* after shifting the top2^{ts} mutant cells to the nonpermissive temperature to prevent product decatenation is reported. The results of such experiments in yeast and a similar set of studies for the Cre-*lox* system in *Escherichia coli* suggest a unifying model for the role of these site-specific recombination systems in DNA plasmid replication.

In order to study the influence of chromatin on site-specific recombination, nucleosomes were reconstituted onto recombination substrates *in vitro* . Using λ Int recombination as a model system the efficiency of recombination and the topology of the products was directly compared to the patterns observed *in vitro* using naked DNA substrates and correlated with earlier studies of the reaction *in vivo*. Chromatin greatly reduced the extent of recombination but did not inhibit it. Furthermore, nucleosomes were observed to absorb free supercoils and thereby change the topological complexity of the products.

F 405 A BACTERIAL MODEL SYSTEM FOR CHROMOSOMAL TARGETING,

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Efficient site-specific chromosomal targeting on the *E. coli* chromosome has been achieved using the FLP site-specific recombination system derived from the yeast 2 μm plasmid. An FRT site to be used as a target is introduced into the chromosome by Tn5 transposition. FLP protein is supplied from a plasmid-borne and regulated FLP gene. The DNA to be integrated is introduced to the cell in the form of a non-replicative, non-self-integrative bacteriophage λ vector (b211 cI857 Oam29 Pam80) containing an FRT site and a selectable marker. We have found that (1) site-specific integration is dependent on the expression of FLP protein and the presence of an FLP Recombination Target (FRT) site on the chromosome, (2) the integration frequency is as high as 10^{-1} targeted transformants per input phage. Non-targeted background integration via homologous or illegitimate recombination was observed at efficiencies about a thousand fold lower, and (3) the integration is reversible. Targeting is independent of *recA* function but the apparent integration frequency is affected by *recA* mutation. We also investigated some parameters that could affect targeting efficiency. Targeting efficiency is strongly affected by the structure of the recombination site and the FLP expression vectors, but transcription and the position of the site on the chromosome seem to have only slight effects.

F 406 Molecular analysis of the promotor enhancer region of IgG1 germline transcripts

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In activated B lymphocytes immunoglobulin class switch recombination is targeted to IgG1 by Interleukin 4. This process is accompanied by molecular changes in the chromatin: Upon induction with Interleukin 4, the DNA of the IgG1 switch region and 5′ thereof becomes demethylated and hypersensitive to DNAseI. Transcription is rapidly initiatet. Subsequently the DNA between the switch regions of IgM and IgG1 loops out and is deleted from the chromosom.
We analysed the 5′IgG1 switch region for DNA binding proteins possibly involved in targeting of switch recombination.We have identified proteins which could be involved in the regulation of this process by gel retardation. Two DNA binding proteins, bind to a region 700 bp upstream of the transcription start sites. One of this proteins is induced by interleukin 4, a second one is B cell specific. Analysis of the promotor region shows 4 additional DNA protein complexes. Experiments are under way to characterise the observed protein complexes further and understand their role in the process of class switch recombination.

F 407 HALF-SITE RECOMBINATIONS MEDIATED BY FLP RECOMBINASE FROM *SACCHAROMYCES CEREVISIAE*,

Marie-Claude Serre, Lei-Zheng and Makkuni Jayaram, Department of Microbiology, University of Texas at Austin, Austin, TX 78746

The Flp recombinase can mediate strand transfer within a half-site or between half-sites to form hairpins, full-sites and pseudo full-sites. The reactions are fairly insensitive to the sequence of the spacers and can tolerate spacer lengths of 6 to 10 nucleotides. In crosses between half-sites and full-sites, synapsis and initial strand exchange can occur by `normal′ or `reverse′ alignment. Selection of proper site orientation during a normal recombination event likely occurs at the branch migration step. `Step-arrest′ mutants of Flp, which are inactive in full-site recombination, show some strand exchange activity with half-sites. The mechanism appears to be similar to the two-step-cleavage and reunion-model accepted for the full-site reaction. Association of Flp with half-sites generate higher order protein-DNA complexes. The step-arrest mutants show specific defects in forming these complexes.

F 408 BASIS FOR RECOMBINATION SELECTIVITY AND THE ROLE OF DNA SUPERCOILING IN GIN RECOMBINATION,

Roland Kanaar[1], Anke Klippel[2], Nancy J. Crisona[1], E. Lynn Zechiedrich[1], Tania N. Gonzalez[1], Regine Kahmann[2], and Nicholas R. Cozzarelli[1], [1]Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley, CA 94720 [2]Institut für Genbiologische Forschung Berlin GmbH, D-1000 Berlin 33, Germany

Recombination by phage Mu Gin is restricted to a substrate containing two inverted recombination sites (gix) and an enhancer sequence, bound by Fis, on the same supercoiled DNA molecule. A direct site substrate is knotted but not recombined. This strict specificity of the reaction is caused by its dependence on both DNA supercoiling and the Fis-enhancer complex. The two trapped (-) supercoils in the Gin synaptic complex preclude deletion and intermolecular fusion. For mutants of Gin that no longer depend on Fis and the enhancer, synaptic complexes trapping no or only 1 (-) supercoil are now competent for recombination. These synaptic complexes can lead to intermolecular fusion and deletion, respectively.
In addition, we present a model for the Gin synaptic complex that explains extensively knotted products generated through processive recombination on different substrates. The model predicts the existence of two synaptic complexes that have the same topology but differ in their geometry.

F 409 IMPROVED YEAST ARTIFICIAL CHROMOSOME VECTORS: *FLP*-MEDIATED YAC CIRCULARIZATION.

Jacques Oberto and Carlo V. Bruschi, Microbiology Program, International Centre for Genetic Engineering and Biotechnology, Trieste I-34012, Italy.

Yeast Artificial Chromosome vectors (YACs) constitute the cloning vehicle of choice to study large genomes. Their ability to carry cloned DNA fragments ranging from 50 KB up to 1 MB simplifies by more than one order of magnitude the establishment of genomic libraries. The genetic information of interest, once cloned on a YAC, is replicated and maintained stably as an additional chromosome in the host, *S. cerevisiae*. The linear structure of the Yeast Artificial Chromosome might hinder further purification or isolation of the YAC from the other yeast chromosomes for subcloning purposes. We therefore propose a new YAC derivative having the property of circularizing itself *in vivo*, after being established as a linear YAC. This process is achieved by using the FRT/FLP site specific recombination system from the endogenous 2 micron yeast plasmid. In its circular form, the YAC carrying the cloned information will be able to transform *E. coli*. In order to ensure proper replication of such a large circular plasmid in the bacterial host, we have substituted the pBR322 replication origin present on the original YACs with the F′ plasmid replicon. The ability of circularizing yeast artificial chromosomes 'at will' will increase the versatility of YAC cloning and enable to study the fate of ring chromosomes in yeast through mitosis and meiosis.

F 410 LIGATION ACTIVITY OF THE FLP RECOMBINASE, Guohua Pan and Paul D. Sadowski, Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada M5S 1A8

The FLP recombinase of the 2 μm plasmid of *Saccharomyces cerevisiae* promotes a multi-step conservative site-specific recombination reaction. Two key steps in this reaction are DNA strand cleavage and strand rejoining (ligation). The cleavage reaction is promoted by a nucleophilic attack of a tyrosine residue (amino acid 343) upon a specific phosphodiester bond that marks the site strand exchange. A transient covalent intermediate between the tyrosine of FLP and the 3'-phosphoryl group is formed. Because the cleavage and ligation steps are closely coupled, it has been difficult to study the ligation reaction independently of the cleavage reaction. We report here the use of a partial FLP recombination target (FRT) site to measure the ligation activity of the FLP recombinase.

The substrate consists of half-FRT site(s) prepared by digesting a FRT site with a mutant FLP protein that generates a large amount of FLP-FRT covalent intermediate. After pronase digestion the half-sites are isolated by PAGE. Using this substrate we find that wild-type FLP protein promotes efficient strand ligation. Interestingly, several recombination-deficient and cleavage-deficient mutant proteins also perform the ligation reaction as well as the wild-type protein. In contrast, two other mutant proteins that cleave the DNA efficiently are unable to catalyse ligation. A mixture of two polypeptides (P13 and P21) derived from proteolysis of FLP also promotes strand ligation. Preliminary analysis of the substrates suggest that the tyrosine of FLP is retained at the 3'-phosphoryl terminus. These results suggest that the 3'-phosphate group is activated by the presence of the tyrosine residue to facilitate nucleophilic attack by the 5'-hydroxyl group. The role of the FLP protein in ligation is to bring together the two termini to initiate this nucleophilic attack. This assay may be applied to other systems that involve a covalent protein-DNA intermediate.

(Supported by the Medical Research Council of Canada.)

F 411 **TWO-DIMENSIONAL BAND SHIFTS,** A. Boffini and P. Prentki, Department of Molecular Biology, University of Geneva, Switzerland.

We have recently developed an approach which permits the rapid isolation of binding sites for a protein within a large set of DNA fragments (*Nucl.Acids.Res.* **19** (1991), 1369-1374). This procedure consists of a simple two-dimensional band shift assay on polyacrylamide gels. In the first dimension, a standard gel retardation electrophoresis is performed at 4°C. In the second dimension, the gel is placed in a horizontal gel apparatus and run at high temperature (60°C) to destabilize the DNA-protein complexes. The previously retarded fragments now migrate according to their real size, and are thus displaced from the diagonal formed by the fragments which were never bound by the protein. This system thus allows the identification of DNA fragments containing one or more specific binding sites for the protein by their migration "off the diagonal". The shifted bands are then amplified by PCR directly from the dried gel, and cloned in a plasmid vector.

We are using this approach to generate, from the *E.coli* chromosome, a library of the binding sites of the protein IHF (Integration Host Factor). The characterization of some of these sites will be presented.

F 412 REACTIONS BETWEEN HALF-FRT SITES AND FULL-FRT SITES: A MODEL SYSTEM FOR ANALYZING EARLY STEPS IN FLP PROTEIN-MEDIATED SITE-SPECIFIC RECOMBINATION, Xiao-Hong Qian, Michael M. Cox and Ross B. Inman, Department of Biochemistry, University of Wisconsin, Madison, WI 53706

The FLP recombination target (FRT) can be cut in half so that only one FLP protein binding site is present (a "half-site"). FLP protein binds the half-sites and joins them into dimeric, asymmetric head-to-head complexes held together chiefly by strong noncovalent interactions. These complexes react with full (normal) FRT sites to generate a variety of products. Analysis of these DNA species reveals that the reaction follows a well-defined reaction pathway that generally parallels the normal reaction pathway. The system is useful in analyzing early steps in recombination, since the identity of the products in a given recombination event unambiguously pinpoints the order in which the cleavage and strand exchange reactions occur. Two conclusions are derived from the present study:
(I) Formation of the dimeric head-to-head complex of half-sites is a prerequisite to further steps in recombination.
(II) The identity of the base pairs at positions 6 and -6 within the FRT site has a subtle effect in directing the first strand exchange event in the reaction to predominantly one of two possible cleavage sites. In addition, results are presented that suggest that a DNA-DNA pairing intermediate involving only two base pairs of the core sequence is formed prior to the first cleavage and strand exchange. DNA-DNA interactions may therefore not be limited to the isomerization step that follows the first strand exchange.

F 413 CRYSTAL STRUCTURE OF $\gamma\delta$ RESOLVASE AND ITS IMPLICATIONS FOR THE MECHANISM OF SITE-SPECIFIC RECOMBINATION

Phoebe A. Rice and Thomas A. Steitz, Dept. of Molecular Biophysics and Biochemsitry and Howard Hughes Medical Institute, Yale University, New Haven, CT 06511.

$\gamma\delta$ resolvase is a 20.5 KDalton site-specific recombination enzyme responsible for the 2nd step in the transposition of the $\gamma\delta$ transposon in E. coli. The substrate for resolvase is the 115 base pair <u>res</u> site, containing 3 inverted repeats to which resolvase binds. All 3 binding sites are required for recombination to occur, but cleavage and religation take place at the center of site I, and only if the 2 <u>res</u> sites are in direct rather than inverted orientation.

The structure of the 15.5 KD catalytic domain has been refined at 2.3 Å resolution. These crystals are space group $C222_1$ and have 3 monomers in the asymmetric unit, related to one another by 2 non-crystallographic 2-fold axes.

We have solved by molecular replacement a second crystal form (space group $P6_422$) in which both the catalytic domain and the intact protein crystallize isomorphously. The DNA binding domain is disordered, but packing analysis shows that there is only a very small region of space where a domain of its size could lie. This model places the DNA binding domain near the active site residue serine 10, and strongly suggests that the catalytic dimer will be arranged such that the binding domain of one monomer is adjacent to the active site of the opposite monomer.

Comparison of the total of 4 independant determinations of the monomer and 3 detetermінations of the solution dimer provided by these 2 crystal forms shows that resolvase is a strikingly flexible molecule. The overall twist of the central beta sheet varies by 10.5°, and the angle at which the 2 helices at the solution dimer interface cross one another is also variable. This flexibility may be critical for the specificity exhibited by resolvase, as it provides a mechanism for the active site to sense the details of the conformation of the DNA to which it is bound.

F 414 NORMAL CODING JOINTS CAN BE FORMED IN CELL LINES FROM **scid** MICE,
David B. Roth, Joanne E. Hesse, and Martin Gellert, Laboratory of Molecular Biology, NIDDK, Bethesda, MD 20892.

Scid is a spontaneous recessive mutation that leads to immunodeficiency resulting from absence of mature B and T cells, and is characterized by defective V(D)J recombination which severely affects the formation of coding joints. Previous work from this laboratory using extrachromosomal substrates demonstrated that lymphoid cells from scid mice form signal joints with relatively normal efficiency; however, coding joints were not detected. In other laboratories, studies using recombination substrates integrated into the chromosome have detected coding joint formation at extremely low frequencies. Sequence analysis of a handful of coding junctions from these cells demonstrated that many coding joints contain large deletions, as expected, but that a few junctions appear normal. Rare, normal coding joints have also been observed at the endogenous Igκ and TCRδ loci in scid mice that are not phenotypically leaky. We have recently found that minor alterations in cell culture conditions allow the recovery of coding joints from scid pre-B and pre-T cell lines transfected with extrachromosomal recombination substrates. Under these conditions, coding joints are formed only 10 to 20 fold less frequently than in wild-type cells. Examination of the coding junctions by restriction and nucleotide sequence analysis revealed both normal and highly deleted junctions. Coding joints were readily detected using recombination substrates that select for formation of a single junction. However, transfection of substrates that select for inversion events, which require formation of both coding and signal joints on the same molecule, demonstrated that inversion occurs at least 100 fold less frequently in scid than in wild-type cell lines.

F 415 SITE-SPECIFIC RECOMBINATIONAL MECHANISM INVOLVED IN DISSEMINATION OF ANTIBIOTIC RESISTANCE GENES.
Paul H. Roy, Luc Bissonnette, Louis Blier, Solange Brassard, Serge Champetier, Marc Ouellette, Roger Parent, Alex Pelletier, Lyson Piché, and Annie Tanguay. Dept. de Biochimie, FSG and Service d'Infectiologie, CHUL, Université Laval, Ste-Foy, Québec.

The roles of R plasmids and of transposons in the dissemination of antibiotic resistance genes are well known. A third mechanism, that of site-specific integration of resistance genes to form strongly expressed resistance operons, is increasingly important. Plasmids of several incompatibility groups, as well as transposons of the Tn21 family, contain a 3.4 kb common region coding for sulfonamide resistance and for a recombinase of the phage integrase family. In all of these plasmids except pVS1, a probable ancestor, one or more resistance genes, without promoters or terminators, are inserted between the sulfonamide resistance promoter and structural gene. In most of these plasmids, an aadA streptomycin/spectinomycin gene is present, indicating an early step in evolution. Other genes which occur in this sequence context are OXA and PSE-type Beta-lactamase genes, aminoglycoside resistance genes, trimethoprim resistance genes, nonenzymatic chloramphenicol resistance, and a novel chloramphenicol acetyltransferase unrelated to that of Tn9. DNA probe results with newly isolated strains indicate that the pVS1 system, unlike other site specific recombination systems, has a considerable potential for tandem insertions. We have also found a similar integration mechanism in Tn7.

F 416 RECOMBINATION MEDIATED BY VACCINIA VIRUS DNA TOPOISOMERASE I IS SEQUENCE-SPECIFIC.
Stewart Shuman, Molecular Biology Program, Sloan-Kettering Institute, New York, NY 10021

Specialized type I topoisomerases catalyze DNA strand transfer during site-specific recombination in prokaryotes and fungi. As a rule, the site-specificity of these systems is determined by the DNA binding and cleavage preference of the topoisomerase per se. The Mr 32,000 topoisomerase I encoded by vaccinia virus (a member of the eukaryotic family of "general" type I enzymes) is also selective in its interaction with DNA; binding and cleavage occur in vitro at a pentameric motif 5'(C/T)CCTT in duplex DNA. Expression of vaccinia virus DNA topoisomerase I in a lambda lysogen of Escherichia coli promotes *int*-independent excisive recombination of the prophage. In order to address whether the topoisomerase directly catalyzes DNA strand transfer in vivo, the recombination junctions of plaque-purified progeny phage were cloned and sequenced. In five of six distinct excision events examined, a topoisomerase cleavage sequence is present in one strand of the DNA duplex of both recombining partners. Recombination entails no duplication, insertion, or deletion of nucleotides at the crossover points, consistent with excision via conservative strand exchange at sites of topoisomerase cleavage. Three of these five recombination events are distinguished by the presence of direct repeats at the parental half-sites that extend beyond the pentameric cleavage motif, suggesting that sequence homology may facilitate excision. The data are consistent with a model in which vaccinia topoisomerase catalyzes reciprocal strand transfer leading to the formation of a non-migrating Holliday junction, the resolution of which can lead to excisive recombination.

F 417 RECOMBINATION POTENTIAL OF THE MURINE HEAVY CHAIN VARIABLE REGION,
Linda F. Van Dyk, and Katheryn Meek, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235

Production of a functional IgH chain requires recombination of the variable gene segments, V_H, D_H, and J_H. The 12/23 rule is generally maintained at the IgH locus, in that gene segments flanked by recombination signal sequences (RSS) with 12 bp spacers (D_H segments) recombine to those with 23 bp spacer RSS (V_H and J_H segments). D_H-J_H rearrangement occurs almost exclusively by direct (as opposed to inverted) joining, resulting in deletion of the DNA between the relevant gene segments. Finally, D_H-J_H rearrangement usually precedes V_H-D_HJ_H rearrangement. We have used PCR amplification of murine bone marrow, spleen, and thymus DNA to study rearrangements which could theoretically occur, but are rarely observed. We isolated various D_H-J_H joints, including direct coding and signal joints, and less frequently, inverted hybrid joints. Also found less frequently, yet consistently, were both inverted and direct D_H-D_H rearrangements, in spite of their breaking the 12/23 rule. Certain potential rearrangements were virtually absent, specifically, those having a D_H segment which had rearranged at the 5' RSS while retaining the 3' RSS. These findings suggest that the observed direct rearrangement results from a recombination bias for utilization of the 3' RSS in D_H-J_H joining. Additionally, V_H-D_H joints (without rearrangement to a J_H segment) were detected, a finding never reported at the IgH locus. These unusual rearrangement products add to the possible generation of diversity and have important implications for the restrictions and sequence of events imposed on immunoglobulin recombination.

F 418 AN ORDERED DISASSEMBLY OF COMPLEXES OF FLP RECOMBINASE AND FRT SITES FOLLOWING RECOMBINATION, Leslie L. Waite and Michael M. Cox, Department of Biochemistry, University of Wisconsin, Madison, WI 53706
The products of FLP-mediated site-specific recombination serve as substrates for additional rounds of recombination under most conditions. We find that if recombination is carried out with two different FRT substrates in different test tubes, and then the two reactions are mixed, cross-reaction between the two reactions is suppressed. This is manifested by a lag in the appearance of expected products of the cross-reaction. The lag increases as the FLP protein concentration increases, suggesting that it is produced by an inhibition of the disassembly of a complex between FLP protein and recombination products. The results suggest that there is an ordered disassembly of FLP protein complexes on FRT sites or recombination products. A key step in the disassembly, probably the dissociation of an FLP monomer, is inhibited by high FLP protein concentrations. High FLP protein concentrations therefore tend to favor reiterative recombination within a single complex.

F 419 EVIDENCE FOR GEOMETRICALLY DISPARATE, BUT TOPOLOGICALLY EQUIVALENT SYNAPTIC COMPLEXES FORMED IN GIN INVERTASE- MEDIATED DNA RECOMBINATION, E. Lynn Zechiedrich, Tania N. Gonzalez, Roland Kanaar, and Nicholas R. Cozzarelli, Department of Cell and Molecular Biology, University of California, Berkeley, CA 94720
Gin invertase from bacteriophage Mu efficiently rearranges the DNA region between its two binding sites- *gix* L and *gix* R. This reaction requires the presence of a host-encoded protein, FIS and its binding site, the enhancer (*sis*). In the present study, we have analyzed the geometry of the synaptic complex. Plasmids were constructed that have varying distances between the three DNA binding sites. These substrates range from having an extremely limited to a very large G region (the distance between *gix* sites). The enhancer distance from the *gix* sites was similarly varied. How the three DNA sites come together dictates what type of product is formed. Thus, by examining the complexity of knotted products created by Gin, we can reconstruct the geometry of the synaptic complexes. Results imply that geometrically disparate complexes are formed. However, they are topologically equivalent. By employing conditions which kinetically block Gin recombination at a double-stranded DNA cleavage intermediate, we have isolated synaptic complexes. Electron Microscopy of these complexes revealed that they were in the configurations predicted by the kinetic data. We conclude that Gin inversion occurs through more than one pathway. Models will be presented.
Supported by NIH GM-14574 (ELZ) and Howard Hughes Graduate Fellowship (TNG).

Transposition

F 420 ASSEMBLY OF THE ACTIVE FORM OF THE MU TRANSPOSASE, Tania A. Baker, Michiyo Mizuuchi and Kiyoshi Mizuuchi, LMB, NIDDK National Institutes of Health, Bethesda MD 20814
The transposase of phage Mu (the MuA protein) promotes all the reactions central to the early stages of transposition. MuA (1) pairs the two ends of the Mu genome, (2) cleaves the donor DNA at the Mu ends and (3) covalently joins the donor DNA to a new target DNA. Both donor DNA cleavage and DNA strand transfer are carried out by MuA protein within the context of a higher order protein-DNA complex. We have recently found a new stable MuA-donor DNA complex which forms prior to DNA cleavage (see poster by Michiyo Mizuuchi). Large amounts of this *stable synapsed complex* (SSC) accumulate in the presence of Ca^{2+} which does not support donor DNA cleavage. This observation allows the structure of this complex to be studied in detail, and the requirements for its assembly to be separated form those needed for the subsequent reaction stages. Protein crosslinking establishes that the stable synaptic complex contains a tetramer of MuA protein simultaneously bound to the two Mu ends; its structure is thus very related to that of the cleaved donor complex (also called the type I complex, Lavoie, BD, Chan BS, Alison RG, and Chaconas G, (1991) EMBO J 10: 3051-3059). Formation of the MuA tetramer, requires a divalent metal ion and donor DNA. The MuA tetramer is assembled from monomers and dimers of MuA protein present in solution. Under normal reaction conditions, tetramerization of the protein appears to be tightly coupled to the pairing of the two Mu ends; appearance of the tetramer requires the two complete Mu end sequences and the internal enhancer (IAS) on a supercoiled DNA. However, by adding solvents that presumably affect the MuA conformation to the reaction, these specific sequence and conformational requirements in the donor DNA are remarkably relaxed. These data indicate that under normal conditions, the principal role of the topological state of the donor DNA and the internal MuA binding sites is to promote assembly of the MuA tetramer. Thus, the MuA protein structure must be significantly altered by interacting with its specific DNA binding sites (in the presence of metal ions) to allow efficient, specific protein oligomerization. The MuA tetramer, whether assembled by interacting with its binding sites or through a "bypass reaction" can pair the two Mu ends and promote the subsequent stages of DNA cleavage and strand transfer.

F 421 HIV DNA INTEGRATION: FUNCTIONALLY CRITICAL INTERACTIONS BETWEEN IN PROTEIN AND DNA, Frederic D. Bushman and Robert Craigie, NIDDK, National Institutes of Health, Bethesda, MD, 20892.

Upon infection of a sensitive cell, a retrovirus such as HIV synthesizes a double stranded DNA copy of the viral RNA genome and integrates that copy into a chromosome of the host. We have found that a single viral protein, the Integration Protein (IN), can carry out the integration of viral DNA in a fully defined in vitro system. Typical assays contain only the IN protein, a model DNA substrate matching the termini of the unintegrated viral DNA, a DNA target, and a suitable reaction buffer. Reaction products made with model substrate DNAs consist of i) DNAs cleaved so as to remove two bases from the 3' end of the viral DNA (a processing reaction known to occur in vivo) and ii) DNA strand transfer products in which the 3' end of the viral DNA is joined to the 5' end of a cut made by IN in the target DNA. This assay has allowed us to carry out a variety of mechanistic studies, and in addition has provided the basis for a rapid assay suitable for screening candidate inhibitors of the integration reaction.

We have determined the position of the IN protein on the viral DNA in "DNA adduct interference" experiments. Donor DNA sequences were treated so as to attach chemical groups such as ethyl groups or primary amines to various points on the DNA chain. Modified DNAs were then assayed for their ability to serve as integration substrates. Results indicate that adducts near but not at the viral DNA end block the integration reaction; the cluster of positions where adducts block the reaction identify key points of interaction between IN protein and the viral DNA ends. Functionally critical interactions are also made to the target DNA near the point of strand transfer. IN protein requires the presence of a nearby DNA end for function, yet IN does not bind to the end directly. Perhaps IN protein i) deforms the DNA in a way that requires a nearby DNA terminus or ii) forms a complex with viral and target DNAs that cannot sterically accommodate an IN binding site embedded in continuous DNA.

F 422 THE BACTERIOPHAGE MU TRANSPOSITION ENHANCER IS REQUIRED FOR SYNAPSIS BUT NOT FOR STRAND CLEAVAGE, George Chaconas and Michael G. Surette, Department of Biochemistry, The University of Western Ontario, London, Ontario N6A 5C1 Canada

The bacteriophage Mu transposition enhancer (1-3) increases the initial rate of the in vitro Mu DNA strand transfer reaction by a factor of 100. The enhancer is located about 1000 base pairs from the left end cut site and contains binding sites for the Mu A protein. The enhancer is also the site of action of the supercoiling relief activity of the E. coli IHF protein (4) which introduces a sharp bend between two Mu A binding sites in the enhancer.

Experiments will be presented which limit the role of the enhancer to the synapsis step of the strand transfer reaction. We have demonstrated strand cleavage at wild type levels in the absence of an enhancer. The role of the enhancer in the synapsis step of the reaction will be discussed.

1)Mizuuchi M. and Mizuuchi K.(1989) Cell 58, 399-408
2)Leung P.C., Teplow D.B. and Harshey R.M. (1989) Nature 338, 656-658
3)Surette M.G., Lavoie B.D. and Chaconas G.(1989) EMBO J. 8, 3483-3489
4)Surette M.G. and Chaconas G.(1989) J. Biol. Chem. 264, 3028-3034

F 423 *TRANS* AND *CIS* REQUIREMENTS FOR INTRON MOBILITY IN A PROKARYOTIC SYSTEM, Jonathan Clyman, John Mueller and Marlene Belfort, Wadsworth Center for Laboratories and Research, New York State Department of Health, Empire State Plaza, Albany, NY 12201

Mobile introns are phylogenetically widespread, having been found in fungal and plant organelles, ciliate nuclei and bacteriophages. Study of the mechanism of intron transfer is hindered in most of these sytems by genetic limitations and by poorly understood recombination pathways. We are therefore exploiting prokaryotic genetics to better define the intron mobility process and have shown that unlike many transposases, the endonuclease that promotes mobility of the bacteriophage T4 td intron has no role in recombination beyond generating a double-strand break. Additionally, transfer of the td intron is promoted by recombinase and 5'-3' exonuclease functions. The role of a 3'-5' exonuclease is also implicated to create a gapped DNA intermediate required for a proposed double-strand-gap repair mechanism for mobility of the td intron.

F 424 ***Purification and Characterization of the Tn5 Inhibitor Protein, p2***
Norberto B. de la Cruz and William S. Reznikoff, Department of Biochemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706

Tn5 is a composite transposon which is found in gram negative bacteria. It is made up of two IS50 sequences which are in inverted orientation and flank a unique region which encodes antibiotic resistances. Tn5 and IS50 transposition rates are very low (10^{-5} per cell per generation) and have been determined genetically to be controlled at various levels. These include: 1. involvement of various host factors, 2. modification of the the IS50 ends by methylation, 3. inhibition of transposase (p1) activity by p2, a natural N-terminal "deletion" of p1 and 4. control of the production of the transposase (p1) and its inhibitor (p2).

In order to understand further how control of transposition is exerted by p1 and p2, we developed a purification protocol using heparin-agarose column chromatography and an in vitro assay for p1 binding to the outside end of IS50.

Recently, we modified the purification procedure for p1 in order to purify p2. We also used the in vitro binding assay to characterize the latter. Here we report some of our findings: 1. P2 can be purified using heparin agarose column chromatography. 2. Under the conditions in which p1 binds to the outside end, we do not detect significant binding by p2. 3. The addition of p2 to the p1 binding reaction enhances the binding of the latter. 4. P2 forms dimers in solution.

These results sugest that p2 inhibits p1 activity through mixed oligomer formation.

F 425 STUDIES ON THE IS*903* TRANSPOSASE. Keith M. Derbyshire and Nigel D.F. Grindley, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510.

IS*903* is a small bacterial insertion sequence of 1057 bp. It has 18bp, perfect, inverted repeats which define the ends of the transposon and it encodes a transposase of 307 amino acids. One of the first steps in the transposition process is the recognition and interaction of the transposase with its inverted repeats. We have begun to analyze this interaction *in vitro* to complement our genetic analyses. We have recently succeeded in over-expressing the transposase in a soluble active form, as a fusion protein with glutathione-S-transferase (Smith & Johnson, 1988 *Gene* **67**:31-40). The fusion protein can be rapidly and efficiently purified using affinity chromatography with immobilized glutathione. The fusion protein specifically binds the inverted repeats of IS*903*. We have examined the interaction of the transposase with the inverted repeats in a number of ways. We have examined the affinity of the protein for wild-type and mutant ends in a retention assay while the protein is still immobilized on a glutathione affinity column. We have also determined the specific DNA contacts by methylation and depurination interference experiments and have correlated this data with *in vivo* experiments that investigated the effect of mutations within the inverted repeat on transposition. The fusion protein can be eluted off the affinity column and used in band shift experiments to retard specifically inverted repeat containing DNA. We will present our analysis of the transposase-inverted repeat interactions.

F 426 INTER - PLASMID TRANSPOSITION OF IS10 IN *Escherichia coli* IS RecA - DEPENDENT AND ENHANCES HOMOLOGOUS RECOMBINATION, Zehava Eichenbaum and Zvi Livneh, Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel.

A new system for assaying inter-plasmid transposition in *Escherichia coli* was developed. The donor plasmid is a pBR322 derivative which carries a single copy of IS10R. The acceptor plasmid is a mini-F derivative which carries the *tet* gene fused to the O_RP_R operator-promoter region from phage 434, and the phage's *cI* repressor gene as a mutational target. Transposition of IS10 into *cI* in cells harboring the two plasmids is detected by analysis of plasmid DNA from tetracycline resistant colonies.

Inter-plasmid IS10 transposition occurred at a frequency of $5x10^{-8}$, and comprised 15-30% of the total tet^R mutations. Transposition was not observed in *recA1*, *recA13* or *ΔrecA* strains indicating dependence on the *recA* gene product. Each IS10 transposition resulted in the fusion of the donor and acceptor plasmids into cointegrate structures. In cells carrying the *recA56* mutation simple IS10 insertions were observed, but fusion of the plasmids was greatly reduced. This suggests that the fused plasmids were generated by a two-stage mechanism which involved an IS10 transposition event followed by homologous recombination. Presumably the RecA56 protein could promote transposition but not homologous recombination, thus separating the two stages. Essentially 100% recombinants were observed in conjunction with IS10 transposition. It is thus suggested that inter-plasmid IS10 transposition greatly stimulates inter-plasmid homologous recombination.

F 427 MECHANISM OF HIV-1 DNA INTEGRATION, Alan Engelman, Kiyoshi Mizuuchi, and Robert Craigie, LMB, NIDDK, NIH, Bethesda, MD 20892
Retroviral DNA integration involves a coordinated set of DNA cutting and joining reactions. Linear viral DNA is cleaved at each 3' end to generate the precursor ends for integration. This cleavage removes two bases from each end and the resulting recessed 3' ends are inserted into target DNA by a DNA strand transfer reaction. Purified HIV-1 integration (IN) protein carries out both of these steps in vitro.
We have observed novel products of the viral DNA cleavage reaction in addition to the expected simple dinucleotide. One of these products is the 3'-5' cyclic form of the dinucleotide; another product results from attachment of glycerol to the dinucleotide. We propose that a critical role of IN protein in the viral DNA cleavage reaction is to strain the phosphodiester bond at the site of cleavage, making it susceptible to attack by any accessible nucleophile. Whereas nucleophilic attack by water generates the simple dinucleotide cleavage product, attack on this bond by the OH group at the 3' end of the viral DNA strand gives rise to the cyclic product.
The stereochemical course of the viral DNA cleavage reaction was analyzed by carrying out reactions with phosphorothioate of defined chirality at the site of cleavage and determining the chirality of this phosphorothioate in the cyclic dinucleotide product. Inversion of chirality was observed. The stereochemical course of the DNA strand transfer reaction also proceeds with inversion of the chirality of phosphorothioate groups at the site of strand transfer in the target DNA. We suggest that the DNA strand transfer reaction utilizes essentially the same chemical mechanism as the cleavage reaction, except that IN protein positions the end of the cleaved viral DNA such that the oxygen atom at the 3' end of the DNA strand accomplishes the nucleophilic attack. The results suggest that both reactions occur by a one-step mechanism without involvement of a covalent intermediate between the viral DNA and IN protein.

F 428 **AVIAN RETROVIRUS INTEGRASE: BIOCHEMICAL PARAMETERS CONTROLLING PAIRWISE INSERTION OF VIRAL DNA TERMINI.** Michael Fitzgerald, Ajaykumar Vora and Duane Grandgenett, Institute for Molecular Virology, St. Louis University Medical Center, 3681 Park Avenue, St. Louis, Missouri 63110

Retroviruses integrate their DNA genome into host cell DNA during infection by non-homologous recombination. The reaction is catalyzed by the viral integrase protein (IN) and is dependent upon the sequences of the inverted repeat at the termini of the linear double-stranded viral molecule. IN first trims the blunt-ended viral DNA molecule on the 3' strands exposing the conserved CA moiety and then joins the trimmed 3' viral strands to the 5' strands of the host DNA. These two steps appear to be spatially and temporally separated during infection. *In vitro*, a similar decoupling of these steps is observed using purified IN.
Using plasmid based long terminal repeat substrates (that mimic the trimmed viral genome) and IN purified from avian myeloblastosis virus, we are studying parameters which affect the biologically relevant pairwise strand transfer reaction. Under investigation are effects of protein/DNA ratio, temperature, pH, salt concentration, and divalent metal ion. These biochemical parameters may influence the apparent noncovalent interactions involved between IN molecules which appear necessary for correct pairwise insertion of viral DNA termini into target DNA.
The termini of avian retrovirus linear DNA form an imperfect inverted repeat when juxtaposed. We have observed that the U3 terminal sequences are more efficient as a substrate than the U5 sequences for the trimming step. To test whether such a difference also holds for the strand transfer reaction, we cloned plasmids with only U3 or U5 termini. Both plasmids are integration competent and data will be presented comparing the efficiencies of the plasmids. An efficient method of subcloning and sequencing the integrants was developed. An analysis of the host site sequence preference and fidelity for host site duplication will be presented using suboptimal and optimal conditions for strand transfer.

F 429 DEVELOPMENT OF A TRANSPOSITION ASSAY FOR LEPIDOPTERAN TRANSPOSONS USING A BACULOVIRUS GENOME AS A TARGET, Malcolm J. Fraser, Kitima Boonvisudhi, Kathleen Pecen, Pamela Brzezinski, Tamara Ciszczon, Lynne Cary, and Heidi Wang, Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556
Several unique features of Baculovirus replication and assembly permit transposon mediated mutagenesis of the virus genome generating viable virus. A recurring spontaneous mutant phenotype called FP can be generated by host transposon insertions within a gene encoding a 25 Kda protein of as yet undefined function. In past analyses we have characterized two transposons inserting within the 25K gene and noted a target site specificity for the insertion event that is common to both types of transposon. PCR analyses of genomic copies of one transposon confirmed that the target site specificity for the insertion event was a quality of the transposon and was not influenced by the virus. This lab is currently adapting the Baculovirus/host system for molecular analyses of the insertion and excision events of these Lepidopteran transposons. In the present report we demonstrate that transposition of plasmid-carried, lacZ-tagged copies of Lepidopteran transposons can be followed into the Baculovirus genome. The insertion event retains the characteristic target site specificity associated with these transposons. This study provides a basis for the continued development of the Baculovirus/host system for analysis of target site-specific insertion of these eukaryotic transposons.

F 430 PROTEIN-DNA INTERACTIONS AT THE ENDS OF MU,
R.M.Harshey, A.Zou, P.C.Leung and C.F.Kuo. Dept.Microbiology, UT Austin, Austin,TX.

Initial events in Mu DNA transposition involve supercoiled Mu DNA-A protein interactions that, in the presence of *E.coli* HU protein, lead to the formation of a very stable nucleoprotein 'synaptic' complex in which the ends are nicked. In order to assess the importance of each of the six *att* sites (three at each Mu *att* end; L1-L3 at *att* L and R1-R3 at *att* R) in transposition, indirect supercoiled DNA-footprinting experiments were carried out. These experiments have revealed that although all six *att* sites are occupied by A within the complex, three of these sites (L2, L3 and R3) are only loosely held. A complex with only three sites occupied (L1, R1 and R2; designated 'core' complex) is extremely stable (in 4M urea, or 2M NaCl, or at 60°C) and is fully competent in the subsequent strand transfer step.

The A protein binds as a monomer to each *att* site, and causes the DNA to bend through approximately 90° at most *att* sites. Hydroxyl radical footprints show that at most sites, the A protein binds to one face of the DNA helix and covers two consecutive major grooves, binding specificity being acheived primarily through the major groove. A pair of DNAase I hypersensitive sites, one on each strand, appear at the back of only one of the two contacted major grooves at most sites and can be correlated with the degree of A protein-induced bend. Hydroxyl radical footprints confirm that at *att* R, the core site R1 would be out of phase with R2. Upon protein binding and bending, a special DNA structure must then be generated at this Mu end. We suggest that such a structure could facilitate the kind of proximity required for fostering interactions between the core sites that would be necessary for stabilizing a higher-order structure adopted by the Mu DNA ends in the synaptic complex.

F 431 Tn*5* Promoted Adjacent Deletions,
Ross A. Jilk, John C. Makris, Lynne Borchardt, and William S. Reznikoff, Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706
Tn*5* is able to promote adjacent deletions which result in the loss of DNA sequences flanking the transposon. The formation of these deletions requires a functional transposase gene, but they occured under conditions which do not allow transposition due to mutations present at a single end. Therefore adjacent deletions are likely to result from abortive transposition events. Two classes of adjacent deletions were observed. Class I deletions extend from a specific hot spot in the gene neighboring the transposon to the precise end of Tn*5*. The hot spot has a homology to the end sequence. Class II deletions extend from varying sequences in the neighboring gene to a position one base away from the end of Tn*5*. The class of deletions that occurs depends upon the structure of second Tn*5* end sequence located 12 kb away from the deletion associated end. A complete removal of the second end sequence and point mutants in the putative transposase binding site of that end result in class I deletion formation suggesting that these adjacent deletions occur when transposase fails to bind to this distant end sequence. Class II deletions are found in conjunction with both a wild type end sequence and a different subset of point mutant end sequences. These results indicate that some form of communication occurs between the Tn*5* end sequences even when they are widely spaced, that this form of communication is transposase dependent, and that different end sequence mutants affect different transposition related functions.

F 432 BIOCHEMICAL AND BIOPHYSICAL STUDIES OF RETROVIRAL INTEGRASE,
Kathryn S. Jones, Mark Andrake, Thomas Laue,[1] and Anna Marie Skalka, Fox Chase Cancer Center, Institute for Cancer Research, Philadelphia, PA 19111, and [1]University of New Hampshire, Durham, NH 03824
Retroviral integrase (IN) is required for the recombination reaction in which the reverse transcribed, double stranded DNA copy of the RNA genome integrates into the host chromosomal DNA. It has been demonstrated that IN alone is sufficient for the major steps of retroviral integration: the processing reaction (the removal of two nucleotides from the 3′ ends of the viral DNA) and the joining reaction (the staggered cleavage of the host DNA, and the subsequent strand joining of these ends to the processed ends of the viral DNA). We are currently investigating the biochemical and biophysical aspects of these reactions.

One powerful approach to the study of these reactions which has not yet been exploited is kinetic analysis. As a first step in these studies, we have demonstrated that the Rous sarcoma virus IN protein is an enzyme capable of catalytic turnover in both the processing and the joining reactions. We are currently performing kinetic studies to determine the turnover number for IN.

In related work, we are investigating the oligomeric composition of the functional native enzyme. Our kinetic analyses indicate that the monomer form of IN has little or no activity for the processing reaction; the functional unit of IN for this reaction appears to be a dimer. Similar studies for the joining reaction are in progress. These biochemical studies are supported by sedimentation analyses, which show that purified IN protein exists as a dimer in solution under a variety of conditions. Other physical studies are underway to enhance our understanding of the active integrase complex and to determine which regions of the IN protein are involved in oligomerization.

F 433 MUTAGENESIS OF RETROVIRAL INTEGRASE IDENTIFIES A CANDIDATE CATALYTIC SERINE,
Richard A. Katz, Joseph P. G. Mack,[1] George Merkel and Anna Marie Skalka, Fox Chase Cancer Center, Institute for Cancer Research, Philadelphia, PA 19111; [1]NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, Frederick, MD 21702
Retroviruses encode a protein, the integrase (IN), that is required for integration of linear viral DNA into the host chromosome. IN, alone, can carry out the reaction *in vitro*. The reaction involves two steps: nicking at the 3′-ends of both viral DNA strands (processing reaction) followed by joining of these ends to new 5′-ends of each strand of host DNA (joining reaction). We have substituted the evolutionarily conserved serine and threonine residues of bacterially-expressed Rous sarcoma virus (RSV) integrase. We find that conservative substitution of RSV serine 85 (S85) eliminates both the *in vitro* processing and joining reactions, but does not affect DNA substrate binding. These results suggest that S85 is directly involved in catalysis, and predicts that IN utilizes a single active site for both the processing and joining reactions. We propose a model in which IN functions as a tetramer to accomplish the four strand cleavages and two strand transfers that occur during the concerted integration reaction. The possible functions of S85 will be discussed.

F 434 SPECIFICITY OF RETROVIRAL DNA INTEGRATION IN VITRO: EFFECT OF CpG METHYLATION,

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To study the basis of observed preference for retroviral DNA integration, we developed a rapid and sensitive PCR-based system which allows us to assess the relative frequency of use of specific bases as targets for the avian leukosis virus in *in vitro* integration system. To test the role of DNA modification in integration specificity, i.e., to test the effect of 5-methylation of cytosine in runs of CpG on the distribution of integration target sites in our *in vitro* system, CpG-containing oligonucleotides were inserted into the target plasmid DNA. The following results were obtained: (1) preferred integration sites were observed and the distribution of integration sites was not uniform along the target DNA. (2) Integration of proviral DNA was observed in both orientations at the preferred sites. (3) Retroviral integration into a target CpG-region occurred on methylated, but not unmethylated target DNAs.

Recently, using LTR fragment as a donor DNA with purified integrase, we obtained evidence that the first two properties appeared to be inherent solely to viral integrase, while integration of the LTR end into a target CpG-region occurred on both methylated and unmethylated target DNAs. In addition to integrase , therefore, other factor(s) seemed to be necessary to confer specificity toward methylated CpG runs.

F 435 DEVELOPMENTAL DNA DELETION EVENTS IN THE CILIATE *EUPLOTES CRASSUS* ARE PRECISE AND GENERATE FREE CIRCLES WITH AN UNUSUAL JUNCTION REGION,

Lawrence A. Klobutcher, S. Lorraine Tausta, Leah R. Turner, and Linda Buckley, Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06030

During its life cycle, the hypotrichous ciliate *Euplotes crassus* transforms a copy of its chromosomal micronuclear genome into a macronucleus that contains thousands of short, linear, transcriptionally active DNA molecules (average size of ~2 kilobase pairs). A large number of DNA breakage and rejoining, or splicing events, also occur during macronuclear development. Two classes of DNA sequences have been defined that are eliminated via DNA breakage and rejoining. The first class are short segments of unique DNA referred to as internal eliminated sequences (IESs). The second class is a highly repetitive group of transposon-like elements with long inverted repeats (~700 bp) referred to as Tec1 elements. Both classes of elements are bounded by a short direct repeat. Recent studies indicate that both IESs and Tec1 element exist as circular forms following their excision from the micronuclear chromosome (Tausta and Klobutcher, *Cell* **59**:1019; Jahn et al., *Cell* **59**:1009). In the current study we have used a polymerase chain reaction (PCR) procedure to characterize the precision of IES and Tec1 excision. For, two Tec1 elements and three IESs, excision events were found to be precise in independent episodes of macronuclear development, and one terminal direct repeat was always retained in the mature macronuclear DNA molecule. Other studies used PCR to characterize the junction region of the free circular IESs. Our results indicate that most junction regions retain both copies of the terminal direct repeat separated by 10 bp that also appear in the mature macronuclear DNA molecule. Two classes of PCR products derived from the circular IESs have been observed, which differ in retaining primarily sequences from the left or the right flanking regions of the IESs. These results indicate that the excision process involves staggered cuts in the DNA and suggests that heteroduplex regions may exist at the junctions of the excised circular DNA segments.

F 436 PARTIAL PURIFICATION AND CHARACTERIZATION OF THE MOLONEY MURINE LEUKEMIA VIRUS INTEGRATION MACHINERY,

Myung Soo Lee, Robert Craigie and Kiyoshi Mizuuchi, Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892

A nucleoprotein complex mediates the integration of retroviral DNA into the host genome, an essential step in the retrovirus life cycle. Such complexes, isolated from cells after infection with Moloney murine leukemia virus, efficiently integrate their endogenous copy of viral DNA into an exogenously introduced target DNA *in vitro*; greater than 50% of the viral DNA within the nucleoprotein complexes can integrate into the target DNA within 2 minutes. Although the presence of either magnesium or manganese ions is absolutely required for the integration reaction to occur, metal ions are not required for maintenance of a functional complex. We have also shown that the viral DNA within the complex can be digested by restriction enzymes without significantly affecting the efficiency of normal pairwise integration of the viral DNA ends. This strongly implies that the two ends of the viral DNA are physically held together by the integration machinery and that the association remains intact after restriction enzyme cleavage. Furthermore, we have obtained a preparation of the nucleoprotein complex that is virtually devoid of both contaminating RNA and free proteins. These complexes do not contain a detectable level of the CA, the major structural protein of the virion. This preparation will allow us to further analyze the protein composition of the functional complex as well as the nucleic acid-protein interactions that contribute to its stability and promote efficient integration of the viral DNA into a target DNA, but prevent integration of the viral DNA into itself.

F 437 THE INTERACTION OF γδ TRANSPOSASE WITH THE TERMINAL INVERTED REPEATS OF THE γδ TRANSPOSABLE ELEMENT,

Earl May and Nigel D. F. Grindley, Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06510.

The γδ transposon is a member of the Tn*3* family of transposable elements and proceeds through a replicative pathway during transposition. Transposase, a 113 kDa protein, mediates cointegration by acting directly at the ends of the element. Another property of the Tn*3* family shared with γδ is that of transposition immunity. A replicon containing a copy of γδ, or just a single end of the element, is "immune" to the insertion of other copies of γδ.

The first step in the project was the saturation mutagenesis of the γδ 35 bp inverted repeat. A mating-out assay was developed to determine the immunity of a target plasmid harboring a mutant end. Transposons were constructed from a subset of these mutant ends and assayed for transposition frequency using a similar mating-out assay. The following conclusions can be drawn from the data obtained to date:

•Most single base substitutions have little effect on immunity or transposition.

•Those mutations that affect immunity cluster in three regions corresponding to the portions of the end at which transposase crosses the major groove of the DNA (Wiater and Grindley, 1990, J. Biol. Chem. 266:1841).

•The strongest affects are seen with mutations in the most internal major groove contact region, base pairs 29 to 33, the weakest at the most external contact region, base pairs 9 to 14.

•Mutations near the outside end positions do not affect immunity, but transposons with these mutant ends do not transpose. These results are consistent with a two-domain model for γδ similar to that proposed for IS*903* (Derbyshire, et al. 1987, Proc. Nat. Acad. Sci. 24:8049), and later for Tn*3* (Ichikawa, et al. 1990, Gene 86:11).

Several mutants have been chosen for binding studies using a gel mobility shift system. The relationships between protein binding, transposition immunity, and cointegration are thus explored.

F 438 Gel assay for VDJ recombination of extrachromosomal substrates. Joseph P Menetski, Kiyoshi Mizuuchi and Martin Gellert; Laboratory of Molecular Biology, NIDDK, NIH. Bethesda, MD 20892:

We have shown that the products of VDJ recombination in extrachromosomal substrates can be observed directly in DNA isolated from lymphoid cell lines after transfection. These data show that transformation of the DNA into bacteria does not alter these products and that the reaction is completed in the lymphoid cell. We also show that the transfected DNA appears in different cellular fractions during the time course of the transfection. Early in the transfection the DNA is mainly found in an insoluble chromatin fraction. Freely diffusible, supercoiled and nicked plasmid molecules are not observed until late in the transfection (17-20 hours). The appearance of plasmid in this fraction is coincident with the onset of recombination. Also, all of the DNA observed in this diffusible fraction has undergone replication. These data may suggest that replicated plasmid must be in this diffusible fraction to undergo recombination. An alternative explanation may be that recombination is occurring in the chromatin fraction which seeds the diffusible fraction with replicating plasmids. Current experiments are planned to address these two possibilities.

F 439 STRUCTURAL ANALYSIS OF THE INITIAL STABLE SYNAPSED COMPLEX AT THE EARLY STAGE OF Mu TRANSPOSITION, Michiyo Mizuuchi, Tania A. Baker and Kiyoshi Mizuuchi, LMB, NIDDK, NIH, Bethesda, MD 20892

Phage Mu transposition occurs through a series of specialized protein-DNA complexes. At least three proteins (MuA, MuB and HU) and three distantly located DNA segments (two Mu ends and the internal activation seqence= IAS) are involved in the formation of these complexes. One of the complexes is the cleaved donor complex (CDC). In this complex the two ends of Mu DNA are synapsed together stably, but non-covalently, by MuA protein, and the donor DNA is cleaved at the ends of the Mu sequences. DNase protection analysis of the CDC showed that only three (L1, R1 and R2) of the six MuA binding sites at the ends of the Mu DNA are protected from nuclease digestion. Approximately 10bp outside of the Mu L and R ends are also protected by MuA in this complex.

By substitution of Ca^{2+} for Mg^{2+} in the reaction, we have recently trapped a stable MuA-DNA complex that is formed prior to the endonucleolytic cleavages at the ends of Mu DNA. This complex accumulates during the incubation in the presence of Ca^{2+}. By subsequently adding Mg^{2+}, this complex can be chased into the CDC. In this complex, as in the CDC, the two Mu ends are stably synapsed together by MuA (refer to the poster by Tania Baker et al.). Nuclease protection analysis of this complex, which we call the stable synapsed complex (SSC), exhibits patterns of protection similar to that of the CDC. The requirments for the formation of the SSC and its physiological significance will be discussed.

F 440 BACTERIOPHAGE MU DNA REPLICATION: POSSIBLE FUNCTION OF TRANSPOSITION PROTEINS AFTER STRAND TRANSFER, Hiroshi Nakai, Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, D.C. 20007.

In the *in vitro* bacteriophage Mu transposition system developed by K. Mizuuchi (1983, Cell **35**, 785-794), the formation of simple inserts and cointegrates is catalyzed. The first phase of this reaction is Mu strand transfer, which can be catalyzed in the presence of three purified proteins: Mu *A* protein, Mu *B* protein, and *Escherichia coli* HU protein. In this process a pair of single-strand DNA transfers, one from each end of Mu DNA, is made to target DNA to create a branched structure with two potential replication forks. Host proteins present in an *E. coli* extract can convert this DNA intermediate into a simple insert or cointegrate. The *A*, *B*, and HU proteins remain bound to the intermediate after catalysis of strand transfer, and this protein-DNA complex has been termed a "type II complex" (Surrette, M. G., Buch, S. J., and Chaconas, C., 1987, Cell **49**, 253-262). Possible effects of these bound proteins in the completion of transposition were examined. The transposition intermediate, either as a type II complex or as deproteinized DNA, was introduced into a reaction mixture containing an *E. coli* extract. In both reactions the completed transposition products were predominantly simple inserts although cointegrate products were also detectable. Extracts of *dnaB*⁻, *dnaC*⁻, *dnaG*⁻, and *dnaE*⁻ *E. coli* strains, which fail to support Mu DNA replication *in vivo*, were tested for their ability to convert the intermediate to simple inserts. Both the type II complex and the deproteinized intermediate were readily converted to simple inserts by *dnaB*⁻, *dnaC*⁻, and *dnaG*⁻ cell extracts. In contrast, only the deproteinized intermediate, and not the type II complex, was converted to a simple insert by the *dnaE*⁻ cell extract (*dnaE* encodes the α subunit of *E. coli* DNA polymerase III holoenzyme). The DNA in the type II complex was not degraded or altered in the presence of the *dnaE*⁻ extracts. These and other results suggest the following: Although DNA polymerase III is not required for the conversion of the deproteinized intermediate to a simple insert, it may play a crucial role in making the type II complex accessible to other host proteins. Proteins contained in the type II complex may serve to protect the DNA from action of host nucleases and replication proteins until the binding of DNA polymerase III holoenzyme to the transposition intermediate.

F 441 REVERSE GENETICS OF *Caenorhabditis elegans*, USING THE Tc1 TRANSPOSON

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An almost complete physical map of the *Caenorhabditis elegans* genome is available, and current efforts are aimed at the determination of the complete DNA sequence of this animal. No method has yet been described for targeted alteration of a chromosomal sequence in a nematode, and this limits functional analysis of the genome. We here present a two-step strategy for reverse genetics of *Caenorhabditis elegans*:

1. Animals are selected that contain a Tc1 insertion in a sequence of interest. This is done in a sib-selection protocol: DNA is analyzed from pools of animals, and sibling pools of a positive pool are further analyzed. Looking at insertions in G protein subunit genes we found that approximately 1 per 40,000 animals per generation contains a new Tc1 insertion per kbp DNA. Tc1 seems to insert quite often into intron sequences, probably because they are rich in TA sequences.
2. Animals with a Tc1 insertion are made transgenic for a DNA segment that corresponds to the region of insertion of Tc1 and that contains a mutation of interest. In their progeny animals are selected that have lost the chromosomal Tc1 element; these have often picked up the mutation from the transgene. This is the result of template directed repair of the double strand break left after Tc1 excision (see Plasterk, EMBO J. **10**, 1919-1925); we find that extrachromosomal transgenic DNA can be used as template for DSB repair.

This strategy can be used to generate point mutations, deletions, insertions, and replacements.

F 442 PURIFICATION AND CHARACTERIZATION OF HIV-1 INTEGRASE EXPRESSED IN E.COLI AND ANALYSIS OF SOME N-TERMINAL MUTATIONS, Karen A. Vincent, Viola Ellison, Samson A. Chow, and Patrick O. Brown, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305
Replication of a retroviral genome is dependent upon integration of the viral DNA into a chromosome of the host cell. The integration reaction is catalyzed by integrase (IN), a viral enzyme. In vitro analyses of the integration reaction using retroviral IN derived from various heterologous expression systems have shown that these enzymes possess all of the activities of viral integrase and that IN is the only protein required for integration. We have expressed HIV-1 IN in E.Coli using the T7 expression system and purified the protein to near homogeneity. By employing an in vitro assay based upon the use of short, double-stranded oligonucleotides as substrates, optimum conditions for the integration reaction were identified. A kinetic analysis of integration/ processing activity is in progress. In addition, we have constructed mutants that contain amino acid substitutions within the N-terminal region of IN. Four of these are located within the conserved HHCC region, a postulated metal ion binding domain. Activity of each of the mutants has been characterized in three assays : integration, processing, and the reverse of the integration reaction (or disintegration). While mutations in some conserved amino acids retain no integration or processing activity, weak activity in both assays was observed in one mutant. All mutants retain some activity in the disintegration assay. The latter result indicates that the active site for DNA cleavage/ligation is probably not located in this domain. However, the integration/processing activities are more sensitive than disintegration to mutation in this region of the protein. Possible explanations for this observation include enhanced binding to the disintegration substrate due to the target sequence component of the substrate or a possible effect of the mutations on protein/protein interaction where this might be required for integration/processing but not for disintegration. Further characterization of these mutants as well as further mutagenesis of HIV-1 IN are in progress.

F 443 SITE-SPECIFIC HYDROLYSIS AND ALCOHOLYSIS OF HIV DNA TERMINI BY THE VIRAL INTEGRASE PROTEIN, Cornelis Vink, Dik C. van Gent and Ronald H.A.Plasterk, Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
Before integration of the human immunodeficiency virus (HIV) DNA, two nucleotides are removed from the 3' ends of the viral DNA by the integrase (IN) protein. We studied the chemistry of this reaction, and found that IN mediates site-specific hydrolysis of a single phosphodiester bond, resulting in release of a dinucleotide. No evidence was found for a covalent linkage between the IN protein and this dinucleotide, suggesting that IN directs a single nucleophilic attack of water at the specific phosphodiester bond. A class of alcohols (including glycerol) can also act as nucleophile in this reaction, and likewise the alcoholic amino acids L-serine and L-threonine can be covalently linked to the dinucleotide. These results are consistent with a one-step mechanism for IN action.
We coupled HIV IN to a solid support and found that the immobilized protein was more stable and more active. The kinetics and specificity of DNA-binding to immobilized IN were investigated.

F 444 THE ROLE OF SUPERCOILING IN TRANSPOSITION OF THE BACTERIOPHAGE MU

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Supercoiling of DNA is required for transposition of the bacteriophage mu. It is now clear that supercoiling of DNA substrates is involved only in the initial stage of the transposition reaction, i.e. the formation of a synaptic complex in which mu ends are nicked. Despite great efforts in mu and in many other systems, the role of supercoiling remains elusive.

We have attempted a different approach to address the problem. Using a simple in vitro assay, we have determined the rate constants for the synaptic complex formation of a set of mini-mu plasmids of different superhelical densities. Our results indicate that the supercoiling energy may be used directly to reduce the activation energy for formation of an intermediate before the mu ends can be nicked by the protein A.

F 445 An N-terminal Deletion Analysis of the Tn5 transposase, Michael D. Weinreich and William S. Reznikoff, Department of Biochemistry, University of Wisconsin, Madison, WI 53706.
The transposase of Tn5 is identical to the element encoded inhibitor protein (which does not promote transposition) except that it contains an additional N-terminal 55 amino acids. We have created a set of N-terminal deletions in this unique region of the transposase to examine the importance of these residues for transposition. Deletion of just 3 amino acids results in a 10-fold loss of transposition activity and deletions removing 7 or more amino acids result in a total loss of transposase function. The first 41 amino acids of the transposase contains a potential helix-turn-helix DNA binding domain and the first α-helix of this predicted domain is amphipathic. We are examining the DNA binding activity of several transposase deletion derivatives in order to test whether removal of this potential HTH domain results in the loss of the specific DNA binding activity of the transposase. We further show that high level production of the transposase is lethal to *E. coli* even in the absence of transposition and that this effect is dependent on the presence of the wild type N-terminal domain, since similar overexpression of the deletion mutants is not lethal. We present several interesting explanations for this phenomenon.

F 446 **Transposase Mutants That Increase the Transposition Frequency of Tn5**, Torsten W. Wiegand and William S. Reznikoff, Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706.
Transposition of Tn5 in *Escherichia coli* is regulated by two transposon encoded proteins: transposase (Tnp), promoting transposition preferentially in cis, and the trans-acting inhibitor (Inh). Two separate transposase mutants were isolated that replace glutamate with lysine at positions 110 (EK110) and at position 345 (EK345). The EK transposase proteins increase the Tn5 transposition frequency 6- to 16-fold in cis and enhance the ability of transposase to act in trans. The purified mutant transposase proteins interact with transposon outside end (OE) DNA differently from the wild-type protein, resulting in the formation of a novel complex in gel retardation assays. During characterization of the transposase proteins in the absence of inhibitor we found that wild-type transposase itself has a transposition inhibiting function and that this inhibition is reduced for the mutant proteins. We propose the existence of two transposase species, one cis-activating and the other trans-inhibiting. The phenotype of the EK transposase mutants can be explained by a shift in the ratio of these two species.

Replication Fork

F 500 THE *E. coli* DnaC PROTEIN REGULATES the DnaB HELICASE in CHROMOSOMAL REPLICATION.

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The DnaC protein of *E. coli* is essential for replication *in vivo* and *in vitro*. In the initiation of replication of a minichromosome at its origin, DnaC delivers the DnaB helicase from a DnaB•DnaC complex to the future replication fork and then departs. However, if an excess of DnaC was present in subsequent steps, it severely inhibited replication by slowing the DnaB helicase at the replication fork. When DnaB was present at a level equimolar with the excess DnaC, the inhibition was relieved, implying that the ratio of DnaC to DnaB is critical for achieving optimal replication activity and avoiding inhibition by DnaC. *In vivo*, overproduction of DnaC slowed cell growth. This slowing was alleviated by overproducing DnaB at the same time. *E. coli* strains with a *dnaCts* gene defective in chromosomal initiation were complemented by the wild-type gene in *trans*. On the other hand, strains with an elongation-defective *dnaCts* gene were not complemented by the wild-type *dnaC* gene. The dominance of the mutant protein suggests that it remains tightly complexed with DnaB at the replication fork, inhibiting elongation even in the presence of the wild-type DnaC.

F 501 THE IDENTIFICATION AND CHARACTERIZATION OF TWO NOVEL DNA HELICASE ACTIVITIES FROM THE YEAST *S. cerevisiae*. Daniel W. Bean and Steven W. Matson, Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280
Two NTP hydrolysis-dependent DNA helicases have been identified and partially purified from protein extracts of *S. cerevisiae*. Yeast helicase I(yHelI) and yeast helicase II(yHelII) have been characterized with respect to both DNA-dependent ATPase and DNA helicase activities. Both enzymes catalyzed the unwinding of duplex DNA and the hydrolysis of ATP in a time and protein concentration dependent reaction. Both yHelI and yHelII utilized either rATP or dATP in unwinding reactions; the other NTPs could not substitute for ATP. In addition each helicase displayed biochemical and physical properties that set it apart from previously described yeast DNA helicases. The enzymes cofractionated on phosphocellulose and ssDNA cellulose, and were resolved on dsDNA cellulose with yHelI eluting at 650 mM NaCl while yHelII eluted at 350 mM NaCl. Further purification of yHelI yielded results suggesting that the active enzyme may be composed of two or more loosely associated polypeptides. Experiments are underway to further address this question. yHelI unwound duplex DNA in a 5' to 3' direction with respect to the strand on which it was bound. The addition of *E.coli* SSB stimulated the unwinding of a 217 bp partial duplex substrate 15-fold. Interestingly, both ssDNA and duplex DNA served as cofactors in the yHelI ATPase reaction. yHelII was further purified on heparin sepharose, anion exchange HPLC and by glycerol gradient sedimentation. The enzymatic activity appears to reside in a 75 kDa polypeptide that sedimented as a monomer in 250 mM NaCl. Further biochemical characterization of this enzyme is in progress.

F 502 STRAND-SPECIFIC RECOGNITION OF A SYNTHETIC REPLICATION FORK BY THE SV40 LARGE TUMOR ANTIGEN, James A. Borowiec and Dhruba SenGupta, Department of Biochemistry, New York University Medical Center, NY, NY 10016

The SV40 large tumor antigen (T antigen) is a required factor in SV40 DNA replication in part due to the intrinsic DNA helicase activity of T antigen. To characterize the interaction of a DNA helicase with DNA during DNA unwinding, we probed a complex formed between T antigen and a synthetic replication fork. The synthetic replication fork was prepared from two partially complementary oligonucleotides that form a 'Y-structure' when annealed. T antigen was found to form a specific complex with the fork in the presence of ATP, but this binding did not require ATP hydrolysis. Enzymatic probing of T antigen bound to the fork revealed that T antigen protected only one of the two strands comprising the fork, that containing a 3' end in the single-stranded portion of the molecule (top strand). Significant protection of the top strand was observed over approximately fourteen nucleotides at the fork, of which ten were in the single-stranded DNA. This binding is consistent with the 3' to 5' directionality of the T antigen DNA helicase activity. The binding of T antigen to fork molecules with different sequences was also examined. These experiments confirmed that T antigen did not bind a particular DNA sequence, but rather recognized a fork-specific element within the DNA molecule.

The binding of T antigen to chemically modified DNA molecules indicated that the primary interaction between T antigen and the synthetic replication fork was through the sugar-phosphate backbone. The importance of the sugar-phosphate backbone was confirmed by interference studies which showed that ethylation of specific phosphates inhibited the denaturation of the replication fork by T antigen. These phosphates were found in a six nucleotide region at the single-strand/double-strand junction on the same strand bound by T antigen. These data suggest that the fork-specific structure recognized by T antigen is the single-strand to double-strand transition (3'→ 5') of the sugar-phosphate backbone within an individual strand.

The oligomeric state of T antigen bound to the fork was examined by a gradient gel analysis. T antigen appeared to bind the fork primarily as a hexamer, although a lesser amount of double hexamer also recognized the fork. Essentially no binding to the fork by T antigen oligomers smaller than hexamer was found. These data lead to a simple model for the interaction of T antigen with the replication fork during DNA helicase action.

F 503 MUTAGENESIS AND FUNCTIONAL COMPLEMENTATION OF *E. coli rnhA* MUTANTS BY RETROVIRAL REVERSE TRANSCRIPTASE, Don G. Ennis and Robert J. Crouch, Laboratory of Molecular Genetics, NICHD, National Institutes of Health, Bethesda, MD 20892.

We are studying the functional organization of reverse transcriptase (RT) from murine lukemia virus (MuLV) and of human immunodeficiency virus (HIV). Molecular and biochemical studies of several different RT have suggested that the polymerase activities reside in the N-terminus and the RNase H activity is in the C-terminal portion the RT enzyme.

To better study mutant RT proteins, we have developed a functional expression system in *E. coli*. Bacterial strains carrying a null mutation of *rnh*, the gene that encodes RNase H, are viable; however, *rnh*⁻ in combination with *recB* (or *recC*) null mutations are inviable. Bacterial strains which carry the *rnh339::cat* and the conditional *recB270*(Ts⁻) mutation are inviable at high temperature (42° C) unless RNase H is supplied from another source. We have identified expression conditions for plasmid encoded RT from MuLV and HIV-1 which permit these strains to grow at 42° C. In contrast, plasmids with deletions in or near the postulated RNase H domain were unable to confer a temperature-resistant phenotype for the same strains. These results indicate that the RNase H activity of both RT enzymes can functionally replace the *E. coli* RNase H, and we anticipate that this system will be a useful tool for functional analysis of the RNase H activity. We have obtained several lines of evidence that our expression conditions of RT also increases the rates of mutagenesis in these bacteria. Further characterization of RT-induced mutagenesis is in progress.

F 504 THE EFFECT OF TRANSCRIPTION UNIT ORIENTATION ON REPLICATION FORK MOVEMENT
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In *E. coli* where multiple rounds of replication occur simultaneously on a single chromosome during rapid growth, it is almost impossible for transcription and replication to occur in isolation of one another. Circumstantial evidence suggests that the consequences of collisions between DNA and RNA polymerases are most severe for genes which are transcribed and replicated in opposite directions. 92 of 97 genes encoding protein synthesis machinery in *Escherichia coli* are oriented so that they are replicated in the same direction as they are transcribed [1]. To directly examine the physical consequences of the interaction between replication and transcription, an inducible replication origin was inserted onto the *E. coli* chromosome upstream or downstream from *rrnB*. One of seven rRNA operons, *rrnB* is easily recognized in chromatin spreads. It is long and densely packed with RNA polymerases, serving as a good region over which to view the interaction between replication and transcription. Chromatin spreads were prepared from cultures 4 to 6 min after induction of replication and positions of replication forks with respect to RNA polymerases were determined by electron microscopy. Replication forks were observed within active genes regardless of the direction in which they were transcribed. In either orientation the DNA polymerase complex appeared to dislodge RNA polymerases in its path. RNA polymerases were seen in front of the replication fork, but not immediately behind it. Replication fork movement was not impeded by RNA polymerases provided replication and transcription were co-directional. In the opposite orientation in contrast to the situation in yeast, the 3′ ends of *E. coli* rRNA genes did not serve as barriers to replication forks approaching from downstream. When DNA and RNA polymerases collided head-on, replication fork movement was, however, significantly retarded. More than two minutes were required to replicate *rrnB* in this orientation compared to a few seconds when the polymerases moved in the same direction. Occasionally altered chromatin structure behind the replication fork suggested that lagging and leading strand synthesis had become completely uncoupled. In these instances it is possible that replication fork movement was completely stalled. Our observations of the interactions between DNA and RNA polymerases suggests that evolutionary selection for the co-directionality of replication and transcription may be based on the need to replicate both halves of the chromosome within a similar time frame.

[1] B.J. Brewer (1989) in *The Bacterial Chromosome*, Dirlca & Riley, eds.
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F 505 INHIBITION OF DNA HELICASE II UNWINDING AND ATPase ACTIVITIES BY DNA INTERACTING LIGANDS: KINETICS AND SPECIFICITY, James W. George,* Sujata Ghate,* Jeffrey M. Besterman# and Steven W. Matson,* *Department of Biology, University of North Carolina, Chapel Hill, NC 27599, #Department of Biology, Glaxo Inc., Research Triangle Park, NC 27709

The effect of several DNA binding ligands and topoisomerase inhibitors on the unwinding activity of *E. coli* DNA helicase II was examined. Mitoxantrone and nogalamycin, the most potent compounds tested, inhibited the unwinding of a partial duplex DNA substrate with an apparent K_i of less than 1 μM. To determine the effect of mitoxantrone on the interaction of helicase II with ssDNA, the DNA-dependent ATPase activity of helicase II was also measured. Mitoxantrone effectively inhibited the DNA-dependent ATPase reaction catalyzed by helicase II in the presence of M13 ssDNA. In contrast, little inhibition of the ATPase reaction was observed using poly dT ssDNA suggesting the inhibition of helicase II by mitoxantrone required secondary structure in the DNA substrate. To directly test the effect of mitoxantrone on the unwinding reaction, in the absence of ssDNA, we performed unwinding reactions with a fully duplex DNA substrate. The apparent K_i of mitoxantrone for inhibition of the unwinding reaction was identical to that measured using the partial duplex DNA substrate. In addition, control experiments suggested that mitoxantrone did not interact directly with helicase II. Taken together the results suggest that, at low drug concentrations, inhibition of the unwinding and ATPase reactions is a result of the intercalation of mitoxantrone into duplex DNA. When helicase II encounters this complex it either dissociates from the substrate or is trapped in a ternary complex. This work may provide a new approach in the characterization of DNA helicases along with a better understanding of how DNA binding ligands function *in vivo*.

F 506 BIOCHEMICAL CHARACTERIZATION OF HUMAN AND YEAST REPLICATION PROTEIN-A: A SINGLE-STRANDED BINDING PROTEIN REQUIRED FOR DNA REPLICATION

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Replication Protein-A (RP-A; also known as Replication Factor-A and human SSB), is a single-stranded DNA-binding protein that is required for SV40 DNA replication *in vitro*. RP-A isolated from both human and yeast cells is a tight complex of 3 subunits (70kD, 32kD, and 14kD) and is phosphorylated in a cell-cycle dependent fashion. Several lines of evidence suggest that RP-A has other functions in addition to coating single-stranded DNA during replication. However, the specific role of this protein in DNA replication is poorly understood.

We have analyzed the biochemical properties of both human and yeast RP-A in order to gain a better understanding of their role(s) in DNA replication. Human RP-A has a high affinity for single-stranded DNA and a very low affinity for RNA and double-stranded DNA. The apparent affinity constant of RP-A for single-stranded DNA is in the range of $10^9\ M^{-1}$. RP-A has a binding site size of approximately 30 nucleotides. The binding of RP-A to single-stranded DNA is not highly cooperative and is partially sequence dependent. The affinity of human RP-A for pyrimidines is approximately 50-fold higher than its affinity for purines. The binding properties of yeast RP-A are similar to those of the human protein. Both yeast and human RP-A bind preferentially to the pyrimidine rich strand of a homologous origin of replication: the ARS307 or the SV40 origin of replication, respectively. Since RP-A probably interacts with other replication proteins, this asymmetric binding suggests that RP-A could play a direct role in the process of initiation of DNA replication. The physiological significance of these and other results will be discussed.

F 507 PROTEIN-PROTEIN, PROTEIN-DNA INTERACTIONS AT THE REPLICATION FORK OF THE BACTERIOPHAGE T7

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Four proteins, T7 gene 5 protein, T7 helicase/primase, T7 gene 2.5 protein, and *Escherichia coli* thioredoxin, account for the fundamental reactions that occur at the replication fork of bacteriophage T7. T7 gene 2.5 protein plays an important role in T7 DNA replication, recombination, and repair. Affinity chromatography using gene 2.5 protein coupled to affi-gel shows the gene 2.5 protein interacts with T7 DNA polymerase and T7 primase/helicase. Steady state fluorescence emission anisotropy gives a dissociation constant of ~1 μM for the complex of T7 gene 2.5 protein and T7 DNA polymerase (the complex of T7 gene 5 protein and *Escherichia coli* thioredoxin). *E. coli* thioredoxin binds tightly to T7 gene 5 protein and confers a high processivity to the polymerization reaction. Nanosecond emission anisotropic analysis suggests that the complex contains one monomer each of gene 2.5 protein, gene 5 protein, and thioredoxin.

Fluorescently labeled thioredoxin and primer/template were used for monitoring protein-protein, protein-DNA interactions at the replication fork of the bacteriophage T7. Fluorescence emission anisotropic measurements give a dissociation constant, 7 nM, for fluorescein-primer/template and T7 DNA polymerase-thioredoxin complex.

F 508 PROCESSIVITY AND PAUSING OF HIV-1 REVERSE TRANSCRIPTASE ON HIV-1 TEMPLATES *IN VITRO*,

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Replication of human immunodeficiency virus type 1 (HIV-1) involves synthesis of double-stranded proviral DNA (9.7 kb) by reverse transcriptase (RT) using both RNA and DNA templates. To better characterize the biochemical events required for efficient proviral DNA synthesis, we are studying polymerization of HIV-1 genomic sequences *in vitro*. DNA from the infectious HIV-1 clone pNL4-3-2 was ligated into a pGEM vector to yield two subclones: "pHIV-RUN" (containing a 1.6 kb *pol* sequence that includes multiple regions of nucleotide runs) and "pHIV-RRE" (containing a 1.8 kb *env* fragment encompassing the Rev response element [RRE]). Single-stranded phagemid DNAs corresponding to viral minus-strand sequences were isolated, hybridized to synthetic oligonucleotide primers, and used as templates for polymerization by recombinant HIV-1 RT (p66/p51). Analyses of the polymerization products by sequencing gels revealed weak, nonspecific pausing in template regions lacking nucleotide runs and apparent secondary structure; maximal processivity in the presence of limiting RT was ~300 nucleotides. In contrast, multiple strong pause sites occurred on the pHIV-RUN template and, as a result, processivity was very low (max. = 125-160 nucleotides). The strongest pausing occurred within or following runs (≥4) of template T residues regardless of primer position and RT concentration. A time course with limiting enzyme showed that RT falls off the template at these sites. Polymerization was almost completely blocked (>95%) at the strongest pause site (a run of 4 T's followed by 4 A's) even in the presence of excess RT or after extended incubation. Polymerization on the pHIV-RRE template revealed another strong pause site opposite position 1 of the first RRE stem-loop, implying that RT also has trouble copying through stable secondary structures. In summary, we show that purified HIV-1 RT exhibits very low processivity on HIV-1 templates and pauses at distinct viral DNA sequences. These data point to possible sites for error-prone polymerization and suggest that accessory factors are required for efficient synthesis of full-length proviral DNA in the cell.

F 509 THE ANTITUMOR AGENT CC-1065 AND ITS ANALOGS INHIBIT HELICASE-CATALYZED UNWINDING OF DUPLEX DNA, Ira P. Maine, Daekyu Sun, Laurence H. Hurley and Thomas Kodadek, Department of Chemistry, University of Texas

In this study, the drug CC-1065, which is a potent antitumor antibiotic produced by *Streptomyces zelensis*, and four of its analogs were bound to tailed oligonucleotides and these molecules were used as substrates for helicase-mediated unwinding. CC-1065 is thought to exert its biological effects by binding covalently to double-stranded DNA and presumably interfering with one or more aspects of cellular DNA metabolism. It was found that the presence of CC-1065 inhibits unwinding catalyzed by *E. coli* helicase II and the bacteriophage T4 dda protein. However, a difference between the results obtained with the two helicases is that all DNAs containing drug are poor substrates for helicase II, but dda protein-mediated unwinding is inhibited only when the drug is attached to the strand from which the helicase initiates unwinding. The implications of these results with respect to the mechanism of action of CC-1065 in vitro are presented on this poster.

F 510 DnaB PROTEIN OF E. COLI: ATP HYDROLYSIS, HELICASE ACTIVITY, AND PRIMASE INTERACTIONS IN TWO DnaB MUTANTS AND AN ISOLATED PROTEIN DOMAIN, Russell Maurer, Paresh Shrimankar, Leif Stordal and Jeffrey Tseng, Department of Molecular Biology and Microbiology, Case Western Reserve University, Cleveland, OH 44106-4960.

The dnaB gene of E. coli encodes an ATP-driven helicase essential for DNA replication. DnaB is also needed for synthesis of Okazaki fragment primers by the dnaG primase. Here we describe the behavior of mutant DnaB proteins (including a domain of DnaB) with respect to these activities. A carboxyl-terminal fragment of DnaB (residues 172-470) is a functional ATPase[1] and helicase. A mutant containing cysteine in place of arginine at residue 231 (within a consensus nucleotide binding motif) attains a hexameric structure, interacts with DNA and nucleotides, and stimulates primase on poly(dT) templates. On the other hand, it is profoundly deficient in ATPase and helicase activities. Curiously, a mutant DnaB in which isoleucine-135 is replaced by asparagine has substantial ssDNA-dependent ATPase activity but severely compromised helicase activity, even though the mutation is outside the domain sufficient for helicase action in isolation. Together, these results emphasize the dependence of helicase action on ATP hydrolysis (not just binding) and suggest that the first 170 residues affect either the proper folding or activity of the carboxyl domain. In addition, detailed analysis of the efficiency of priming and the length distribution of primers made under the influence of wild type DnaB and the two substitution mutants suggests that DnaB remains in contact with primase throughout primer synthesis and precipitates the termination of primer RNA through an event linked to ATP hydrolysis.

[1] Nakayama et al., J. Biol. Chem. 259, 88-96 (1984).

F 511 PURIFICATION AND PROPERTIES OF A DROSOPHILA SINGLE STRANDED DNA BINDING PROTEIN (D-SSB).
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Single stranded DNA binding proteins have been shown to be required for recombination, replication and repair. The examination of the biochemical and physical properties of SSB's from E.coli and T4 phage and their interaction with associated proteins in replication and recombination has led to mechanistic insight into these processes. Recently eukaryotic SSBs (RF-A, RP-A, H-SSB) which seem to serve a similar function have been purified.

Drosophila melanogaster embryos provide a rich source of eukaryotic replication proteins. In addition, replication in *Drosophila* shows both developmental and tissue specific control and is amenable to genetic analysis to assess *in vivo* function. We have identified an SSB protein in *Drosophila* embryo extracts by its stimulation of DNA polymerase α activity on singly primed templates and have purified it to 90-95% homogeneity. *Drosophila* SSB (D-SSB) is similar to both the yeast and human SSB's. It is a trimer composed of 70 Kd, 32 Kd, and 11 Kd subunits, binds tightly to single stranded DNA, and increases the processivity of nucleotide polymerization of its cognate DNA polymerase, DNA polymerase α. Preliminary fluorescence quenching measurements of the association of D-SSB with nucleic acids will be presented.

F 512 INTERACTION OF PRIMASE AND TOPOISOMERASE DURING LAGGING STRAND DNA SYNTHESIS OF PHAGE T4, AND A RECOMBINATIONAL BYPASS MECHANISM, Gisela Mosig, Gene Lin and Ardith Chang, Department of Molecular Biology, Vanderbilt University, Nashville, TN 37235

Phage T4 codes for most of its replication and recombination genes, including primase and a type II topoisomerase(1,2). Both primase- and topoisomerase -mutants have a DNA-Delay phenotype but they are viable(3). We have shown that the primase mutants replicate at first, as expected, by displacement synthesis. Subsequent double strand replication requires a bypass mechanism to copy the displaced strand. This bypass mechanism depends critically on T4 endonuclease VII(4) (which cuts recombinational junctions,5). We will present five lines of evidence for interactions of topoisomerase and primase:

1) The topoisomerase mutants produce similar large displacement loops or -forks as early replicative intermediates, as the primase mutants.
2) The late double-strand DNA replication of the topoisomerase mutants depends on the same bypass mechanism (reqiring the recombination endonuclease VII), as that used in the primase mutants.
3)There is allele-specific interaction of primase and topoisomerase mutations.
4) T4 topoisomerase specific antiserum co-precipitates primase in extracts of T4 infected cells.
5) DNA replication of both topoisomerase and primase mutants is inhibited by novobiocin, but not by mutations in the host's DNA gyrase. Replication of both mutants is at first partially inibited and later on stimulated by the presence of UvsX protein, a RecA analogue of T4.

When both primase and topoisomerase are functioning, endoVII is not required for recombination dependent T4 DNA replication.
We will discuss a replication-recombination model in which T4 topoisomerase is required for conventional priming of Okazaki pieces. Endo VII can bypass this requirement because it generates substituting DNA primers from recombinational intermediates. The DNA-Delay phenotype of the primase and topoisomerase mutants is a consequence of the complex regulation of gene 49 (endoVII) expression (6).

(1) Nossal, N.G. and B. M. Alberts 1983: Bacteriophage T4 pp. 71-81.
(2) Kreuzer, K. and W.-M. Huang 1983: Bacteriophage T4 pp. 90-96.
(3) Epstein , R.H. et al. 1963 Cold Spring Harbor Symposium 28, 375-392. (4)Mosig, G. et al. The New Biologist, in press). (5) Kemper, B. et al. 1984 Cold Spring Harbor Symposium 48, 815-825. (6) Barth, K. et al. 1988, Genetics 120, 329-343.

F 513 EXPRESSION OF DNA PRIMASE USING BACULOVIRUS VECTORS; Heinz-Peter Nasheuer[1], Andrea Brückner[1], Silke Dehde[1] & Ben Y. Tseng[2]; Institut für Biochemie[1], Univ. München, Karlstr. 23, W-8000 München 2, FRG, and Genta Inc.[2], 3550 General Atomic Court, San Diego, CA 92121

DNA primase is essential for the initiation of DNA replication and the synthesis of the lagging strand. To investigate its enzymatic mechanisms in detail, we expressed the murine primase subunits, p48 and p58, in the Baculovirus expression system. Each primase cDNA (1,2) was cloned into the transfer vector pVL1393. The expression of both proteins was maximal after 70h. The active DNA primase was expressed by coinfecting Sf9 insect cells with Baculovirus harboring the primase p48 or p58 genes. The recombinant DNA primase was then purified from cytosolic extracts by ion exchange chromatography. The purified DNA primase fractions were free of cellular RNA polymerases. The RNA primers synthesized by the recombinant DNA primase are comparable to the products synthesized by the DNA primase of the bovine DNA polymerase α–DNA primase complex. The p48 subunit of the purified murine DNA primase was radioactively labeled by photo-crosslinking with ^{32}P αATP. These results show that the expressed DNA primase behaves essentially the same as the primase of DNA polymerase α–DNA primase complex.

1. Prussak, C.E., M.T. Almazan & B.Y. Tseng (1989) J. Biol. Chem. 264, 4957-4963
2. B. Tseng, pers. communication.

This work was supported by the DFG (Fa 138/3-5 and Fa 138/3-6).

F 514 SYNTHESIS OF SV40 DNA PRIMERS AND THEIR MATURATION INTO AN OKAZAKI PIECE HAVE DISTINCT ATP-REQUIREMENTS, T. Nethanel, T.Zlotkin and G. Kaufmann, Department of Biochemistry, Tel Aviv University, Ramat Aviv, Israel 69978.

It is not fully understood how do the three replicases, α, δ and ϵ (1), share functions in eukaryotic chromosome replication. We have previously proposed that two replicases partake in lagging strand synthesis. Accordingly, pol α-primase deposits an array of short RNA-DNA precursors reaching ca. 36 bases (**DNA primers**). Next, DNA primers assemble into an Okazaki piece following removal of intervening RNA and gap-filling by pol δ or ϵ (2,3). This **Nested Discontinuity model** has been inferred from the configuration of DNA primers in replicating SV40 DNA and from differential effects of specific inhibitors and antibodies on the two steps of Okazaki piece synthesis (2-4). ATP depletion caused accumulation of incomplete DNA primers of 25 bases. The nonhydrolysable analog AMP-PNP enabled their chase into DNA primers of 36 bases which still faced short gaps at the 3'-ends. These DNA primers could be further chased into Okazaki pieces upon restoration of ATP. The chase was blocked by aphidicolin but not by BuPdGTP. These data indicate that ATP acts as an effector during DNA primer formation, whereas the ensuing gap-filling reaction depends on an ATPase. Such requirements hint at possible roles of RF-C and PCNA (5) in successive steps of Okazaki piece synthesis.

1. Morrison et al., Cell **62**:1143, 1990.
2. Nethanel and Kaufmann, J.Virol **64**:5812, 1990.
3. Nethanel et al., J. Virol. **64**:2867, 1988.
4. Bullock et al., Mol.Cell. Biol. **11**:2350, 1991.
5. Tsurimoto and Stillman, J. Biol. Chem. **266**:1950,1961; 1991.

F 515 A Wild-type DNA ligase I Gene Is Expressed in Bloom's Syndrome Cells. John H. J. Petrini, Kristin G. Huwiler, and David T. Weaver
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Alteration of DNA ligase I activity is a consistent biochemical feature of Bloom's syndrome cells. In Bloom's syndrome cells, DNA ligase I activity is either reduced and abnormally thermolabile, or is present in an anamolously dimeric form. In order to assess the role of DNA ligase function in the etiology of Bloom's syndrome, we have cloned the DNA ligase I cDNA from normal human cells by a degenerate PCR strategy based on conserved regions of the *S. cerevisiae* and *S. Pombe* DNA ligase genes. Human DNA ligase I cDNAs from normal and Bloom's syndrome cells complemented a *S. cerevisiae* DNA ligase mutation, and protein extracts prepared from normal and Bloom's syndrome *S. cerevisiae* transformants contained comparable levels of DNA ligase I activity. DNA sequencing and Northern blot analysis of DNA ligase I expression in two Bloom's syndrome cell lines representing each of the two aberrant DNA ligase I molecular phenotypes demonstrates that this gene is unchanged in Bloom's syndrome cells.

Thus, a distinct gene product which interacts with DNA ligase in normal cells, but is absent or reduced in Bloom's syndrome, may be responsible for the observed reduction in DNA ligase I activity associated with this chromosomal breakage syndrome. In order to test this hypothesis, we are using antisense phosphorothioate oligonucletides specific for DNA ligase I to modulate the level of DNA ligase I activity and thereby simulate the Bloom's syndrome phenotype in otherwise normal cells.

F 516 ***Saccharomyces cerevisiae*** **MUTANTS AFFECTING THE STRUCTURE OR EXPRESSION OF THE DNA POLYMERASE α-PRIMASE GENES.**

Paolo Plevani, Antonella Pizzagalli, Corrado Santocanale, Marco Muzi Falconi, Simonetta Piatti, Anna Piseri, Daniele Derossi, Maria Pia Longhese, Remo Tazzi, Francesca Locati and Giovanni Lucchini, Dipartimento di Genetica e di Biologia dei Microrganismi, Via Celoria 26, Milano, Italy. The products of the *POL1*, *PRI1* and *PRI2* yeast genes encoding, respectively, the DNA polymerase α and the p48 and p58 primase subunits have been shown to share extensive homology with their mammalian counterparts. However, despite the high degree of homology, null or conditional mutations in one of the two yeast primase genes can not be complemented *in vivo* by the expression of the corresponding mouse genes. Characterization of mutants altered in conserved residues of the primase subunits have allowed us to assess the essential role of this enzyme in mitotic and premeiotic DNA replication. Furthermore, these mutants show both an hyper-recombination and mutator phenotype and their partial biochemical characterization has provided some insight on the protein-protein interactions among the polymerase-primase polypeptides. We have previously shown that the expression of the *POL1*, *PRI1* and *PRI2* genes is transcriptionally regulated during the mitotic cell cycle. Characterization of trans-acting mutations altering the expression of a *POL1-lacZ* fusion has identified two classes of recessive mutations, suggesting that both negative and positive trans-acting factors are involved in *POL1* transcriptional control.

F 517 PHOSPHORYLATION IS REQUIRED FOR MAMMALIAN DNA LIGASE I ACTIVITY. Claude Prigent*, Ken-ichi Kodama*, Dana Lasko** and Tomas Lindahl*, *Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts., EN6 3LD, United Kingdom, **Ludwig Institute for Cancer Reasearch and Department of Medicine, McGill University, Montréal, Québec, Canada.

DNA ligase I was purified to homogeneity from calf thymus in order to raise a polyclonal antiserum. Immunoblot analysis of crude extracts of mammalian cells revealed a doublet of two polypeptides very similar in size, strongly suggesting that the protein was phosphorylated. DNA ligase I protein immunoprecipitated from cells metabolically labelled with radioactive inorganic phosphorus has been identified as a phosphoprotein. Phosphorylation modifications of DNA ligase I appear to be essential for the enzyme activity: (1) Extensively dephosphorylated purified bovine DNA ligase I is essentially inactive *in vitro*; (2) the human DNA ligase I full-length cDNA is not able to rescue a DNA ligase mutant of *E. coli*; (3) also when the protein is over-expressed in *E. coli*, it is inactive *in vitro*; (4) DNA ligase I activity is enhanced *in vitro* after phosphorylation by casein kinase II; (5) DNA ligase I over-expressed in *E. coli* becomes active after phosphorylation by CKII. Several potential CKII sites were predicted from the human DNA ligase I amino-acid sequence and analysis of the phosphorylated protein revealed that CKII phosphorylates several serine residues. When the DNA ligase I C-terminal catalytic domain is over-expressed in *E. coli*, it is active, suggesting that the N-terminal domain of the full length protein negatively controls the ligase activity and that this negative controle is released after phosphorylation by CKII.

F 518 THE FATE OF PARENTAL NUCLEOSOMES DURING SV40 DNA REPLICATION, Sandra K. Randall and Thomas J. Kelly, Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205

Most studies of SV40 DNA replication have made use of naked DNA templates. Our aim is to extend this system to the analysis of the replication of chromatin templates. The fate of parental nucleosomes during the replication of chromatin templates was studied using a modification of the cell-free SV40 DNA replication system. When chromatin templates containing the SV40 origin were replicated *in vitro*, the progeny DNA retained a nucleosomal organization. The topoisomer distribution of the progeny was intermediate to the input templates and that of fully relaxed DNA, consistent with the partitioning of roughly half the nucleosomes to each daughter duplex. We determined whether the nucleosomes associated with the progeny molecules resulted from displacement of parental histones during replication followed by reassembly onto the newly synthesized DNA. When replication reactions were carried out in the presence of a variety of added control templates, only the progeny of the chromatin templates retained a nucleosomal structure. The progeny of the control DNA molecules added to the same reaction mixture were not assembled into chromatin. Our data indicates that parental nucleosomes remain associated with the replicating molecules and are transferred to the progeny molecules without displacement into solution. To account for these observations we propose a simple model in which nucleosomes ahead of the fork are transferred intact to the newly synthesized daughter duplexes.

F 519 THE SRS2 GENE OF S. CEREVISIAE ENCODES A NOVEL DNA HELICASE AND THE SUBCELLULAR LOCALIZATION OF THE PROTEIN IS CONTROLLED BY A NUCLEAR LOCALIZATION SIGNAL AT THE CARBOXYL TERMINUS, Lei Rong and Hannah Klein, Department of Biochemistry, New York University Medical Ceter, 550 First Ave. New York, NY 10016

Genetic studies have shown that the SRS2 gene has an important role in DNA repair and recombination in mitotic cells of S. cerevisiae. The sequence of the gene has the hallmarks of an ATP-dependent DNA helicase. Biochemical characterization of the SRS2 protein expressed and purified in E.coli has demonstrated that SRS2 gene encodes a bona fide DNA helicase.
The purified SRS2 protein exhibits ssDNA-dependent ATPase activity. It also catalyzes the unwinding of duplex DNA in the presence of ATP or dATP. Further studies with regard to enzyme kinetics, directionality of unwinding and cofactor requirements will be presented. Mutants of SRS2 isolated on the basis of a repair phenotype have been characterized genetically and the mutants have been mapped and sequenced. Proteins from some of these mutants have been examined for the ATPase and helicase activities.
A sequence KKKSK at the C-terminus of the protein has been identified as being responsible for the nuclear localization of the SRS2 gene product. Deletion of the sequence results in the mislocalization of the protein in the cytoplasm. The C-terminal 150 amino acids including this NLS signal, when fused to β-galactosidase, directs the fusion protein to the nucleus.

F 520 HIV REVERSE TRANSCRIPTASE: EVIDENCE FOR A FUNCTIONALLY ASYMMETRIC DIMER, Roberta L. Thimmig and Charles S. McHenry, Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Science Center, Denver, CO 80262.

The reverse transcriptase, composed of two subunits, p51 and p66, is responsible for the replication of HIV. Plasmids were constructed to contain the sequences encoding the subunits with stop codons placed immediately after the carboxyl-terminal codons of p51 and p66. Authentic p51 and p66 subunits have been produced using a strategy where the *E. coli* methionine aminopeptidase removes the amino-terminal methionine revealing an amino-terminal proline, the same residue found in the authentic viral protein. Levels of production were 2% and 4% of total cell protein for p66 and p51, respectively. The subunits were individually purified by ammonium sulfate precipitation and by chromatography on DEAE Sephacyl, Q-Sepharose, and single-stranded DNA cellulose.

Having the individual subunits available provided an opportunity to compare their functional properties alone and in the reconstituted heterodimer. The individual subunits show marked differences in reverse transcriptase activity under varying salt concentrations. Differences in reverse transcriptase activity of p51 and p66 on different templates were observed. The p66 subunit was 7-fold more active on poly(rA)(dT) and p51 was 2-fold more active on poly(dC)(dG). Mixing the subunits results in activity that could only be attributed to heterodimer formation. The heterodimeric enzyme did not display the salt and template specificity of either of the individual subunits. Interpreted in the light of the asymmetric dimer hypothesis initially formulated for the *E. coli* replicative apparatus (Johanson,K., and McHenry, C. (1984) JBC **259**, 4589-4595, McHenry, C. (1991) JBC, **266**, 19127-19130), these data suggest that HIV reverse transcriptase may also be a *functionally* asymmetric dimer with distinguishable (+) and (-) strand polymerases.

F 521 EUKARYOTIC DNA LIGASES, Alan E. Tomkinson, *Deborah E. Barnes, Errol C. Friedberg and *Tomas Lindahl, Department of Pathology, University of Texas Southwestern Medical Center at Dallas, TX 75235-9072 and *Imperial Cancer Research Fund, Clare Hall Laboratories, Blanche Lane, South Mimms, Herts EN6 3LD, UK.

Genetic and biochemical studies in *Escherichia coli* and *Saccharomyces cerevisiae* have demonstrated that DNA ligases are essential for DNA replication, DNA repair and genetic recombination. In these organisms, only one species of DNA ligase has been identified. In contrast, mammalian cells contain three distinct enzymes. Mammalian DNA ligase I, the major activity in rapidly proliferating cells and tissues, has been purified and characterised. The complementation of the conditional lethal phenotype of a *Saccharomyces cerevisiae cdc9* DNA ligase mutant by human DNA ligase I cDNA indicates that this enzyme functions in DNA replication.

The cellular functions of mammalian DNA ligases II and III cannot be determined solely from their biochemical properties. The multiplicity of DNA ligases is being investigated in *Saccharomyces cerevisiae* since this eukaryotic organism is highly amenable to genetic analysis. The DNA ligase encoded by the *CDC9* gene has been purified to homogeneity. An additional DNA ligase activity that has similar properties to mammalian DNA ligases II and III has been detected and is being further characterised.

Altered DNA ligation has been observed in human cell lines derived from patients with clinical symptoms of immunodeficiency and cellular hypersensitivity to DNA damaging agents. The autosomal recessive disease, Bloom's syndrome, is not caused by a mutation in the DNA ligase I gene. However mutations have been detected in the DNA ligase I gene in a cell line derived from a severely immunodeficient Anglo-Saxon female, and these alterations account for the malfunctioning DNA ligase I enzyme in these cells.

F 522 MONOCLONAL ANTIBODIES AGAINST DNA-DEPENDENT ATPASE A INHIBIT DNA SYNTHESIS IN *XENOPUS* OOCYTES. Patrick A. Truman*, Timothy M. Jenkins†, Larry D. Mesner*, Eric J. Ackerman† and Joel W. Hockensmith*, *Department of Biochemistry, University of Virginia Health Sciences Center, Charlottesville, VA 22902 and †Genetics and Biochemistry Branch, NIDDK, NIH, Bethesda, MD 20892.

We have developed 18 monoclonal antibodies (MAbs) against calf thymus DNA-dependent ATPase A. Six MAbs detect three different antigens in a crude oocyte homogenate. Fractionation of the homogenate through a number of purification steps, including denatured calf thymus DNA-cellulose, results in the detection of a high molecular weight antigen (~200kd) that cross reacts with all six MAbs. In addition the chromatographic behavior of the antigen is identical to ATPase A. Twelve of the MAbs were individually injected into *Xenopus* oocyte nuclei along with single-stranded M13 DNA and α-^{32}P dCTP and monitored for conversion to double-stranded DNA. Five of the MAbs inhibited DNA synthesis with the response varying from partial to complete inhibition. None of the inhibitory MAbs have associated nuclease activity. Three of these five MAbs cross-react on western blots of partially purified fractions.

F 523 CHARACTERIZATION OF DNA HELICASE ε FROM CALF THYMUS. John J. Turchi[1,2], Richard S. Murant[1], Gregg Siegal[1], and Robert A. Bambara[1,2]. Department of Biochemistry[1] and Cancer Center[2], University of Rochester, Rochester, NY 14642.

A DNA helicase has been purified to approximately 80% homogeneity from calf thymus. The helicase co-chromatographs with both DNA polymerases ε and ε* on a variety of chromatography matricies and is termed DNA helicase ε. A combination of SDS polyacrylamide gel electrophoresis, direct α-[^{32}P]ATP crosslinking, glycerol gradient sedimentation, and gel filtration chromatography indicate that the helicase is a monomer of 95-105 kDa. ε helicase translocates 3' to 5' with respect to the template strand on which it is bound. The standard DNA helicase assay measures the displacement of a labeled 25mer annealed to M13mp18. We have observed displacement of fragments up to 157 nucleotides in length in a standard helicase assay. Analysis of displacement of larger DNA fragments will also be presented. A complete duplex of 128 base pairs cannot be melted by the helicase demonstrating the necessity of a single strand DNA region to which the helicase must bind. The rate of displacement of a primer consisting of an 18 nucleotide complementary region followed by a 12 nucleotide 3' non-complementary region is identical to that for the 18 nucleotide primer alone. The rate of displacement of a 12 nucleotide 5' mismatch followed by an 18 nucleotide duplex was 2.5 fold slower compared to the 18 nucleotide primer alone. This was demonstrated in both time course experiments and enzyme titrations. The helicase is also capable of displacing RNA:DNA heteroduplexes. Experiments designed to determine whether a functional complex between ε helicase and DNA polymerase ε exists will also be presented. The association of DNA polymerase ε with DNA helicase ε suggests that it may be involved in DNA repair and/or DNA replication.

F 524 A HUMAN DNA HELICASE PHOSPHORYLATED BY CDC2 KINASE, N. Tuteja, R. Tuteja, K. Rahman and A. Falaschi, International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012, Trieste, Italy.

DNA helicases are known to unwind DNA to provide single-stranded templates for DNA replication, repair, recombination and transcription. We have reported earlier (Tuteja et al. Nucleic Acid Res. 18, 6785-6792, 1990) the existence of at least four different molecular species of DNA helicases in HeLa Cells, namely HDH I, II, III and IV as well as purification of HDH I. Recently (Tuteja et al. Nucleic Acid Res. 19, 3613-3618, 1991) we have also purified HDH IV from HeLa Cells. The apparent molecular weight of HDH I is 65 kDa, it requires a divalent cation for activity ($Mg^{2+}>Mn^{2+}>Ca^{2+}$) and the direction of unwinding is 3' to 5'. HDH IV has the apparent molecular weight of 100 kDa, it requires divalent cation for activity ($Mg^{2+}=Mn^{2+}=Zn^{2+}$) and it unwinds DNA by moving in the 5' to 3' direction along the bound strand. Both enzymes also unwind RNA:DNA hybrid.
We have tested the HDH I and HDH IV as substrates for cdc 2 kinase (provided by G. Draetta) and we found that only HDH IV can be phosphorylated by cdc 2 kinase. Our preliminary finding shows that this phosphorylation of HDH IV by cdc 2 kinase as well as subsequent dephosporylation by acid or alkaline phosphatases has no effect on helicase activity.

F 525 ASYMMETRIC DIMERIC DNA POLYMERASE III HOLOENZYME AT THE *E. COLI* DNA REPLICATION FORK. Carol A. Wu,[1] Ellen L. Zechner,[1,2] A. John Hughes, Jr.,[3] Jo Anna Reems,[3] Mary A. Franden,[3] Charles McHenry,[3] and Kenneth J. Marians,[1,2] [1]Program in Molecular Biology, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, and [2]Graduate Program in Molecular Biology, Cornell Graduate School of Medical Sciences, New York, NY; and [3]Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center, Denver, CO.

Individually purified subunits have been used to reconstitute the action of the *E. coli* DNA polymerase III holoenzyme at a replication fork formed in the presence of the primosome, SSB and a tailed form II DNA template. Complete activity could be reproduced with a combination of the Pol III core, the γδ complex, and the β subunit. However, coordinated leading- and lagging-strand synthesis could be supported by the combination of Pol III core and β. Dilution experiments with active replication forks suggested that the lagging-strand Pol III core remained associated continuously with the replication fork through multiple cycles of Okazaki fragment synthesis. Since the lagging-strand Pol III core must dissociate from the 3' end of the completed Okazaki fragment, this indicates that its association with the fork is via protein-protein interactions, suggesting that it forms a dimeric complex with the leading-strand Pol III core. An asymmetry in the action of the subunits was revealed under conditions (high ionic strength) that were presumably destabilizing to the integrity of the replication fork. Under these conditions, τ acted to stimulate DNA synthesis only when the primase was present (i. e., when lagging-strand DNA synthesis was ongoing). This stimulation was reflected by an inhibition of the formation of aberrantly small Okazaki fragments. Similarly-sized fragments were manufactured by replication forks assembled at a limiting concentration of the Pol III core. These fragments were not arrayed in tandom along the lagging-strand DNA template, but were separated by large gaps. However, long Okazaki fragments could be produced under these conditions if the concentration of primase was lowered. Thus, the production of small Okazaki fragments resulted from a delay in transit of the lagging-strand Pol III core from the Okazaki fragment to the new primer and τ appears to accelerate this transition.

F 526 A MURINE MULTIPROTEIN COMPLEX MEDIATES POLYOMAVIRUS DNA REPLICATION IN VITRO.

Y. Wu[1], K. Lawlor[2], R.J. Hickey[1], H.L. Ozer[2], Fang Yu[1] and L.H. Malkas[1], [1]University of Maryland School of Medicine, Dept. of Pharmacology, Molecular and Cellular Biology Program, Program in Oncology, Baltimore, MD, [2]UMDNJ-New Jersey Medical School, Dept. of Microbiology and Molecular Genetics, Newark, NJ.

We have identified and partially purified a multiprotein complex of associated DNA replication protein activities from the murine mammary carcinoma cell line (FM3A) using a series of centrifugation and ion-exchange chromatography steps. Proteins and enzymatic activities identified thus far as being associated with the mouse cell multiprotein complex include DNA polymerase, DNA primase, proliferating cell nuclear antigen (PCNA), DNA ligase, DNA unwinding activity and DNA topoisomerase I and II. The sedimentation coefficient of the complex is 18-20S, as determined by sucrose density gradient centrifugation. We have also demonstrated that the integrity of the murine cell complex is maintained after treatment with various agents, such as detergents, salt, and nucleases suggesting that the association of the complex proteins with one another is independent of non-specific interaction with other cellular components. Most importantly, we have demonstrated that this complex of proteins is fully competent to replicate murine polyomavirus DNA in vitro.

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F 527 KINETICS OF ATP HYDROLYSIS DURING THE HELICASE II - PROMOTED UNWINDING OF DUPLEX DNA,

J. G. Yodh and F. R. Bryant. The Department of Biochemistry, The Johns Hopkins School of Hygeine and Public Health, Baltimore, MD 21205.

The ATP hydrolysis activity of E. coli DNA Helicase II (uvrD protein) was examined in the presence of linear single- (ss) and double- (ds) ϕX DNA. The ssDNA-dependent ATPase reaction followed a linear time course until ATP was depleted, whereas reactions containing dsDNA exhibited an initial lag phase before the linear phase was attained. Order of addition experiments established that the lag phase did not represent a rate-limiting preassociation event between helicase and any other component of the reaction. We have proposed that the nonlinear phase of the dsDNA-dependent ATPase reaction reflects the ATP-dependent conversion of dsDNA to ssDNA by Helicase II. Agarose gel assays have confirmed that the DNA is being unwound during the lag phase of the ATPase reaction, and the kinetics of appearance of completely unwound DNA in this assay are discussed in terms of a model in which the transient phase of the dsDNA-dependent ATPase reaction corresponds to a rate-limiting propagation of unwinding event. Experiments which analyze the dependence of ATP hydrolysis activity on several factors including helicase, Mg, and ATP concentrations, unwindability of the DNA cofactor, and length of duplex DNA will be presented in support of this model.

Fidelity Mechanisms

F 528 CONSTRUCTION OF A PLASMID SYSTEM TO ASSAY THE FIDELITY OF *ORIC* DNA REPLICATION *IN VITRO*.

Masahiro Akiyama[1], Hisaji Maki[2], Mutsuo Sekiguchi[2] and Arthur Kornberg[1]. [1]Department of Biochemistry, Stanford University Medical Center, Stanford, California, USA. [2]Department of Biochemistry, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

DNA polymerase mechanisms for high fidelity replication include base selection and editing functions. Errors, which are rare, are the source of spontaneous mutations. To understand their biochemical basis, we assess the fidelity of DNA polymerase at a replication folk, where initiation and elongation of DNA chains are catalyzed by multisubunit enzyme assembles. Replication at the unique *E. coli* chromosomal origin (*oriC*) has been reconstituted *in vitro* with purified enzymes (*oriC* recon). We have developed an assay system to detect the fidelity of the *oriC* recon by replicating the *oriC* plasmid *in vitro* and isolating the mutations in the *rpsL* gene encoding a 10-kDa ribosomal protein. With the *rpsL* gene, Maki et al. have isolated various kinds of mutations *in vivo* (J. Mol. Biol., in press). Taking advantages of their system, isolation of the mutated gene (*rpsL*$^-$) from the replicated plasmids is achieved simply by transforming the *rpsL*$^-$ cells with the replicated plasmids and selecting the transformants with streptomycin: *rpsL*$^-$ cells that carry the *rpsL*$^-$ but not the *rpsL*$^+$ plasmid grow on streptomycin plates. Our system has three advantages: (1) mutations caused by the replication machinery assembled at *oriC* *in vitro* can be compared with those observed *in vivo*, (2) synthetic DNA, amplified 20-fold by rolling circle replication, improves detection of rare mutations, and (3) forward mutations (from *rpsL*$^+$ to *rpsL*$^-$) are readily measured.

F 529 THE MUTATIONAL MECHANISM OF HIV-1 REVERSE TRANSCRIPTASE

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HIV-1 RT is highly error-prone during DNA-dependent DNA synthesis *in vitro* using an M13mp2 template. DNA sequence analysis of mutants revealed that the majority of errors are base substitutions and single base frameshifts located in several hot spots. A common feature of the hot spots is that all are homopolymeric nucleotide sequences. We proposed three mechanisms to be responsible for this unique error specificity: template-primer slippage resulting in frameshift errors, transient misalignment resulting in some base substitutions and simple miscoding leading to the remaining base substitutions. We also observed a correlation between termination probability and minus-one-base frameshifts. In the present study we have examined the proposed mechanisms and further analysed the relationship between termination probability and frameshift fidelity. For this purpose phenotypically silent, single-nucleotide changes were introduced into the lacZα target sequence to test specific predictions of the slippage, dislocation and miscoding models. The results demonstrate that single base changes have substantial effects on the termination probabilities and on frameshift error rates at the hot spots, but that the relationship between the two properties is complex. Furthermore the changes in mutant error rates and specificity at the hot spots support the involvement of all three proposed models in generating errors at these sites. For example, disruption of a homopolymeric sequence resulted in a loss of a frameshift hot spot, consistent with the slippage model; a change of the base adjacent to the 5'-most base of a homopolymeric sequence resulted in a change of base substitution specificity at the 5' position of this sequence, consistent with the dislocation model. Thus, HIV-1 RT is error-prone for mistakes initiated by misalignment and is the only replicative polymerase identified to date that generates base substitutions by dislocation.

F 530 EUKARYOTIC DNA POLYMERASE AMINO ACID SEQUENCE REQUIRED FOR 3'-5' EXONUCLEASE ACTIVITY.

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We have identified an amino-proximal sequence, Phe-Asp-Ile-Glu-Thr, in *Saccharomyces cerevisiae* DNA polymerase II (ε) that is almost identical to the 'Exo 1' motif in the 3'-5' exonuclease active site of *Escherichia coli* DNA polymerase I. Similar motifs were identified by amino acid sequence alignment in related, aphidicolin-sensitive DNA polymerases. Substitution of Ala for the Asp and Glu residues in the motif reduced the exonuclease activity of partially purified DNA polymerase II at least 100-fold while preserving the polymerase activity. Yeast strains expressing the exonuclease-deficient DNA polymerase II had on average about a 20-fold increase in spontaneous mutation rate, consistent with a presumed proofreading role *in vivo*. In multiple amino acid sequence alignments of this and the 'Exo 2' and 'Exo 3' motifs recognized previously, five residues of the 3'-5' exonuclease active site of *E. coli* DNA polymerase I appeared to be invariant in aphidicolin-sensitive DNA polymerases known to possess 3'-5' proofreading exonuclease activity. None of these residues, however, appeared to be identifiable in the catalytic subunits of human, yeast or *Drosophila* α DNA polymerases.

F 531 A CELL-FREE SYSTEM FOR UV MUTAGENESIS IN *Escherichia coli* : INVOLVEMENT OF DNA REPLICATION AND REPAIR,

Orna Cohen-Fix and Zvi Livneh, Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel.

The development of cell-free assay systems has facilitated the elucidation of the mechanisms of a variety of biological processes. It enabled their detailed biochemical analysis including the isolation and characterization of components involved in these processes.

We report here the development of a cell-free assay system for UV mutagenesis. The assay consists of two stages: 1. An *in vitro* stage which involves incubation of a UV-irradiated plasmid carrying the *cro* gene, with a soluble protein extract prepared from *Escherichia coli* cells. 2. Detection of mutations produced in the *in vitro* stage by a subsequent bio-assay step. It involves transformation of an indicator strain with plasmid DNA that was isolated from the reaction mixture followed by scoring mutant colonies on indicator plates. This assay system enabled us to identify two UV mutagenesis pathways: 1. A pathway that depends on DNA replication and requires the *recA* and *umuC* gene products, known to be required for UV mutagenesis *in vivo*. 2. A pathway that depends on DNA repair, but not on DNA replication. DNA sequence analysis of *in vitro* generated UV-mutations revealed a spectrum typical of *in vivo* UV mutations.

The newly developed cell-free system will hopefully enable a detailed biochemical analysis of UV mutagenesis, and the elucidation of its molecular mechanism.

F 532 Constitutive *umuDC* expression is sufficient to restore UV survival and mutagenesis in *E. coli lexA* Ind⁻ bacteria

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We have isolated *umuDCo*^{c} mutation on a low copy number plasmid in order to study the amounts of UmuD and UmuC proteins required in SOS mutagenesis in combination with RecA protein.

The *o*^{c} mutation has two effects: (i) it reduces the binding affinity for LexA repressor by changing the SOS box, (ii) it increases expression of the *umuDC* operon by improving the homology with the -10 consensus promoter sequence.

We found that constitutive synthesis of UmuDC proteins restores SOS mutagenesis in *lexA* (Ind⁻) bacteria and that this effect is independent of the level of RecA protein. Constitutive synthesis of RecA protein does not render cells UV mutable if the amount of UmuDC proteins is limiting. We cannot exclude that other LexA-controlled proteins at their basal level may be involved in SOS mutagenesis. We postulate that SOS mutagenesis is governed by RecA and UmuD'C proteins forming a complex at the damaged site. The complex straightens out bent DNA to permit uninterrupted replication.

F 533 THE FIDELITY OF EXONUCLEASE-DEFICIENT KLENOW POLYMERASE IS INCREASED BY LOW REACTION pH

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The accuracy of DNA replication stems largely from the intrinsic ability of a DNA polymerase to differentiate correct from incorrect DNA structures. However, the precise polymerase-DNA interactions which govern the processes of nucleotide discrimination and productive extension synthesis are, as yet, unknown. Focusing on the exonuclease-deficient Klenow polymerase as a model enzyme, we demonstrate that a low reaction pH (6.2) increases fidelity 10-fold for both base substitution and frameshift errors relative to physiological pH, as measured using *in vitro* M13mp2 fidelity assays. While the overall processivity of the enzyme is slightly enhanced at low pH, the pattern of termination of synthesis along the M13mp2 DNA template differs significantly as a function of reaction pH. Lowering the reaction pH to 6.2 did not result in decreased frequencies of base misinsertions relative to pH 7.6, as measured by steady-state kinetics for three mispairs (G•dATP, T•dGTP, and A•dCTP), suggesting that the observed increase in fidelity at low pH is due to increased discrimination by the polymerase against the utilization of mispaired or misaligned primer-termini. As a first approach towards understanding which component of the E•DNA•dNTP complex is differentially affected by pH, we are measuring the pH response of exonuclease-deficient Klenow polymerase derivatives which have amino acid substitutions within the proposed polymerase active site. The response in fidelity to changing reaction pH has also been observed for several distinct 3'→5' exonuclease-deficient polymerases, including the HIV-1 reverse transcriptase, yeast DNA polymerase I, and the Taq polymerase. These experiments demonstrate that under specific reaction conditions the degree of base selectivity inherent to the polymerase catalytic domain can be as high as 10^{-6}.

F 534 *ESCHERICHIA COLI* ANTIMUTATOR MUTANTS IN THE ALPHA SUBUNIT OF DNA POLYMERASE III , Iwona Fijalkowska and Roel M. Schaaper, Laboratory of Molecular Genetics, NIEHS, Research Triangle Park, NC 27709

To gain further insight into the role of DNA replication errors in mutagenesis, we undertook a search for *E. coli* strains with an antimutator phenotype based on increased accuracy of DNA replication. We used localized mutagenesis of the *dnaE/dnaQ* region of the *E. coli* chromosome in strains defective in DNA mismatch repair (*mutL*). In this background mutations can be assumed to result from DNA replication errors. Mutagenized colonies were screened for antimutator phenotype using a papillation assay based on the reversion of a *gal* mutation. Among 10,000 colonies, seven putative antimutators were obtained whose level of papillation was reduced 5- to 30-fold. These isolates also displayed decreased mutation frequencies for several other markers. Mapping of the mutations by P1 transduction and complementation assays showed them to reside in the *dnaE* gene, which encodes the alpha subunit of DNA Polymerase III. We expect that the antimutators will be useful in studying the precise mechanism(s) by which DNA polymerases avoid errors, as well as in determining the role of DNA replication errors in mutagenesis.

F 535 THE VECTORIAL MOVEMENT OF THE *E.coli* UVRABC ENDONUCLEASE DURING THE TRACKING OF DNA IN ITS SEARCH FOR DAMAGED SITES. L. Grossman, L..Claassen, S.Mazur, E-Y.Oh, T.Seeley, E. Hildebrand, J-T. Wang,and S. Thiagalingam. Department of Biochemistry. The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD 21205.
The ability of the *Escherichia coli* Uvr proteins to interact with a damaged site on DNA, because of their intrinsically reduced discrimination capabilities, *requires translocation of the complex* from an undamaged site to one that is damaged. The $UvrA_2B$ complex functions as a 5'--->3' helicase producing waves of supercoiling ahead (+) and behind (-) it. The overall reaction requires ATP whose site and mode of participation in repair can be summarized as follows: the binding of ATP to either UvrA or UvrB results in protein-protein and protein-DNA **association** reactions whereas ATP hydrolysis is required for all of the **dissociation** reactions. The direction of the ATP<==>ADP+Pi equilibrium, therefore, can influence the tracking of DNA *in search for damaged* sites. When such sites are encountered by $UvrA_2B$ complexes, there is a 10^3 increase in association constant which stabilizes the nucleoprotein complexes. The driving force, or power stroke, for vectorial movement is derived from a cryptic ATPase associated with UvrB. The introduction of fluorescent amino acids or derivatized amino acids at the active site has revealed the conformational changes on UvrB as it translocates along the DNA in the presence of $UvrA_2$. The directed, deletion and random-directed mutagenesis studies which have led to this model of vectorial movement will be presented.

F 536 METHYL-DIRECTED MISMATCH "REPAIR" OF UV PHOTOPRODUCTS IN NONREPLICATING PHAGE DNA SIMULATES HOMOLOGOUS RECOMBINATION, John B. Hays and Wen-Yang Feng, Department of Agricultural Chemistry, Oregon State University, Corvallis, OR 97331.
Recombination of nonreplicating phage lambda DNA is negligible in "repressed" infections of *E. coli* homoimmune lysogens, but recombinant frequencies are stimulated several hundred-fold by prior UV irradiation of phages. We have recently demonstrated that in *uvrA* bacteria most UV-stimulated recombination of nonreplicating phage appears to result from processing of UV photoproducts by the *E. coli* GATC-methyl-directed mismatch repair system [Feng, W.-Y. and Hays, J.B. (1991) *Genetics*, in press (December)]: recombination mostly requires host MutH, L and S products and GATC-undermethylated phage DNA, and during repressed infections, UV-irradiated phage DNA undergoes a loss of biological activity ("restriction") that similarly depends on MutHLS function and undermethylation.

We find MutHLS-dependent UV-stimulated recombination in *uvrA* (but otherwise wild-type) bacteria to require both bacterial RecBCD and RecF activity: recombination was negligible in *recBCF* mutants, and rates of recombination in *recBC* or *recF* mutants were 1/6 of rec^+ rates. *recJ* mutants, which lack a ssDNA 5' → 3' exonuclease thought to be involved in mismatch repair, show a 30-min delay in appearance of recombinants, but finally achieve wild-type rates. Mismatch repair directed towards nascent DNA strands, at UV-photoproduct sites in newly replicated bacterial chromosomal DNA, might play a role in UV mutagenesis. To test this hypothesis, we are measuring effects of *mutS* mutations, in *uvrB* bacteria, on UV-induced reversion of various *lacZ* codon-461 mutants, known to revert via specific transition and transversion pathways [Cupples, G.G. and Miller, J.H. (1989), PNAS 86:5345-5349]. Although a *mut* mutation increased UV-induced reversion of several codon-461 mutations (about 2- to 4-fold), it decreased UV-dependent reversion of one mutation.

F 537 ENZYMOLOGICAL STUDIES ON THE VSR GENE PRODUCT OF *E. COLI* K-12

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The *vsr* gene of *Escherichia coli* K-12 is known to be required for very short patch (VSP) DNA mismatch correction [1]. We have fused this gene to that of β-lactamase (*bla*). The chimeric gene was expressed in *E. coli* and the product purified to homogeneity exploiting affinity of β-lactamase to immobilized phenylboronate. The Vsr moiety of the fusion protein can be liberated by site-specific protease cleavage. Enzymatic properties of the Vsr protein as well as those of the β-lactamase/Vsr fusion protein were investigated using a series of chemically synthesized oligonucleotides as substrates. Results obtained illustrate how the Vsr protein initiates very short patch DNA mismatch repair.

[1] Sohail, A., Lieb, M., Dar, M., & Bhagwat, A. S. (1990) *J. Bacteriol.* 172, 4214-4221.

F 538 "EXPRESSION, PURIFICATION, AND CHARACTERIZATION OF AN *ESCHERICHIA COLI* PHAGE T5 DNA POLYMERASE 3'-5' EXONUCLEASE MUTANT."

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The T5 DNA polymerase has been purified from an overproducing strain of *E. coli* to apparent homogeneity as judged by SDS-PAGE. Amino acid residues Asp_{164} and Glu_{166} were altered by site-directed mutagenesis to Ala_{164} and Ala_{166} respectively. The mutations were confirmed by DNA sequence analysis. The polymerase activity from an overproducer of these mutations was purified 150-fold by the sequence of heparin agarose cation exchange-affinity chromatography, single-strand DNA agarose affinity chromatography, Toyopearl butyl 650S hydrophobic interaction chromatography and Mono-Q FPLC anion exchange chromatography. The homogeneous product is free of both 3'-5' and 5'-3' exonucleolytic activity as well as any endonucleolytic activity. The exonuclease mutant does not turnover alpha-phosphate labeled nucleotides. The processivity of the T5 DNA polymerase is at least 7250 bp, corresponding to a full length M13mp19 DNA template. The effect of the mutations on the fidelity of DNA synthesis as compared to the wild type enzyme will be presented.

F 539 A MAMMALIAN GENE CORRECTS CONSTITUTIVE ABNORMALITIES OF REPLICATION IN A POST REPLICATION REPAIR DEFECTIVE ANIMAL CELL MUTANT, AND THEREBY INCREASES RESISTANCE TO UV AND MNU BY DIRECTING ERROR-PRONE DNA REPLICATION BYPASS, Robert T. Johnson[1], Simon D. Bouffler[1], C. Stephen Downes[1], Darren B. Godfrey[1], Anderson J. Ryan[1], Helen R. Strutt[1], Daniel R. Catchpoole[2] and Bernard W. Stewart[2], [1]CRC DNA Repair Group, Department of Zoology, Cambridge University, Cambridge CB2 3EJ, UK and [2]Children's Leukaemia and Cancer Research Unit, Prince of Wales Children's Hospital, Sydney, N.S.W. 2031, Australia.
The excision repair competent SV40 transformed Indian muntjac cell line, SVM, is hypersensitive to the cytotoxic effects of UV light and methylnitrosourea (MNU), both of which induce defective maturation of daughter strand DNA. SVM thus displays a post replication repair defect. Transfection of wild type mouse DNA into SVM together with a selectable marker (pSV2neo), followed by G418 and UV selection, resulted in transformants with significant recovery of UV and MNU resistance, an associated improvement of PRR, but with high levels of induced mutation. The gene has been cloned by cosmid rescue, biological activity lying in a 9 kb fragment.

Using benzoylated-DEAE cellulose (BND cellulose) chromatography we have examined replication-associated structural change in DNA in undamaged SVM and a normal muntjac cell. This procedure permits quantitation of the proportion of single strand DNA, and also measurement of the single stranded lengths. With no damage ^{3}H-thymidine, newly incorporated into either cell type, was immediately associated with two classes of single stranded DNA: short (200 nucleotides), and long (1-4kb); pulse chase studies showed that these regions were unusually long-lived in SVM. Defective maturation of DNA in SVM was confirmed by agarose gel electrophoresis studies which showed that SVM accumulates a replication intermediate in the size range 50-100kb. This constitutive defect in SVM, partly corrected in the transformants, identifies it as a replication mutant and suggests that defective PRR, evident in SVM cells after DNA damage, may be consequent upon a genetic defect associated with the processing of newly incorporated polynucleotides into mature DNA.

F 540 GENE CONVERSION ACCOUNTS FOR THE PRODUCTIVE REARRANGEMENT OF PILIN GENES IN *NEISSERIA GONORRHOEAE*, Michael Koomey, Deborah DeRyckere, Peter Lauer and Qian Yu Zhang, Department of Microbiology and Immunology, University of Michigan School of Medicine, Ann Arbor, Mi. 48109
Combinatorial diversification and antigenic variation of gonococcal pili/pilin results from homologous recombination involving a single complete pilin gene (expression locus) and multiple, variant-encoding partial pilin genes (silent loci). In this system, recombination is non-reciprocal with the partial pilin genes acting as donors and the expression locus being solely a recipient such that the repertoire of variant alleles remains unperturbed. We investigated the nature of these rearrangements and the possible contribution of transformation (uptake of exogenous DNA) to the process by constructing gonococcal strains carrying defined frameshift, missense and nonsense mutations within the pilin expression locus. Reversion to a pilus+ phenotype requires correction of the lesion and thus provides a simple means of scoring productive recombination and antigenic variation. Assessment of the rates of reversion and the levels of competence displayed by the mutants revealed a discrepancy between their low degree of transformability and the high frequency with which revertants arose. Similarly, the presence of DNAse during cultivation had no significant quantitative effect on the rates of productive recombination. Through the use of one mutant in which a frameshift mutation encompassed the introduction of a novel restriction endonuclease site, revertants could be easily scored by Southern blotting pattern as to whether recombination was reciprocal or not, thus avoiding impediments associated with allele-specific oligonucleotide probes. In all cases, regardless of conditions, the rearrangements were non-reciprocal. These results indicate that gene conversion is responsible for antigenic variation of pili/pilin in gonococci.

F 541 ASYMMETRIC HIV-1 REVERSE TRANSCRIPTASE ERROR RATES WITH RNA AND DNA TEMPLATES.
Thomas A. Kunkel, Jayne C. Boyer and Katarzyna Bebenek, Lab of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709, U.S.A.
One source of sequence diversity in the type I human immunodeficiency virus (HIV-1) may be inaccurate DNA replication by reverse transcriptase (RT). This enzyme, which lacks a 3'→5' exonuclease proofreading activity for correcting polymerization errors, has been shown to be error-prone during DNA-dependent DNA synthesis *in vitro* in several model systems. While these studies are relevant for second-strand DNA-dependent DNA synthesis during the virus life cycle, estimates of the fidelity of first-strand synthesis, i.e., RNA-dependent DNA synthesis, have been reported only using a synthetic polynucleotide substrate. We have developed a system to determine the fidelity of RNA-dependent DNA synthesis with natural RNA, and use it to directly compare the fidelity of HIV-1 RT with RNA and DNA templates of the same sequence. At DNA template nucleotides where HIV-1 RT is unusually error-prone for base substitutions and single-base deletions, cDNA synthesis with an RNA template is more accurate. The asymmetries in HIV-1 RT error rates with RNA versus DNA suggests that replication errors during first- and second-strand synthesis may not contribute equally to the final mutation rate and renowned sequence diversity of HIV-1. The data also suggest that the interactions between HIV-1 reverse transcriptase and a template-primer are substantially different for RNA versus DNA with respect to generating and/or utilizing both misaligned and mispaired substrates.

F 542 IMMUNODETECTION OF THE MISMATCH REPAIR PROTEIN PMS1 FROM *S. CEREVISIAE*, Robert Lahue, Anna Barcelo, and Erica Selva, Dept. of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655

An important pathway for the correction of mismatched DNA base pairs in *S. cerevisiae* requires the products of the *PMS1*, *PMS2*, and *PMS3* genes (Williamson *et al.* *Genetics* 110:609, 1985). As a first step in the biochemical analysis of these proteins, we have raised antibodies against the N-terminal portion of Pms1. The antigen, a 40 kD polypeptide, was overexpressed in *E. coli* and partially purified. Amino terminal sequencing of the 40 kD polypeptide confirmed identity with the N-terminal region of Pms1. Some of this material was further fractionated on an SDS-polyacrylamide gel and used to immunize rabbits. We are currently testing the resulting polyclonal antiserum by immunoblotting yeast extracts that are either devoid of Pms1 or overproducing full-length Pms1 (predicted molecular weight of 103 kD). As a tool for characterizing native Pms1, we are also producing monoclonal antibodies against a non-denatured sample of the 40 kD fragment. The results of both immunological approaches towards analysis of Pms1 will be reported.

This work was supported by NIH grant GM 44824 and by American Cancer Society award JFRA-327.

F 543 STYRENE ADDUCTS AT ADENINE N^6 BUT NOT GUANINE N^2 CAUSE SEVERE BLOCKAGE OF DNA REPLICATION *in vitro*. Gary J. Latham†, Constance M. Harris‡, Liang Zhou‡, Thomas M. Harris‡, and R. Stephen Lloyd†. Center in Molecular Toxicology, Departments of Biochemistry† and Chemistry‡, Vanderbilt University, Nashville TN 37232-0146.

Deoxyoligonucleotides bearing structurally specific adducts of styrene oxide on N^2 of guanine and N^6 of adenine were prepared by the reaction of phenylglycinol with oligonucleotides containing 2'-deoxy-2-fluoroinosine and 6-chloropurine deoxyriboside, respectively [Harris, C. M., *et al.* (1991) *J. Am. Chem. Soc.* *113*, 4328-4329]. This procedure yields styrene oxide adducts in a completely regio- and stereospecific manner, even in oligonucleotides which contain multiple guanine or adenine residues. In order to deduce the biological fate of specific adducts, two 11-base human N-ras gene oligonucleotides, one centered at codon 12 with a styrene adduct at the N^2 of guanine and the other centered at codon 61 with a styrene adduct at N^6 of adenine, were ligated into M13mp7L1/L2. Transformation of the unmodified and styrene-modified ras-12 sequences into *E. coli* UT481 (wild type) and AB2480 (uvrA⁻ recA⁻) cells generated an equal frequency of plaques and no enhanced frequency of mutations. However, transformation of the styrene-modified ras-61 sequence into UT481 and AB2480 cells generated 10^4-fold fewer plaques than wild type. The ligation efficiencies for modified and unmodified oligonucleotides were shown to be equal, leading to the conclusion that the N^6 styrene adduct poses a lethal block to the replication machinery. Further studies utilizing induction of the SOS response to evaluate the degree of replication blockage as well as transformation with other adducts at both adenine sites in codon 61 are underway in an effort to evaluate the consequences of such lesions more precisely. [Supported by USPHS grants ES05509 and ES05355]

F 544 DELETIONS IN *Escherichia coli* CHIMERIC PLASMIDS: ROLE OF REPLICATION ORIGIN, REPLICATION TERMINATOR AND TRANSCRIPTION. B. Michel, H. Bierne, D. Vilette, M. Uzest and S.D. Ehrlich. Laboratoire de Génétique Microbienne, I.N.R.A., 78352 Jouy en Josas cedex, France.

Hybrids composed of *Escherichia coli* phage M13 and plasmid pBR322 are not viable in *E. coli* and undergo deletions to yield viable progeny. One deletion endpoint is invariably at the nick in the phage replication origin and the other is frequently in particular plasmid regions, termed deletion hotspots (DHS). We report here that a DHS can be created by arresting DNA synthesis at a replication terminator or by binding repressors on cognate operators and that DHS distribution can be affected by transcription from strong promotors.
(1) *ter*B, an *E. coli* replication termination signal was inserted in a M13/pBR322 hybrid devoid of any other DHS. Interaction of properly oriented *ter*B and the host protein Tus is known to arrest replication forks. In Tus⁺ cells up to 80% of deletions ended at *ter*B, provided that it was oriented to arrest pBR322 replication forks. *ter*B in the opposite orientation was 20-fold less efficient DHS. No deletions ended at *ter*B in Tus⁻ cells. The position of *ter*B and the flanking sequences had little effect on the DHS activity.
(2) Promotor/operator regions of P_L or P_{tac} were inserted in similar M13/pBR322 hybrids. In the presence of cognate repressors >30 % of deletion endpoints were localised in the vicinity of the operators. In control plasmids devoid of these regions, deletion endpoints were scattered over a large portion of the molecule. Repressor/operator interaction therefore generates a DHS, as does Tus/*ter*B interaction.
(3) Transcription from P_L or P_{tac} in the direction opposite to pBR322 leading strand synthesis generated deletion endpoints downstream from promotors in ≈90 % of cases. The endpoints were not constrained to the transcribed region of the molecule. In contrast, when the transcription and replication progressed in the same direction the deletion endpoints were evenly distributed upstream and downstream of promotors.
Our experiments lead us to conclude that the frequency with which a DNA region undergoes deletions depends on its replication and transcription status.

F 545 RECOMBINATION VIA TEMPLATE-SWITCHING DURING DNA SYNTHESIS BY *TAQ* DNA POLYMERASE I, Shannon J. Odelberg[1], Robert B. Weiss[1,2], Akira Hata[2], and Ray White[1,2], [1]Department of Human Genetics and [2]Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT 84112

Recombinant molecules are often generated during the polymerase chain reaction (PCR) when partially homologous templates are available (1-3). It has been suggested that these recombinants are a result of truncated extension products annealing to partially homologous templates during subsequent PCR cycles (1,3). However, we demonstrate that at least some of these recombinants are generated during a single round of primer extension in the absence of heat denaturation. Furthermore, recombination is reduced several-fold when the complementary template strands are physically separated by attachment to different sets of streptavidin magnetic beads. These results support the hypothesis that either *Taq* DNA polymerase or the 3'-end of the nascent extending strand switches templates during DNA synthesis. This template-switching process appears to be contingent upon interaction between the two complementary template strands, thus allowing the switch to occur without requiring the polymerase or 3'-end to jump a considerable distance to the second template.

We are continuing our investigation of this recombinational process by determining whether other related polymerases, e.g., the Klenow fragment, T7 DNA polymerase, and T5 DNA polymerase, exhibit this template-switching phenomenon.

REFERENCES

1. Saiki *et al.* (1988) *Science* **239**, 487-491.
2. Paabo *et al.* (1990) *J. Biol. Chem.* **265**, 4718-4721.
3. Meyerhans *et al.* (1990) *Nucleic Acids Res.* **18**, 1687-1691.

F 546 FIDELITY MECHANISM OF HUMAN DNA POL α,
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The polymerization of incorrect or analogue nucleotides into DNA requires misinsertion and subsequent elongation from the 3'terminal mispair. We have examined the two steps required for incorporation of araCMP into DNA at internucleotide linkages by DNA pol α. The oligonucleotide products of DNA pol α catalyzed reactions were analyzed on polyacrylamide gels to measure insertion of araCMP, extension from an araCMP 3'terminus, and binding of the enzyme to an araCMP 3'terminus. The rate of araCMP insertion is about 5-fold slower than dCMP insertion at the same site. Extension from an araCMP 3'terminus by addition of the next complementary nucleotide is about 1000-fold slower than extension from a dCMP 3'terminus. To determine if slow extension from the araCMP 3'terminus results from slow phosphodiester bond formation oligonucleotide primers with araCMP positioned at the 3'terminus were elongated by addition of the α-phosphorothioate analogue of the next complementary nucleotide. The elemental effect on the rate of extension from araCMP is 6-fold indicating that chemical bond formation is partially rate limiting. Competitive binding analysis using oligonucleotide primers with araCMP, ddCMP, and dCMP 3'termini was performed to assess the relative binding affinity of DNA pol α for the araCMP 3'terminus. These results indicate that the enzyme binds poorly to an araCMP 3'terminus. Taken together the relatively small elemental effect and the apparent low binding affinity suggest that DNA pol α discriminates against araCMP incorporation into DNA at internucleotide positions by a mechanism that promotes dissociation of the enzyme upon araCMP insertion. (Supported by American Cancer Society grant CH-508)

F 547 REPAIR OF MISMATCHES DURING ILLEGITIMATE RECOMBINATION,
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Microhomologies are commonly found in junctions generated by illegitimate recombination *in vivo* and *in vitro*. During recombination processes, DNA ends with protruding single-strands may form overlap structures which can be set by single fortuitously matching basepairs (Pfeiffer & Vielmetter 1988, NAR 16:907) and which are most probably transiently stabilized by yet unknown alignment factors (Thode et al. 1990, Cell 60:921). Due to the overlap structures which determine final junctional repair patterns, the junctions often contain mismatches. Here, we investigate whether mismatch repair is part of the joining mechanism in extracts from *X. laevis* eggs which efficiently join nonhomologous DNA ends of any structure and sequence *in vitro*. Joining was monitored by the recircularization of double restriction cut plasmid DNA. Presence or absence of mismatches within junctions was analyzed by denaturing gradient gel electrophoresis (DGGE).

The results show that mismatches 3' adjacent to a gap have to be removed since they cannot serve as primers for DNA fill-in synthesis. In contrast, mismatches that are flanked by proper basematches on both sides neither restrain gap fill-in nor nick ligation and are maintained during the joining process. For mismatches immediately 3' or 5' of a nick, obviously an equilibrium exists between mismatch repair and the ligation of the mispaired structure. Since the partially purified DNA ligase activity from the egg extract is not able to ligate such unrepaired structures the necessity for a stabilizing alignment factor seems to be inevitable.

These results lead to the conclusion that mismatches are often maintained within junctions after illegitimate recombination. These mismatches have to be repaired by independent mechanisms that might also act in homologous recombination and in the repair of mismatched bases that have escaped proofreading during replication.

F 548 A DAMAGE-SPECIFIC DNA-BINDING PROTEIN FROM PRIMATE CELLS: PURIFICATION AND CHARACTERIZATION
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We have recently identified (Hirschfeld et al., Mol.Cell.Biol. 10: 2041, 1990) a constitutive damage-specific DNA-binding (DDB) protein from monkey (CV-1) cells with high affinity for UV-dsDNA and enhanced expression in UV-pretreated cells. We have purified (Abramic' et al., J. Biol. Chem., in press) the DDB protein using hydroxylapatite, phosphocellulose, MonoS, and DNA-affinity columns. Both the constitutive and UV-induced DDB activity is heterogeneous with regard to charge, and separation of three forms of protein was obtained on a phosphocellulose column. SDS-PAGE of purified preparations demonstrated that >90% of all three forms is a protein of ~126 kDa. DDB proteins showed similar binding affinity for DNA regardless of their purity: UV-dsDNA> >dsDNA, with no detectable band shift with either ssDNA or UV-ssDNA. In the Southern-Western and competition types of DNA-binding experiments, DDB proteins also showed binding to UV-ssDNA. Thus, the DDB protein(s) might be recognizing a specific photoproduct or distortion in UV-dsDNA and UV-ssDNA; however, for strong binding to DNA, both hypothetical domains of the DDB protein, one that recognizes a modified DNA and the other that interacts with intact dsDNA, are needed.
DDB proteins with similar binding characteristics were detected in various human cell lines, but not in cells from some DNA repair-deficient patients [xeroderma pigmentosum, group E (Protic' at al., Mol. Toxicol. 2:255, 1989]. DDB protein-like activities can also be detected in various eucaryotic cell lines and tissues. Our results suggest that the constitutive presence of DDB-like proteins cannot be directly correlated with the cell's capacity for excision repair or photoreactivation. Nevertheless, repair-deficient cells show significantly slower kinetics of DDB protein recovery after UV in comparison to repair-proficient cells. This damage-recognition protein, alone or in conjunction with other subunits, may be of general importance for the initial recognition of DNA damage as part of a mammalian DNA repair complex.

F 549 IN VITRO TRANSLESION REPLICATION PAST SITE SPECIFIC ABASIC SITES & PYRIMIDINE DIMERS,
Malini Rajagopalan*, Sunye Kwack*, Chi Z. Lu*, Mike O'Donnell#, Myron F. Goodman§, Christopher W. Lawrence¶, & Harrison Echols*. From the * Dept. of Mol. & Cell Biology, Univ.of California, Berkeley, CA 94720., # Microbiol. Dept., Cornell Univ. Medical College, NY,NY 10021., § Dept.of Biological Sciences, Univ. of Southern California, Los Angeles, CA 90089., ¶ Dept. of Biophys. & Biochem., Univ. of Rochester School of Medicine & Dentistry, Rochester,NY 14642.

We are interested in understanding at a biochemical level how mutations are introduced at the site of lesions when the SOS response is induced in *E.coli*. SOS mutagenesis presumably occurs by a translesion replication process involving DNA pol III holoenzyme (HE), RecA, UmuC,and UmuD' proteins. We have set up an in vitro system to study the replicative bypass of site-specific lesions on DNA, such as abasic sites and pyrimidine dimers. We have found that the replication of DNA pol III HE of E.coli is completely blocked by such lesions. However, a reconstituted DNA pol III HE (α.HE) lacking ε, the editing subunit misincorporated opposite abasic sites and pyrimidine dimers, but did not bypass these lesions to any significant extent. Purified α subunit also replicated opposite and bypassed (to small extent), whereas the replication of the αε subassembly was completely blocked by such lesions. We infer from these data that inhibiting the exonuclease of DNA pol III HE might be necessary but not sufficient for translesion replication. In an analogous situation, replication of T7 DNA polymerase was completely blocked by abasic sites and pyrimidine dimers, whereas the genetically engineered T7 DNA polymerase lacking the editing function almost completely bypassed these lesions. Therefore, the extent to which proofreading enhances the inhibition of DNA polymerization opposite lesions is different for different polymerases.

A UmuC protein preparation enhanced the bypass of pyrimidine dimers and abasic sites by α subunit, αε subassembly and α.HE. Further, processivity assays with α subunit and αε subassembly of DNA pol III suggest that the UmuC protein preparation increases processivity of the DNA polymerase III.

F 550 DNA POLYMERASE PAUSING CORRELATES TO PREFERRED SITES FOR INITIATION OF DELETIONS AND COMPLEX MUTATIONS MEDIATED BY MISALIGNED PAIRING BETWEEN EITHER DIRECTLY REPEATED OR PALINDROMIC DNA SEQUENCES, Lynn S. Ripley, Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, NJ 07103.
The specificity of mutations arising during *in vitro* polymerization reactions that depend upon misaligned DNA intermediates are influenced by DNA polymerase. For example, DNA polymerase I of *Escherichia coli* differs from its Klenow fragment derivative in the specificity of deletions arising from misalignments between palindromic DNA sequences, while the specificity of 1 bp deletions, which are not mediated by complementary, primer misalignments, are unaffected.[1] A complete analysis of all the possibilities for misalignment leading to detectable mutations in the system has been coupled to a semi-quantitative analysis of pausing on the DNA.[2] The results show that pausing is important to defining the most frequent sites at which mutations are initiated and that other factors are important in defining where the DNA strand realigns. It is the realignment step which seems to be the most important factor contributing to differences between E. coli DNA polymerase I and its Klenow fragment. These results offer an excellent rationale for the observation that *in vivo* mutation frequencies depending on misalignments are often inconsistent with estimates of the stability of the misaligned intermediate and can be differentially influenced by specific mutant DNA polymerases.

[1]Papanicolaou, C. & Ripley, L.S. (1989) J. Mol. Biol. 207: 335-353.
[2]Papanicolaou, C. & Ripley, L.S. (1991) J. Mol. Biol. 221: (In Press).

F 551 FIDELITY OF DNA REPLICATION DURING LEADING AND LAGGING STRAND SYNTHESIS IN HUMAN CELL EXTRACTS, John D. Roberts, David C. Thomas, Dinh C. Nguyen and Thomas A. Kunkel, Laboratory of Molecular Genetics, NIEHS, Research Triangle Park, NC 27709
We have examined the fidelity of leading and lagging strand DNA synthesis during SV40-origin-dependent, bidirectional DNA replication in extracts of human HeLa cells. Fidelity measurements were made with both a forward assay that detects many types of errors and a reversion assay that focuses on minus 1-base frameshift events. Two M13mp2 vectors having the SV40 origin on opposite sides of the *lacZα* mutational target were constructed with each target. Replication of these vectors in reactions with dNTP pool imbalances allows one to determine error rates for both leading and lagging strand synthesis. The fidelity of the system is quite high for -1-base frameshifts in runs consisting of three to seven A • T base pairs. Imbalanced nucleotide pools that contain low levels of dATP or dTTP lead to increased frequencies of minus 1-base frameshifts in the run. Exonucleolytic proofreading appears to enhance the fidelity of synthesis with regards to -1-base frameshifts, but the overall error rates are similar for leading and lagging strand synthesis. The dNTP pool biases also increase the frequency of other errors at sites near the A • T runs. DNA sequence analysis is underway to determine the nature of these errors, their relative frequency during leading and lagging strand synthesis, and the ability of exonucleolytic proofreading activities within the extract to suppress them. The overall fidelity of replication in the forward assay is also high but is altered specifically by the dNTP pool imbalances. Using one of the two M13mp2 vectors, substantial site-dependent differences in error rates are observed for identical mispairs at different locations within the target. These differences could be due to effects of the surrounding sequence, to differences in the fidelity of leading versus lagging strand DNA replication, or both. We are currently using the alternate origin-containing vector to distinguish among these possibilities.

F 552 A SUPPRESSOR OF *ESCHERICHIA COLI* MUTATOR *mutD5* RESIDING IN THE *dnaE* GENE, Roel M. Schaaper, Laboratory of Molecular Genetics, NIEHS, Research Triangle Park, NC 27709.

The *Escherichia coli* mutator *mutD5* is a mutator whose strength is moderate when growing in minimal medium but very strong when growing in rich medium. The primary defect of this strain resides in the *dnaQ* gene, encoding the ε (exonucleolytic proofreading) subunit of the DNA polymerase III holoenzyme. In one of our *mutD5* strains we discovered a mutation that suppressed the mutability of *mutD5*. Interestingly, suppression was strong in minimal medium but only weak in rich medium. The mutation was localized to the *dnaE* gene, encoding the α (polymerase) subunit of the DNA polymerase III holoenzyme. The mutation, termed *dnaE910*, also produced an antimutator effect against *mutL*, *mutT* or *dnaQ49* mutators. The results suggest that *dnaE910* encodes an antimutator DNA polymerase, whose effect might be mediated by improved insertion fidelity or by increased proofreading *via* its effect on the exonuclease activity. Possible mechanisms to explain how the suppression of *mutD5* can be strong in minimal medium but only weak in rich medium will be discussed.

F 553 EFFECTS OF 3'- 5' EXONUCLEASE ACTIVITY ON FIDELITY AND PRIMER DEGRADATION DURING THE POLYMERASE CHAIN REACTION, Dan D. Shoemaker, Kirk B. Nielson and Eric J. Mathur, Bioseparations R&D, Stratagene Inc, 11099 North Torrey Pines Road, La Jolla, CA 92037.
Several independent studies suggest that the 3' to 5' exonuclease dependent proofreading activity enhances fidelity by selectively excising mismatched nucleotides during DNA synthesis. The demand for high fidelity DNA synthesis during the polymerase chain reaction (PCR) has generated considerable interest in thermostable DNA polymerases which possess a 3' to 5' proofreading exonuclease activity. However, in addition to increasing fidelity, the 3' to 5' exonuclease activity also recognizes single-stranded oligonucleotide primers as substrates.
To analyze the mechanism of 3' to 5' exonuclease activity at the molecular level, our laboratory has designed an assay which determines how DNA polymerases with proofreading activity respond to mismatched 3' termini. In addition, we have developed a genetic assay to measure the fidelity of DNA polymerases during PCR. The assay is based on the amplification of the gene sequences coding for the lac repressor protein. Following PCR, the amplification products are cloned into a lambda phage vector, packaged and plated on a chromogenic substrate. Errors incurred during the amplification process which result in nonfunctional repressor protein will appear as blue plaques. Thus, the mutation frequency can be defined as the proportion of blue (mutant) plaques to the total number of plaques scored.
One of the drawbacks associated with 3' to 5' exonuclease activity is the single-stranded degradation of oligonucleotide primers. We have devised several assays which elucidate the fate of oligonucleotide primers during PCR. The results from these studies will allow the effects of primer degradation to be minimized during high fidelity PCR.

F 554 RECOMBINATION AND REPLICATION ERRORS ASSOCIATED WITH DOUBLE STRAND BREAK REPAIR

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We have used the gene for the HO double strand endonuclease under the control of a regulatable promoter and a synthetic recognition site for HO-endonuclease to generate DNA breaks at a defined position in *S. cerevisiae*. We have used this system to monitor the consequences of repair of the break in the interval containing the cut. The genetic system consists of the TRP1 and HIS3 genes inserted into chromosome III between the CRY1 and MAT loci. The HO recognition site was inserted into a polylinker region between trp1 and his3. The coupling of the CRY1 and MAT alleles relative to each other and to the selected recombinant allele can be established without tetrad dissection distinguishing several classes of recombinants.

Double strand breaks can act as donors. As expected, very efficient gene conversion in the trp1-his3 interval is induced by HO-endonuclease cleavage. Double strand gap filling (recipient) events predominate, but recombinants that result from the formation of heteroduplex adjacent to the cut are produced. These include events in which the chromosome with the HO site is the apparent genetic donor.

Replication errors accompany gap repair. In diploid cells undergoing repair of HO induced double strand breaks, the reversion rate of a homozygous trp1 mutation adjacent to the cut site is 100 fold higher than for uninduced cells. This suggests that the DNA polymerase that is involved in the repair synthesis has a higher error rate than the normal DNA replication machinery.

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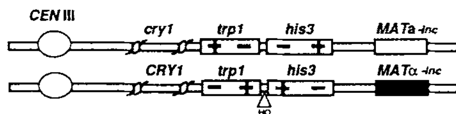

F 555 MOLECULAR MECHANISMS OF DNA SYNTHESIS: ROLE OF SUGAR PHOSPHATE BACKBONE IN ABASIC SITE MUTAGENESIS

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We have investigated the molecular mechanisms of DNA synthesis using site specific mutagenesis techniques. Nucleotide units with abasic properties were synthesized chemically with abasic sites placed at defined positions of a double strand plasmid vector. Following transformation of *E. coli*, progeny plasmids were recovered and subjected to DNA sequence analysis. Elimination of the base while retaining the ring structure of the sugar phosphate backbone led to dAMP incorporation opposite abasic site (60%), dGMP incorporation (<10%) and deletions (30%). Conversion of the backbone to a linear structure resulted in an increase in the number of deletions (>90%) at the expense of dAMP incorporation; dGMP incorporation was not affected. These results indicate that the "A rule" applies only when the deoxyribose ring is intact.

F 556 FIDELITY OF MAMMALIAN DNA REPLICATION AND REPLICATIVE DNA POLYMERASES

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Based on recent models, two or more DNA polymerases may be required for the high fidelity semiconservative DNA replication of eukaryotic genomes. We directly compared the fidelity of SV40 origin-dependent DNA replication in human cell extracts to the fidelity of mammalian DNA polymerases α, δ and ε in a forward assay using *lacZα* of M13mp2 as a reporter gene (*Biochemistry*, in press). Their fidelity, in decreasing order, is replication ≥ pol ε > pol δ > pol α. DNA sequence analysis of mutants derived from reactions using HeLa cell extracts suggests that replication is accurate when considering single-base substitutions, frameshifts and larger deletions. The exonuclease-containing calf thymus pol ε is also highly accurate. When a high concentration of dNTPs and dGMP are included in the pol ε reaction, both base substitution and frameshift error rates increase, suggesting that exonucleolytic proofreading contributes fidelity for both classes of errors. Exonuclease-containing calf thymus pol δ, which requires PCNA for efficient synthesis, is significantly less accurate than pol ε. In contrast to pol ε, pol δ generates errors during synthesis at a relatively modest concentration of dNTPs (100 μM) and the error rate did not increase upon addition of AMP. Thus, we have been unable to demonstrate that exonucleolytic proofreading contributes to fidelity during synthesis by pol δ. The four-subunit DNA polymerase α-DNA primase complex from both HeLa cells and calf thymus is the least accurate replicative polymerase. Sequence analysis of independent mutants generated by each polymerase shows that they all produce single-base substitution and frameshift errors, as well as larger deletions. However, the three enzymes have distinctly different error rates and specificities. These observations have implications for their roles in the various stages of DNA replication and will be discussed.

F 557 Construction of a *umuDC* operon substitution mutation in *Escherichia coli.*

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Inducible mutagenesis of *Escherichia coli* requires the products of the *umuDC* operon. *E. coli* strains possessing mutations in either *umuD* or *umuC* fail to exhibit the increase in mutation frequency normally seen after *E. coli* is exposed to a variety of DNA replication-inhibiting agents. Only nine chromosomal *umu* mutants have previously been described. Of these, all of the six original mutants are missense mutations. Of the insertion mutations, two have been mapped to the carboxyl-terminal of *umuC*, and one to the amino terminal of *umuD*. Although mutagenesis functions have been compromised, it is conceivable that all of the existing mutants possess partial or full activity of other, as yet uncharacterized UmuDC functions. Similar to *E. coli umuDC* mutants, a number of enterobacteria are not inducible for mutagenesis. However, many of these enterobacteria possess genes homologous to the *E. coli* or *S. typhimurium umu* operons, and also express a protein homologous to the *E. coli* UmuD protein. It has been suggested that these strains may have retained the *umu* operon in their genomes through some function (other than inducible mutagenesis) that proved advantageous in evolutionary terms. These data, together with the lack of any evidence for a chromosomal deletion in *E. coli* of the *umuDC* genes, have increased speculation that the Umu proteins may perform some function essential for cell survival.

To test if the Umu proteins are indeed essential, we have utilized a specialized transducing λ phage to delete the *umuDC* operon of *Escherichia coli* and have replaced it with the *chloramphenicol acetyltransferase* gene. The Δ*(umuDC)595::cat* mutation was subsequently transferred by generalized P1 transduction into a variety of genetic backgrounds. Since all of these strains are viable, it is concluded that the UmuD,C proteins, which are normally required for inducible mutagenesis, are not essential for cell survival.

General Recombination

F 600 Purification and characterization of the Bacillus subtilis 168 RecR protein.
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The recR (previously termed recM) gene of Bacillus subtilis encodes a product that is involved in DNA repair and recombination.
To assign a biochemical function to RecR protein, the recR gene was cloned in a plasmid vector which utilized the strong T7ϕ10 promoter to overproduce the protein. Purification of RecR (specifically labeled with [^{35}S]met.) was performed by monitoring the radioactively labeled material.
The purified RecR protein has a M_r of 24,000, as determined by SDS-PAGE. N-terminal protein sequence analysis was consitent with the amino acid sequence that was predicted from the DNA sequence of the recR gene. The RecR polypeptide bound to single-stranded and double-stranded DNA in a filter binding assay. RecR is able to bind DNA with a low and a high affinity, depending on experimental conditions. Competition experiments demostrated that RecR binds preferentially, to double-stranded DNA.

F 601 A YEAST DEOXYRIBOENDONUCLEASE REQUIRED FOR DOUBLE STRAND BREAK REPAIR IS A MEMBER OF A GENE FAMILY.
David G. Burbee and Barbara Armstrong. *Biosciences* Division, General Atomics, P.O. Box 85608, San Diego, California, 92186-9784.

We are currently sequencing the 3 kb yeast gene that we believe encodes an endonuclease required for double strand break repair and sporulation in the yeast Saccharomyces cerevisiae. This gene is also involved with, but is not absolutely required for, resistance to DNA alkylating agents and intrachromosmal recombination. We will present the DNA sequence of the nuclease gene and further characterizations of the nuclease deletion phenotype.
In the process of attempting to clone the entire gene, we isolated a second yeast gene that appears to share abundant sequence homology with the endonuclease gene. This gene (LLN1) is also being sequenced and appears to be somewhat smaller than the endonuclease gene. We are now preparing lln1 deletions to determine if it is also involved in DNA metabolism in Saccharomyces.

F 602 PURIFICATION AND CHARACTERIZATION OF A MAMMALIAN PUTATIVE RECOMBINATION ENDO-EXONUCLEASE,
Terry Y.-K. Chow and Chantal Couture, Dept Nuclear Medicine and Radiobiology, University of Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4.

Studies with a specific group of deoxyribonuclease, the Escherichia coli recBCD-like deoxyribonuclease (endo-exonuclease) in Saccharomyces cerevisiae and Neurospora crassa, have indicated its importance in DNA recombination and repair processes. The endo-exonuclease from the various eukaryotic sources studied so far, have demonstrated that they are highly conserved and shared common antigenic epitopes with the E. coli recC protein.
Utilizing an affinity column specific for the endo-exonuclease, we have purified a similar protein from the monkey cell, CV-1, and its level is induced in the transformed cell line, COS-1. The enzyme has a molecular weight of 65,000 and has activity toward both single- and double-stranded DNA. The ssDNase activity is endonucleolytic and nonprocessive, whereas the dsDNase activity is exonucleolytic and processive. The pH optimum for ssDNase is 8 and for dsDNase is 7.5. Both nuclease activities require divalent metal ion for activity and exhibit the same kinetics of heat inactivation. The purified protein binds to and cleaves synthetic Holliday junction substrate. The overall enzymatic characteristics of the mammalian protein is very similar to the endo-exonucleases that purified from the fungi Neurospora crassa, Aspergillus nudilans, and Saccharomyces cerevisiae. (Supported by NCIC and FRSQ)

F 603 *A CRYPTIC ELEMENT IN NOCARDIOFORM BACTERIA* GENERATES E. COLI AMPICILLIN RESISTANCE PLASMIDS

Eric R Dabbs and Selwyn Quan

Genetics Department, University of the Witwatersrand, Johannesburg, RSA

The origin of most naturally occurring plasmids is obscure. In observations that may throw light upon this process, we find that chromosomal DNA from a number of strains of *nocardioform bacteria can transform* E. coli *to give rise* to plasmids conferring high-level ampicillin resistance. Southern blot analysis confirmed that the plasmid sequences were present in the DNA of donor strains. The determinant was cryptic in these Gram-positive bacteria, since they (like E. coli) were inhibited by 2ug/ml ampicillin, whereas the plasmids conferred resistance to at least one thousand fold higher concentrations of the antibiotic. Also, by several criteria, the DNA generating the plasmids was apparently not present as a circular supercoiled molecule in nocardioforms. A 15kb segment of DNA capable of giving rise to the E. coli plasmids has been cloned in a nocardioform background from one of strains studied, and is presently being characterized.

F 604 DEVELOPMENT OF A CELLULAR ASSAY TO STUDY CLASS SWITCH RECOMBINATION IN MURINE B CELLS, Gregory Daniels and Michael Lieber, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305.
Immunoglobulin class switch recombination is one of two site-directed rearrangement reactions that is responsible for immunoglobulin gene assembly (the other reaction is V(D)J recombination). Class switch recombination changes the heavy chain from Igμ to γ1, γ2a, γ2b, γ3, α or ε, and is directed by 2 to 10 kb switch regions. Each switch region contains units of 10 to 50 base pairs in length repeated in a direct fashion over the entire region. The exact sequence of these repeating units is quite different between the different switch regions. Utilizing these sequences, we have reconstructed this region-specific reaction on extrachromosomal substrates. Replication of these substrates is controlled by the presence of the polyoma viral origin and large T antigen. Introducing these substrate molecules into murine cell lines, we have defined the importance of lineage specificity, switch region length dependence, transcription, and replication. Important implications in the regulation of the process are inferred both with respect to class targeting and recombination specificity.

F 605 RNA-MEDIATED RECOMBINATION in *Saccharomyces cerevisiae*, Leslie K. Derr, David J. Garfinkel and Jeffrey N. Strathern, Laboratory of Eukaryotic Gene Expression, NCI-FCRDC, Frederick, MD 21702
The existence of processed pseudogenes suggests that RNAs can act as intermediates in recombination. We have developed an extremely sensitive assay system in yeast *Saccharomyces cerevisiae* that has allowed us to detect RNA-mediated recombination and distinguish these events from events not involving an RNA intermediate. The assay is based on a plasmid-borne *his3* gene with an artificial intron inserted in an antisense orientation relative to the *HIS3* promoter (thereby inactivating *his3*). Transcripts can initiate at both the normal *HIS3* promoter and at a *GAL1* promoter placed at the 3' end of *his3*. Transcripts initiating at *GAL1* can be spliced and are then equivalent to an antisense copy of a functional *HIS3* gene. The formation of $His3^+$ prototrophs is RNA-mediated and results from two distinct recombinational events, homologous, RNA-mediated recombination and cDNA gene formation. Additionally, the formation of $His3^+$ prototrophs requires expression of the retrotransposon Ty. The chromosomal $His3^+$ prototrophs show many of the hallmarks of naturally occurring pseudogenes. They have inserted at novel sites in the chromosome, lack introns and possess poly(A) tracts, additionally their 5' ends correspond with the site of initiation of the *GAL1* transcript. We are presently examining the role of cis-acting sequences and trans-acting factors (both cellular and Ty) required for the efficient formation of $His3^+$ prototrophs.
Research is supported in part by the National Cancer Institute, DHHS under contract No. N01-C0-74101 with ABL and a Damon Runyon-Walter Winchell Cancer Research Fund Fellowship, DRG-1114.

F 606 DIFFERENTIAL DNA STRAND DEGRADATION BY THE RECBCD ENZYME OF E.COLI, Dan A. Dixon and Stephen C. Kowalczykowski, Department of Microbiology, University of California, Davis, Davis, CA 95616
The RecBCD enzyme of E. coli is a multifunctional enzyme that is required for homologous recombination. To explain the products of an in vitro recombination reaction involving RecA, RecBCD, and SSB proteins with linear dsDNA and supercoiled DNA, differential degradation of the 5' and 3'-terminal DNA strands by RecBCD enzyme was proposed. Direct experimental examination of this suggestion was carried out by analyzing of the ssDNA produced during the unwinding of dsDNA by RecBCD enzyme. At saturating RecBCD enzyme concentration, the unwinding of 5' end-labeled dsDNA results primarily in a ssDNA fragment whose size is one-half the length of the linear DNA molecule and which retains the 5'-terminal end derived from the entry site for the RecBCD enzyme. When the dsDNA is labeled at the 3'-end, only acid soluble DNA fragments are detected. Such a pattern of both 5' and 3'-terminal fragment production would result if each dsDNA end is being unwound and degraded by RecBCD enzyme, with the 3'-terminal end being degraded at a greater frequency than the 5'-terminal end; the resultant half-length 5'-labeled ssDNA strand is produced when two RecBCD enzymes, unwinding from each dsDNA end, meet at the center of the DNA molecule. Consistent with this interpretation, when subsaturating RecBCD enzyme concentrations are used, full-length ssDNA is produced in unwinding reactions using either 5' or 3' end-labeled dsDNA. This is the result expected due to the 3'-terminal strand being degraded and the 5'-terminal strand being left largely intact. This difference in strand degradation is apparent in the presence of SSB protein, when the Mg^{+2} concentration is greater than the ATP concentration, or when the nuclease inhibitor, Ca^{+2}, is absent. This differential DNA strand degradation and the interaction between RecBCD enzyme and the recombination hotspot Chi to attenuate the 3'-terminal DNA strand specific nuclease activity of RecBCD enzyme are discussed.

F 607 HERPES SIMPLEX VIRUS 1 RECOMBINATION: ROLE OF DNA REPLICATION AND VIRAL a SEQUENCES, Rebecca Ellis Dutch, Robert C. Bruckner and I.R. Lehman, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305
During the course of infection, elements of the herpes simplex virus 1 (HSV-1) genome undergo inversion, a process that is believed to occur through the viral *a* sequences. To investigate the mechanism of this recombinational event, we have developed an assay that detects the deletion of DNA segments flanked by directly repeated *a* sequences in plasmids transiently maintained in Vero cells. With this assay, we have observed a high frequency of recombination (approximately 8%) in plasmids that undergo replication in HSV-1 infected cells. We also find a low level of recombination between *a* sequences in plasmids introduced into uninfected cells, and in unreplicated plasmids in HSV-1 infected cells. In replicating plasmids, recombination between *a* sequences occurs at twice the frequency seen with directly repeated copies of a different sequence of similar size. Recombination between *a* sequences appears to occur at approximately the same time as replication, suggesting that the processes of replication and recombination are closely linked.

F 608 LARGE SCALE REARRANGMENTS OF THE *E. coli* GENOME CAUSED BY RECOMBINATION BETWEEN RNA OPERONS AND Tn*10*, INTEGRATED UPSTREAM OF THE *udp* GENE.

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Tn*10* -induced mutants of *E.coli* were selected by their ability to overexpress uridine phosphorylase (Udp). Lack of complementation of a closely linked *metE* mutation in P1-transduction experiments indicates that the mutation caused significantly large replacement of genetic material. Physical mapping of eight mutant strains, using pulse field gel electrophoresis of *Not*I-generated fragments, made it possible to localize the studied rearrangments. Blot-hybridization with rrn, udp and metE DNA-probes demonstrated that seven of eight mutant strains harbor a large inversion of the *metE-rrnD* segment covering 12% of the *E.coli* chromosome. In the inversions the udp gene was moved downstream of the *rrnD* promoter. When similar mutations were selected in a strain carrying natural *rrnD-rrnE* inversion (a derivative of *W3110*), the Udp-overexpressing phenotype was due to a *metE-rrnD/E Tn10*-induced inversion. In all studied variants this initial rearrangment was followed by a duplication of the chromosomal region between *metE* and *rrnD/E*. Blot-hybridizations of genomic DNA from randomly chosen *E.coli* lab strains showed naturally occurring recombination between rrn operons followed by long-range genome rearrangments that may play an important role in *E.coli* genome plasticity. The genetic and physical maps of *E.coli* were correlated in the 85-86 min region and the relative orientation of S and T *Not*I fragments on the physical map has been established.

F 609 RECOMBINATION-DEFICIENT MUTANTS OF *SALMONELLA TYPHIMURIUM*,

Timothy P. Galitski and John R. Roth, Biology Dept., University of Utah, Salt Lake City, UT 84112.

The genomes of *Salmonella* and *Escherichia* are highly plastic. About 10% of the cells in any bacterial culture carry a duplication. Duplications can provide significant growth advantages, but segregate to return the cell to haploidy. The events that form and remove duplications share features with recombinational repair events. Duplications are thought to arise by sister strand exchanges between separated homologous sequences in direct orientation (e.g. *rrn*, Rhs, IS, REP) and are lost by exchanges between the repeated copies. Both events are independent of *recBCD* function and may involve a single non-conservative (half-reciprocal) exchange. In order to study internal recombination events that may resemble the natural function of the recombination system, we have developed a screen for identifying mutants of *Salmonella typhimurium* that are deficient in the ability to segregate (and presumably to form) duplications. We have constructed duplications with a lac operon fusion at the join point. During colony growth, recombination between the duplicated segments results in haploid Lac$^-$ segregant sectors that can easily be scored on indicator plates. Using these strains we have found that *recB, C, D, F* and *J* mutations do not affect duplication segregation, while *recA* mutations prevent segregation. We have isolated mutant types other than *recA* that appear to reduce recombination between direct chromosomal repeats.

F 610 THE CHI SEQUENCE PROTECTS AGAINST RECBCD DEGRADATION OF DNA *IN VIVO*.

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Plasmids which replicate via a rolling circle mechanism (ssDNA plasmids) have as replication products principally monomeric dsDNA and ssDNA in *rec*$^+$ cells. If a ssDNA plasmid contains a foreign DNA insert, notably rich in GC, it produces predominantly high molecular weight linear multimers (HMW)[1]. However, in a *recBC* mutant, even a ssDNA plasmid without a foreign DNA insert makes HMW.
The inserted DNA which causes the appearance of HMW in *rec*$^+$ *Escherichia coli* has been identified to be the **CHI** sequence, 5'GCTGGTGG3'. Replication of ssDNA plasmids produces a displaced 5' plus strand which, once converted to double-stranded DNA, provides a possible means of access for RecBCD. In an inducible system, we show that HMW is generated only when **CHI** is oriented for recognition by this putative mode of RecBCD entry. Further *in vivo* experiments in which RecBCD exonuclease activity is turned on after conditional inactivation by the lambda *gam* product indicate that **CHI** sequences **block** degradation of HMW by RecBCD enzyme. Our results illustrate that the previously proposed roles of **CHI** as the site which i) inactivates RecBCD exonuclease activity[2], and ii) acts as a recombination hot spot[3], are separable. By analogy with our results, we suggest that a principal role of **CHI** in the survival of lambda *red*$^-$*gam*$^-$ mutants in wild type strains is to protect rolling circle concatamers (i.e., late replication) from degradation by RecBCD.

1. Gruss and Ehrlich 1988. J. Bact. 170:1183-1190; Dabert, P., Ehrlich, S.D. and A. Gruss, submitted
2. Thaler, D., et al. 1989. Genome 31:53-67.
3. Stahl, F., Crasemann, J. and M. Stahl. 1975. J. Mol. Biol. 94:203-212; Taylor, A., Schultz, D., Ponticelli, A. and G. Smith. 1985. Cell 41:153-163.

F 611 PHYSICAL ANALYSIS OF HO-INDUCED DOUBLE-STRAND BREAK RECOMBINATION IN YEAST,

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Galactose induction of the site-specific yeast HO endonulcease provides a synchronous initiation of double-strand breaks (DSB) so that recombination intermediates can be followed in vivo. HO-induced recombination events are slow, requiring 30-60 min. HO cleavage is followed by slow (1-2 nt/sec) but extensive 5' to 3' exonucleolytic digestion to create long single-stranded regions. The rate of exonuclease digestion is reduced in a *rad50* deletion strain, though recombination still occurs slowly. In *rad52* strains, degradation is much more rapid and extensive. *rad52* strains cannot complete gene conversion events but do form deletions between flanking homologous sequences at a low frequency that is dependent on the size of flanking homologous regions. The molecular defects caused by mutations in other radiation-sensitivity and DNA replication/repair genes will be reported.

In contrast to the extensive 5' to 3' exonulcease digestion, a marker 4 bp away from the 3' end of a DSB is rartely lost during recombination. A single base pair substitution in the Z region of *MAT*a is retained in 85% of switches to *MAT*α in a *pms1* strain that is unable to correct single base pair mismatches in heteroduplex DNA.

Intrachromosomal DSB repair occurs by two competing mechanisms: gap repair and single-strand annealing (SSA). Increasing the distance between two recombining regions delays the kinetics of deletion formation by SSA without affecting the kinetics of gene conversions arising from gap-repair. The efficiency of deletion formation is proportional to the size of the shared homology, from 90 bp 1.2 kb. In plasmids containing a direct repeat of the lacZ regions, gap repair almost always occurs by gene conversion without crossing-over; but in plasmids with the same sequences in inverted orientation, crossing-over accompanies gap repair about half of the time.

F 612 INVESTIGATION OF DEFECTIVE DOUBLE STRAND DNA BREAK REPAIR AND RECOMBINATION IN A MUTANT MUNTJAC CELL LINE.

Mathew Hall, Anderson J. Ryan, Simon Bouffler, Amanda Evans and Robert T. Johnson, C.R.C. Mammalian DNA Repair Group, Dept. of Zoology, University of Cambridge, UK.

An SV40-transformed Indian muntjac cell line (SVM) has been shown to be hypersensitive to the cytotoxic action of a range of simple alkylating agents when compared to a spontaneously transformed muntjac cell line (DM). Whereas the sensitivity of SVM to methyl nitrosurea, and similar agents, can be attributed to a lack of O^6methylguanine methyltransferase activity, sensitivity to dimethylsulphate (DMS) can be explained by the induction of large numbers of unrepaired DNA double strand breaks (DSBs). It is possible that SVM is defective in DNA DSB rejoining and/or recombination, by analogy with other cell mutants that are sensitive to DNA damaging agents. The CHO mutant, EM9, is defective in homologous recombination but possesses a relatively normal ability to seal DNA DSBs, while an ataxia telangiectasia cell line (AT5BIVA) is found to be grossly deficient in DNA break sealing ability but demonstrates wild type capacity for homologous recombination. To determine whether the abnormality expressed by SVM is primarily a break-joining or a recombination defect, we have generated cell lines (DP2,DP21,DS1) from SVM, transfected with human DNA, and selected for resistance to DMS cytotoxicity.

To assess break joining in the various cell lines, plasmids linearised within the coding region of a selectable gene (gpt) were transfected into cells and the fidelity of restriction endonuclease break joining was measured by functional testing of the selectable gene. Homologous recombination was investigated using modified pSV2gpt plasmids with complementary deletions in the gpt coding sequence, but also possessing a 320bp region of homology within the gene. Following co-transfection of the deletion plasmids into cells, homologous recombination must occur to produce an intact selectable gene, allowing an assessment of the relative efficiency of recombination. Data will be presented for SVM and DM in order to determine the primary defect in DNA DSB processing, and also for partially corrected transfectant lines derived from SVM.

F 613 IDENTIFICATION OF A 5'-3'EXONUCLEASE ACTIVE IN RECOMBINATION IN YEAST NUCLEAR EXTRACTS,

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We have developed a biochemical assay which detects recombination between two linear DNA substrates with an overlapping region of terminal homology. When these two substrates are incubated with yeast nuclear extract, they are found to efficiently recombine at the region of homology to produce a joint molecule. To date, we have extensively characterized this reaction. We have identified the presence of a 5'-3' exonuclease in the extracts which acts on the DNA substrates and is likely to be responsible for the formation of the observed product. We propose that the exonuclease degrades each substrate to reveal regions of complementary sequence which are then able to anneal and form the product. We have further characterized the reaction to determine the optimal length of homology and the effect of a block of nonhomology on the terminus of one or both substrates. We have also tested extracts made from recombination-defective strains. To date, all mutant strains tested have been active in catalyzing product formation, indicating that the reaction is likely to be dependent upon a novel gene product.

F 614 A 45kd PROTEIN BINDS TO THE MAJOR BREAKPOINT REGION OF THE BCL-2 ONCOGENE,

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The translocation t(14;18) in human follicular lymphoma juxtaposes the BCL-2 oncogene with one of 6 JH regions of the immunoglobulin (Ig) heavy chain locus. This illegitimate recombination takes place at the time of the D-J rearrangement in early B cells. While the break on chr.14 seems to be mediated by V(D)J recombinase, the molecular basis of the break on chr.18 is unclear. Interestingly, 70% of the BCL-2 breakpoints are clustered within only 150bp in the major breakpoint region (mbr). We have investigated this region and found that it is preferentially cleaved by an endogenous nuclease activity present in early B cells. Moreover, a 45kd protein (bp45) from these nuclear extracts binds to a homopurine-homopyrimidine tract at the 5'border of the mbr. Localization of the binding sites as well as the tissue distribution of bp45 suggest that this protein-DNA interaction is directly or indirectly involved in the translocation t(14;18).

F 615 CHARACTERIZATION OF THE NUCLEASE ACTIVITY OF YEAST SEP1 WITH RESPECT TO ITS STRAND EXCHANGE ACTIVITY

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Strand exchange protein 1 (Sep1) from Saccharomyces cerevisiae catalyzes the formation of heteroduplex DNA molecules from single-strand circles and homologous linear double stranded DNA in vitro. In such reactions synapsis is accomplished by the pairing of single-strand circular DNA with 3' single-strand tails on the double-strand molecules generated from limited digestion by the intrinsic 5'-3' exonuclease of Sep1. Under conditions where the nuclease and strand exchange activities of Sep1 can be functionally separated, the nuclease has been shown to be necessary for synapsis but is dispensible for strand exchange and displacement.

The nuclease activity of Sep1 has been more extensively characterized. The exonuclease of Sep1 has limited processivity on single- and double-strand DNA in vitro, releasing only 50 to 70 nucleotides as mononucleotides before falling off the substrate. This extent of digestion is similar to the apparent binding site size of Sep1 on single-strand DNA. Fluorescence spectroscopy of Sep1-ethenoDNA complexes indicated an apparent site size of 70 nucleotides. Alternatively, an apparent site size of 100 nucleotides has been determined by an exonuclease competition assay using native single-strand DNA. Because Sep1 does not act as a nuclease on linear duplex DNA if it is prebound to single-strand DNA, a saturation point for binding to single-strand DNA can be measured. The titration point observed for the saturation of single-strand DNA by Sep1 in these two assays is similar to the titration point for Sep1 at which strand exchange can be detected: one monomer per 70 nucleotides of single-strand DNA. These results support a model in which as Sep1 is titrated into a strand exchange reaction it first coats the single-strand molecules. Only when the single-strand molecules are saturated is there free Sep1 to act as a nuclease to digest the ends of the double-strand molecules to initiate pairing However, the optimum stoichiometry for joint molecule formation is approximately one monomer per 35 nucleotides, twice the saturation point. One interesting possibility is that strand exchange requires the dimerization of Sep1 on the single-strand circular DNA to drive branch migration.

F 616 ANALYSIS OF THE YEAST HYPER-RECOMBINATION GENE HPR1,
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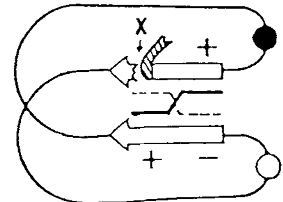
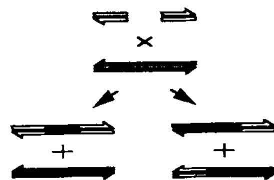
The HPR1 gene was defined by the hpr1-1 mutation which results in a specific increase in intrachromatid excision recombination of tandem duplications with no effect on homolog or sister chromatid recombination. The predicted amino acid sequence shows significant homology to the yeast DNA topoisomerase I gene and also to the mammalian RAG1 gene required for immunoglobulin V(D)J recombination. It is unclear whether HPR1 has topoisomerase activity. There is no obvious active site tyrosine in the sequence and mutagenesis of a potential active site tyrosine has no phenotype. Strains carrying a deletion of the HPR1 gene show no altered growth on campothecin and the hyper-recombination phenotype is not complemented by overexpression of topoisomerase I. Although the haploid strains carrying a deletion of the HPR1 gene have only a slightly reduced growth rate as compared to wild type, these strains require functional topoisomerase genes and grow extremely poorly when the double mutant hpr1 top is formed. The slow growth phenotype is seen when the hpr1 mutation is combined with a mutation in either TOP1, TOP2, or TOP3. hpr1 top1 strains grow slowly but at variable rates, with isogenic derivatives showing viability ranging from close to wild type to inviable. The cells that grow slowly accumulate buds that do not contain nuclei. Both hpr1 and hpr1 top1 strains have cell-autonomous mating defects which are due in part to inappropriate expression of cell-type specific genes required for the mating process. hpr1 and hpr1 top1 strains are also defective in the expression of genes unrelated to mating and results of expression of constitutive and inducible genes in these strains will be presented.

F 617 DOUBLE-STRAND BREAK REPAIR BY *E. coli* AND λ: UNCOUPLING OF CONSERVATIVE (TWO-PRODUCT) REACTION AND NON-CONSERVATIVE (ONE-PRODUCT) REACTION,
Ichizo Kobayashi+, *, Noriko K. Takahashi*, Luo Si-Qin+, and Hiroshi Yoshikura*. +: Dept Molecular Biology, Institute of Medical Science, Univ Tokyo, Shiroganedai, Tokyo 108. *: Dept Bacteriology, Medical School, Univ Tokyo, Tokyo 113, Japan.
A double-strand (ds) gap in a duplex DNA is repaired through gene conversion copying a homologous duplex in RecE pathway of *E. coli* (I.K. & N.T. Genetics 119:751) and in Red pathway of λ (N.T. & I.K. PNAS 87:2790) as predicted by the double-strand break repair models. A ds break might also stimulate recombination in a non-conservative way (one product from two parents) as we demonstrated for RecF pathway (N. T. et al., in prep. K. Y. et al., in prep.). We employed plasmid substrates that allow recovery of both the recombination products and found that conservative (two products from two parents) double-strand break repair is a major route of recombination triggerred by a ds break in RecE and Red pathways. Attachment of short non-homologous DNA to either one of the two ends at the double-strand gap (Figure) decreased conservative ds gap repair but left a high level of non-conservative recombination. These results indicate that the end plays different roles in the two routes.



F 618 DOUBLE-STRAND BREAK REPAIR BY *E. coli*: recA AFFECTS ASSOCIATION OF FLANKING EXCHANGE:
Kohji Kusano+, Noriko K. Takahashi*, Shigemi Aizawa+, Hiroshi Yoshikura*, and Ichizo Kobayashi+,*. +: Dept Molecular Biology, Institute of Medical Science, Univ Tokyo, Shiroganedai, Tokyo 108, Japan. *: Dept Bacteriology, Medical School, Univ Tokyo, Tokyo 113, Japan.
A double-strand (ds) gap in a duplex DNA is repaired through gene conversion copying a homologous duplex in RecE pathway of *E. coli* (I.K. & N.T. Genetics 119:751) as predicted by the ds break repair models. This gap repair was often accompanied by crossing-over (reciprocal exchange) of the flanking sequences (Figure). We found that several *recA* alleles cause strong bias in the choice between the non-crossing-over type and the crossing-over type. This indicates that these two routes are not equivalent.

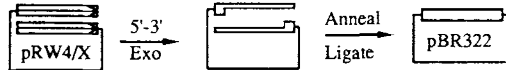


A mutation in *recE* gene, which lies in Rac prophage, abolished the reaction. A *rec*+ strain lacking Rac can promote ds gap repair when a part of it is expressed from a plasmid. We infer that this part codes for proteins equivalent to λ redα exonuclease and λ redβ annealase, which promote ds gap repair (N.T. & I.K. PNAS 87:2790). We found that *recBC, recD, recJ, recN,* and *ruvC* gene functions are not essential in *recBC sbcA* background. We failed to detect repair of a ds gap of 300 bp or a simple ds break in *recBC*⁻, *recD*⁻, and *recF*⁻ strains (N.T. et al. submitted).

F 619 THE CHROMOSOME TRANSMISSION GENE *CHL15* IS REQUIRED FOR DNA METABOLISM IN THE YEAST *SACCHAROMYCES CEREVISIAE*
V. Larionov[1] N. Kouprina[1], V. Zakharyev[2], F. Spencer[3], P. Hieter[3]. [1]Inst. of Cytology, St. Petersburg, USSR 194064; present address: NIEHS, Box 12233, Research Triangle Park, NC. 27709; [2]V.A. Engelhardt Inst. Molecular Biology, Moscow, 117984; [3]Dept. Molecular Biol.& Genetics, Johns Hopkins Medical School, Baltimore, MD, 21205.
The *CHL15* gene was previously identified in a screen for mutants with increased rates of mitotic loss of either chromosome III and artificial chromosomes. Mutations in the *CHL15* gene lead to a 100-fold increase in the rate of chromosome loss/cell division and a 200-fold increase in the rate of marker homozygosis on this chromosome by mitotic recombination. Analysis of the segregation properties of circular minichromosomes and chromosome fragments indicated that sister chromatid loss (1:0 segregation) is the predominant mode of chromosome destabilization in the *chl15-1* mutant. A genomic clone of *CHL15* was isolated and used to map its physical position on chromosome XVI. Nucleotide sequence analysis of *CHL15* revealed a 2.8 kb open reading frame with a 105 kD predicted protein sequence. At the N-terminal region of the protein sequence, zinc-finger motifs were found which define potential DNA binding domains. The C-terminal region of the predicted protein displayed a similarity to a sequences of regulatory proteins known as the helix-loop-helix (HLH) proteins. Data on partial deletion analysis suggest that the HLH domain is essential for function of the *CHL15* gene product. Analysis of sequences upstream of the *CHL15* ORF revealed the presence of the hexamer, ACGCGT, a sequence that controls both the periodic expression and coordinate regulation of DNA synthesis genes in budding yeast. Since disruption of the *CHL15* coding sequence results in inviability, the *CHL15* gene is essential for mitotic cell division. *chl-15 rad52* double mutants were slow growing as compared to either single mutant suggesting a role for *CHL15* in DNA metabolism. We suggest that *CHL15* is a new essential DNA synthesis gene in yeast.

F 620 HOMOLOGOUS RECOMBINATION IN A *XENOPUS* OOCYTE CELL FREE SYSTEM: ROLE OF A 5'→3' EXONUCLEASE, Chris W. Lehman and Dana Carroll, Dept. of Biochemistry, University of Utah Medical School, Salt Lake City, UT 84132.

DNA injected into the nuclei of *Xenopus laevis* oocytes recombines efficiently, with ng quantities of substrate processed in each oocyte. A requirement for this reaction is the presence of homologous sequences at or near molecular ends. This process operates via a resection/annealing mechanism where the first step utilizes a 5'→3' exonuclease. The reaction is depicted in the figure below for the substrate we use most commonly.



We have demonstrated recombination in cell free extracts made from oocyte nuclei (Lehman and Carroll (91) Proc. Natl. Acad. Sci. USA in press). The *in vitro* reaction, like that *in vivo*, produces completed recombinant products having covalently closed strands. This cell free system has allowed studies of the recombination mechanism, and catalysts required for each step in the process. The complete reaction requires the addition of a hydrolyzable nucleotide triphosphate, whereas the exonuclease does not. Also the exonuclease requires Mg^{+2} or Mn^{+2} for activity. We have recently begun to purify the strand specific exonuclease which catalyzes the first step of the recombination reaction.

F 621 OVERPRODUCTION AND PURIFICATION OF THE RecG NUCLEASE OF *E. coli*, Robert G. Lloyd and Gary J. Sharples, Department of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK.

The *recG* locus is required for recombination and DNA repair in *Escherichia coli*. The gene was identified by Storm et al. (1) some twenty years ago but received no further attention until Lloyd and Buckman (2) described a mini-kan insertion (*recG258*) at the same locus. The insertion, which is located at minute 82.1 on the genetic map, confers a slight deficiency in recombination and a modest sensitivity to DNA damage. These effects are seen in *recBC⁺sbc⁺*, *recBC sbcA*, and *recBC sbcBC* strains. Mutation of *recG* has no effect on plasmid recombination in a *recBC⁺sbc⁺* background, and *recG* is not required for induction of the SOS response.

Our interest in *recG* has been stimulated by a functional overlap with the *ruv* genes. Strains carrying the *recG* insertion in combination with a *ruv* (*A,B* or*C*) mutation are very deficient in recombination, much more so than strains carrying these mutations alone, and are extremely sensitive to UV light (3). This means that *recG* probably has a more critical role in recombination than is indicated by the properties of *recG* single mutants. It also provides a clue as to the function of the gene product since the *ruv* genes act late in recombination to resolve Holliday-junctions (4).

Nucleotide sequencing identified *recG* as the fourth gene in the *spoT* operon. It encodes a polypeptide of 76 kDa which shows homologies to ATPases and helicases. We have constructed a plasmid for the over-expression of RecG from a phage T7 promoter and have purified the protein. As predicted by the sequence, RecG is a DNA-dependent ATPase. It is also a nuclease. Further properties of this protein will be presented.

1. Storm, P.K., *et al. Mutat. Res.* **13**, 9-17 (1971).
2. Lloyd, R.G. & Buckman, C. *J. Bacteriol.* **173**, 1004-1011 (1991).
3. Lloyd, R.G. *J. Bacteriol.* **173**, 5414-5418 (1991).
4. Connolly, B., *et al. Proc. Natl. Acad. Sci.* **88**, 6063-6067 (1991).

F 622 **Function of a ϕX174 Type Primosome and Its Primosomal Proteins in Plasmid and Chromosomal Replication**

Hisao Masai, Yoshihisa Kubota, Kiyoatsu Umemura and Ken-ichi Arai
Department of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo, JAPAN

A primosome can be assembled either at duplex replication origins (*ori*) or on a *ssi* (*s*ingle-*s*tranded *i*nitiation sequence) containing *pas* (*p*rimosome *a*ssembly *s*ite). The *E. coli* chromosome and λ phage replicons relies on the primosomes assembled at the duplex *ori*, *oriC* and *ori*λ, for bidirectional replication, whereas ColE1 and ColE2 type replicons replicate dependent on the ϕX174 type primosome assembled on the n'-*pas* downstream of the *ori*. Several replicons including F, R6K, and Rts1 contain a pas adjacent to the *ori* and we have proposed that a primosome may be assembled on these *pas* after the initial replication fork migrates through it.[1]

R1 plasmid contains a G site, a *ssi* on which primase synthesizes a unique RNA primer, downstream of the *ori* (*oriR*) and it provides the leading strand initiation site. Deletion of this sequence results in reduction of *in vitro* replication activity by 70-80% probably due to inefficient leading strand initiation. Introduction of a n'-*pas* in place of this G site restores the replicative activity and the recovery is mediated by a ϕX174 type primosome assembled on the introduced *pas*. However, this *pas*-containing R1 derivative replicates unidirectionally, which suggests that the primosome cannot migrate past the *oriR*. Helicase assays using partial heteroduplexes containing the *oriR* sequence in the duplex region indicate that *repA* initiator protein bound to the *oriR* blocks the helicase activity of the principal primosomal protein, *dnaB*.

Proteins n' and n are the components of the ϕX174 type primosome and are encoded by *priA* (88.7 min) and *priB* (95 min), respectively, on the *E. coli* chromosome. ColE1 type plasmids such as pBR322 and RSF1030 cannot replicate in a *priA* disruption strain, ΔpriA, whereas a pBR322 derivative containing the A site that directs assembly of an ABC type primosome[2] can replicate in this mutant strain. We have concluded that, in replication of ColE1 type plasmids, n' protein is required solely for generation of the "ϕX174 type replication fork" and that it can be functionally replaced by the "ABC type replication fork". R1, *oriC*, R6K, miniF, pSC101, RK2, and Rts1 plasmids can be transformed into the ΔpriA, indicating that n' protein is dispensable for replication of these plasmids. Although the *priA* function is not essential for survival of *E. coli* cells, stable DNA replication observed constitutively under *rnh⁻* background is impaired by the *priA* disruption. This result suggests that the ϕX174 type primosome may function in the *recA*-dependent constitutive stable DNA replication. Similar analysis is being carried out with the *priB* gene.

1) H. Masai *et al.* J. Biol. Chem. **265**, 15124-15133 (1990)
2) H. Masai *et al.* J. Biol. Chem. **265**, 15134-15144 (1990)

F 623 KIN17, A DNA-BINDING NUCLEAR PROTEIN FROM MOUSE SEROLOGICALLY RELATED TO RECA, Alexander Mazin, Jaime F. Angulo, Valérie Schreiber*, Josiane Menissier de Murcia*, Gilbert de Murcia* and Raymond Devoret, Groupe d'Etude "Mutagénèse et Cancérogénèse", Laboratoire d'Enzymologie, CNRS, F-91198 GIF-SUR-YVETTE, FRANCE, *Laboratoire de Biochimie II. Institut de Biologie Moléculaire et Cellulaire, CNRS, 15 rue René Descartes, F-67084 STRASBOURG, FRANCE

We have cloned a mouse cDNA called *KIN17*, which codes for a mouse protein of 44.6 kDa, strongly reactive with anti-recA antibodies. The structure of the protein displays a 39-residue region homologous to RecA protein in the C-terminal extremity, a zinc-binding motif and several nuclear localization signals (Angulo et al., 1991, NAR, in press).

We have produced kin17 protein in *E. coli*, purified it and shown that kin17 protein binds to zinc ions and to double-stranded DNA. There seems to be at least two regions of the protein involved in DNA-binding. We propose that along with the zinc-finger the region homologous to RecA is involved in kin17-DNA interaction. We showed that kin17 is a nuclear protein: bipartite nuclear localization signal of kin17 protein is sufficient to direct the enzyme beta-galactosidase in the nucleus of HeLa cells. Genomic sequences homologous to *KIN17* cDNA were detected in rat and human DNAs suggesting that *KIN17* gene is conserved among mammals.

Taken together these results confirm that kin17 is a nuclear protein implicated in DNA transactions. We put forward the hypothesis that functional domains of recA protein may have burst out and spread to mammalian proteins involved in DNA metabolism.

F 624 MECHANISTIC CONSTRAINTS LIMIT INVERSION FORMATION IN *S. TYPHIMURIUM*, Lynn Miesel, Anca Segall, and John Roth, Department of Biology, University of Utah, Salt Lake City, UT 84112

Inverse order repeated sequences at some separated sites in the same chromosome can recombine to generate an inversion; the inverting segments are termed permissive intervals. When the same repeated sequences are placed at other sites, recombination occurs but no inversions are found among the recombinants; these chromosomal segments are termed nonpermissive.

The difference between permissive and nonpermissive intervals would be explained if inversion of the non-permissive intervals is lethal to the cell so that inversion recombinants would escape detection. A second possibility is that chromosomal position constrains the recombination mechanism, preventing the formation of inversions at nonpermissive intervals.

To determine if inversions of nonpermissive segments are lethal, we have constructed inversions of two intervals that were classified as non-permissive based on intrachomosomal recombination tests. The method involves a two-fragment transductional cross which directs formation of an inversion with prespecified endpoints. We have constructed inversions for two segments, *his - trp* and *his - cysA*. Although both intervals were classified as nonpermissive, neither constructed inversion is lethal. This result suggests that, for these two intervals, the failure to recover inversions by intrachromosomal recombination is not due to lethality of the product. This suggests that mechanistic barriers limit inversion formation.

Since inversion formation requires a conservative exchange in a rather short region of homology and since the exchange is dependent on RecBC function, we tested the possibility that the RecBCD exonuclease might limit inversions if its activity were excessive at some positions. We isolated and tested the effect of *recD* mutations on inversion formation. Nonpermissive intervals were not made permissive by a *recD* mutation.

To seek mutants that permit inversion of nonpermissive intervals, we have constructed a selection system by which separated homologies can only recombine (to Lac^+) by inversion formation. Preliminary results suggest that nonpermissive intervals actually do invert but at a frequency about 10^3-fold lower than permissive intervals. We are looking for mutations that increase the frequency of "nonpermissive" inversions and are trying to understand their rarity in terms of mechanistic constraints.

F 625 EVIDENCE FOR TETRASTRANDED DNA: A ROLE IN RECOMBINATION, A. Richard Morgan, Janusz Chlebek, Doug Scraba and Roger Bradley, Dept. of Biochemistry, Univ. of Alberta, Edmonton, Alberta, Canada.

Base tetrads formed by identical bps, $\frac{A:T}{T:A}$ and $\frac{C:G}{G:C}$ are isomorphous, with dyad symmetry. They have been found in cocrystals of simple derivatives of the bases. One bp binds to its identical partner through the major groove (pRecA binds to the narrow groove of duplex DNA). Such base tetrads can readily exchange W-C partners using the hydrogen bonds between the W-C bps as pivots. Such structures suggest a role in recombination. In order to form tetrastranded DNA between two homologous duplexes (tetraplexes), the plectonemic problem must be overcome. Since negatively supercoiled circular DNA has plectonemic right-handed turns, these turns are topologically equivalent to tetraplex. The free energy of supercoiling (torsional) may force the formation of tetraplexes (structural supercoils with $\sigma = -1$, but no torsion). Thus palindromic circular DNAs constructed *in vitro* were found to give rise by several criteria to intramolecular tetraplex DNAs. These could strand-exchange to give Y structures which finally form cruciforms. By EM, there were frequently two cruciforms observed on one molecule, as expected for tetraplexes formed at each dyad followed by strand exchange. For models involving melting of palindromes at the dyad axis and cruciform branch migration to a relaxed circle two cruciforms should not form. To prevent cruciformation and to phase supercoiling, inverted repeat circular DNAs were constructed with curved DNA separating the repeats. These very rapidly formed tetraplex DNA which could again strand exchange but only from the interior of the tetraplex. Strand exchange gave rise to Holliday cross-overs which were resolved by T7 endonuclease I, a Holliday cross-over resolvase. Topoisomerase I, however, did not relax the tetraplexes since there is no torsional stress in the circular DNA. *[The support of ARM and DS by MRC Canada is acknowledged.]*

F 626 PURIFICATION OF A NEW MITOTIC STRAND EXCHANGE PROTEIN FROM SACCHAROMYCES CEREVISIAE, David Norris and Richard Kolodner, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

The SEP1 strand exchange protein/exonuclease of Saccharomyces cerevisiae catalyzes the formation of heteroduplex DNA joints between single-stranded circular and homologous duplex DNA in vitro. To find other proteins involved in hDNA formation in Saccharomyces cerevisiae, we have looked for factors which permit strand exchange to occur when SEP1 is present at suboptimal concentrations. One such protein, named SF1 for *S*timulatory *F*actor *1*, has been purified to homogeneity from mitotic extracts. In the presence of this new protein, the concentration of SEP1 could be reduced over 300-fold before the rate and extent of joint molecule formation decreased below that seen in the unstimulated reaction. However, to see this level of stimulation, SF1 itself had to be present at high stoichiometries (1 SF1 monomer per 20 nucleotides of single-stranded DNA). This ratio of SF1 to SEP1, as well as the observation that SF1 actively renatures homologous single-stranded DNA in the absence of SEP1, suggested that the bulk of joint molecule formation in the stimulated reaction was promoted by SF1. This interpretation has been supported by recent experiments which show that SF1 no longer requires SEP1 to catalyze strand exchange when the linear substrate is lightly pre-digested with a 5' to 3' exonuclease. Therefore, SF1 is a new yeast strand exchange protein which we have renamed SEP2. However, unlike other strand exchange proteins such as RecA or UvsX, SEP2 requires that the duplex DNA substrate have a short single-stranded tail, a structure which has been seen at recombination initiators in yeast.

We have recently cloned and sequenced the gene for SEP2. The gene encodes a protein of approximately 55,000 daltons which has little homolgy to any other protein in the PIR databases. Genetic analysis indicates that the *Δsep2* mutation, while generating no strong phenotypes on its own, shows interesting interactions with a *Δsep1* mutation. For instance, the *Δsep2* mutation suppresses the mitotic growth phenotypes and uv-sensitivity of *Δsep1* mutants. However, the *Δsep2* mutation enhances the meiotic phenotypes of the *Δsep1* mutant, i.e., the *Δsep1*, *Δsep2* double mutant, unlike the *Δsep1* mutant, loses viability during sporulation, suggesting a lethal block in meiotic recombination.

F 627 *RNC1* codes for a novel yeast endo-exonuclease that plays a role in recombination and repair. Ed Perkins, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences (NIEHS), P.O. Box 12233, Research Triangle Park, NC 27709, Terry Y.-K. Chow, Univ. of Sherbrooke, Quebec, Michael Resnick, NIEHS

The levels of the yeast endo-exonuclease RhoNUC have previously been shown to be influenced by the *RAD52* gene thus suggesting a role for RhoNUC in DNA repair (1). The gene, *RNC1*, encoding RhoNUC from S. *cerevisiae* has been identified and cloned (2). DNA sequence analysis reveals an open reading frame of 1458 nucleotides which matches partial amino acid sequence analysis of purified RhoNUC. The N-terminal portion of the protein shares significant homology with the human *rho* oncogene, a member of the *ras* gene family. In addition, the protein exhibits 17% identity and 43% similarity with the E. *coli* recC protein. Thus RhoNUC, or *rho* associated nuclease, is a chimeric protein containing both a GTP binding domain as well as a nuclease domain. OFAGE analysis places RNC1 on chromosome XI.

Deletion-disruption of *RNC1* reveals that it is not essential for mitotic growth, meiotic development nor are cells sensitive to UV or ionizing radiation. When *RNC1* is cloned into a high-copy plasmid, an increase in MMS resistance is observed. However, this increase in resistance is dependent upon a functional *RAD52* gene. When *RNC1* is placed under the control of the *GAL1* promoter and the cells are grown in galactose containing media, the cells become enlarged and their nuclei abnormal. This *RNC1* overexpression does not appear to generate doublestrand breaks since induction of *RNC1* in strains containing a *RAD52* deletion does not result in lethality.

Strains containing a disruption of *RNC1* do not affect mitotic heteroallelic interchromasomal recombination or recombination between direct repeats in a plasmid recombination system or when the repeats are placed on a chromosome. In strains containing only a complete deletion of *RAD52*, the levels of direct repeat recombination are lowered. However, the lowered levels of recombination in this *rad52* mutant is largely rescued in a *rad52 rnc1* double mutant. These results suggest that *RNC1* plays a role in repair and recombination. Furthermore, given the homology with *ras/rho* proteins *RNC1* may play a role in cellular signalling.

1) Chow, T. Y.-K. and Resnick, M. Mol. Gen. Genet. 211(1998) 41.
2) Chow, T. Y.-K., Perkins, E. and Resnick, M. (1991) submitted.

F 628 THE FORMATION OF LARGE DUPLICATIONS IN THE E COLI CHROMOSOME IS CONTROLLED BY THE MISMATCH REPAIR SYSTEM. Marie-Agnès Petit, Joan Dimpfl, Miro Radman* and Hatch Echols. Department of Molecular and Cell Biology, University of California, Berkeley CA 94720. *Institut Jacques Monod, Université Paris VII, France. Excessive recombination between repeated, interspersed, and diverged DNA sequences is a potential source of genomic instability. We have investigated the possibility that a mechanism exists to suppress genetic exchange between these quasi-homologous (homeologous) sequences. We examined the role of the general mismatch repair system of *E. coli* because previous work has shown that the mismatch repair pathway functions as a barrier to inter-species recombination between *E. coli* and *Salmonella typhimurium*. The formation of large duplications by homeologous recombination in *E. coli* was increased som e ten-fold by mutations in the *mutL* and *mutS* genes that encode the mismatch recognition proteins. These findings indicate that the mismatch recognition proteins act to prevent excessive intrachromosomal exchanges. We conclude that mismatch repair proteins serve as general controllers of the fidelity of genetic inheritance, acting to suppress chromosomal rearrangements as well as point mutations.

F 629 RECOMBINATION BETWEEN HOMEOLOGOUS (DIVERGED) DNA SEQUENCES IN YEAST DURING PLASMID TRANSFORMATION. Scott D. Priebe and Michael A. Resnick , Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 and Torsten Nilsson-Tillgren, Institute of Genetics, University of Copenhagen, Denmark.
Sequence divergence is expected to impede recombination efficiency because of constraints inherent in the biochemical reactions. Ancillary genetic factors such as DNA-mismatch repair are also expected to affect recombination between homeologous DNAs. To investigate recombination between homeologous DNAs, episomal or integrating plasmids bearing a double-strand break or gap within a cloned *HIS4* gene of *Saccharomyces cerevisiae* were used to transform *S. cerevisiae* haploids. Recipient strains had either the natural CHR III or CHR III from *S. carlsbergensis*. DNA from these two yeasts is estimated to be 80-85% homologous in coding regions. The strains were either proficient or deficient (*pms1*) in DNA-mismatch repair. Transformation of episomal and integrating plasmids, gapped or broken in *HIS4*, was mediated by homeologous chromosomal DNA at 1 to 10% of the frequency observed for homologous chromosomal DNA. Restriction site differences between the homeologous *HIS4* regions were used to examine gene conversion tract length associated with recombinational repair of both episomal and integrating plasmids in relation to DNA-mismatch repair. Surprisingly, neither transformation frequency nor conversion tract length appeared to be *PMS1*-dependent. Most episomal plasmids recovered from transformants had conversion tracts that covered either just the original gap or the original gap plus sequences extending from one side only. Preliminary examination of plasmid integrants revealed little gene conversion associated with the integration; although when conversion occurred, the tracts were sometimes over 1kb and occurred at both ends of the plasmid insert in some cases.

F 630 RECOMBINATION AMONG RABBIT IMMUNOGLOBULIN KAPPA ALLOTYPE LIGHT CHAIN GENES, Carl Ritter and Ben Wolf, Departments of Animal Biology and Pathobiology, University of Pennsylvania Veterinary School, Philadelphia, PA 19104
Rabbit immunoglobulin (Ig) kappa (κ) light chain allotype genes provide an important model for antibody diversification. Their polymorphic variants: b4,b5,b6 and b9 are inherited as codominant alleles. Additional, non-allelic (latent) variants have been induced in our laboratory by infecting rabbits with Trypanosoma brucei. We have analyzed full length Ig-κ light chain mRNAs to study variation in their degrees of recombination for both hypervariable regions and V-J coding joints. In homozygous b4,b9 rabbits expressing latent b5 and b6 allotypes cDNAs were reverse transcribed from spleen mRNAs using either a b5 or a b6 primer and amplified by PCR. The PCR products contained identical, or near-identical leader regions. Vκ sequences from one rabbit contained variable CDR I, II and III, while Vκ sequences from a second rabbit were variable only in CDR III. Vκ-Jκ joints and J region sequences were similar to those which have been published. The Cκ region sequences were either all b4, or all b9, or contained long stretches of both b4 and b9 or b4+b5+b9. The multiple allotype patterns of these Cκ sequences resemble those in rabbit latent Cκ genes which we have previously determined (Wolf, etal, submitted). It seems likely that the mRNAs, from which our cDNAs were made, were themselves transcribed on allotype gene templates which have multiple Cκ composite allotype sequences. Supported by NSF DCB 9005021.

F 631 A REQUIREMENT FOR THE YEAST ARG4 INITIATOR OF MEIOTIC RECOMBINATION TO BE LOCALIZED IN A NON-TRANSCRIBED REGION, E. Rocco, B. de Massy and A. Nicolas, Institut de Génétique et Microbiologie, Bât. 400, Université Paris-Sud, 91405 Orsay Cedex, France.
The 5' region of the ARG4 gene of Saccharomyces cerevisiae contains an initiation site for meiotic gene conversion (1) and is the site of a specific double-strand break (DSB) during meiosis (2). The sequence necessary for the activity of this site has been mapped by deletion analysis between positions -319 and -37 of the ARG4 promoter. To test whether this sequence is sufficient for initiation of meiotic recombination, we inverted on the chromosome different DNA fragments including this region and the ARG4 coding sequence. Surprisingly, some inversions result in the loss of the normal recombination properties and double-strand break formation.The high conversion frequency drops at least 10 fold. To understand the cause of this inhibition of recombination, we examined the level of ARG4 transcripts in these diploids by Northern blot analysis and found a correlation between the inhibition of recombination and the presence of flanking transcription overlapping the initiation site for gene conversion. The activity of the site was restored by the insertion of a functional transcription terminator. We conclude that this initiator of meiotic gene conversion is active only when localized in a non-transcribed region.

1) Nicolas A. et al. (1989): An initiation site for meiotic gene conversion in the yeast Saccharomyces cerevisiae. Nature 338, 35-39.
2) Sun, H. et al. (1989) Double-strand breaks at an initiation site for meiotic gene conversion. Nature 338, 87-90.

F 632 RECOMBINATIONAL MECHANISMS OF PILIN ANTIGENIC VARIATION IN *NEISSERIA GONORRHOEAE*,

Hank Seifert, Leslie Hughes, and Kris Hoikka, Department of Microbiology-Immunology, Northwestern University, Chicago, IL 60611.

Antigenic variation of the gonococcal major pilus subunit (pilin) has evolved to promote avoidance of immune surveillance and to alter pilus receptor binding. Pilin antigenic variation occurs when a variant DNA sequence is transferred from one of several silent loci to the single expression locus in a nonreciprocal, *recA*-dependent manner. Two hypotheses have been proposed to account for pilin antigenic variation. The first mechanism invokes a gene conversion mechanism to account for the nonreciprocal nature of these recombination events. The second mechanism proposes that antigenic variation occurs through DNA transfer between bacterial cells. In this model, silent pilin sequences released from an autolysed sibling are taken up from the medium by DNA transformation and recombined with the expressed gene. This model is consistent with the autolytic properties and constitutive transformation competence demonstrated by the gonococcus. Several previous studies have demonstrated that inhibiting DNA transformation reduces the level of apparent nonreciprocal pilin recombination.

We have developed a PCR-based recombination assay to analyze mechanisms of pilin recombination. This assay uses a primer specific for the expression locus and a primer specific for a silent variant to detect recombination of that variant sequence into the expression locus. Amplification is performed on pilin cDNA, and a level of PCR product is produced proportional to the amount of starting mRNA that encodes the variant sequence. Using cDNA avoids the production of PCR products resulting from *in vitro* recombination during PCR. This PCR-based pilin recombination assay has been used to demonstrate that DNaseI in the growth medium reduces the amount of pilin recombination. We are also beginning to characterize a putative *cis*-acting site that shows homology to known site-specific recombination systems. The results from these studies will be presented.

F 633 TOPOISOMERASES MEDIATE DRUG-INDUCED RECOMBINATION IN SV40,

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Topoisomerases inhibitors and DNA polymerase inhibitors cause rapid topological and recombinational changes in replicating SV40 chromosomes. Disrupted topoisomerase action appears to be involved in the recombinational events following exposure to these drugs. In all cases, dramatic topological and structural events precede the recombinational events. The specific topoisomerase poison camptothecin causes rapid and extensive breakage of moving DNA replication forks on both leading and lagging strand sides. This fork breakage detaches replication bubbles and generates aberrant sigma forms and linear forms. All of the aberrant intermediates are crosslinked to protein (topoisomerase I). In a subset of the replication intermediates with broken forks, a ligation occurs between a labeled nascent strand and an unlabeled parental strand at the site of a fork break. This can be considered a half-completed sister chromatid exchange. Higher molecular weight aberrant forms of unknown structure are also produced from replication intermediates by camptothecin exposure. Exposure to specific type II topoisomerase inhibitors slows the completion of the latest replication intermediates and causes accumulations of highly catenated daughter chromosomes. In the case of topoisomerase II poisons, these aberrant forms are crosslinked to protein (topoisomerase II). Replication fork breakage and recombination and ligation of nascent strands to parental strands also occurs, but at much lower levels than seen with the type I topoisomerase poison camptothecin. Inhibition of DNA polymerase with aphidicolin causes rapid and extensive compaction of replication intermediates (40S forms) which immediately break down due to the formation of protein-associated DNA strand breaks. This rapid initial breakdown is followed by replication fork breakage, ligation of nascent strands to parental strands and continued recombinational change to generate high molecular weight forms of unknown structure. Both novobiocin and proflavine inhibit the formation of protein-associated DNA strand breaks in the rapid initial phase of 40S breakdown.

F 634 HOLLIDAY-STRUCTURE RESOLVING T4 ENDONUCLEASE VII INITIATES THE CORRECTION OF MISMATCHED BASES THROUGH THE COMBINED ACTION OF T4 DNA-POLYMERASE AND T4 DNA-LIGASE IN SYNTHETIC OLIGONUCLEOTIDES in vitro.

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Endonuclease VII is the product of gene 49 of bacteriophage T4. It resolves Holliday-structures and a broad range of branched DNAs. Here we show that the enzyme can also detect mismatches and single nucleotide insertions in synthetic oligonucleotides by cleaving both strands of the DNA in a nick and counter-nick fashion. A limited number of nicks were found within 6 nucleotides 3' adjacent to the mismatch site. By comparing efficiencies of cleavage among the mismatches three classes were observed: All G-containing mismatches were cleaved with low efficiencies. A/A, A/C, C/A, C/C and T/T belong to an intermediate class. C/C and C/T show the highest efficiencies of cleavage. In heteroduplexes with single nucleotide additions pyrimidines were better substrates than purines with G the least efficiently recognized nucleotide. With one exception (T/C) the determination of melting profiles of all 12 mismatch substrates by DGGE analysis revealed a perfect match with the measured cleavage efficiencies. When a C/C mismatch was treated with endonuclease VII in the presence of T4 DNA-polymerase and T4 DNA-ligase in vitro, the time lag between ick and counter-nick was sufficient to allow complete repair of the mismatch in either strand, and repair tracks were found in DNA dumpbell substrates. The result point to a further role of endonuclease VII in phage T4 development as an initiator for mismatch repair.

F 635 A *recA* mutant deficient in a late step of homologous recombination

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Recombination between two duplicated portions of the *lac* operon is reduced 500-fold in *recA423* (Arg169 to His) bacteria. *recA423* mutant is also deficient in duplication formation. In contrast, there is normal integration of a marker upon conjugation with an Hfr donor. In another recombination assay, λbio plated on *recA423* as well as on $recA^+$ indicator bacteria. Efficient plating of λbio involves one cross-over required for phage DNA dimer formation.

recA423 bacteria display a protease constitutive phenotype; they are UV sensitive and impaired in replication restart.

Purified RecA423 protein binds normally to single-stranded DNA and can accommodate 3 single strands as wild type RecA does. Yet, binding of RecA423 protein to double-stranded DNA is slow.

recA423 has a recombination phenotype analogous to Ruv^-. We postulate that RecA423 is deficient in a recombination late step that might be the resolution of Holliday junctions.

F 636 CLONING OF 46 AND 47, TWO GENES IMPLICATED IN INITIATION OF HOMOLOGOUS RECOMBINATION IN BACTERIOPHAGE T4. Katherine Stemke-Hale and Thomas Kodadek. Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, TX 78712.

Genetic studies have implicated two genes, 46 and 47, as being involved in controlling the initiation event for homologous recombination in bacteriophage T4. Since single-stranded DNA is believed to be the active intermediate in recombination, the most straightforward explanation is that the proteins encoded by these genes, which are thought to form a complex, either are a nuclease or control a nuclease activity.

Using PCR, I have succeeded in cloning both genes individually into the expression vector pKK223-3. Because Taq polmerase has a low fidelity, both clones were verified by dideoxy sequencing.

Each protein was over-expressed to over 10% of the total cell mass. The gp47 has been purified to approximately 98% homogeneity, as determined by Coomassie Blue staining. Since there are no known activities for the 47 protein, purification was followed by molecular weight on a 10% SDS-polyacrylamide gel. After the protein had been purified, the first 15 amino acids were sequenced by Edman degradation to verify that the correct protein had been purified. Purification of the 46 protein is underway.

F 637 THE CRYSTAL STRUCTURE OF E. coli recA PROTEIN Randall M. Story and Thomas A. Steitz. Dept. of Molecular Biophysics and Howard Hughes Medical Institute, Yale University, New Haven CT 06511

We have determined the crystal structure of *E. coli* recA protein and have refined the structure at 2.3 Å resolution.

The major domain of recA protein contains an 8-stranded β-sheet, part of which consists of parallel strands flanked by helices that form a nucleotide binding site. The ATP binding site has been located by diffusing ADP into the crystal. RecA protein belongs to the family of proteins containing the phosphate-binding loop motif GXXXXGKT. Binding of phosphates of the nucleotide is identical to other known structures containing this motif, however binding of the rest of the nucleotide differs.

A 6_1 helical filament of recA molecules is found in the crystals (space group $P6_1$) that closely resembles the EM structure of recA/DNA filaments determined at low resolution (Egelman and Stasiak, JMB 191: 677). Knowledge of the polymeric structure of recA protein allows us to begin dissecting the many activities of recA protein (DNA binding, strand exchange, ATP hydrolysis, repressor binding, etc.) in the proper structural context of the recA filament.

The close proximity of the known ATPase active site and likely site(s) for DNA binding suggests a model for the allosteric coupling between ATP and DNA binding.

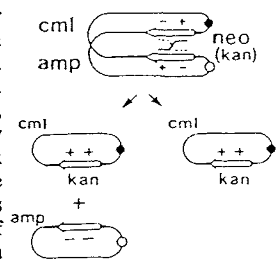
F 638 DOUBLE-STRAND BREAK REPAIR AND RECOMBINATION CATALYZED BY NUCLEAR EXTRACTS OF *SACCHAROMYCES CEREVISIAE*. L. Symington and R. Johnson, Institute of Cancer Research and Dept. of Microbiology, Columbia University, 701 W. 168th St., New York, NY 10032, (212) 305 7753.

DNA damaging agents, such as ionizing radiation, generate double-stranded breaks in DNA. Since the integrity of the chromosomes is destroyed by this type of damage, repair of the broken ends is an essential cellular process. Genetic studies in yeast have shown that the predominant pathway of double-strand break repair involves homologous recombination. To examine the biochemical events involved in double-strand break repair, we have developed a cell-free system to study this reaction (Symington [1991] EMBO J. 10: 987-996). We have shown that yeast nuclear extracts catalyze double-strand break repair and recombination of plasmid DNA substrates. Repair events are detected amongst reaction products by Southern blot hybridization, or by PCR. The reaction is stimulated by a double-strand break within homologous sequences and proceeds by a mechanism that involves branched DNA intermediates. In addition to pairing events that generate crossovers, the formation of inverted repeats (head-to-head and tail-to-tail joined products) is also detected. We have examined extracts prepared from yeast DNA repair defective strains and found that several of the members of the *RAD52* group have decreased activity *in vitro*.

We have recently developed a simple method for generating figure-of-eight molecules *in vitro*. These substrates are cleaved at the Holliday junction by yeast extracts to produce the expected products. We are currently fractionating extracts to purify the resolving activity.

F 639 **Non-conservative homologous recombination in *E. coli*** Noriko K. Takahashi*, Kenji Yamamoto*, Yoshihiro Kitamura*, Luo Si-Qin+, Hiroshi Yoshikura*, and Ichizo Kobayashi*,+. *: Dept Bacteriology, Medical School, Univ Tokyo, Tokyo 113. +: Dept Molecular Biology, Institute of Medical Science, Univ Tokyo, Shiroganedai, Tokyo 108 Japan

We asked whether homologous recombination between two duplex DNA molecules generates two DNA duplexes (*conservative*) or, alternatively, it generates only one recombinant DNA duplex (*non-conservative*). We employed plasmid substrates that should allow recovery of both the recombination products (Figure).



We obtained evidence that recombination is non-conservative in *E. coli* RecF pathway. A double-strand break increased this non-conservative recombination. If a non-conservative recombination event leaves an end, this end may stimulate another event. This "successive half crossing-over" model can explain why apparent plasmid gene conversion is not accompanied by reciprocal exchange of the flanking sequences in RecF pathway (K.Y. et al. submitted). It can explain origins of plasmid linear multimers and of transcribable recombination products, mating-type switching in yeast and recombination in mammalian somatic cells (I.K. Adv. Biophysics 28: in press).

We also observed non-conservative homologous recombination with a double-strand break in a *recD⁻* strain.

F 640 GENETIC ANALYSIS OF THE SACCHAROMYCES CEREVISIAE STRAND EXCHANGE PROTEIN SEP1, Daniel X. Tishkoff and Richard D. Kolodner, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, 02115

Sep1 (Strand Exchange Protein 1) from *Saccharomyces cerevisiae* has been shown to catalyze *in vitro* a number of homologous pairing reactions similar to those catalyzed by the RecA protein of *E. coli*. In addition to its homologous pairing activities, Sep1 also contains an intrinsic 5' to 3' exonuclease activity (A.W. Johnson and R.D. Kolodner, 1991, J. Biol. Chem. 266: 14046-14054) which is necessary for synapsis but not strand exchange. These activities suggest that Sep1 may be involved in catalyzing recombination events *in vivo*.

The *SEP1* gene has been cloned, and several Tn10*LUK* insertion mutations have been characterized in a SK1 background (D.X. Tishkoff, A.W. Johnson, and R.D. Kolodner, 1991, Mol. Cell. Biol. 11: 2593-2608). These mutants display several phenotypes including slow growth, sporulation deficiency, slightly decreased mitotic gene conversion rates, increased meiotic induction of gene conversion, and increased genetic mapping distance (cM) and reduced positive interference as measured between several markers on chromosome III. The sporulation defect was not alleviated when *sep1* mutations were combined with *spo13* or *spo13rad50* mutations. This suggests that *SEP1* does not act in the *RAD50/RAD52* recombination pathway.

In order to more thoroughly characterize the recombination phenotypes of *sep1* mutants, *sep1Δ* (deletion) mutants were constructed in a background which permitted the measurement of various gene converion and crossover events at several loci. Mitotic rates of gene conversion were again found to be slightly reduced at most loci tested, and rates of mitotic crossing over were also found to be slightly reduced. However in meiotic return to growth experiments, both the maximum fold of induction and the maximum induced frequency of recombination between *his7* and *lys2* heteroalleles were found to be reduced 3-5 fold in *sep1Δ* mutants relative to wild type. Additionally, maximum fold of induction and induced frequency of crossing over were reduced at least 15 fold in *sep1Δ* mutants. Both the biochemical activities and the genetic analysis of Sep1 are consistent with it having a role in recombination. Experiments are now under way to examine synaptonemal complex and the formation of double-strand breaks and crossover products at a recombination hotspot during meiosis in *sep1* mutants.

F 641 GENETIC ANALYSES OF *recF*, *recR* and *recO* GENE FUNCTIONS AS REVEALED BY THEIR INTERACTION WITH MUTATNT *recA* ALLELES OF *Escherichia coli*, Tzu-Chien V. Wang, Hai-Yin Chang and Jai-Li Hung, Department of Molecular Biology, Chang Gung Medical College, Kwei-San, Tao-Yuan, Taiwan

The *recF*, *recR* and *recO* genes were originally identified as those affecting the RecF pathway of recombination. Several lines of evidence suggest that these genes may belong to the same epistatic group. In the present work, the abilities of *recA718*, *recA720*, *recA730*, *recA750* and two known *recA*(Srf) alleles (*recA2020* and *recA441*) to act as suppressor for *recF*, *recR* and *recO* mutations were examined by studying their effects on the UV radiation sensitivities in *uvrA* cells. With the exception of *recA718*, all the other *recA* alleles examined in this work are able to suppress the UV radiation sensitivities caused by *recF*, *recR* and *recO* mutations. The suppression by *recA750* was minimal. The suppression of *recF* and *recR* by other *recA* alleles was more pronounced, but none of them could exert a full suppression. On the other hand, the *recA730* and *recA441* were able to fully suppress the UV sensitivity of *recO* cells, while the *recA720* and *recA2020* only partially suppress the UV sensitivity of *recO* cells. The observations that certain *recA* alleles could act as suppressor for *recF*, *recR* and *recO* mutations suggest that the *recF*, *recR* and *recO* gene products may function as a complex. From the known functional changes caused by the mutant *recA* alleles, the functions of the putative RecFRO complex are deduced.

F 642 GENE CONVERSION IN ALLOTYPIC AND ISOTYPIC RABBIT Ig KAPPA LIGHT CHAIN GENES, Benjamin Wolf, Carl Ritter and Jun Long Liu, Departments of Pathobiology and Animal Biology, University of Pennsylvania Veterinary School, Philadelphia, PA 19104

Two Ig isotypes κ-1 and κ-2 are present in the rabbit while only one is found in the mouse or human. In addition, each of the rabbit κ genes is polymorphic. The Cκ-1 locus is expressed as four (nominal) allelic products, b4,b5,b6 and b9 which are inherited in a codominant autosomal manner while the κ-2 locus is expressed as two allotypic forms. Although nominal Cκ-1 genes have been cloned and sequenced and their function described, genes encoding Cκ-1 nonallelic allotypic variants have neither been cloned nor sequenced nor is their function known. In an attempt to isolate these latent allotype genes we have used the polymerase chain reaction (PCR) to copy b5 latent sequences from b5 and b6 - latent allotype expressing homozygous b4 rabbits. The PCR products were not coding genes, but pseudogenes having sequences which categorized them as likely donors in gene conversion. Alignment of the pseudogenes at the AAAA motif (nt 160-164) of the Cκ coding region yielded maximum homology between pseudogenes and coding genes. Several pseudogenes contained long (~150 nt) Cκ-1 homologous sequences, one of which was broken only by a short region of Cκ-2 homology. Two pseudogenes had nearly equal contiguous κ-1 and κ-2 sequences while in another the κ-2 to κ-1 ratio was 2:1. Another pseudogene sequence contained a singular latent κ-1 b9 segment which correlated with the latent allotype secreted from the lymph node cells from which the DNA had been isolated. Segments of b4,b5 and b6 latent allotype genes were present in the individual pseudogenes. If these mosaic genes were coding genes, the gene products would have serological characteristics of latent allotypes. These are the first reported sequences which might account for the origin, through gene conversion, of rabbit Cκ allotypic and isotypic genes. Supported by NSF DCB 9005021.

F 643 FORMATION OF PARANEMIC JOINTS PROMOTED BY RECA-PROTEIN, Brian C. Wong, Sung-Kay Chiu and Samson A. Chow, Department of Biochemistry, University of Hong Kong, Hong Kong.

In the absence of free ends, RecA protein pairs single strands with homologous duplex DNA to form paranemic joints in which the strands are homologously aligned but not truly intertwined. In this study, we examined the effects of length of homology and DNA topology on RecA-protein promoted paranemic joint formation. Paranemic joints were formed between circular single strands and circular or linear double-stranded DNA from chimeric plasmids or phage M13Gori1. In these chimeric constructs, various lengths of DNA sequences (59 to 6,407 bp) homologous to the single strand were flanked by heterologous DNA, thus preventing true intertwining of the paired strands. The extent of joint formation was measured by a nitrocellulose filter binding assay. When the chimeric double-stranded DNA substrate was linear, no significant formation of paranemic joints was detected when the length of homology shared with the single strand was 496 bp or less. Between 662 and 6,407 bp of homology, the formation of paranemic joints increased proportionally to the length of homology. With negatively superhelical substrates, the minimal length of homology required for significant formation of paranemic joints decreased to 138 bp; formation of joints increased with length of homology but the maximal efficiency was reached at a homology length of 2,200 bp. For the lengths of homology that we examined, the pairing reaction using circular single strands was more efficient when the double-stranded DNA substrate was negatively supercoiled rather than linear; whereas relaxed, closed circular duplex DNA was the least efficient in forming paranemic joints. The stimulation by negative superhelicity on paranemic joint formation was exerted during the initial stage of the pairing reaction. Once the paranemic joint was formed, the paired structure could be maintained in the absence of negative superhelicity. These results indicate that both the length of homology and the topology of the DNA substrate can influencethe efficiency of paranemic joint formation promoted by RecA protein.

F 644 NON-HOMOLOGOUS END-JOINING IN HUMAN CELLS,

C. S. H. Young[1], Myra Derbyshire[2], Andrea Nicolás[1], and Richard Fishel[3], [1]Department of Microbiology, Columbia University, New York, NY 10032, [2]Laboratory of Chromosome Biology, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick MD 21702, [3]Department of Microbiology and Molecular Genetics, University of Vermont Medical School, Burlington, VT 05405.

An end-joining activity, which mimics the type of reaction observed in the non-homologous integration of transfected DNA in mammalian cells, has been identified and partially characterized *in vivo* and *in vitro*. When human cells are transfected with left and right terminal fragments of adenovirus containing internal non-complementary ends, the junctions formed in the viral genomes usually display sequence loss of a few nucleotides. A mechanism for the generation of these non-homologous junctions, which involves a nuclease (most likely with 3' to 5' polarity) and a ligase, can be incorporated into an end-joining model that is consistent with these data. We have begun to characterize from human cells a crude nuclear extract, and a partially purified protein fraction, both of which perform an efficient end-joining reaction. We term this activity NHR ligase for "non-homologous recombination ligase". Both the extract and the purified fraction promote the formation of head-to-head (H:H) and tail-to-tail (T:T) multimers from blunt-ended substrates. The junctions are not recuttable with the restriction enzyme used to create the substrate, but the loss of nucleotides is restricted to the very end, and follows a uniform but asymmetric pattern of loss. The partially purified fraction contains an ATP-dependent ligase, a 3' to 5' exonuclease, and a pairing function, and the end-joined products retain the 5'-phosphates. The activities are present in a protein complex of some 500kDa, as measured by exclusion chromatography. A model that involves a complex of homologous and non-homologous recombination functions to perform the end-joining will be presented.

Cell Cycle Regulation

F 645 UNREQUITED RECOMBINATIONAL REPAIR: A STORY OF CELL CYCLE ARREST AND LETHALITY INDUCED BY A NONCHROMOSOMAL DNA LESION IN YEAST,

Craig B. Bennett, Alice L. Lewis, Kristin K. Baldwin and Michael A. Resnick, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences (NIH), PO Box 12233, Research Triangle Park, NC 27709.

Cells of the yeast *Saccharomyces cerevisiae* are delayed in G-2 following chromosomal DNA damage. The arrest which is *RAD9* dependent (1) suggests a signalling mechanism(s) between chromosomal lesions and cell cycling so as to allow for recombinational repair. We examined the "global" consequences of an enzymatically induced double-strand break at a YZ (from *MATYZ*) sequence in a nonyeast region of a dispensible plasmid, thereby preventing recombinational repair of the break. The unrepairable DSB causes a permanent *RAD9* independent arrest in over one-third of the cells at a stage other than G-2. The remaining cells of a RAD^+ strain give rise primarily to microcolonies containing permanently arrested cells. Based on the profound effects of a single nonchromosomal DNA lesion, this system provides a convenient means for studying the *trans*-signalling effects of a DNA lesion and the relationship with recombination as well as for designing strategies to modulate cell proliferation.

1. Weinert & Hartwell, *Science 24* , 317 (1987)

F 646 REVERSIBLE SHUT-OFF OF INITIATION OF CHROMOSOME REPLICATION IN *ESCHERICHIA COLI*: EFFECTS ON GROWTH, CELL DIVISION AND NUCLEOID DISTRIBUTION,

Rolf Bernander, Thomas Åkerlund and Kurt Nordström, Department of Microbiology, Biomedical Center, Uppsala University, Box 581, S-751 23, Uppsala, Sweden.

In *E. coli* strain EC::71CW, the plasmid R1 derivative pOU71 is inserted into the chromosome such that *oriC* is inactivated, and replication instead starts at the R1 origin of pOU71. In EC::71CW, the rate of chromosome replication can be controlled by growth temperature, since the frequency of initiation of replication from pOU71 is temperature-dependent. At low temperature (below 35°C) the chromosome copy number is close to wild type, whereas with increasing temperature the replication initiation frequency gradually increases, and high temperatures are inhibitory to growth due to over-replication of the chromosome. Initiation of replication of plasmid R1 is mainly controlled at the post-transcriptional level, through RNA-RNA interaction. A small antisense RNA, CopA, specifically binds to the mRNA for the initiator protein RepA, thereby inhibiting initiation of replication by preventing expression of RepA. Plasmid pOU420 produces large amounts of CopA at 36°C but very small amounts at 39°C. By introducing pOU420 into strain EC::71CW it was, therefore, possible to specifically turn off chromosome replication by lowering the growth temperature from 39°C to 36°C. In contrast to other methods of inhibiting chromosome replication, *e.g.* those that use antibiotics or *dnaA*(ts) alleles, the cellular physiology is not otherwise disturbed. Thus, temperatures below 39°C result in under-replication of the chromosome, whereas growth above 40°C lead to over-replication, making the strain suitable for analysis of how variations in the efficiency of chromosome replication affect growth, cell division, and nucleoid distribution.

Growth was found to continue, although at a lower rate, when initiation of chromosome replication in EC::71CW was inhibited by shifting the growth temperature from 39°C to 36°C. After runout of chromosome replication cell division was inhibited, resulting in elongation of the cells. DAPI staining revealed that at this stage most cells contained a single, centrally located nucleoid. Inhibition of replication, therefore, leads to inhibition of cell division also when other cellular processes function normally. The replication block was found to be fully reversible, and removal of the block by increasing the growth temperature resulted in a resumption of cell division approximately one hour after the shift. The approach allows investigation of the coordination mechanisms between chromosome replication and cell division in *E. coli*, as well as of the rules that govern septum localization.

F 647 *DMC1* (DISRUPTED MEIOTIC cDNA) : A MEIOSIS-SPECIFIC YEAST HOMOLOGUE OF *E. COLI recA* IS REQUIRED FOR RECOMBINATION, NORMAL SYNAPTONEMAL COMPLEX FORMATION, AND PROGRESSION FROM PROPHASE TO METAPHASE I.

Douglas K. Bishop, Demian Park, Liuzhong Xu, and Nancy Kleckner. Department of Biochemistry and Molecular Biology. Harvard University, Cambridge, MA 02138

Analysis of a *S. cereviesiae* meiosis-specific cDNA library by Tn10::LUK mutagenesis has identified a new meiosis-specific gene, *DMC1*, that is essential for chromosome metabolism during meiotic prophase and is a structural and evolutionary homologue of *E. coli* RecA.

The *DMC1* gene encodes a 33 kd protein which is 26% identical to *E. coli* RecA in a region spanning 238 of 334 amino acids. The crystal structure of *E. coli* RecA protein has been determined (R. Story, I. Weber, and T. Steitz) and alignment of the Dmc1 primary sequence to the a-carbon trace of recA has revealed several features which demonstrate structural homology between Dmc1 and RecA (R. Story, D.K.B., N.K., and T. Steitz).

The *DMC1* transcript is absent from mitotic cells and is dramatically induced at the beginning of meiotic prophase. *dmc1Δ* mutants are not defective in spontaneous or UV-induced mitotic recombination and are not mms sensitive. *dmc1* mutants do have several important meiotic defects:

1. Recombination defects: recombination associated site-specific DSBs are made at the same time as in wild type cells. However the breaks never disappear; 5' ends at the break site are subject to extensive degradation while 3' ends remain intact. The yield of reciprocal recombination products for a pair of markers which flank the DSB site is dramatically reduced. Commitment to interchromasomal gene conversion at *HIS4* is reduced 5 to 15 fold while commitment to intrachromasomal "pop-out" events is increased. These defects are consistant with the proposal that *dmc1* mutants are deficient in strand exchange activity.

2. Pairing defect: full length SC is not formed, although short patches of SC are observable. Together with the recombination defect this result confirms and extends earlier work in which repair of DSBs and synapsis were shown to be inter-related (reviewed in Kleckner et al., (1991) CSHSQB, in press)

3. Cell cycle defect: *dmc1* mutants arrest in meiotic prophase prior to the formation of the meiosis I spindle. This result suggests that there is a direct link between prophase chromosome metabolism and the rest of the meiotic cell cycle. Consistant with arrest before MI spindle formation, dmc1 mutants are not supressed by the spo13 mutation.

Homology between a different yeast gene, *RAD51* and *recA*, was discovered concurrently and independently by A. Shinohara and T. Ogawa; together we have found that the *RAD51* and *DMC1* orfs are 45% identical showing that a repair protein and a meiosis-specific protein are related. These two genes are the first eukaryotic homologues of recA to be identified.

F 648 Differential Replication Timing of Human rRNA Genes in Transformed Versus Normal Cells. R. Blelloch[1,3], R. Scott[2,3] and J.M. Vos[1,2,3] (1)Curriculum in Genetics, (2)Dept. of Biochemistry and Biophysics and (3)Lineberger Comprehensive Cancer Center, U. of North Carolina, Chapel Hill, NC 27599

Previous mammalian studies showed replication timing of actively transcribed genes typically occurring within the first half of S phase. An interesting exception was the housekeeping rRNA genes which were found to replicate throughout S. Since these data were obtained in transformed human cells, we investigated whether this unusual replication timing of the rRNA genes related to the transformation process. Neoplastic T98G glioblastoma cells and WI38 normal fibroblasts synchronized by serum starvation were pulsed with BrdU at different time points in S phase and heavy-light DNA was purified by density centrifugation. Replicated DNA was analyzed by either Southern blot or quantitative PCR to compare the replication rate of individual genes relative to the genome overall. T98G, like previously examined established cell lines, replicated the rRNA loci throughout S phase. These cells replicated the active DHFR locus in early S and the silent β-globin locus in late S indicating that the altered replication timing is specific for the rRNA genes. Unlike T98G, WI38 normal fibroblasts replicated the rRNA loci predominantly in late S. We will describe a novel approach to dissect the molecular mechanisms responsible for the altered replication timing of rRNA genes. We will also discuss potential models for the replication switch.

F 649 DNA REPLICATION AND TRANSCRIPTION TAKE PLACE AT DISTINCT SITES IN VIRUS INFECTED CELLS, Julia Bosher and Ronald T. Hay, Department of Biochemistry and Microbiology, University of St. Andrews, Scotland, KY16 9AL, UK.

It has long been known that in adenovirus (Ad) infected cells viral replication proteins are not evenly distributed throughout the nucleus but are localised in discrete nuclear sites which were thought to be the sites of viral DNA synthesis. We have previously demonstrated that the virus coded DNA binding protein (DBP) colocalises with the cellular protein nuclear factor I (NFI) in Ad2 infected cells and that this colocalisation is a specific targeting event that reflects the requirement for NFI in Ad2 DNA replication. NFI is not colocalised with DBP in Ad4 infected cells; Ad4 does not require NFI for viral DNA replication and does not have an NFI binding site within its origin of DNA replication. Incorporation of 5-bromodeoxyuridine into replicating DNA in vivo and visualisation by immunofluorescence revealed that NFI and DBP are indeed colocalising at sites of viral DNA replication. In contrast another cellular transcription factor, NF-κB, and the viral transcriptional activator, E1A are not present at the sites of viral DNA replication but do colocalise at a different subset of nuclear sites. Incorporation of biotinylated UTP into messenger RNA in vitro and visualisation by fluorescence microscopy revealed that NF-κB and E1A are colocalising at sites of viral transcription. Thus viral DNA replication and transcription take place at distinct sites in .Ad infected cell nuclei.

F 650 CELL CYCLE REGULATION OF THE CATALYTIC SUBUNITS OF DNA POLYMERASES α AND δ FROM FISSION YEAST, Dominique Bouvier, Véronique Damagnez, Georges Pignède, Jeanne Tillit, Anne-Marie De Recondo and Giuseppe Baldacci, CNRS-UPR 272, Villejuif, France

We are interested in the structure, expression and regulation of the genes and proteins involved in DNA replication in fission yeast *S. pombe* (Damagnez *et al.* Mol. Gen. Genet. 1991, *226*, 182-189; Damagnez *et al.* Nucl. Acids Res. 1991, *19*, 1089-1104; Pignède et al. J. Mol. Biol. 1991, *222*, in press; Bouvier et al. Exp. Cell Res. 1991, in press). We have isolated the *S. pombe POL1* and *POL3* genes by sequence similarity with the homologous *S. cerevisiae* genes encoding the catalytic subunits of DNA polymerases α and δ, respectively. We sequenced the *S. pombe POL1* and *POL3* genes (both containing a single intron in their 5' regions), we studied their transcription and we characterized the function of their products. Pol α from *S. pombe* (37% identical to *S. cerevisiae* pol α and 31% to human pol α) shows five amino acid blocks specific of eukaryotic DNA polymerases α known to date. Pol δ from *S. pombe* shows a central region 60% identical to *S. cerevisiae* pol δ. Rabbit polyclonal antibodies raised against a pol δ/β-galactosidase fusion protein detect in mammalian cells a 140 kDa protein which, in regenerating rat liver, is exclusively associated with cell proliferation. Rabbit polyclonal antibodies raised against pol α- or pol δ-fusion proteins partially inhibit the aphidicolin-sensitive DNA polymerase activity of *S. pombe* cell extracts. A striking difference with *S. cerevisiae* is the absence of a strong increase of transcription of both these genes at the G1/S phase transition. Our results also indicate that the catalytic subunit of DNA polymerase α (170 kDa) increases in late G1/S and that a second protein form of 165 kDa is specific of late G1 and S phases. This cell cycle-dependent post-translational modification of pol α will be discussed in the context of the overall regulation of DNA replication.

F 651 Minichromosome replication in living *E. coli* cells and in a computer model, *Bjarke Bak Christensen, Tove Atlung and Flemming G. Hansen, Department of Microbiologi The Technical University of Denmark DK-2800 Lyngby.*

The initiation titration model proposed by F.G.Hansen and T. Atlung, is formulated to explain the initiation control of the *E. coli* chromosome. The model is based on an initiator protein (DnaA) and its DNA binding sites (DnaA-boxes). The DnaA-boxes are assumed to be high affinity sites for binding of DnaA protein and will titrate newly synthesized DnaA as long as there are free DnaA-boxes. Initiation of replication occurs when the high affinity sites are saturated and DnaA protein will be 'free' to interact with lower affinity sites in oriC. An important factor is an eclipse period after initiation, where *oriC* is hemimethylated and thus prevented from reinitiation.

This work has focussed on the replication behavior of minichromosomes, such as their high copy number, and their ability to initiate simultaneously with the chromosomal origin.

I introduced the minichromosomes in the computer version of the initiator titration model assuming them to be identical to the chromosomal origin. At cell division minichromosomes where distributed randomly between each daughter cell. If a daughter cell is without minichromosome, it was not allowed to grow any longer.

Our simulations showed that, when minichromosomes are introduced to a exponentially growing "computerculture" at either very low level or very high level, the copy number will, after several generations, reach a steady state number. This steady state number (in the model) was extremely dependent on the rate of methylation.

These simulations explain an experimental observation saying that there are about 3% of overreplication from minichromosomes, when these are introduced at very low level.

F 652 CELL-CYCLE REGULATION OF GENETIC RECOMBINATION AND RECOMBINATION-DEPENDENT RADIATION REPAIR IN HUMAN CELLS, Richard Fishel[1], David Kaplan[2], and Deborah Morrison[2], [1]Department of Microbiology and Molecular Genetics, University of Vermont Medical School, Burlington, VT 05405, [2]Molecular Mechanisms of Carcinogenisis Laboratory, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702.
Cell division in eukaryotes is tightly coupled to the completion of DNA replication. Furthermore, several genetic results have suggested that genetic recombination may be regulated during the cell cycle in normal mitotic cells. These observations have suggested that there are important "checkpoints" in the DNA metabolic processes that sense the completion of chromosome replication and recombination. Eukaryotic cells appear to perform this remarkable function by precisely controlled post-translational modification events, primarily through phosphorylation. We have been studying protein components from human cells that appear to be involved in genetic recombination in human cells. *HPP-1* is required for the homologous pairing of DNA. *hRP-A* is a single-stranded binding holoprotein consisting of three peptides capable of stimulating HPP-1-dependent homologous pairing and strand exchange 70-fold: a 70-kDa single-stranded binding peptide, a 32-kDa phosphoprotein of unknown function, and a 14-kDa peptide of unknown function. Finally, a potent blunt-end DNA joining (*NHR-ligase*) activity appears to mimic the nonhomologous recombination reaction. Recently, we have found that the essential replication holoprotein complex hRP-A may play a central role in the regulation of the recombination process via post-translational phosphorylation of the hRP-A(32) subunit. Furthermore, DNA damage elicits a hyperphosphorylation response that may inhibit normal DNA replication (and therefore cell division) while inducing the recombination/repair response. Our results appear to suggest that hRP-A occupies a critical "checkpoint" that controls passage through the normal cell cycle as well as functioning to regulate reentry into the cell cycle following DNA damage.
Research sponsored in part by the National Cancer Institute, DHHS, under contract No. N01-CO-74101 with ABL.

F 653 PLASMID REPLICATION DURING THE BACTERIAL CELL CYCLE, J. D Keasling, B. O. Palsson, and S. Cooper, Department of Chemical Engineering, University of Michigan, Ann Arbor, MI 48109-2136 and Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-0620

Plasmid replication during the *Escherichia coli* division cycle has been investigated using the membrane-elution technique to produce cells labelled at different times during the division cycle and subsequent quantitation of the label incorporated into the plasmids. The results indicate that the naturally-occurring, low-copy F plasmid and P1 prophage replicate in a cell-cycle-specific manner, with replication occurring approximately one-half generation between subsequent rounds of chromosome replication initiation. When the cell-cycle age of replication of the F plasmid is determined over a range of growth rates, it is found that the cell size at which low-copy plasmid replication occurs follows a pattern similar to that for replication of the bacterial chromosome - initiation occurs when a constant mass per origin is achieved - except that the initiation mass per origin is different for the low-copy plasmids than for the chromosome origin. The origin responsible for cell-cycle-specific replication has been determined by successively eliminating the three origins on the F plasmid. It has been found that two of the three origins are necessary for cell-cycle-specific plasmid replication. One of these origins initiates replication in a cell-cycle-independent manner when the other origin is removed. In contrast, the membrane-elution technique reveals that the naturally-occurring, high-copy R6K, pSC101, and ColE1-type plasmids replicate throughout the cell cycle.

F 654 A *DROSOPHILA* FUNCTIONAL HOMOLOG OF THE *S. cerevisiae* G1 CYCLINS. Elaine E. Lahue[1], Albert V. Smith[1,2], and Terry L. Orr-Weaver[1,2], [1]Whitehead Institute and [2] Department of Biology, M.I.T., Cambridge, MA 02142

Three *S. cerevisiae* G1 cyclins (*CLN1, CLN2, CLN3*) have been shown to trigger entry into S phase (1). These three genes are functionally redundant for S-phase entry as deletion of all three cyclins is necessary to arrest the cells in G1. We screened a *Drosophila* Kc cell cDNA library (an embryonically derived tissue culture line) in a *S cerevisiae* strain triply mutated for the G1 cyclins. Growth was maintained in the mutant yeast strain by the presence of *CLN3* on a *URA3* marked vector. We transformed this strain with the Kc cell cDNA library which had been constructed in a high copy yeast vector. Expression of the cDNAs was under the control of the strong and constituitive *ADH1* promotor. After plating all transformants onto 5-FOA medium to select for loss of the *URA3* marked *CLN3* containing plasmid, one transformant was obtained which rescued growth of the triply mutated *S. cerevisiae* strain. This cDNA, designated *CLNDm*, is present in single copy in the *Drosophila* genome and encodes a mRNA of approximately 1.2 Kb with an open reading frame encoding a protein of approximately 31 Kd. This putative *Drosophila* G1 cyclin has homology to other members of the cyclin family, with the strongest homologies found between other G1 cyclins. Of the published cyclin sequences, *CLNDm* is most similar to the mouse cyl3 sequence (2) and the human cyclin D sequence, both of which were isolated in screens for G1 cyclins. *CLNDm* mRNA expression correlates with periods of maximal cell division throughout *Drosophila* development, with the transcript most abundant in early embryos, and low in larvae, pupae, and adults.

1. Richardson, H. E., Wittenberg, C., Cross, F., and Reed, S.I. (1989) Cell 59: 1127-1133.
2. Matsushime, H., Roussel, M., Ashmun, R., and Sherr, C. (1991) Cell 65: 701-713.
3. Xiong, Y., Connolly, T., Futcher, B., and Beach, D. (1991) Cell 65: 691-699.

F 655 **Role of the mioC promoter in Escherichia coli chromosome and minichromosome replication.** **Anders Løbner-Olesen[1] and Erik Boye[2]. [1]Dept. of Microbiology, The Technical University of Denmark, DK-2800 Lyngby - Copenhagen, Denmark and [2]Dept. of Biophysics, The Norwegian Radium Hospital, 0310 Oslo, Norway.**

Replication of the Escherichia coli chromosome initiates at a fixed point, oriC, and proceeds bidirectionally toward the terminus region located opposite on the chromosome. Plasmids replicating autonomously from oriC, minichromosomes, have been isolated and were shown to have the same requirements for initiation of replication as the chromosome: DnaA protein, de novo protein synthesis and RNA polymerase mediated transcription. minichromosomes replicate in synchrony with the chromosome, and each plasmid is only replicated once per cell cycle. Therefore minichromosomes have been considered as good tools for studying initiation of chromosome replication.

A cis acting region covering the mioC promoter has been identified as essential for proper minichromosome replication. We have constructed well defined chromosomal mioC mutations and showed that the resultant mutant strains are normal with respect to a number of cell cycle parameters including the DNA concentration, origin content and single cell initiation synchrony.

Similarities and differences between minichromosomes and the chromosome with respect to the mioC promoter are summarized and the role of the mioC promoter in minichromosome and chromosome replication is discussed.

F 656 DEVELOPMENTAL EXPRESSION OF DROSOPHILA RRP1.

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Rrpl was originally purified from Drosophila embryos using a strand transfer assay. Subsequent analysis of the protein revealed Rrpl to contain two domains, an N-terminal domain unrelated to any known proteins and a C-terminal domain with striking homology to two DNA repair nucleases, E. coli exonuclease III and Streptococcus pneumoniae exonuclease A. The 3' exonuclease and apurinic/apyrimidinic activity of Rrpl has been directly demonstrated.

We are examining the expression of Rrpl during Drosophila development. Rrpl protein is rare, and unusually active amoung strand transferases. Northern hybridization analysis indicates that Rrpl mRNA is a very low abundance message. It is detectable at all stages examined, which may reflect an involvement of the protein in DNA repair. Interestingly, the mRNA is expressed maternally, and is most abundant in pre-blastoderm embryos and in adult females. Rrpl mRNA is up to ten times more concentrated in early embryos and adult females than in larvae, pupae or adult males. DNA repair proteins may be more abundant in the rapidly dividing cells of early embryos. In addition, meiotic recombination in Drosophila takes place only in females, and the high concentration of mRNA in female adults (which are about 50% oocytes by mass) and early embryos may reflect a role for the protein.

F 657 THE RAD3⁺ GENE OF SCHIZOSACCHAROMYCES POMBE IS INVOLVED IN CELL CYCLE CHECKPOINT FUNCTIONS AND IN DNA REPAIR,

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Checkpoint functions ensure that entry into and exit from mitosis occur only after the execution of an orderly progression of events. For example, checkpoints a) delay mitosis or arrest cells in G2 phase of the cell cycle in response to DNA damage, b) maintain the dependence of mitosis on the completion of chromosome replication and c) make the exit from mitosis dependent upon the completion of spindle assembly and proper chromosome segregation. An analysis of radiation sensitive (rad) mutants of S. pombe, uncovered genes involved in at least two of the checkpoint functions mentioned above. The mutant, rad3-136 is sensitive to UV and gamma radiation, refractory to caffeine sensitization after exposure to UV light and exhibits a reduced frequency of UV-induced forward mutation compared to wild-type cells. In addition, rad3-136 is deficient in two checkpoint functions, G2 arrest following gamma-irradiation and the coupling of DNA synthesis to mitosis. An S. pombe genomic clone that restores resistance to UV and gamma radiation completely restores the missing checkpoint functions when introduced into the rad3-136 mutant. There also appears to be a role for the rad3⁺ gene in DNA repair. Similar studies are ongoing in the S. pombe rad1 mutant.

F 658 Cdc7 AND Cdc28 PROTEIN KINASES INTERACT TO REGULATE THE G1 TO S PHASE TRANSITION,

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Cdc7 protein kinase phosphorylates both ser and thr residues in histone H1 and is nuclear (1). Cdc7 is required for the G1 to S phase transition and acts downstream of Cdc28 protein kinase in the mitotic cycle. Overexpression of Cdc7 is not detrimental to wild-type cell division (1). However, Cdc7 overexpression produces synthetic lethality in a *cdc28-1* mutant that is defective at the G1 to S transition and executes START poorly. In contrast, a *cdc28-1N* mutant that executes the G2 to M transition poorly is unaffected. Cdc7 polypeptide is required for the effect but it can be inactive. The overexpressed inactive Cdc7 will also cause lethality in a *cdc7* mutant but not in a *cdc8* (dTMP kinase) mutant. Overexpressed Cdc7 may act as a competitive inhibitor of Cdc28, which is less active in the *cdc28* mutant at the permissive temperature. Alternatively, Cdc7 and Cdc28 may compete for a limiting factor and inactive Cdc7 may compete with the active Cdc7 for this factor. These results support the hypotheses that Cdc7 may be a substrate for Cdc28 or that both kinases may be regulated by another protein, possibly another kinase during the G1 phase. Direct biochemical evidence will be presented for these hypotheses.

We have cloned wild-type genes that in high copy Alleviate the Cdc7-Inhibition phenotype (*ASI* genes). One clone, *ASI1* alleviates the inhibition in both *cdc7* and *cdc28* mutants. Overexpression of Cdc7 and temperature-sensitivity are unaffected. The *ASI1* plasmid may overproduce this limiting factor. Experiments are underway to test this possibility. *ASI1* maps on chromosome IV. Other genes important for the G1 to S phase transition that do not affect this phenotype are *CLN1* and *DBF4*.

1. Hollingsworth, R.E., Jr. and Sclafani, R.A. Proc. Natl. Acad. Sci. USA 87, 6272-6276 (1990).

F 659 *MCM2* AND *MCM3* ARE INVOLVED IN EARLY STEPS OF DNA REPLICATION IN YEAST,

Hong Yan, Susan Gibson, and Bik-kwoon Tye, Department of Biochemistry, cell, and Molecular Biology, Cornell University, Ithaca, NY 14853

MCM2 and *MCM3* are two essential genes important for the function of ARSs in yeast. Mutants defective in Mcm2 and Mcm3 show an ARS-specific minichromosome maintenance defect. In addition, these mutants exhibit a premitotic cell cycle-arrest and an increase in mitotic chromosome loss and recombination. Genetic analyses suggest that they play interacting or complementary roles in DNA replication. Double mutants of mcm2-1 and mcm3-1 is inviable at the permissive temperature for each of the single mutants. In addition, overproduction of Mcm3 is lethal in *mcm2-1* at 37°C, while overproduction of Mcm2 can partially complement the *mcm3-1* mutation at the nonpermissive temperature.

Mcm2 and Mcm3 show striking sequence homology to each other. This homology also extends to another *S. cerevisiae* protein, Cdc46; two *S. pombe* proteins, Cdc21 and Nda4; and two mammalian proteins, the human and mouse P1 proteins. Interestingly, these other proteins have also been implicated to play a role in replication initiation. Furthermore, *CDC46* complements *mcm5*, another ARS-specific minichromosome maintenance mutant isolated in this lab. Therefore Mcm2 and Mcm3 may be members of a family of proteins involved in replication initiation.

Mcm2 and Mcm3 are localized in the nucleus in a cell cycle-dependent manner similar to that of Cdc46. They enter the nucleus at the end of mitosis, persist there throughout G1 phase, and disappear from it at the G1/S transition point. This property is consistent with their being replication initiation proteins that function only once per cell cycle. Studies using Mcm3-LacZ fusion proteins indicate that amino acid residues 312 to 786 of Mcm3 contain the critical information for cell cycle-dependent nuclear localization.

Late Abstracts

RESOLVASE-CATALYSED SITE-SPECIFIC RECOMBINATION, Shahnaz Akhter, David G. Blake, Martin R. Boocock, Sally-J Rowland, David J. Sherratt, W. Marshall Stark, Mark Watson and Ai-Li Yang, Institute of Genetics, Glasgow University, Glasgow, G11 5JS

For efficient resolvase-catalysed recombination, the *res* sites must normally be in direct repeat on the same supercoiled molecule, and the product is normally a singly interlinked catenane. We previously proposed that the basis for this selectivity is a strict requirement for an interwrapped synaptic structure with a defined local conformation. Formation of this intermediate and/or strand exchange within it are only favourable when *res* sites collide with initial topologies that allow interwrapping of the sites with no entanglement of the DNA outside the synapse. We tested this proposal using a catenated, non-supercoiled substrate in which two topologically non-equivalent intermolecular recombination events are possible. The model predicts that only one of these events is likely to occur, and the topology of the likely product. Both predictions were found to be correct.

We have undertaken a detailed examination of the effects of varying the length of DNA between two closely spaced directly repeated *res* sites. There is a clear dependence on the helical phase of the recombining sites. We will discuss the implications of these results regarding the processes of synapsis and strand exchange.

The *Staphylococcus aureus* transposon Tn552 encodes a protein with homology to Tn3 resolvase, and has an internal *res* site. The sequence of this *res* has unusual features, in particular a very long spacing between subsites I and II. Also, the protein sequence has more homology with the DNA invertases than other resolvases, and the recombination system was first characterised for its ability to catalyse inversion *in vivo*. We have now reconstituted the system *in vitro*, and we will present our initial results on its properties.

INTERACTION OF MU REPRESSOR AND ESCHERICHIA COLI INTEGRATION HOST FACTOR WITH MU OPERATOR DNA IN VITRO, R.Alazard, M.Betermier, M.Chandler, Molecular Genetics and Microbiology, CRBGC du CNRS, 118 Rte de Narbonne, 31062 Toulouse Cedex, FRANCE.

Both lytic and lysogenic growth of bacteriophage Mu involve transposition. The choice between these pathways is controlled by a 200 bp region at about 1 kb from the left end of the phage genome. This region contains the operator sites O1, O2 and O3 partially overlapping two divergent promoters, Pe and Pc. Transcription from Pe specifies early lytic functions whereas transcription from Pc leads to the synthesis of the phage repressor protein, c. This region also carries a site for the Integration host factor (IHF) located between operator sites O1 and O2. This host protein exerts its effect on Mu growth at several levels.

It has been demonstrated that IHF preferentially enhances transcription from Pe over that from Pc on supercoiled plasmid DNA in vitro and in vivo. On the other hand, the presence of a *binding site between O1 and O2 suggests that IHF may influence* c binding at these sites. We used gel retardation and DNA footprinting techniques to analyse the binding of c and IHF to a linear 287 bp operator fragment. We demonstrate that IHF does not assist c binding but significantly stabilises the complex formed by c and its cognate binding sites O1 and O2. O3 is not implicated in the IHF stabilised complex. The results also indicate that binding of both proteins induces a large conformational change (bending) in the DNA fragment. We are at present analysing these interactions using small supercoiled DNA minicircles generated in vivo by a novel technique.

IHF binding to the control region thus appears to have two opposing effects: stimulation of transcription in the absence of c and stimulation of repression in its presence. Our results are discussed in term of a model in which IHF stabilises transcription patterns once the choice between lysogeny and lytic growth has been made.

CLONING AND OVEREXPRESSION OF YEAST DNA POLYMERASE δ IN *E. coli*, William Clay Brown, Martin Budd, and Judith L. Campbell, Division of Biology, 147-75CH, California Institute of Technology, Pasadena, CA 91125.

Three nuclear DNA polymerases; α, δ and ε have been characterized and shown to be essential in *Saccharomyces cerevisiae*. Previous work has shown that DNA polymerase δ was encoded by *CDC2* (*POL3*). We and others (Simon *et al*, 1991; P. Burgers, per. comm.) have observed that *POL3*-containing plasmids are unstable in *E. coli*. Overlapping fragments of the gene could be stably cloned when out of frame suggesting that uncontrolled expression is responsible for the instability, thus DNA polymerase δ must be toxic to *E. coli*. A new T7 vector, pT7SC, has been constructed which suppresses inappropriate expression prior to induction by infection with T7 phage containing amber mutations in several critical replication genes. The infection by T7 phage appears to be essential to successful production of stable protein as induction of the plasmid in cells containing a chromosomal copy of the T7 RNA polymerase gene led to degradation of DNA polymerase δ. Degradation also occurred in *lon*$^-$, *hsp r*$^-$ cells induced by infection with M13 containing the T7 RNA polymerase gene.

The full length *POL3* gene and a fragment beginning at the Nde I site are both stable in pT7SC but readily undergo insertions and deletions in pT7-7, the parent vector. These plasmids yield high levels of 124 kD and 97 kD polypeptide respectively upon infection with mutant phage. A small amount of the 124 kD protein is soluble and appears in extracts, however the bulk of the 124 kD and all of the 97 kD polypeptide is found in inclusion bodies. Protein purified from the inclusion bodies and dialyzed against urea for renaturation displays polymerase activity and is aphidicolin sensitive. A construct is currently being prepared which begins at the Eco RV site of *POL3*. This will encode an 83 kD form of the protein which lacks the putative exonuclease I, I' and II regions (Simon *et al*, 1991). The activities of all three forms of DNA Polymerase δ will be compared.

Reference:

Simon, M., Giot, L., and Faye, G. (1991) *EMBO J.* **10**, 2165-2170.

THE MINIF PLASMID CcdB KILLER PROTEIN IS A POISON OF E. COLI TOPOISOMERASE II. M. Couturier and P. Bernard. Department of Molecular Biology, Université libre de Bruxelles, Rhode Saint Genèse, B-1640, Belgium.

To ensure its stability, the F plasmid uses a series of "stabilization cassettes" which act synergetically. The ccd mechanism (ccd for control of cell death) is one such cassette. It contributes to the plasmid's high stability by killing bacteria that have not inherited a plasmid copy, after bacterial division. The ccd locus contains two genes, ccdA and ccdB, which encode proteins of 72 and 101 residues, respectively. Despite their small size, these proteins are multifunctional. They are involved in the repression of their own synthesis and in a bacterial killing/antikilling mechanism. The CcdB gene product is a potent cell-killing protein, the activity of which is negatively regulated by the CcdA protein.

Bacterial mutants resistant to the lethal effect of the CcdB cytotoxic protein have been isolated. The mutations are located in the *gyrA* gene. One of the mutant genes has been cloned and located at the nucleotide level by genetic reconstruction and sequencing. This mutation produces an Arg462 → Cys substitution in the amino-acid sequence of the GyrA polypeptide. Hence, the mutation was called gyrA462. The gyrA462 mutation suppresses both cell killing and SOS activation, normally promoted by the CcdB protein. As for gyrase nalidixic acid resistant mutations, the sensitive phenotype was shown to dominate over the resistant phenotype. This suggests that the CcdB protein is more than a mere inhibitor of an essential function but a poison of that function. One of the most prominent cellular effect of topoisomerase II poison is an important breakage of double strand DNA, revealed upon treatment with a protein denaturant. We show that, *in vivo*, the CcdB protein is responsible for *gyrase-mediated plasmid DNA breakage. We conclude that the* CcdB protein is a topoisomerase II poison which exerts its action, like quinolone antibiotics and a variety of antitumoral drugs, by trapping a cleavable topoisomerase II-DNA complex.

The origin binding protein of Herpes Simplex virus type 1 and its interaction with a viral origin of replication, Per Elias, Claes Gustafsson, Ola Hammarsten and David Åberg, Department of Medical Biochemistry, University of Göteborg, S-400 33 Göteborg, Sweden

We have previously shown that native OBP, purified as a dimer, binds cooperatively to at least two sites within ori_S inducing a conformational change in the AT-rich central region of the ori_S palindrome. We have now observed that the C-terminal fragment of OBP can be expressed in E. coli and purified as monomer. This fragment binds DNA with the same sequence specificity and affinity as native OBP indicating that protein-protein interactions between the N-terminal parts of OBP are responsible for the formation of dimers as well as the cooperative binding to ori_S.

The interaction of OBP with its recognition sequence is rapidly and completely inhibited by NEM. However, a preformed OBP-DNA complex is stable even during prolonged treatment with NEM. Studies with mutant forms of OBP indicate that the NEM reactive residues are present outside the DNA binding domain indicating an interaction between the N-terminal ATP hydrolyzing domain and the C-terminal DNA-binding domain of OBP.

Mutant forms of OBP that are deficient in the hydrolysis of ATP have been used to monitor the role of ATP/ADP in DNA binding as well as protein-protein interaction. A model of OBP-ori_S interaction will be discussed on these observations.

FUNCTIONAL DOMAINS OF YEAST DNA POLYMERASE α
Suzanne Elsasser and Judith L. Campbell, Department of Biology, California Institute of Technology, Pasadena, CA.

Yeast DNA polymerase α, which has been shown to be indispensable for replication, shares six regions of homology with a large class of polymerases referred to as the α polymerases. Among those in this class are the replicative yeast DNA polymerases α, δ, and ε, and viral polymerases including HSV polymerase. Functional assignments of the conserved domains have been approached through the recovery of mutations in the HSV polymerase showing altered sensitivity to drugs and nucleotide analogs. The majority of the isolated mutations map to domains II and III, *implicating these regions in nucleotide binding.*

Two site directed mutations have been prepared in the yeast DNA polymerase α, one each in domains II and III. The domain II mutation involves a change in tyrosine 843 to histidine, and is patterned after a mutation conferring aphidicolin resistance and phosphonoacetic acid hypersensitivity to HSV polymerase. The domain III mutation occurs at asparagine 948, changing this residue to a serine. The analogous HSV mutation confers acyclovir resistance. The point mutants have been cloned into a galactose inducible expression system to enable overproduction and quantitative purification. The biochemistry of the mutant proteins is being characterized.

TRANSPOSITION OF IS1, J.M.Escoubas, D.Zerbib, D.Lane, D.Galas, and M.Chandler. Molecular Genetics and Microbiology, CRBCG du CNRS, 118 Rte de Narbonne, 31062 Toulouse Cedex, FRANCE. and * Molecular Biology, University of California, Los Angeles CA 90089-1481,

The insertion sequence *IS1* is a small (768bp) and genetically compact transposable element found in many gram-negative enterobacteria. Two open reading frames located on the same coding strand are essential for *IS1* transposition activity. The product of the upstream frame, a small basic protein (InsA), binds to the inverted repeats which define the ends of the element, represses both *IS1* gene expression, and appears to inhibit transposition. The downstream frame (InsB') would be translated in reading phase -1 compared to InsA. We have been unable to detect a product of this reading frame but have demonstrated that InsB' is expressed as part of a fusion protein InsAB'. InsAB' is generated by a retroviral-like -1 translational frameshift which occurs in the 3' end of *insA* in a region of overlap between the two frames. Constitutive production of this protein results in an increase of more than 10^3 fold in transposition frequency. Indeed we have constructed derivatives which exhibit a transposition frequency of 2% when measured in the mating-out assay. The results we have obtained are consistent with the idea that *IS1* transposition levels are determined primarily at the translational level by the ratio of InsA and InsAB', thus providing a system to control transposition levels of elements which might insert into highly transcribed genes. We are at present exploiting the capacity of InsAB' to strongly induce the SOS system in *E.coli* to isolate mutants in the protein.

IS*1* transposition generates two types of product: direct insertions and stable cointegrates. Our results suggest that overproduction of the InsAB' protein leads to a strong preponderance of cointegrates compared to direct insertions and that derivatives carrying this mutation undergo high levels of IS*1*-mediated deletions (a phenomenon generally thought to involve intramolecular cointegrate formation). Moreover, it can be shown that there is a strong preference for the InsAB' protein to act on the IS*1* end (IRR) proximal to the 3' end of its gene.

CHARACTERIZATION OF *rdil-1*, A RECOMBINATION MUTANT OF *SACCHAROMYCES CEREVISIAE* THAT FAILS TO REGULATE *DIN* AND IS DEFECTIVE IN MINICHROMOSOME TRANSMISSION. Michael T. Fasullo[1,2] and Rodney Rothstein[1], [1]Department of Genetics, College of Physicians and Surgeons, New York, NY 10032 and [2]Department of Radiotherapy, Loyola University Chicago, Maywood, IL 60153. DNA damage stimulates recombination, damage-inducible genes *(DIN)* and arrests the cell cycle. We have been screening mutants of *Saccharomyces cerevisiae* that are defective in damage stimulated sister-chromatid recombination, for defects in induction of *DIN* or in arrest of the cell cycle. *rdil-1* (regulation of damage induction), a temperature-sensitive mutant, is defective in damage-stimulated sister-chromatid recombination and is constitutive for *DIN1*, the regulatory subunit of ribonucleotide reductase. *DIN1* is still damage-inducible by DNA damaging agents in this mutant. At both 30°C and 37°C, cells form multibudded extensions in which many buds contain no nuclei and others contain multiple nuclei. A centromere containing mini-chromosome, YCp50*SUP11*, fails to segregate properly at the permissive temperature. At the restrictive temperature, a diploid homozygous for the *rdil-1* mutation fails to sporulate. Cell division cycle (cdc) mutants with similar growth phenotypes *(cdc4, 34,* and *53)* fail to complement the *rdil-1* mutation. Interestingly, *rdil-1* partially suppresses the uv sensitivity conferred by a *rad9::URA3* disruption (blocked in the damage-induced cell cycle arrest at the G2-M transition) in a *rdil-1, rad9* double mutant. Our current hypothesis is that the *rdil-1* mutant is defective in DNA replication and the wild-type gene is currently being cloned.

GENE EXPRESION AND TRANSPOSITION OF IS911, A MEMBER OF THE IS3 FAMILY OF INSERTION SEQUENCES, O.Fayet, P.Polard, M.F.Prère, and M.Chandler. Molecular Genetics and Microbiology, CRBGC du CNRS, 118 Rte de Narbonne, 31062 Toulouse Cedex, FRANCE,

IS911 is a 1250bp long insertion sequence originally isolated from *S.dysenteriae*. It is a member of a widely distributed family of sequences (the *IS3* family) which is composed of at least 20 members. *IS911* shares several characteristics with the retroviral family of transposable elements. These include the terminal nucleotides 5'-TG....CA-3' and an open reading frame whose translation product exhibits striking sequence similarities to retroviral integrase proteins.

Like the unrelated insertion sequence IS*1*, IS*911* carries two consecutive open reading frames (ORFs) in the 0 and -1 phases respectively. These frames give rise to three protein species: the product of the upstream frame, ORFA, the product of the downstream frame, ORFB, and a fusion product of the upstream and downstream frames, ORFAB, generated by a retroviral-like -1 translational frameshift. Translation of ORFB occurs from an ATT initiation codon. The only other known example of initiation at an ATT codon in prokaryotes is that of translation initiation factor IF3, suggesting that expression of ORFB may be intimately coupled to the physiological state of the cell.

Overproduction of these proteins *in vivo* in the presence of suitable IS*911*-based transposon substrates results in a variety of unusual stable and unstable recombination products. These include transposon circles, recircularised donor plasmid molecules, and linear DNA fragments. We have determined the IS*911* proteins and the IS911 sequences required in cis to generate each of these products and are examining the relevance of these forms in the IS*911* transposition mechanism.

INTERVIRAL RECOMBINATION IN INFECTIONS OF NON PERMISSIVE RAT CELLS BY POLYOMAVIRUS. Michele M. Fluck, Hong Hwa Chen and David L. Hacker. Department of Microbiology, Michigan State University, East Lansing, MI. 48823-1101.

We have previously documented the existence of homologous interviral recombination (frequency of 30 % over a distance of 1.7 kb) coincidental with the integration of the viral genome in the process of neoplastic transformation of rat cells by polyomavirus. Experiments with large T-antigen temperature sensitive mutants provide negative evidence for a role of large T in the recombination process and suggest that viral DNA synthesis is not required. In half of the cases, double recombination events, compatible with gene conversion events, were observed. The results also demonstrate that recombination is not a post-integration event. Targeted recombination experiments between an exogenous viral genome and integrated viral sequences suggest that such events occur at about 1/10th the frequency of exchanges between two viral genomes recombining in the process of integration. Finally, a gradient of recombination was observed with a minimum in the nucleosome free enhancer region, and a maximum representing a 60-fold increase, in the early region approximately 2 kb away.

BIOCHEMICAL CHARACTERIZATION OF MUTANT FORMS OF dnaA PROTEIN IN INITIATION OF DNA REPLICATION, Jon M. Kaguni, Theodore R. Hupp, Kevin M. Carr, Cynthia A. Petersen, Jaroslaw Marszalek, and Carla Margules, Dept. of Biochemistry, Michigan State University, East Lansing, MI 48824

Chromosomal replication in *Escherichia coli* appears to be regulated at or prior to the initiation of a cycle of chromosomal replication. Of the many proteins involved in this process, dnaA protein has been the recent focus of study due to its involvement in initiation from the chromosomal origin, *oriC*. Recent work in our laboratory has centered on biochemical characterization of mutant forms of dnaA protein to include the *dnaA46*, *dnaA5* and *dnaA204* gene products. The *dnaA46* and *dnaA5* alleles are associated with a defect in the proper timing of initiation *in vivo*. These mutant forms of dnaA protein differ from the wild type counterpart in: 1) thermolabile activity in an *oriC* plasmid replication system dependent on a crude enzyme fraction, 2) altered binding to DNAs containing dnaA protein binding sites, 3) inactivity in unwinding of *oriC*, and 4) inactivity in *oriC* plasmid replication systems dependent on purified enzymes. The inactivity in replication systems dependent on purified enzymes was restored by addition of a crude enzyme fraction. dnaK and grpE heat shock proteins were identified which confer replication activity to dnaA5 protein in the purified enzyme system. The effect of these heat shock proteins on dnaA5 and dnaA46 protein was separated from the event of DNA synthesis by incubation in two stages of activation followed by DNA replication. Activation of dnaA5 and dnaA46 protein by heat shock proteins was thermolabile suggesting that the temperature sensitivity of *dnaA5* and *dnaA46* mutants is related to this interaction. A third heat shock protein, dnaJ protein, interferes with the activation of dnaA5 protein during the first stage, but not during the second stage of incubation. The modification by these heat shock proteins, the nature of which has not been determined, may regulate the replication activity of $dnaA^+$ protein and as a consequence, contribute to regulation of DNA replication.

MITOCHONDRIAL DNA POLYMERASE: MECHANISM AND STRUCTURE, Laurie S. Kaguni, Matthew W. Olson, Andrea J. VonTom, Catherine M. Wernette and Rhoderick H. Elder, Department of Biochemistry, Michigan State University, East Lansing, MI 48824.

Near-homogeneous γ polymerase from *Drosophila* embryos is a heterodimer comprising two catalytic activities in subunits of 125 and 35 kilodaltons. The polymerase function resides in the large subunit. No subunit assignment for the 3' → 5' exonuclease function has yet been made, nor has the role of the 35 kDa subunit been defined. However, highly specific polyclonal antiserum developed against the native enzyme coprecipitates stoichiometrically the two subunits from crude enzyme fractions, and inhibits strongly both the DNA polymerase and exonuclease activities. Immunological and structural studies indicate that the two subunits are intact and distinct.

Drosophila γ polymerase incorporates nucleotides by a quasi-processive mechanism. Under the standard condition of 120 mM KCl which is optimal for DNA synthetic rate on primed single-stranded DNA, the enzyme polymerizes in bursts of ~30 nucleotides, producing DNA strands with an average length of ~150 nucleotides. Remarkably, at low salt concentrations largely suboptimal for DNA synthetic rate, Pol γ is highly processive and capable of polymerizing through sites of stable secondary structure to replicate fully M13 DNA. In general, reagents which increase DNA synthetic rate decrease processivity. In contrast, *E. coli* single-stranded DNA binding protein, low $MgCl_2$ concentrations and glycerol stimulate DNA synthesis while promoting processive DNA strand synthesis.

The DNA polymerase and 3' → 5' exonuclease activities of *Drosophila* γ polymerase function coordinately to replicate DNA with a high degree of accuracy. Mechanistic studies suggest separate active sites for the two functions, and a higher efficiency of excision of mispaired nucleotides in the "stationary" versus "polymerization" mode.

STRUCTURE-FUNCTION RELATIONSHIPS IN INSECT DNA POLYMERASE-PRIMASE,

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DNA polymerase-primase and primase-free DNA polymerase α have been purified from young larvae of the mosquito, *Aedes aegypti*, and their structural features compared to the comparable enzyme forms isolated from embryos of the fruit fly, *Drosophila melanogaster*. Mosquito DNA polymerase-primase was purified 3600-fold by chromatography of a larval S-100 fraction on phosphocellulose, followed by DEAE-Spectra gel, hydroxylapatite and single-stranded DNA agarose chromatography and glycerol gradient sedimentation. The resulting enzyme fraction comprises six polypeptides of 165, 107, 80, 76, 55 and 40 kilodaltons. A near-homogeneous form of the mosquito DNA polymerase α which is devoid of DNA primase activity was purified by a similar scheme omitting the DEAE-Spectra gel step, and contains only two polypeptides of 107 and 76 kDa. Both enzyme forms are sensitive to aphidicolin ($I_{50} \sim 1$ μg/ml) and butylphenyl dGTP ($I_{50} < 0.1$ μM).

Mosquito DNA polymerase-primase and primase-free Pol α are antigenically related to DNA polymerase α from the fruit fly. Polyclonal antiserum developed against the *Drosophila* α polymerase, which contains a 182 kDa polymerase catalytic subunit, recognizes the 165, 107 and 76 kDa polypeptides in the mosquito enzymes, indicating that the 107 and 76 kDa species are likely products of *in vitro* proteolysis. Further, polyclonal antiserum developed against the near-homogeneous mosquito primase-free Pol α, which contains only the 107 and 76 kDa species, recognizes the 165 kDa polypeptide both in the crude phosphocellulose fraction of DNA polymerase-primase, and in the corresponding fraction of the primase-free Pol α. Peptide mapping experiments to evaluate the structural features of the common epitopes between the mosquito and fruit fly enzymes are underway.

RECOMBINATION IN THE TERMINUS REGION OF THE E. coli CHROMOSOME.

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Two intense recombination activities have been recently observed in the terminus region. It has been proposed that site-specific recombination, implicating the *dif* site and the XerC recombinase, might participate to daughter chromosome resolution (Clerget; Blakely *et al*; Kuempel *et al*, 1991, The New Biologist 8:780-811). The second observation is that excisive, RecA dependent, recombination occurs between directly repeated sequences installed between *dif* and a neighbor terminator sequence, at a rate consistent with an involvment of homologous recombination at each termination step (Louarn *et al*, 1991, J. Bacteriol, 173:5097). We will present further informations on "terminal recombination" and its control (role of RecBCD, effect of *tus* deletion or pause site displacement). Recent observations illustrating the possibility of a cooperation at the *dif* site between RecA-directed and XerC-dependent recombination pathways will be discussed.

COORDINATION OF 3′ → 5′ EXONUCLEASE AND DNA POLYMERASE FUNCTION IN *DROSOPHILA* MITOCHONDRIAL DNA POLYMERASE,

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A mispair-specific 3′ → 5′ exonuclease copurifies quantitatively with the near-homogeneous *Drosophila* γ polymerase. The exonuclease and polymerase exhibit similar reaction requirements and optima, suggesting functional coordination of their activities. Under non-polymerization conditions, the 3′ → 5′ exonuclease hydrolyzes 3′-terminal mispairs ~15-fold more efficiently than 3′-terminal base pairs on primed single-stranded DNA substrates, while it does not discriminate between any of three specific mispairs (dAMP:dAMP, dGMP:dGMP, dGMP:dAMP). Under polymerization conditions, γ polymerase does not extend a 3′-terminal mispair from the "stationary" state, even in the presence of a large excess of the next nucleotide. Instead, 3′-terminal mispairs are hydrolyzed quantitatively by the 3′ → 5′ exonuclease over the reaction time course. During DNA synthesis by γ polymerase in the "polymerization" mode, limited misincorporation and subsequent mispair extension do occur. Here, it appears that misincorporation and not mispair extension is rate-limiting. Primer-template challenge experiments demonstrate that the mechanism of primer-template transfer from the 3′ → 5′ exonuclease active site to the DNA polymerase active site is exclusively intermolecular: transfer from the exonuclease to polymerase mode requires dissociation and reassociation of mitochondrial DNA polymerase.

INITIAL BINDING OF 2′-DEOXYNUCLEOSIDE 5′-TRIPHOSPHATES TO HIV-1 REVERSE TRANSCRIPTASE,

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HIV-1 reverse transcriptase, a heterodimer consisting of two polypeptide chains of 66 and 51 kDa, fluoresces due to the presence of 36 trp residues. Association of 2′-deoxynucleoside 5′-triphosphates (dNTPs) with the enzyme results in a decrease in the intensity of the emission spectrum that can be used to calculate apparent K_d values. The K_d values of the four natural dNTPs to the free enzyme range from 36.7 ± 3.9 μM for dTTP to 47.3 ± 3.9 μM for dATP. The enzyme shows no preference for purine or pyrimidine nucleotides. Hill coefficients and the results of dual ligand titration experiments demonstrate that the free enzyme possesses a single dNTP binding site for which the four natural dNTP substrates compete. Photoaffinity labelling experiments with [α-32P]dTTP indicate this site to be on the 66kDa polypeptide. The presence of template-primers does not result in selective binding of the complementary dNTP, indicating that Watson-Crick base pairing is not involved in the initial binding reaction. The driving force for the association of the ligands with the enzyme is hydrophobic. Approximately 14% of the binding energy is derived from electrostatic interactions. Although Mg^{+2} is required for catalytic activity, it is not absolutely required for initial binding.

BASE SEQUENCE PREFERENCES AT DRUG-INDUCED CLEAVAGE SITES OF DNA TOPOISOMERASES SUGGEST A COMMON MODEL FOR DRUG ACTION.

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The DNA cleavage sites induced by topoisomerase II inhibitors generally are different for each class of drug. We have determined the DNA sequence locations of a large number of drug-stimulated cleavage sites of topoisomerase II, and find that the results provide a clue to the possible structure of the complexes and the origin of the drug-specific differences. We find that cleavage enhancements by doxorubicin, amsacrine (*m*-AMSA) and VM-26, which are representative of different classes of topoisomerase II inhibitors, have strong dependence on the bases directly at the sites of cleavage. The preferred bases are either at the 3' terminus (doxorubicin and VM-26) or at the 5' terminus (*m*-AMSA). They are: A for doxorubin (Capranico et al., *Nucleic Acids Res.*, 1990, 18: 6611), A for *m*-AMSA, and C for VM-26. Additional base preferences exist in the immediate neighborhood of the break sites, but these are relatively weak. Topoisomerase I-induced DNA breaks stimulated by camptothecin were also analyzed. The results suggest that camptothecin blocks topoisomerase I preferentially at cleavage sites that have a G at the 5' terminus. Experiments using oligonucleotides containing a strong SV40 cleavage site (Jaxel et al., *Nucl. Acids Res.*1989, 23: 11157) also show that camptothecin-induced cleavage is greatest when a G is at the 5' DNA terminus of the enzyme cleavage site and is smallest when it is a T. Therefore, we propose that camptothecin interacts directly with this G. Taken together, these observations suggest a common model for enzyme inhibition by topoisomerase I and II inhibitors. Stabilization of enzyme-DNA cleavage complexes may result from stacking between the drug and one of the base pairs at a DNA break site. DNA sequence selectivity of drug action may result from preferential stacking of drug polycyclic ring systems with specific bases.

MOLECULAR ANALYSIS OF THE LACTOCOCCUS LACTIS CONJUGATIVE TRANSPOSON TN5276 AND ITS MECHANISM OF EXCISION AND INSERTION,

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The conjugative transposon Tn5276, found in the chromosome of Lactococcus lactis NIZO R5, encodes the ability to ferment sucrose and the production of and immunity to the lantibiotic nisin. This 70-kilobase (kb) element can be conjugally transferred to other L. lactis strains and it integrates at various loci in the genome of the recipient.

The nucleotide sequences of the termini of Tn5276 show no homology to the ends of the known conjugative transposons Tn916 from Enterococcus faecalis and Tn1545 from Streptococcus pneumoniae. Unlike most transposons, Tn5276 lacks an inverted repeat at its termini. Its ends are asymmetric and AT-rich.

The mechanism of excision and insertion of Tn5276 is currently under investigation. We have found an open reading frame near the right end of Tn5276 with an amino acid sequence that shows the characteristics of the Int-related family of site-specific recombinases. The involvement of this ORF, together with a putative Xis-like ORF found upstream of it, in the excision and/or integration of Tn5276 is being studied. We have shown the existence of a circular, non-replicative intermediate of Tn5276. This intermediate is believed to be transferred in the conjugation process. Further investigations will show if the mechanism of excision and insertion of Tn5276 resembles those of the lambdoid phages and of the conjugative transposons Tn916 and Tn1545.

INFLUENCE OF DNA POLYMERASE ON THE SPECIFICITY AND FIDELITY OF BASE SELECTION.

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Mutations may arise during genomic replication as a result of DNA polymerase (pol) errors. The frequency and types of errors are determined, in part, by the ability of pols to utilize "inappropriate" dNTPs and primer•template structures as substrates. To better characterize parameters that influence substrate recognition and pol fidelity, we have compared HIV-1, FIV, MLV and AMV retroviral pols, T7 DNA pol (Sequenase) and pol-α's from calf thymus and human lymphoblasts for their abilities to form different nucleotide mispairs *in vitro* in the absence of 3'→5' exonucleolytic proofreading. The kinetics of nucleotide misincorporation were measured opposite a single template site (#587) on four different oligonucleotide-primed øX174 DNAs ($^{5'...TATCC}_{3'...ATAGGNTGTTT...5'}$; N^{587}= A, C, G or T) with identical surrounding sequences (5,385 bases). A comparison of the relative frequencies of incorporation (f_{inc}) of the 12 possible mispairs revealed: 1) all pols, except MLV, preferentially form $G_t \cdot T$ and/or $T_t \cdot G$ mispairs ("$_t$" refers to the template base); 2) none of the pols form $C_t \cdot C$ or $T_t \cdot T$ mispairs efficiently; 3) pols have unique strand preferences for mispairs, e.g., $G_t \cdot T > T_t \cdot G$ (HIV, FIV and T7), $G_t \cdot T < T_t \cdot G$ (MLV) and $G_t \cdot T \approx T_t \cdot G$ (calf pol-α); 4) distinguishing mispair preferences are exhibited by calf pol-α ($A_t \cdot A$, $T_t \cdot C$ and $C_t \cdot T$), MLV ($A_t \cdot A$) and T7 ($G_t \cdot G$); 5) pols differentially form $G_t \cdot A$ mispairs by a presumed dislocation mechanism (HIV ≈ FIV > T7 ≈ MLV ≈ human pol-α > AMV ≈ calf pol-α), and; 6) the fidelities of homologous pols differ by as much as 200-fold ($f_{inc(G_t \cdot T)} = 3 \times 10^{-4}$ for HIV and 1×10^{-6} for MLV). These data show that different DNA polymerases form mispairs with unique preferences and suggest that structural differences in and around the active sites of these enzymes contribute to base selection and fidelity.

UNCOUPLING OF DNAB HELICASE AND POL III HOLOENZYME CAN ACCOUNT FOR REPLICATION PAST BULKY LESIONS,

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This model is concerned with the replication of DNA molecules with bulky lesions such as pyrimidine dimers and many other mutagenic and carcinogenic chemical adducts. The original studies on the properties of newly-synthesized DNA in excision-defective *E. coli* were interpreted as demonstrating that all the new DNA was present in short pieces comparable to the distance between template strand lesions and that these short strands were converted to high molecular weight DNA by a process called postreplication repair, recombinational repair or daughter strand gap repair. Since the original studies were done long ago, many details of DNA replication have been elucidated; it is now clear that the replication fork is a complex multiprotein machine that is far more complicated than simply being a DNA polymerase. While daughter strand gaps generated from lesions on the lagging strand template are easy to understand because the lagging strand is normally synthesized discontinuously, daughter strand gaps generated from bulky adducts in the leading strand template are much more problematic. For example, some recent models imply that the leading strand must be synthesized continuously, and that a replisome arrested at a bulky lesion in the leading strand template can continue only when the leading strand is extended past the lesion by some sort of bypass synthesis. This hypothesis is apparently inconsistent with the original conclusion that daughter strand gaps are present in all the newly synthesized DNA. The specific model being proposed here is that the helicase and the polymerase at the replication fork become uncoupled when the DnaB helicase proceeds past leading strand lesions while the Pol III holoenzyme stalls at the lesion to generate a single-strand region extending between the helicase and the polymerase. A priming event can then occur on this newly-generated single-stranded segment to restart the leading strand. The likely roles for RecA and UmuCD' will be considered.

E.COLI XERC RECOMBINASE IS REQUIRED FOR BOTH PLASMID STABILITY AND CHROMOSOMAL SEGREGATION, David J. Sherratt, Garry W. Blakely, Sean Colloms, Gerhard May and Jennifer Roberts, Institute of Genetics, Glasgow University, Glasgow, G11 5JS

E.coli XerC recombinase belongs to the integrase family of site-specific recombinases and is required for multicopy plasmid stability and normal segregation of replicated chromosomes to daughter cells. Plasmid and chromosomal sites for XerC recombination show similarities in their crossover regions but differ in their requirement for accessory sequences and accessory proteins. For example, XerC recombination at the sites present in natural multicopy plasmids shows strong selectivity for intramolecular resolution; multimers are efficiently converted to monomers, whereas monomers are not recombined inter-molecularly to multimers. This recombination requires PepA and ArgR proteins as accessory factors and some 200 bp of specific sequence 5' of the crossover site. In contrast, XerC recombination at *dif*, a 32 bp sequence derived from close to the replication terminus of the *E.coli* chromosome, does not require ArgR, PepA or the accessory sequences 5' of the recombination site when the site is contained within a plasmid. This recombination shows no selectivity for intramolecular resolution, occuring both inter- and intra-molecularly. We are dissecting the molecular mechanisms that allow XerC to show these different requirements and properties at the two types of recombination site. Additionally, we are trying to understand the precise role of XerC and its relatives in the metabolism of bacterial chromosomes.