NUCLEASES: STRUCTURE, FUNCTION AND BIOLOGICAL ROLES

Organizers: R. Stephen Lloyd, Stuart Linn and Richard J. Roberts

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Recombination Nucleases

K 001 FUNCTIONAL ORGANIZATION OF HIV INTEGRASE PROTEIN: A 136 AMINO ACID DOMAIN IS NECESSARY AND SUFFICIENT FOR CATALYSIS OF PHOSPHORYLTRANSFER

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Integration of a DNA copy of the HIV genome into a chromosome of the host cell is an essential step in the HIV replication cycle. The integrase protein encoded by the virus mediates the key steps that insert the viral DNA into the host genome. Purified HIV integrase protein has two *distinct* biochemical activities: 3' processing (the site-specific nuclease activity that cleaves the 3' ends of the viral DNA prior to integration) and DNA strand transfer (the cleavage and ligation activity that inserts the viral DNA into host DNA). Integrase also catalyzes an apparent reversal of the DNA strand transfer reaction, termed disintegration. The stereochemical course of both the 3' processing and DNA strand transfer reactions has been previously analyzed. The results support the view that both reactions occur by a one-step transesterification mechanism. The finding that a number of different nucleophiles can participate in the 3' processing reaction suggests that a primary function of integrase is to render the phosphodiester bond at the site of cleavage susceptible to nucleophilic attack. Although the 3' processing and DNA strand transfer reactions might at first sight seem to be very dissimilar, a simple model can explain how these reactions might be catalyzed by a common active site in integrase protein.

Comparison of amino acid sequences among retroviral integrase proteins has identified two distinct conserved motifs. A pair of His and Cys residues are invariant in the amino-terminal region. The central part of integrase contains another motif, termed D,D-35-E, that includes these three acidic amino acids as an invariant feature. In order to analyze the functional organization of HIV integrase protein we changed these highly conserved amino acid residues and also made variants of the protein with deletions extending from the amino and/or carboxyl terminus. These proteins have been purified and assayed for their *in vitro* biochemical activities.

Mutation of the conserved His and Cys residues in the amino-terminal part of integrase severely diminished both 3' processing and DNA strand transfer activities but had much lesser effects on the disintegration reaction. In contrast to the relative tolerance of the His-Cys region to mutation, two the amino acid substitutions in the central core of integrase, D116N and E152Q, completely abolished detectable activity in all three *in vitro* assays. Substitution of other invariant residues in this region also drastically affected the activities of the protein.

We have constructed a set of mutant HIV integrase proteins with deletions extending from the amino and/or carboxyl terminus. The smallest of these proteins (amino acids 50-212 and 50-186) are still active in the "disintegration" assay conclusively demonstrating that this central part of the protein is sufficient for catalysis of phosphoryltransfer. We interpret the failure of these proteins to catalyze the 3' processing or DNA strand transfer reactions to indicates that other parts of integrase mediate protein-protein and/or protein-DNA interactions that are necessary for 3' processing and DNA strand transfer, but not for disintegration. Experiments are in progress to test this hypothesis and identify the interactions involved

K 002 HO ENDONUCLEASE AND OTHER EXO- AND ENDO-NUCLEASES INVOLVED IN SITE-SPECIFIC HOMOLOGOUS RECOMBINATION IN *SACCHAROMYCES CEREVISIAE*

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Yeast mating type (*MAT*) switching is initiated by the site-specific HO endonuclease that creates a double-strand break (DSB) with 3' overhanging ends. *MAT* switching occurs by gene conversion in which a- or α-specific sequences at *MAT* are replaced by new sequences copied from one of two unexpressed donor loci. Using a galactose-inducible HO gene, we have physically monitored the molecular events during recombination using Southern blot and PCR assays. *MAT* switching takes nearly one hour to complete, during which time we can observe several intermediate steps, including a slow (1-2 bp/sec) 5' to 3' exonuclease that creates 3' single-stranded tails that then invade the donor locus and begin new DNA synthesis by primer extension. In contrast, there is apparently no 3' to 5' exonuclease activity, as a mutation only 8 bp from the 3' end is nearly always preserved during recombination.

When the HO cut site is inserted into other DNA sequences, we observe both gene conversion (GC) and a highly efficient alternative pathway of repair, i.e., recombination of homologous sequences flanking the DSB. This repair occurs by single-strand annealing (SSA), facilitated by the 5' to 3' exonuclease to expose complementary strands.

The role of a number of genes important in DNA repair and replication can be assessed by examining the molecular events that occur in various mutant strains. A deletion of the *RAD50* gene markedly reduces 5' to 3' exonuclease activity but still permits (delayed) completion of GC and SSA. A deletion of *RAD51* (which is homologous to E. coli recA) blocks early in *MAT* switching, before strand invasion and primer extension, but still permits SSA repair of broken chromosomes. Surprisingly, *rad51* strains also permit GC, with and without associated crossing-over, on plasmid substrates carrying two copies of the lacZ gene. The possibility that there is an alternative repair pathway for plasmids is under investigation. A deletion of *RAD52* prevents GC in all substrates but permits a reduced amount of SSA, with an increased requirement for the lengths of flanking homology. Deletions of *RAD54*, *RAD55* and *RAD57* block early in *MAT* switching; their effects of HO-induced plasmid recombination and SSA will be reported. Other genes involved in DNA repair and replication are also being studied.

RAD1 has long been known to be involved in the excision repair of UV photodimers but exhibits little effect either on the repair of chromosomal DSBs created by X-rays or by HO during *MAT* switching. However, *RAD1* is required for both GC and SSA whenever non--homologous DNA must be removed from the ends of the DSB before homologous recombination can be completed.

K 003 NOVEL HOMOLOGOUS PAIRING PROTEINS FROM *SACCHAROMYCES CEREVISIAE* AND *ESCHERICHIA COLI*

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Much of our knowledge of how proteins promote homologous pairing reactions come from studies of the *E. coli* RecA protein. However, recent studies have led to the discovery of a different class of homologous pairing activities that function in conjunction with exonucleases.

S. cerevisiae Sep1 protein 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 is a 175,000 Mr protein having two independent activities; homologous pairing activity and a 5' to 3' exo activity. The exo activity degraded RNA, ssDNA and dsDNA. In 20 min strand exchange reactions where up to 7250 bases of heteroduplex is formed 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 exo III or T7 gene6 exo, then Sep1 promoted homologous pairing and strand exchange when its nuclease activity was inhibited. Under nuclease active and nuclease inactive conditions, a 20 base homologous ss tail on the linear dsDNA substrate was required for strand exchange. Extensive regions of heteroduplex DNA were formed by a protein promoted strand displacement reaction yielding partially exchanged molecules and full length ds circular products. These data suggest the exo activity of Sep1 is required for initiation of pairing by producing a short ss tail on the linear duplex substrate and this determines the polarity of the reaction when the linear dsDNA has blunt ends.

sep1 mutations caused a defect in meiosis as *sep1* mutants did not sporulate and arrested in pachytene. DNA replication and commitment to recombination occurred with normal kinetics. Gene conversion was either increased up to 3 to 5 fold or decreased up to 5 fold depending on the strain and locus examined. Physical analysis of the formation and repair of meiosis-specific ds-breaks and meiotic crossing over demonstrated that ds-breaks were formed but not repaired and crossing over was almost entirely eliminated in *sep1* mutants. This suggests that Sep1 is important for meiotic recombination.

Recombination of plasmid DNAs and recombination of bacteriophage λ *red* mutants in *recB recC sbcA E. coli* mutants, where the *recE* region is expressed, does not require *recA*. We have shown the *recE* region encodes exo VIII, a processive 5' to 3' dsDNA-specific exonuclease. A.J. Clark and colleagues have shown the *recE* region contains two genes, *recE* and *recT*, and that exo VIII is likely produced by translational frame shifting that fuses the N-terminal 97 kDa *recE* gene product to the C-terminal 33 kDa *recT* gene product. We found a 33 kDa protein was co-expressed with both exo VIII and a truncated version of exo VIII, pRac3 exo, when they were overproduced. The 33 kDa protein was purified and demonstrated to be encoded by *recT*. RecT protein bound to ssDNA and also promoted the renaturation of complementary ssDNA. A combination of exo VIII and RecT protein promoted homologous pairing and strand exchange of circular ssDNA and homologous linear dsDNA with a 5' to 3' polarity with respect to the displaced strand. If the linear dsDNA was resected with exo III or T7 gene6 exo, then RecT protein promoted homologous pairing and strand exchange by itself. The observation that exo VIII and RecT protein promote homologous pairing and strand exchange appears to explain how some recombination reactions are independent of *recA* when the *recE* region is expressed.

These studies were supported by NIH Grants GM26017 and GM29383 to R.K. and NIH Postdoctoral Fellowship GM13594 to A.J.

K 004 NUCLEASES AND HOMOLOGOUS RECOMBINATION IN *ESCHERICHIA COLI* AND *SCHIZOSACCHAROMYCES POMBE*. Gerald R. Smith, Susan K. Amundsen, Sathyam Ganesan, Philippe Szankasi, and Andrew F. Taylor, Fred Hutchinson Cancer Research Center, Seattle, WA 98104.

RecBCD enzyme of *E. coli* has ATP-dependent DNA unwinding and nuclease activities; it is required for the major (RecBCD) pathway of homologous recombination involving a linear ds DNA molecule (1). In the presence of Mg^{++}, but absence of ATP, RecBCD enzyme forms a tight complex with a ds DNA end (2). In this complex the terminal 16-17 and 20-21 nucleotides of the 3' and 5' ended strands, respectively, are protected from DNase I digestion. UV irradiation crosslinks RecB to the 3' ended strand and RecC+RecD to the 3' ended strand. These and other results have suggested a mechanistic model for RecBCD enzyme's unwinding of DNA with the formation of asymmetric ss DNA loop-tail structures. During its unwinding of DNA RecBCD enzyme cuts at high frequency a DNA strand containing the Chi hotspot (5' G-C-T-G-G-T-G-G 3'); upon cutting at a Chi site the enzyme loses the ability to detectably cut at another Chi site on the same or a second DNA molecule and the ability to unwind a second DNA molecule (3). Cutting at exactly one Chi site near each end of a linear DNA molecule provides an enzymatic basis for guaranteeing even numbers of exchanges, and hence viability, in recombination between a linear chromosomal fragment and a circular complete chromosome (4). The physical basis of RecBCD enzyme's change upon cutting at Chi is unknown but may be the loss or alteration of a 22-23 nucleotide-long RNA molecule that co-purifies with RecBCD enzyme: DNA unwinding and Chi cutting are also lost when RecBCD enzyme is treated with RNase. Controls show that the effect of RNase is on RecBCD enzyme and not on the DNA substrate or the assay. At least 17 nucleotides of the RNA have a unique sequence, with which we are seeking the putative *E. coli* gene encoding this RNA and an *E. coli* mutant lacking it to determine the role of this RNA in RecBCD enzyme's activities and in *E. coli* recombination.

Recombination in eukaryotes has been proposed to require nucleases that make ss nicks or ds breaks or that enlarge these to ss or ds gaps. To test this proposal, we have purified two nucleases from *S. pombe* (5) and cloned the genes encoding them. ExoI digests ds DNA from 5' ends and generates 3' tails, putative recombination intermediates; it also digests nicked (but not intact) circular DNA. ExoI has a molecular mass of 36 kDa, has a K_M of 10 nM for DNA ends, and is induced 5-fold during meiosis. ExoII digests ss DNA from the 5' end; it has a molecular mass of 134 kDa, has a K_M of 36 pM for DNA ends, and is at most only slightly induced during meiosis. The deduced amino acid sequence of ExoII is about 50% homologous to that of the Sep1 protein of *Sacchromyces cerevisiae*, which has DNA strand transfer and nuclease activities. The nuclease acitivites of ExoI and ExoII are analogous to those of phage lambda exonuclease and *E. coli* RecJ protein, which are required for the Red and RecF recombination pathways, respectively. We are using the cloned *exo1* and *exo2* genes to generate *S. pombe* mutants lacking these nucleases, to determine their roles in recombination.

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K 005 RESOLUTION OF HOLLIDAY JUNCTIONS IN VITRO, Stephen C. West, Richard Bennett, Fiona Benson, Hazel Dunderdale and Helena Hyde, Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts EN6 3LD, U.K.

The *ruv* locus of the *E. coli* chromosome encodes three proteins required for the processing of recombination intermediates and the formation of recombinant DNA products. One of these proteins, RuvC, resolves Holliday junctions by specific endonucleolytic cleavage. The RuvC protein is a Holliday junction-specific endonuclease that shows little activity on single- or double-stranded DNA. Studies with synthetic Holliday junctions show that it is highly specific for cross-over points that contain three or four DNA strands. The interaction of RuvC with a synthetic Holliday junction has been studied in detail. Under conditions in which resolution is not permitted (low magnesium ion concentration), the protein binds Holliday junctions to form a protein-DNA complex, the structure of which has been probed by DNase I and hydroxyl radical footprinting. Binding occurs at the central core of the junction and results in a hypersensitivity to hydroxyl radical attack. The hypersensitivity is observed in only two of the four DNA strands at the junction point. When resolution is allowed, the Holliday junctions are cleaved by the introduction of nicks at sites that are diammetrically opposed in the two other DNA strands. Although junction binding is structure-specific, the RuvC protein shows a weak sequence specificity and cleaves DNA at the 3'-side of thymine residues. Most importantly, resolution is dependent upon the presence of homologous DNA sequences at the core of the junction, indicating that specific contacts may be required for the correct positioning of the DNA strands within the active site of the enzyme. These features distinguish the RuvC Holliday junction resolvase from bacteriophage T4 endonuclease VII and T7 endonuclease I, both of which resolve junctions in vitro, and is likely to reflect the specialised role that RuvC plays in recombination and the recombinational repair of DNA damage. The in vitro properties of RuvC protein will be compared with those of the bacteriophage and mammalian resolvases.

Repair Nucleases-I

K 006 MULTIFUNCTIONAL AP ENDONUCLEASES: TWO FAMILIES OF DIESTERASE/PHOSPHATASES, Bruce Demple, Dindial Ramotar and Rachel Lillis, Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115

Abasic (AP) sites and fragmentation products of deoxyribose in DNA are formed by many mutagenic and cell-killing agents. These lesions represent losses of genetic information and must be removed to preserve genetic integrity. The repair of these damages is initiated by a class of enzymes commonly called AP endonucleases, but which also harbor a specific, limited 3'-exonuclease activity that removes nucleotide fragments from DNA, and DNA 3'-phosphatase activity. Two families of these AP endonucleases have recently been defined, based on both enzymatic and molecular genetic comparisons. One family includes endonuclease IV of *E. coli* and Apn1 protein of *S. cerevisiae*, both of which are zinc-containing hydrolytic enzymes. The activity of endonuclease IV may be regulated post-translationally by another transition metal, manganese, and is controlled transcriptionally by the oxidative stress-inducible *soxRS* system. The biological DNA repair roles of both endonuclease IV and its yeast homolog, Apn1, have been demonstrated *in vivo*. Both proteins remove potentially lethal 3'-damages formed by oxidative attack, and lethal alkylation damages, evidently AP sites. Apn1 is the only nuclear AP endonuclease/3'-repair diesterase that we have detected in yeast. The Apn1 protein also plays a clear role in maintaining genetic stability in yeast: Apn1-deficient strains have a substantially elevated spontaneous mutation rate, due presumably to the endogenous DNA damages normally handled by Apn1. The phenotypes of Apn1-deficient yeast cannot be complemented efficiently by *E. coli* endonuclease IV, which is localized mostly to mitochondria in yeast.

The major AP endonuclease of human cells, encoded by the *APE* gene, belongs to a different protein family that includes similar enzymes from other mammals, Drosophila Rrp1 protein, ExoA protein of *S. pneumoniae* and *E. coli* exonuclease III. A second, evidently unrelated AP endonuclease/3'-repair diesterase has been extensively purified from HeLa cells. The *APE*-encoded AP endonuclease is a relatively weak 3'-diesterase/3'-phosphatase, in contrast to exonuclease III, for which the AP endonuclease, 3'-diesterase and 3'-phosphatase activities are about equal. It is possible that <8 amino acid substitutions account for this difference, which probably does not arise from a change in the DNA cleavage mechanism. The *APE* gene is compact (5 exons, 4 small introns) and maps to the 14q11.2-12 locus in the human genome. No genetic diseases that seem related to AP endonuclease deficiency map to this locus. The biological role of human AP endonuclease is being addressed by manipulation of the enzyme levels in cultured cells.

K 007 NUCLEOTIDE EXCISION REPAIR: A STUDY IN VECTORIAL MOVEMENT

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The Escherichia coli UvrABC endonuclease system is involved in the dual incision of DNAs damaged by a wide variety of chemically unrelated damage in which one break occurs 7-8 nucleotides 5' and the second 3-4 nucleotides 3' to the same damage. These sites of breakage are essentially invariant regardless of the primary chemical nature of the genotoxic damage. Even though this is an energetically favorable reaction ATP is required for incision. In approaching the role(s) of ATP and the question of substrate specificity it became apparent that the Uvr A_2B complex could not locate damaged sites by passive diffusion because of the small difference in association constants between undamaged and damaged sites ($\sim 10^3$). Translocation to damaged sites is achieved as a consequence of this multiprotein complex which acts as a helicase unwinding small DNA regions in a 5'-->3' direction reforming hydrogen bonds behind it and generating, in the process, waves of supercoiling. In all likelihood this supercoiling qualifies the Uvr repair system to interact with those other biological processes which supercoil such as transcription and replication. To this end the T7 and E. coli RNA polymerases, in the presence of their specific promoters, interact with the Uvr A_2B complex in both supercoiling and transcription providing a unique "landing or start site". Damaged regions act as the termination site for the repair complex. The Uvr A_2B protein complex represses RNA polymerase-catalyzed chain elongation but not its initiation. ATP provides the energy for these pre-incision reactions by directing protein-protein and nucleoprotein associations through nucleotide binding whereas macromolecular dissociations are driven by ATP hydrolysis reactions. ATP hydrolysis is not required for the dual endonucleolytic reactions in incision once a damaged site is identified. The mechanisms of translocation are being studied by steady state and time-resolved fluorescence spectroscopy of appropriately derivatized UvrB and by circular dichroism of UvrA proteins mutagenized in zinc finger motifs and helix-turn-helix regions.

K 008 BASE EXCISION REPAIR OF PURINES DAMAGED BY FREE RADICAL GENERATING SYSTEMS OR ALKYLATING AGENTS,

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Free radical generating systems induce in DNA a variety of damaged bases including, 8-oxopurines and formamidopyrimidine (Fapy) derivatives. Alternatively 7-methylguanine can also yield a Fapy derivative. Fapy lesions inhibit in vitro DNA synthesis by DNA polymerase I which stops one base before the lesion present in the template[1,2]. Fapy lesions and 8-hydroxyguanine as well as many other lesions are excised in E. coli by the Fpg protein[3]. The structural gene coding for this protein has been cloned and sequenced[4]. The Fpg protein has a zinc finger consensus sequence and contains one zinc atom per molecule[5]. The Fpg protein has an associated abasic site nicking activity which incises the DNA both at the 3' and 5' side of the abasic site leaving a gap[6]. The Fpg protein also catalyses the excision of 5' terminal deoxyribose phosphate (dRp) from DNA[7].
We have further investigated the role of the 6 cysteines present in the Fpg protein. Non conservative site-directed mutagenesis in the four cysteine residues of the Zn finger consensus sequence of the Fpg protein have approximately 1000 fold lower Fapy-DNA glycosylase, C8 oxoguanine DNA glycosylase and dRPase activity. The ß-lyase activity being lower.
We have purified more than 10.000 times, a protein from calf thymus as an enzymatic activity which removes Fapy residues, and incises DNA containing C8-oxoguanine residues and DNA containing abasic sites. Fpg protein in E. coli as well as in mammals contributes to cellular processes that counteract the delerious effects of reactive oxygen species and possibly plays a role in the excision of dRp residues.
3-methyladenine-DNA glycosylase is active on the repair of some lesions induced in DNA by alkylating agents. We have cloned expressed and purified this protein from rat and human cells[8]. The human enzyme has been obtained as a truncated protein. However it exhibits physiological properties in vivo and is stable in vitro where its enzymological properties have been compared to the full length protein.

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K 009 REPAIR ENDONUCLEASES/GLYCOSYLASES THAT RECOGNIZE OXIDATIVE MODIFICATIONS OF PYRIMIDINES,

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Oxidative DNA damages are recognized by DNA N-glycosylses, DNA N-glycosylses with associated apurinic/apyrimidinic (AP) lyase activity, and AP endonucleases. Since oxidative lesions are the most common cellular lesions, organisms have evolved numerous complementary systems to cope with them. In Escherichia coli, the recognition of oxidized pyrimidines is accomplished by endonucleases III and VIII. The former is the abundant activity accounting for more than 90% of the cellular thymine glycol DNA glycosylase activity. Both enzymes have associated AP lyase activities with endonuclease III catalyzing a ß elimination reaction and endonuclease VIII, a ßδ elimination. Endonucleases III and VIII exhibit broad substrate specificities including ring fragmentation, contraction and saturation products of pyrimidines, as well as alkoxyamine modified AP sites. All are removed by cleavage of the N-glycosyl bond followed by a concerted enzyme-catalyzed ß or ßδ elimination resulting in a strand break with a blocked 3' terminus.

Substrate specificity was further examined with oligonucleotides containing an internucleotide pyrophosphate (P-P) bond 3' or 5' to the lesion and either adjacent to or one base removed from the damage. As expected, DNA containing a unique abasic site with a pyrophosphate bond 3' to the damage was not a substrate for endonuclease III; however a 5' P-P also inhibited the reaction. Interestingly, DNA containing a P-P bond 3' or 5' to an abasic site was a good substrate for both endonuclease VIII and FAPY DNA glycosylase. Since the lyase activities leave a blocked 3' terminus, a 5' AP endonuclease, such as endo IV or exo III, is essential for preparing a substrate for DNA polymerase. These activities are also influenced by the presence of adjacent internucleotide P-P bonds.

The processing of strand breaks, abasic sites, and base damages has also been studied in vivo using a duplex ØX transfecting DNA containing the lesion of interest and E. coli hosts containing or lacking the appropriate base excision repair enzyme. In all cases, the in vivo results are congruent with the in vitro predictions.

Replication Nucleases-II

K 010 CONTRIBUTIONS OF EXONUCLEASE ACTIVITY AND LOCAL DNA SEQUENCE TO THE FIDELITY OF SYNTHESIS BY T4 DNA POLYMERASE, Myron F. Goodman and Linda B. Bloom, University of Southern California, Department ofBiological Sciences, Los Angeles, CA 90089-1340.

Exonucleolytic proofreading during DNA synthesis increases the fidelity of sythesis by a factor of about 10 to 1000 depending on local DNA sequence and the particular enzyme. Specific interactions between DNA primer/template and DNA polymerase/exonuclease are likely to contribute to the efficiency of exonucleolytic removal and give rise to nonrandom mutations. The contributions made by DNA sequence and exonuclease properties to the efficiency of mismatch excision for wild-type and mutant T4 DNA polymerases have been examined and will be compared to the effects of nearest neighbor base stacking on nucleotide insertion. The fluorescent base analog, 2-aminopurine (2-AP), which forms a "correct" Watson-Crick type base pair with thymidine was used as a probe to measure the presteady state kinetics of nucleotide removal from both correct base pairs and mispairs. Kinetics of exonucleolytic removal by T4 DNA polymerase of 2-AP correctly paired with T and mispaired with C, G, and A were measured by monitoring the increase in fluorescence as 2-AP was excised and released as dAPMP. The efficiency of removal of dAPMP was examined as a function of 5'-nearest neighbor base stacking partner and within different local sequences which varied in their G•C content. Wild-type T4 DNA polymerase excised dAPMP from a 2-AP•T base pair at a rate of about 0.5 s^{-1}. This rate changes by about a factor of 3 as a function of 5'-nearest neighbor base stacking partner. Polymerase and exonuclease activities of well-defined T4 DNA polymerase mutants were characterized to determine how polymerase and exonuclease properties contribute to the fidelity of DNA synthesis. The dynamics of 2-AP in DNA primer/templates of different sequence were analyzed using time-resolved fluorescent techniques and anisotropy measurements. We analyzed these data for correlations between the structure and dynamics of DNA primer/templates and the polymerase and exonuclease activities of mutant and wild type T4 DNA polymerases within these sequence contexts. The measurements indicate that 2-AP exists in two distinct states within a primer/template corresponding to a "melted out" state where 2-AP is not fully base paired with greater degrees of rotational freedom and to a fully base paired "annealed" state.

K 011 MOLECULAR CHARACTERIZATION OF THE EXONUCLEASE ACTIVITY OF T4 DNA POLYMERASE, Michael K. Reddy, Steven E. Weitzel and Peter H. von Hippel, Institute of Molecular Biology, University of Oregon, Eugene, OR, 97403

The enzymatic replication of the bacteriophage T4 DNA genome is among the most accurate of all known biological processes. A significant portion of this fidelity is derived from the very potent exonuclease activity of T4 DNA polymerase. We have investigated this activity further using a three-pronged approach: first, on a functional level by monitoring exonuclease processivity; next, on a structural level by partial proteolysis of the intact polymerase; and finally, on a chemical level by probing the size of the exonuclease active site. By carrying out single-turnover experiments we have demonstrated that T4 DNA polymerase can switch intramolecularly between exonuclease and synthesis modes. That is, the pathway of proofreading is processive. We have observed that T4 DNA polymerase binds with equal affinities to base-paired and mismatched primer-template DNA substrates. This result is consistent with the notion that the refractory synthesis exhibited by T4 DNA polymerase upon encountering a mismatched primer terminus is a manifestation of kinetic barriers and not of an intrinsic inability of T4 DNA polymerase to bind to mismatched termini.

We have also monitored exonuclease processivity using an oligonucleotide of defined sequence as a substrate in different contexts. These experiments were performed by comparing the extent of processive digestion of the DNA, free in solution as a single-strand, as well as in a base-paired conformation as a primer-template structure that mimics lagging strand synthesis, and lastly in a DNA construct of the type that polymerase encounters during leading strand synthesis (*i.e.*, a miniature version of a DNA replication fork). Finally, we have probed the size of the exonuclease active site *indirectly* by monitoring exonuclease processivity on different combinations of multiply-mismatched primer-template substrates, and *directly* by the UV laser-light cross-linking of oligonucleotides to T4 DNA polymerase. Our composite results suggest that the exonuclease active site of the T4 DNA polymerase -- while bound to a primer-template substrate -- optimally accommodates two residues of single-stranded DNA at the 3'-end of the primer. The above battery of approaches has also been used with proteolytic fragments of the intact T4 DNA polymerase that retain the exonuclease activity but are incapable of DNA synthesis.

K 012 **GENETIC DISSECTION OF DNA POLYMERASE EXONUCLEASE ACTIVITY,** **L. J. Reha-Krantz, A. Atrazhev, L. Kihn, and R. L. Nonay, Department of Genetics, University of Alberta, Edmonton, Alberta, Canada.**

Genetic studies of bacteriophage T4 DNA polymerase provide the means to study the dynamics of DNA polymerase function. Since the early 1960s, more than 100 mutations have been isolated in the T4 DNA polymerase gene. Of particular interest are mutant DNA polymerases that replicate DNA with either reduced or increased accuracy because studies of these mutant DNA polymerases can provide information about function as well as insights into the mechanism that coordinates polymerase and 3'→5' exonuclease activities. Genetic and biochemical data amassed thus far are consistent with a mechanism for coordinating synthetic and proofreading activities that is based on a competition between polymerase and exonuclease active centers for binding the primer-terminus. For example, if a wrong nucleotide is incorporated, the polymerase active center is predicted to have reduced affinity for the mispaired primer-terminus while the exonuclease active center is predicted to have increased affinity which then results in proofreading. Thus, relative abilities of polymerase and exonuclease active centers to interact with the primer-terminus determines whether synthesis or proofreading activities are favored. DNA polymerase mutations that weaken or enhance interactions of polymerase or exonuclease active centers with the primer-terminus also affect the relative balance of polymerase and exonuclease activities. Recent genetic analyses and protein chemistry experiments will be presented that indicate an interdependence between polymerase and 3'→5' exonuclease activities.

Topoisomerases

K 013 STRUCTURES AND FUNCTIONS OF DNA TOPOISOMERASES, Tao Hsieh, Maxwell Lee, Donna Crenshaw, Sheryl Brown, and Alice Chen, Biochemistry Department, Duke University Medical Center, Durham, NC 27710

Two areas of research will be covered: functional domains of DNA topoisomerase II and the genetic analysis on the topoisomerase I functions in *Drosophila melanogaster*. Linker-insertion and serial deletion mutants were employed to investigate the domain structures of *Drosophila* topoisomerase II and their corresponding functions. The hydrophilic tail of this enzyme is not required for the catalytic activities, while it likely plays a critical role in the biological functions of this enzyme inside the cell. The cloning and mapping of *Drosophila* topoisomerase I have resulted in the isolation of mutants with deficiencies in the *Top1* locus. Genetic analysis indicated that *Top1* is essential for the growth and development of a multicellular organism like *Drosophila*. The possible maternal and zygotic functions of *Top1* will be discussed.

K 014 TOPOISOMERASE TERNARY CLEAVABLE COMPLEXES INDUCED BY INTERCALATORS AND GROOVE BINDERS, Allan Y. Chen, Barbara Gatto, Chiang Yu, and Leroy F. Liu, Johns Hopkins Medical School, MD 21205

Recent studies have demonstrated a dichotomy in the mode of drug-DNA interaction in trapping topoisomerase cleavable complexes; DNA intercalators have been shown to trap topoisomerase II cleavable complexes while DNA minor groove binding drugs trap topoisomerase I cleavable complexes. Models are proposed to explain this apparent dichotomy.

K 015 DNA GYRASE: STRUCTURE AND MECHANISM, Anthony Maxwell[1], Janid A. Ali[1], Andrew D. Bates[1], Paul M. Cullis[2], Alison J. Howells[1], Andrew P. Jackson[1], David P. Weiner[1,2], Gideon J. Davies[3], Eleanor J. Dodson[3], Guy Dodson[3], and Dale B. Wigley[3], Departments of [1]Biochemistry and [2]Chemistry, University of Leicester, U.K. and [3]Department of Chemistry, University of York, U.K.

DNA gyrase is the enzyme from bacteria which catalyses the introduction of negative supercoils into DNA using the free energy of ATP hydrolysis. The enzyme from *Escherichia coli* consists of two proteins, A and B, of M.W. 97 and 90kDa respectively. The active enzyme is an A_2B_2 complex. The mechanism of DNA supercoiling involves the wrapping of a segment of DNA around the A_2B_2 complex and the passage of another piece of DNA through a double-stranded break in the wrapped segment. Catalytic supercoiling requires ATP hydrolysis, but limited supercoiling can be achieved in the presence of the non-hydrolysable ATP analogue ADPNP. It is thought that the A subunit is largely involved in the DNA breakage-reunion aspects of supercoiling and that the B subunit is the site of ATP hydrolysis.

Recent studies have shown that both the A and B subunits comprise functional domains. A 43kDa N-terminal fragment of the B protein has been shown to be responsible for ATP hydrolysis. This fragment has been crystallised in the presence of ADPNP and its structure solved to 2.5Å resolution. The protein is a dimer with a molecule of ADPNP bound to the N-terminal region of each monomer, and contains a large cavity running through the dimer between the C-terminal regions. This hole could form a DNA-binding site and may be involved in the strand-passage aspect of the supercoiling reaction.

Studies of the hydrolysis of ATP by the 43kDa fragment show that the protein is only active as a dimer and a model consistent with the ATPase kinetics has been derived. This model suggests that product (ADP and Pi) release is the rate-limiting step in the gyrase supercoiling reaction. Using site-directed mutagenesis, the catalytic residue involved in the ATPase reaction has been identified. Studies of the ATPase and supercoiling reactions of gyrase (A_2B_2) suggest that the limit of supercoiling of the gyrase reaction is determined by the free energy available from ATP hydrolysis.

K 016 THE DNA CLEAVAGE/RELIGATION REACTION OF TOPOISOMERASE II, N. Osheroff, K.C. Gale, S.J. Froelich-Ammon, A.H. Corbett, and E.L. Zechiedrich, Dept. of Biochemistry, Vanderbilt Univ. School of Medicine, Nashville, TN 37232-0146.

The ability to cleave and religate DNA is central to all of the catalytic functions of topoisomerase II. These functions can be broken into two broad categories based on the nature of the DNA cleavage/religation cycle employed by the enzyme. The first, which is the interconversion of different topological forms of double-stranded DNA (i.e., supercoiled → relaxed, knotted ⇄ unknotted, or catenated ⇄ decatenated), relies on a tightly coupled intramolecular cycle. In this cycle, topoisomerase II cleaves a double-stranded DNA helix, passes an intact helix through the opening in the nucleic acid backbone, and reseals the original break. Kinetic studies indicate that during its intramolecular cycle the enzyme cleaves (and religates) double-stranded DNA by making (and rejoining) two coordinated and sequential single-stranded nicks in the nucleic acid backbone. Furthermore, it appears that the presence of the passing DNA helix is a prerequisite for the efficient cleavage of the original helix. The second catalytic function of topoisomerase II revolves around the enzyme's potential for mediating nucleic acid recombination. This function relies on an intermolecular DNA cleavage/religation cycle in which topoisomerase II breaks one molecule of DNA but rejoins it to a second nucleic acid molecule. While such intermolecular events are rare when double-stranded DNA substrates are used, they are relatively common when unusual DNA structures (double-stranded/single-stranded hybrids, hairpins, etc.) are employed. Since the intermolecular nature of recombination uncouples the DNA cleavage and religation events, both reactions are accessible for mechanistic analysis. The first substrate used for such an analysis was the (+) strand of ØX174. Topoisomerase II cleaved the circular phage DNA in a spontaneous and time dependent fashion. Following cleavage, the enzyme was covalently joined to the newly generated 5'-terminus of the linear ØX174 molecule. Moreover, topoisomerase II was able to join this cleaved DNA to the 3'-OH of a double-stranded (blunt-ended or 5'- or 3'-overhanging), single-stranded, or nicked oligonucleotide acceptor molecule via an intermolecular ligation event. Divalent magnesium was required for intermolecular ligation, but the enzyme's ATP cofactor was not. Recently, an oligonucleotide-based model system was developed to examine more precisely the interaction of topoisomerase II with unusual DNA structures. The model oligonucleotide is a 51-mer (bases 3941-3991 in the (+) strand of ØX174) that contains a 17-base palindrome. This 51-mer is an efficient cleavage substrate and is being used to define sequence/structure requirements of topoisomerase II-mediated DNA recombination. Supported by NIH Grant GM33944 and ACS Faculty Research Award FRA-370.

K 017 BIOLOGICAL ROLES OF TOPOISOMERASE IV IN *S. TYPHIMURIUM*, Molly B. Schmid, David J. Sekula, Amy L. Springer, Princeton University, Princeton, NJ 08544.

Bacteria possess four topoisomerases - two type 1 enzymes (topoisomerases I and III) and two type 2 enzymes (topoisomerase II (DNA gyrase) and topoisomerase IV). Both of the type 2 enzymes are essential for the growth of *E. coli* or *S. typhimurium*. Topoisomerase IV is composed of two subunits, encoded by the genes *parC* and *parE*, which show strong similarity in both DNA and amino acid sequence to the genes encoding gyrase, *gyrA* and *gyrB*. We have a large number of conditional lethal mutations in the *parC* and *parE* genes, and in the linked gene, *parF*. The conditional lethal mutants were noticed because they cannot segregate daughter nucleoids at the non-permissive temperature. These mutants allow us to assess the *in vivo* consequences of inactivating topoisomerase IV, and compare these phenotypes to those caused by inactivating DNA gyrase. In this way, we hope to describe the biological division of labor between the related enzymes, DNA gyrase and topoisomerase IV.

Conditional mutations in *parC* and *parE* do not change the superhelicity of the reporter plasmid pBR322, unlike alleles of *gyrA*. These mutations cause small differences in the transcription of certain mudlac fusions, but these fusions are not those that are strongly altered by inhibiting DNA gyrase. Mutations in *parC* and *parE* do not alter the spontaneous duplication frequency, a measure of pseudo-legitimate recombination. In addition, *parC* and *parE* mutations do not block DNA replication after shift to non-permissive conditions. Thus, topoisomerase IV plays no obvious role in initiation or elongation of DNA replication, homologous or non-homologous recombination, transcription or maintenance of global superhelicity. These results suggest that topoisomerase IV is a highly specialized topoisomerase, specific for the task of aiding nucleoid partitioning. Presumably, topoisomerase IV allows partitioning by decatenating the daughter nucleoids, which then allows faithful segregation. This presumption has been substatiated by the finding that a portion of the cell's pBR322 is catenated in *parC* and *parE* mutants after a shift to non-permissive conditions[1].

Some topological tasks may be accomplished by more than one topoisomerase. We are assessing functional redundancy in the topoisomerases by construction of *gyrA parCE* and *topA parCE* double mutants.

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Restriction Nucleases

K 018 EVOLUTION OF DNA SEQUENCE SPECIFICITY IN TYPE 1C RESTRICTION AND MODIFICATION SYSTEMS, Clive Price[1], Marcel Gubler[1], Diego Braguglia[1], Jennifer Meister[1], Chiara Tyndall[1], Andrzej Piekarowicz[2] and Thomas A Bickle[1], [1]Department of Microbiology, Biozentrum, Basel University, Switzerland, [2]Institute of Microbiology, Warsaw University, Poland.

The type I restriction-modification (R-M) systems of the *Enterbacteriaceae* form three genetically complementing families, one of which is the type IC family. All type I R-M systems recognize DNA sequences that are asymmetric and split into two by a spacer that can have any sequence but which, for a given R-M system, has a fixed length. The specific DNA-binding components of both restriction asnd modification enzymes are the products of the *hsdS* genes, which form complexes with the *hsdM* and *hsdR* gene products. Within a family of type I systems the *hsdS* genes have a characteristic structure with two variable regions, each coding a protein domain (called Target Recognition Domains, TRD) that recognizes one half of the recognition sequence, and two (three in one case) conserved regions that are thought to code regions of the protein that are important for protein-protein interactions.The type IA and IC R-M systems are so far the only DNA binding proteins that have been shown to alter their binding specificity by natural means. Type IA enzymes can reassort their TRDs by recombination within a conserved region of the *hsdS* gene, leading to the production of enzymes that have new, hybrid recognition sequences. Type IC systems (and, presumably, type IB systems, although it has not yet been demonstrated) can also change their specificity in this way. In addition type IC systems can change the length of the central conserved region of the *hsdS* gene by unequal crossing over at a 12 bp long repeated sequence. This leads to enzymes that recognize the same specific sequence but with different lengths of non-specific spacer. We have discovered yet a third way in which type IC enzymes can change their specificity. We have recovered a mutant that has a transposon inserted in the middle of the *hsdS* gene and which nevertheless produces an active R-M system with altered sequence specificity. The structure of the mutant enzyme and the sequence that it recognizes will be presented. The sequence is the only known type I recognition sequence that is palindromic. Finally, we will present data on a type IC R-M system of as yet unknown DNA specificity that appears to be on a transposable element. This system is particularly interesting because it is closely integrated, both genetically and physically, with an RNA restriction system that is specific for cells infected by T even phages.

K 019 DNA RECOGNITION BY *Eco*RV, Stephen E. Halford, Geoffrey S. Baldwin, and I. Barry Vipond, University of Bristol, Department of Biochemistry, Centre for Molecular Recognition, Bristol BS8 1TD, U.K.

In the presence of magnesium ions, the *Eco*RV restriction endonuclease cleaves DNA specifically at its recognition sequence, GATATC. Sequences that differ from the recognition site by one base pair are cleaved at least a million times more slowly. Yet, in binding to DNA in the absence of magnesium ions, the *Eco*RV restriction enzyme shows no sequence specificity. The protein binds all sequences with equal affinity, and it can transfer readily from one site to another along the DNA molecule without dissociating from the DNA. However, the DNA cleavage activity that is observed at any particular sequence is a function of the fractional saturation of the relevant enzyme-DNA complex with magnesium ions. The observed difference in cleavage rates is due to the fact that the *Eco*RV enzyme has a high affinity for magnesium when it is located at its recognition site on DNA, but it has a low affinity for magnesium when it is located on any other DNA sequence. Once it has bound the metal ion, the intrinsic activity of the *Eco*RV enzyme at noncognate sites is similar to that at the recognition site.

This mechanism can be correlated to the crystal structures of the *Eco*RV endonuclease that have been determined by F. K. Winkler (Basel, Switzerland). Three structures were solved: the free enzyme in the absence of DNA; the specific complex at its recognition sequence; a nonspecific complex at a different DNA sequence. The specific complex displays a large number of sequence-specific interactions between the protein and the DNA. These are missing in the nonspecific complex. But the additional interactions that are seen in the specific complex contribute nothing to the net free energy change for DNA binding. Instead, all of the energy from the specific interactions is used to distort the DNA, and in other conformational changes: the structure of the specific DNA bound to *Eco*RV is extensively distorted while the nonspecific DNA is close to B-form. The distortion results in only the specific complex being able to bind magnesium ions and to catalyse the reaction. The communication between DNA recognition and catalysis thus seems to be mediated by the structure of the DNA itself. This model is currently being tested by mutational analyses of *Eco*RV and by using fluorescence methods to monitor the conformational changes in the DNA and in the protein.

K 020 TYPE I RESTRICTION ENZYMES OF ENTERIC BACTERIA, N E Murray[1], V A Barcus[1], A J B Campbell[1], D T F Dryden[1], J E Kelleher[1], L M Powell[1], D Willcock[1] and P M Sharp[2], [1]Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland, [2]Department of Genetics, Trinity College, Dublin 2, Ireland.

The multifunctional type I restriction and modification (R/M) enzymes are encoded by three genes (*hsdR*, *hsdM* and *hsdS*). These enzymes recognize asymmetric, bipartite target sequences and their alternative responses of either restriction or modification are determined by the methylation state of the target sequence. Three discrete families of type I R/M systems have been described (A, B and C), and we now present evidence for a fourth. This new system and the enzymes of families IA and IB are all encoded by allelic genes. Members of a family are highly conserved, but differ in their specificity (S) subunits; each of two recognition domains of the S subunit confers specificity for one of the two components of the target sequence. Members of different families are dissimilar in all of their three subunits. Even when encoded by allelic genes, amino acid identity may be only ~20%. The diversity of type I R/M systems will be reviewed in relation to their natural distribution and their evolutionary relationships. Horizontal transfer of *hsd* genes between species and frequency-dependent selection for alternative specificities are invoked to explain the high levels of variability.

Comparative analyses of polypeptides identify conserved sequences within the M polypeptides common to methyltransferases (mtases) in general and adenine mtases in particular, and within the R polypeptides features in common with ATP-dependent helicases. We have made mutations in some of these conserved regions and have isolated other mutants on the basis of a change in response to the methylation state of the target sequence. The latter mutations mimic the effect of a phage encoded polypeptide (Ral) that can enhance modification and ameliorate restriction. The mtase component of *Eco*K, the type I R/M system found in *E. coli* K-12, has been purified and characterized, particularly in terms of binding to target sequences and to the cofactor S-adenosyl methionine. The properties of the wild-type mtase and some mutant derivatives will be reported.

K 021 MCRBC, A NOVEL MULTISUBUNIT GTP-DEPENDENT RESTRICTION ENDONUCLEASE. Elisabeth A. Raleigh, Deborah Dila, Ellen Sutherland, Julia Kelleher, Laurie Moran, Barton Slatko and Pam Briggs. New England Biolabs, 32 Tozer Rd, Beverly, Ma. 01915.

The McrBC system is one of four restriction systems used by *E. coli* K-12 to monitor the origin of invading DNA and determine its fate. Like McrA and Mrr, the McrBC system is specific for modified DNA. 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. A few modified targets are sensitive to restriction mediated by *mcrB* in the absence of *mcrC*.

Three proteins are expressed from the two genes. Only two of the three are required for *in vitro* activity. The *in vitro* cleavage activity reflects the *in vivo* properties of the system in its requirement for a modified substrate and in the spectrum of site-specific modifications that are sensitive to cleavage. GTP is required for cleavage. Non-hydrolysable analogues of GTP inhibit the reaction, as does ATP. Our current model is that cleavage requires the sequence $R^mC(N_{40-80})R^mC$, with multiple cleavage positions on both strands distributed within the spacer region.

The roles played by the two proteins are under investigation genetically. Twelve *mcrB* mutants with dominant phenotypes have been isolated. These fall into three phenotypic classes. All are defective in McrC-dependent restriction; they differ in their ability *in vivo* to inhibit restriction by wild type genes *in trans* and in their ability to carry out McrC-independent restriction. Sequence analysis reveals that each class corresponds to a particular portion of the protein, one of which is the GTP-binding site motif identified in the polypeptide sequence of McrB.

Fungal Nucleases/Mitochondrial Nucleases

K 022 THE CLONING AND SEQUENCING OF THE SACCHAROMYCES AND NEUROSPORA ENDO-EXONUCLEASE GENE. Terry Y.-K. Chow, Dept. Nuclear Medicine and Radiobiology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4.

Recent findings with endo-exonucleases from various organisms indicates that the protein plays an important role in DNA recombination/repair. The enzymological characteristics of the endo-exonucleases isolated so far are similar.[1,2,3,4] The genes that code for the endo-exonucleases from Saccharomyces and Neurospora have recently been cloned and sequenced. The yeast endo-exonuclease protein sequence has identified it as a chimeric protein consisting of a N-terminal Rho oncogene motif and a C-terminal nuclease domain[4]. Protein sequence comparisons of the endo-exonucleases shows that they shared regions of similarity between them and with the E. coli recC protein. Overexpression of the yeast endo-exonuclease leads to increased survival of the yeast cells following exposure to MMS, ionizing radiation, and to the anticancer drugs Melphalan and BCNU. Overexpression of the Neurospora endo-exonuclease in the E. coli XL-1 strain results in an increase survival following UV radiation.

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K 023 ENDO-EXONUCLEASES FROM *E.coli* TO MAN: PROPERTIES, FUNCTIONS, PROCESSING AND HOMOLOGIES. Murray J. Fraser, Children's Leukaemia and Cancer Research Centre, Randwick, N.S.W., Australia, 2031.

Endo-exonucleases (EEs) have been purified and characterized from *Neurospora crassa* (1,2), *Aspergillus nidulans* (3), *Saccharomyces cerevisiae* (4,5), monkey CV-1 cells (6) and recently in this laboratory from human leukaemic CCRF-CEM cells (unpublished). EEs have Mg^{++}(Mn^{++})-dependent single-strand (ss) specific endonuclease activity with DNA and RNA and processive exonuclease activity with ss-DNA and double-strand (ds) DNA which release oligonucleotides with 5'-P and 3'-OH termini. The *N. crassa* and *A. nidulans* EEs act on linear ds-DNA in the presence of a low concentration of Mg^{++} to yield a high level of ss-breaks and a low level of site-specific ds-breaks (2,3). The enzymological properties of the EEs were so similar to those of the major recombination nuclease of *E. coli*, the *recBCD* nuclease (7,8), that a test their possible relatedness was prompted using antibody raised to the purified *N. crassa* EE. Such an antibody has now been shown to cross-react with the EEs derived from *A. nidulans*, *S. cerevisiae*, monkey CV-1 cells and human CEM cells (unpublished) but not with other available nucleases (3,6,9). The antibody also reacted specifically with the *E. coli recC* protein and a low level homology was detected between the *S. cerevisiae* mitochondrial EE and the *recC* polypeptide (10). These findings implicate EEs in recombination and recombinational (ds-break) DNA repair. The rad52 mutant of *S. cerevisiae* and the *uvs-3* mutant of *N. crassa* are repair deficient and show poor expression of the nuclear EEs (4,11).

Antibody to the *N. crassa* EE completely inhibited the ss-DNase activity in fresh extracts of mitochondria of *N. crassa* (9) and *S. cerevisiae* (5) and a deletion in the NUC1 gene, the structural gene for the mitochondrial EE of the yeast, was shown by Zassenhaus *et al.* (12) to eliminate all detectable mitochondrial DNase activity. The antibody also cross-reacted with an endonuclease in mitochondria of mouse plasmacytoma cells (13), possibly a proteolytic derivative of an EE. Several active polypeptides were detected by activity gel analysis in fresh extracts of *N. crassa* mitchondria, the largest of which was 66 kDa in size and disappeared on aging the extract, an indication of limited proteolysis.

Activity gel analysis has also revealed the presence of three active polypeptides in extracts of *N. crassa* nuclei (11), one of the 76 kDa tightly bound to the nuclear matrix and two of 43 and 37 kDa released from chromatin immunoprecipitated or inhibited by antibody to the purified 31 kDa *N. crassa* EE. In addition, an inactive but trypsin-activatable EE of 93 kDa has also been detected in *N. crassa*, a putative precursor of the active forms (9). Precursor EE has now also been detected in nuclei of human leukaemic CEM cells as have various active polypeptides, including one of about 76 kDa. A major fraction of the EE in CEM cell nuclei was lost in response to cytotoxic doses of dexamethasone or etoposide (VP-16) which trigger the chromatin DNA fragmentation associated with apoptosis. However, which active forms are involved in the DNA fragmentation is not yet known. This role of EE is formally equivalent to that of the *recBCD* nuclease in the "reckless" genomic DNA degradation in heavily damaged *E. coli*.

Although it was expected that some strong homologies might be observed among the EEs, surprisingly, low overall homologies have been found on aligning the amino acid sequences predicted from the cloned genes. A scheme of possible relatedness will be presented.

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K 024 THE MITOCHONDRIAL ENDONUCLEASE OF BOVINE-HEART AND ITS POTENTIAL ROLE IN THE METABOLISM OF THE MITOCHONDRIAL GENOME, Robert Low, Kathryn Houmiel, Mariana Gerschenson, and William Parks, Department of Pathology and Program in Molecular Biology, University of Colorado Health Sciences Center, Denver, Colorado 80262.

Mitochondria of mammalian tissues contain a potent, nuclearly-encoded endonuclease activity which becomes soluble once the mitochondrial membranes are disrupted using detergent. This endonuclease has been previously purified about 10,000 fold to near homogeneity from the mitochondria of bovine heart.[1] The endonuclease requires Mg^{+2} or Mn^{+2} for activity and is stimulated 10-20 fold by major membrane phospholipids phosphatidylcholine and phosphatidylethanolamine *in vitro*,[2] a property characteristic of enzyme activities that are membrane associated. Although the enzyme can extensively degrade both duplex and single-stranded DNA into small, acid-soluble fragments *in vitro* at low ionic strength, at moderate ionic strength the endonuclease does attack nucleotide sequences in a highly nonrandom fashion. In fact the purified enzyme shows a striking preference to nick within one site in the nucleotide sequence of mtDNA, an unusual sequence of 12 consecutive guanine and complementary cytosine residues near the origin of mtDNA replication.[3] This preference of the mt endonuclease to nick within G tracts strongly resembles the sequence specificity of a known nuclear endonuclease, endonuclease G.[4] The purified mt endo and endo G, partially purified from calf-thymus nuclei, are being compared to assess whether the two activities are identical. The mt endonuclease, however, is not a nuclear contaminant. The enzyme activity remains with the mitochondria following three successive sedimentation runs of the organelles through linear sucrose gradients. Furthermore, the endonuclease activity can be identified in a stable complex of mtDNA and mtDNA binding proteins which is gently isolated from disrupted mitochondria and that is substantially free of contaminants of nuclear DNA. Although the function of this mt endonuclease remains unknown, the level of the endonuclease activity among the mitochondria of different tissues varies nearly 200-fold and in a manner which predicts that the enzyme may serve *in vivo* to facilitate the removal of oxidative damage in mtDNA incurred from oxygen radicals emerging from the respiratory chain.[5] Among the major organs in the adult rat, the specific activity of the mt endonuclease (per mg of mt protein or per mtDNA circle) is highest in the heart and lowest in the liver and spleen. The identification of factors that regulate the activity and specificity of the endonuclease *in vivo* will be important to learn whether the enzyme actually participates in a repair pathway for DNA damage as the variations in activity would suggest.

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K 025 A MITOCHONDRIAL MULTI-SITE SPECIFIC ENDONUCLEASE, ENDO.*SceI*, OF *SACCHAROMYCES CEREVISIAE*, Takehiko Shibata, Nobuhiro Morishima, Kei-ichi Nakagawa and Katsumi Kawasaki, The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-01, Japan

There are two classes of sequence specific endonucleases active on double-stranded DNA in eukaryotic cells. Endonucleases in one class exhibit very strict site-specificity which allows to cut DNA at only one (or a few) site among whole genome. These endonucleases have been shown to initiate site-specific genetic recombination *in vivo* including "intron homing." Endonucleases in the other class introduces numbers of double-stranded breaks in various double-stranded DNA like bacterial restriction endonucleases. Until now, three endonucleases were biochemically or genetically identified to belong to the latter class; *i.e.* Endo.*SceI* and Endo.*SceII* of *Saccharomyces cerevisiae*, and Endo.*SuvI* from *Saccharomyces uvarum* (*S. carlsbergensis*) (see ref. 1). Active form of Endo.*SceI* is heterodimer of 75kDa- and 50kDa-subunits and localized in mitochondria (2). Endo.*SceI* and Endo.*SuvI* contain the 50kDa-subunit, and each of them is a product of allele of a mitochondrial gene (*ENS2*) (1). The difference in sequence specificity between Endo.*SceI* and Endo.*SuvI* is caused by the substitution of two amino acids (1). The cutting sites of Endo.*SceI* have complex features, and the cutting creates staggered ends, 4 bases protruding at 3' ends (3). These are common features of all sequence-specific endonucleases from the yeast. Another common feature is the presence of two clusters of partially conserved 12 amino acid-sequences. We looked at events around a genetic marker (Oli^r) which was located at *ca.* 200 bps from a cutting site for Endo.*SceI* in a mitochondrial gene, *oli2*. The site was shown to be partially cleaved *in vivo* in mitotic cells having active Endo.*SceI*. We found mating-dependent introduction of a double-stranded break at the cutting site in *oli2* gene in a haploid lacking Endo.*SceI* upon the mating with a partner having active Endo.*SceI*. At the same time, we observed polarized gene conversion at *oli2* locus (4). The disparity in conversion depends on the presence of active Endo.*SceI* in a parent and the difference in the sensitivity of the cutting sites to Endo.*SceI* between parental strains. Endo.*SceI* cuts mitochondrial DNA at more than 30 sites *in vitro*, and we detected *in vivo* cutting at the cutting sites for Endo.*SceI* other than that in *oli2* in haploid or diploid cells having active Endo.*SceI*. These results suggest that the recombination induced by Endo.*SceI* is probably homologous one rather than site-specific recombination. The subunit structure and the mechanism to protect genomic DNA from complete cleavage by the endonuclease are different between Endo.*SceI* and endonucleases of the other class. The 75kDa-subunit of Endo.*SceI* is a HSP70 which is involved in the import of protein into mitochondria (5). This suggests the regulatory role of the larger subunit of Endo.*SceI* *in vivo*. Although Endo.*SceI* is dispensable, but we found another site-specific endonuclease from *S. cerevisiae* lacking a gene for Endo.*SceI* (Ohta, K., Nicolas, A., Keszenman-Pereyra, D. and T. S.). Thus, mitochondria have multiple species of site-specific endonucleases, and that the endonucleases or the recombination induced by them play a basic role in this organella.

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K 026 THE THREE-DIMENSIONAL STRUCTURES OF PENICILLIUM P1 AND ASPERGILLUS S1 NUCLEASE, Dietrich Suck[1], Roberto Dominguez[1], Armin Lahm[2], and Anne Volbeda[3], [1]EMBL, Heidelberg, Germany, [2]IRBM, Rome, Italy, [3]LIP, Grenoble, France.

The zinc dependent nucleases P1 from Penicillium citrinum and S1 from Aspergillus oryzae show a strong preference for single-stranded substrates and cleave DNA as well as RNA in an essentially unspecific reaction. The 3D-structure of P1, a glycoprotein of 270 amino acid residues, has been determined at 2.8Å resolution from tetragonal (1) and more recently at 2.2Å from orthorhombic crystals. The predominantly α-helical protein has a fold very similar to that of phospholipase C from Bacillus cereus despite very limited sequence homology, which together with the conserved zinc ion cluster strongly suggests an evolutionary relationship of the enzymes. A cluster of three zinc ions is located at the bottom of the active site cleft, inaccessible to double-stranded DNA or RNA, readily explaining the preference for single-stranded substrates. Two nucleotide binding sites approximately 20Å apart have been identified by soaking crystals with thiophosphorylated substrate analogues. At both sites an adenine base is bound in a hydrophobic pocket, formed by a Phe and a Val at the active site and two Tyr residues at the other site. The binding of the nucleotide suggests a mechanism involving nucleophilic attack by a zinc-activated water molecule. The 3D-structure of S1 nuclease was derived by comparative modelling based on the refined P1 structure. As expected from the close sequence homology - 50% of the residues are identical with only a one-residue deletion in S1 - the two proteins have nearly identical backbone conformations. An interesting difference between the two enzymes is the absence in S1 of the second nucleotide binding site found in P1 crystals, which may indicate different binding modes for longer substrates. Electrostatic potential calculations provide a possible explanation for the pH and ionic strength dependence of the activities of the two enzymes.

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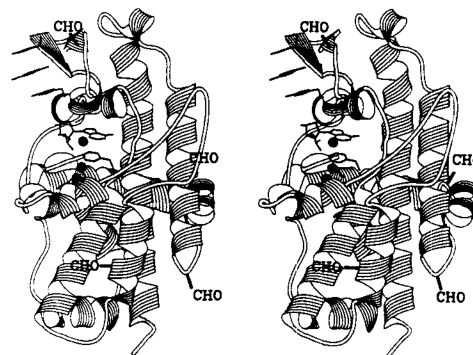

Stereo ribbon plot of P1 nuclease

Structure/Function Relationships in Nuclease Mechanisms

K 027 CLEAVAGE OF PHOSPHODIESTER BONDS: PRINCIPLES AND ENZYMATIC EXAMPLES, John A. Gerlt, Stephen P. Hale, Leslie B. Poole, Medha Bhagwat, Deborah Loveys, Aparna Kolhekar, and Abhijit Mazumder, Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742

We are attempting to understand the mechanistic principles by which the cleavage of phosphodiester bonds in DNA catalyzed by nucleases overcome an extremely large rate retardation caused by the monoanionic charge of the phosphodiester linkage. The rapid enzymatic cleavage of phosphodiester bonds may occur by either of two mechanisms: hydrolysis accompanied by P-O bond cleavage, and β-elimination accompanied by C-O bond cleavage. The reaction catalyzed by Staphylococcal nuclease is an example of the hydrolytic mechanism; the reactions catalyzed by the β-lyase activity of various N-glycosylases/endonucleases in DNA repair are examples of the β-elimination mechanism.

High resolution x-ray structures are available for Staphylococcal nuclease (SNase) so that the catalytic residues likely to be involved in the hydrolysis reaction can be identified. SNase requires Ca^{2+} for activity, and Glu 43 has been proposed to be an essential general basic catalyst. Detailed kinetic studies of wild type SNase and of sequence variants of a mutant in which an Ω-loop adjacent to the active site (residues 44 - 49) has been deleted lead to the following conclusions about wild type SNase: 1) the rate determining step of the reaction is dependent upon pH, with a chemical process being rate determining at neutral pH and a physical process being rate determining at high pH; and 2) the hydrolysis reaction appears to be specific- (i.e., OH^-), not general-, base catalyzed. In contrast, the rate limiting steps in the reactions catalyzed by the deletion mutants and various site-directed mutants of SNase are chemical processes, with the reactions again appearing to be specific-base catalyzed. The large rate acceleration characteristic of SNase ($\geq 10^{16}$) can, in fact, be quantitatively rationalized by electrophilic catalysis in which an unstable, dianionic phosphorane intermediate is stabilized by coordination/hydrogen bonding to the metal ion and amino acid functional groups.

The β-elimination reactions catalyzed by N-glycosylases involve abstraction of a very weakly acidic proton adjacent to the carbonyl group of an aldehyde (α-proton). We propose that the β-elimination reactions involve stepwise mechanisms in which the first step is concerted general base-catalyzed abstraction of the α-proton and general acid-catalyzed protonation of the aldehyde carbonyl group to form an enol intermediate; the second step is vinylogous elimination of the 3'-phosphate group. Formation of an enol rather than an enolate (carbanion) intermediate enhances the acidity of the α-proton and allows rapid abstraction of the α-proton to be rationalized. Alternatively, the aldehyde may form an imine with an active site residue, thereby enhancing the acidity of the α-proton.

We conclude that electrophilic catalysis is quantitatively sufficient to explain both types of enzyme-catalyzed cleavage of the phosphodiester bonds in DNA. (Supported by NIH GM-34572 and GM-34574.)

K 028 MECHANISM OF ENDONUCLEASE V - MEDIATED INCISION AT PYRIMIDINE DIMER SITES - IDENTIFICATION OF THE ACTIVE SITE RESIDUE., R. Stephen Lloyd[1], Robert D. Schrock, III[2], Mary Lou Augustine[1], and Marion L. Dodson[1], [1]John Sealy Center for Molecular Science, The University of Texas Medical Branch, Galveston, TX 77550, [2]Department of Biochemistry and Biophysics, University of California, School of Medicine, San Francisco, CA 94143-0448.

Bacteriophage T4 endonuclease V initiates the DNA repair process at sites of UV-induced cyclobutane pyrimidine dimers. This initiation of repair involves four sequential activities of this 16 kdal protein: a salt-dependent linear diffusion on nontarget DNA, a pyrimidine dimer-specific binding, a pyrimidine dimer-specific DNA glycosylase activity and an abasic (AP) lyase activity. Although the two catalytic activities have been shown to be differentially affected by either mutations within the enzyme or alterations in reaction parameters (salt, pH, temperature, length of DNA substrate), recent studies have demonstrated that the primary amine of the processed N-terminus of endonuclease V is responsible for both activities (1,2).
Endonuclease V was chemically modified by reductive methylation of primary amino groups. Using [^{14}C] formaldehyde in the presence of sodium cyanoborohydride, it was discovered that the first methylation event per enzyme molecule inactivates both the glycosylase and AP lyase activities. Pyrimidine dimer specific binding was not eradicated with these low levels of modification but could be eliminated with higher levels of methylation. Endonuclease V that had been modified with <2 CH_3 per molecule was digested with V8 protease and the resulting peptides separated by HPLC. The radiolabel was found exclusively on the N-terminal peptide. Neither this peptide nor the intact CH_3-modified enzyme could be sequenced by Edman degradation.

Conclusive evidence that the N-terminus was the preferentially labeled primary amino group within the molecule was obtained by [^{13}C] NMR. The resonances for the α and ε [^{13}C] methyl amino groups were assigned by pH titration of these groups using two differentially modified preparations of endonuclease V. A chemical mechanism is proposed in which the αNH_2 moiety is located within a fixed position within the enzyme and acts as a nucleophile for attack at deoxyribose C-1'. Cleavage of the glycosylic bond may be facilitated by the protonation of the 5' pyrimidine of the dimer. A covalent protonated Schiff base is postulated as an intermediate that can either proceed to cleave the phosphodiester backbone by way of β-elimination yielding an α, β unsaturated aldehyde or dissociate to create an apyrimidinic site. Evidence to support the existence of a protonated Schiff intermediate was obtained by trapping a covalent protein-DNA intermediate with sodium borohydride or sodium cyanide. Additional studies on the role of the αNH_2 group in enzyme catalysis involved site-directed mutagenesis of the N-terminal region. Although amino acid substitutions were generally well tolerated at the N-terminus, either the deletion or addition of amino acid residues resulted in dramatic losses in both catalytic activities. Supported by ES04091.

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Special Workshop: Nomenclature

K 029 ALL DNA NICKING ENZYMES ARE NOT NUCLEASES, Walter G. Verly, Liège University, Belgium.

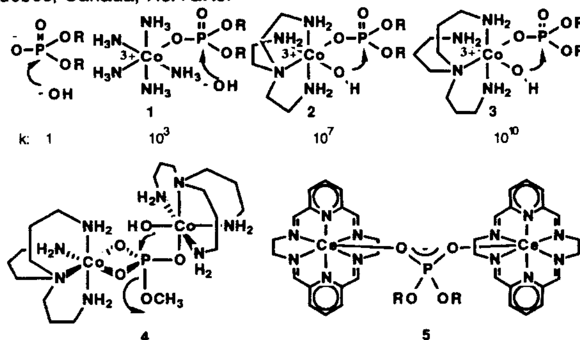
The mechanism of strand nicking 3' to an AP (apurinic/apyrimidinic) site was found not to be a hydrolysis, but a β-elimination leaving a 3'-terminal unsaturated sugar and a 5'-phosphate The enzymes catalyzing such a reaction are not hydrolases, but lyases. Nucleases are hydrolases. Thus the enzymes nicking 3' to AP sites cannot be called AP endonucleases; we proposed to name them AP lyases (EC 4.2.99.18).

Many AP lyases are also DNA-glycosylases. Erroneous appelations like M.luteus UV endonuclease, T4 UV endonuclease, and E.coli endonuclease III should be cancelled. We proposed to name these enzymes rather after their DNA-glycosylase specificity and to speak of the AP lyase activity of these DNA-glycosylases.

Synthetic Nucleases

K 030 ARTIFICIAL HYDROLYTIC METALLO-NUCLEASES, Mariusz Banaszczyk, Jung Hee Kim, Bryan Takasaki, Vrej Jubian, Jik Chin, Department of Chemistry, McGill University, Montreal, Quebec, Canada, H3A 2K6.

Nature has developed many hydrolytic metalloenzymes. They have evolved to hydrolyze some of the most important molecules of life including proteins, membranes and DNA. Most DNases as well as ribozymes are activated by metal ions. In the field of artificial hydrolytic metalloenzymes, the ultimate challenge is to hydrolyze the phosphate diester backbone of DNA. In neutral water phosphate diesters are by far the most stable compared to esters, nitriles, amides, phosphate monoesters and phophate triesters. The hydroxide rate for even the highly activated phosphate diester, bis-(p-nitrophenyl)phosphate (BNPP), is ten times slower than the hyroxide rate for acetamide hydrolysis. In this sense, it is not surprising that nature chose the phosphate diester linkage for preserving the genetic material. The half-life for hydrolytic cleavage of the phosphate diester bonds in DNA has been estimated to be in the order of 200 million years (pH 7, 25 °C). Many DNases hydrolyze DNA within seconds.
In order to hydrolyze the phosphate diester backbone of DNA within hours at neutral pH and ambient temperature, a 10^{12} fold rate-acceleration would be required. Single Lewis acid activation (1) of phosphate diesters with metal complexes gives up to 10^3 fold rate-acceleration for the hydrolysis reaction. Joint Lewis acid activation and intramolecular metal-hydroxide activation (2 and 3) gives up to 10^{10} fold rate-acceleration for hydrolyzing both activated and unactivated phosphate diesters.
Interestingly the efficiency of the intramolecular metal-hydroxide activation increases dramatically with decrease in the value of the O-Co-O bond angle. This angle decreases in size with increase in the N-Co-N bond angle directly opposite the O-Co-O bond angle. Consequently, 3 is about 300 times more reactive than 2 for hydrolyzing phosphate diesters. One way to obtain greater than 10^{10} fold rate-acceleration for hydrolyzing phosphate diesters would be to go from bifunctional activation (joint Lewis acid and metal-hydroxide activation) to multifuctional activation. Tetrafunctional activation (triple Lewis acid and metal-hydroxide activation (4)) has been achieved in rapidly hydrolyzing phosphate monoesters at neutral pH and ambient temperature.



In contrast to DNA hydrolysis, RNA hydrolysis does not require intramolecular metal-hydroxide activation. The 2'-OH group in RNA which acts as a highly efficient intramolecular nucleophile renders the metal-hydroxide ineffective. Hence for RNA hydrolysis, double Lewis acid activation is preferred over joint Lewis acid and metal-hydroxide activation. For DNA hydrolysis, greatest rate-acceleration is obtained by combining double Lewis acid activation and intramolecular metal-hydroxide activation.

K 031 HIGH-SPECIFICITY SEMI-SYNTHETIC NUCLEASES: DESIGN, CONSTRUCTION, AND APPLICATION TO LARGE GENOMES
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Synthetic or semi-synthetic site-specific nucleases can be constructed by covalently attaching a nucleolytic chelator-metal complex to a sequence-specific DNA binding molecule (*J. Amer. Chem. Soc.*, 104:6861, 1982; *Science* 238:645, 1987; *Science* 238:1129, 1987; *Science* 237:1197, 1987; *Proc. Natl. Acad. Sci. USA* 87:2882, 1990). However, previously reported synthetic or semi-synthetic nucleases have exhibited significant non-specific DNA cleavage activity (i.e., they have exhibited significant DNA cleavage activity at DNA sites other than the specific DNA recognition site).

In this report, we describe an approach to construct novel semi-synthetic nucleases that are able to cleave DNA when bound at the specific DNA recognition site, but that are not able to cleave DNA when bound at non-specific DNA sites. The approach is to incorporate a nucleolytic chelator-metal complex at an amino acid of a sequence-specific DNA binding protein that--due to differential DNA bending--is close to DNA in the specific protein-DNA complex, but is not close to DNA in the non-specific protein-DNA complex.

CAP sharply bends DNA in the specific CAP-DNA complex (*Nature* 308: 509, 1984; *EMBO J.* 3:2873, 1984; *Science* 253:1001, 1991). CAP does not sharply bend DNA in the non-specific CAP-DNA complex (*Biochem.* 18:255, 1979; *Nucleic Acids Res.* 7:1699, 1979). As a result, several amino acids of CAP are close to DNA in the specific CAP-DNA complex, but are not close to DNA in the non-specific CAP-DNA complex: i.e, amino acids 24 to 26 and amino acids 89 to 91. We have constructed a semi-synthetic nuclease by incorporation of 1,10-phenanthroline:copper at an amino acid of CAP that is close to DNA in the specific CAP-DNA complex, but is not close to DNA in the non-specific CAP-DNA complex: i.e., at amino acid 26 of CAP. The semi-synthetic nuclease is: [(acetyl-glycyl-5-amino-1,10-phenanthroline:copper)-Cys26;Ser178]CAP ("[(OP6)26]CAP").

We have assayed the DNA-cleavage activity of [(OP6)26]CAP using 7.1 kb, 49 kb, and 4,700 kb DNA substrates having the DNA recognition site for CAP (i.e., recombinant bacteriophage M13 genomic DNA, recombinant bacteriophage lambda genomic DNA, and *Escherichia coli* genomic DNA). [(OP6)26]CAP cleaves each of the DNA substrates within the DNA recognition site(s) for CAP. DNA cleavage is highly efficient and highly specific.

[(OP6)26]CAP has applications in chromosome mapping, chromosome sequencing, and chromosome cloning. We are applying [(OP6)26]CAP to analysis of several prokaryotic and eukaryotic genomes.

K 032 SITE-SPECIFIC NUCLEASES: DESIGN AND APPLICATIONS, David S. Sigman, Department of Biological Chemistry, School of Medicine, Department of Chemistry and Biochemistry, and Molecular Biology Institute, U.C.L.A., Los Angeles, CA, 90024-1318

The chemical nuclease activity of 1,10-phenanthroline-copper can be targeted linking it to ligands such as nucleic acids and proteins (1). Sequence-specific double-stranded scission of duplex DNA scission can be accomplished with OP-derivatized RNA within R-loops. R-loop directed scission is the first method for DNA scission applicable to *any* sequence. One application is the determination of the distance between two marker DNA sequences within a target.

The lambda phage cro protein and the *E. coli* trp repressor have been converted into site-specific scission reagents by a combination of site-directed mutagenesis and chemical modification. To prepare these chimeric proteins, a sequence position was identified which was accessible to the minor groove but did not contribute to the binding affinity or specificity. A cysteine residue is introduced by site-directed mutagenesis and then alkylated by 5-iodoacetamido-1,10-phenanthroline. The minor groove reactivity of 1,10-phenanthroline-copper is useful because many DNA binding proteins require major groove contacts for recognition (2). A chemical nuclease in the minor groove will not interfere with binding.

The cro protein was derivatized at the C-terminus and efficiently made single-stranded nicks in double stranded DNA but was less effective in making double-stranded breaks (3). In contrast, the *E. coli* trp repressor, which was mutagenized at cysteine-49, gave a high yield (50%) of double-stranded breaks after a 20 minute reaction. Since sequence position 49 is close to the protein's C_2 dyad axis and adjacent to the minor groove, *trp* repressor E49C-OP reacts with the operator DNA near the binding site of this symmetry locus of the protein. The patterns of scission of the *trp R, aroH* and *trpEDCBA* operators a) confirm the orientation of the repressor to the operator predicted from the X-ray study of a cocrystal (4); and b) supports the model for tandem binding of the repressor to the *trp R, aroH* and *trpEDCBA* operators based on DNase I footprinting and methylation interference (5). In addition to providing a novel approach to studying the interactions of DNA binding proteins, 1,10-phenanthroline-derivatized proteins such as *trp* repressor E49C-OP may be useful as rare cutters in the analysis of high molecular weight DNAs especially if their binding specificities can be altered.

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Ribonucleases

K 033 MULTIPLE EXORIBONUCLEASES AND THEIR ROLE IN tRNA METABOLISM, Murray P. Deutscher, Nina B. Reuven, Karen O. Kelly, and Zhongwei Li, Dept. of Biochemistry, University of Connecticut Health Center, Farmington, CT 06030.

Seven exoribonucleases, distinct both biochemically and genetically, are now known to be present in *Escherichia coli*. These are polynucleotide phosphorylase (PNPase), RNase II, RNase D, RNase BN, RNase T, RNase PH and RNase R. Six of the enzymes (all except RNase R) are able to act on tRNA precursors *in vitro* releasing nucleoside 5' monophosphates or diphosphates (PNPase and RNase PH). Elucidation of the role of these enzymes *in vivo* has proven difficult because they have overlapping specificities such that removal of any one of them, or even of multiple RNases, generally has little effect on cells. It was known that cells lacking RNase II and PNPase are inviable due to effects on mRNA metabolism, and it has now been shown that cells lacking RNases II, D, BN, T and PH also are inviable. Strains have been constructed that contain only one of the five enzymes missing in the latter strain, and in each case such cells are viable, although they grow with different degrees of effectiveness: RNase T^+ > RNase PH^+ > RNase D^+ > RNase II^+ > RNase BN^+. These observations indicate that the five exoribonucleases functionally overlap *in vivo*, and that any one of them can take over the functions of the others, although to different levels of efficiency. The involvement of each of these enzymes in tRNA maturation was investigated using a series of RNase-deficient strains and a quantitative suppression assay. Our results indicate that each of the five RNases, and perhaps PNPase as well, contribute to the synthesis of mature, functional $tRNA^{Tyr}$ su_3^+ *in vivo*. For this particular precursor, removal of RNase T and RNase PH has the greatest effect on maturation, although removal of other RNases also leads to a loss of some processing activity, and each one of the enzymes, by itself, allows tRNA processing to proceed *in vivo*. Processing of the precursor to $tRNA^{Tyr}$ su_3^+ *in vitro* in mutant extracts confirmed the importance of RNases T and PH, and suggested that RNase PH was most effective in removing nucleotides +3 to +6 or 7 at the 3' terminus, whereas RNase T removes the last 1 or 2 residues closest to the -CCA sequence. Studies to identify the processing intermediates accumulating in the various RNase-deficient strains are underway.

K 034 ENZYMES INVOLVED *IN THE* DECAY OF mRNA IN *ESCHERICHIA COLI*, Sidney R. Kushner, Rong-Fu Wang, Eileen O'Hara, Stephanie Yancey, Caroline Ingle, Julia Chekanova, Laurie Granger, Fred Zheng, Qi Liu, Francis Meffen, Katherine Armstrong, Marti Aldea and Paul Babitzke, Department of Genetics, University of Georgia, Athens, Georgia 30602.

The analysis of mRNA turnover in *Escherichia coli* has focused primarily on four nucleolytic activities, polynucleotide phosphorylase (PNPase), ribonuclease II (RNase II), ribonuclease III (RNase III) and ribonuclease E (RNase E). PNPase and RNase II both work in the 3' to 5' direction to degrade single-stranded RNA one nucleotide at a time. RNase III is an endonuclease that has been shown to be involved in the processing of ribosomal RNAs and intercistronic regions of certain polycistronic mRNAs. RNase E is apparently required for the normal processing of 5S ribosomal RNA as well as a limited number of cleavages in a variety of *E. coli* and bacteriophage T4 mRNAs. In our laboratory we have shown that while mRNA decay is significantly altered in the absence of these four enzymes, full length mRNAs are still being degraded, in most cases into discrete breakdown products. As a result of these observations, we have attempted to identify new enzymes involved in mRNA decay. Initially employing a genetic approach, we have identified a series of new genes, called *mrs* (*m*essenger *R*NA *s*tability), which dramatically alter the decay of total pulse-labeled RNA as well as specific transcripts. Three of the genes have been cloned and their nucleotide sequences determined. The *mrsA* and *mrsC* genes are essential for cell viability. In contrast, the deletion of *mrsF* leads *to a doubling* of the cell's generation time but does not lead to inviability. Multiple mutants with combinations of mutations in either *mrsA*, *mrsC*, *mrsF* and RNase E, PNPase or RNase II have average mRNA half-lives in the range from 25-65 minutes. Of particular interest is the fact that *mrsC* encodes an ATPase that has significant homology to an unique family of eukaryotic ATP binding proteins. In addition, the MrsA protein contains an helix-turn-helix motif that is homologous to the lambda CI protein. The MrsF protein appears to have nucleolytic activity on mRNAs in vitro. The significance of these findings relative to the overall pathway of mRNA turnover will be discussed. (This work was supported in part by a grant from the National Institutes of General Medical Sciences (GM28760) to S.R. K.)

K 035 SUBSTRATE RECOGNITION AND PROCESSING CATALYSIS BY E. COLI RIBONUCLEASE III, Allen W. Nicholson, Honglin Li, Kejing Zhang and Bhadrani S. Chelladurai, Department of Biological Sciences, Wayne State University, Detroit, MI.

Escherichia coli ribonuclease III (RNase III, E.C. 3.1.24) is a double-stranded (ds)RNA-specific endoribonuclease which is prominently involved in the metabolism of cellular, viral and plasmid-encoded transcripts. RNase III processing can regulate gene expression by altering mRNA translational efficiencies, initiating mRNA turnover, or degrading sense-antisense RNA-RNA duplexes. Moreover, in one instance RNase III may act as an RNA-binding regulatory protein. Little is known of the RNase III processing signal identity elements or the mechanism of processing catalysis. These current problems are the focus of our research. We are analyzing the processing reactivities of small (approx. 40-60 nt) RNA substrates that carry the T7 phage R1.1 RNase III processing signal -- an irregular RNA hairpin with an asymmetric internal loop. R1.1 RNA and its sequence variants can be readily synthesized by in vitro transcription of DNA oligonucleotides. We have shown that a conserved sequence element ("CUU/GAA box") in the R1.1 upper stem is not required for accurate in vitro cleavage (1). Also, the R1.1 internal loop can be altered to double-stranded form without inhibiting cleavage; however, similar to the cutting pattern of dsRNA substrates, a second cleavage site appears, opposite the first site. The R1.1 upper stem can be lengthened or shortened without shifting the cleavage site, although reactivity is sharply diminished with the shortened upper stems. The R1.1 lower stem can also be shortened without changing the cleavage site; however, there is evidence that simultaneously altering the upper and lower stems abolishes reactivity. Hydroxyl radical cleavage protection and phosphate ethylation interference assays indicate that RNase III contacts R1.1 RNA in extended directions on both sides of the cleavage site. Taken together these results indicate that RNase III does not measure the length of dsRNA to select the cleavage site, nor does only a single sequence element suffice to dictate binding specificity. Rather, the enzyme recognizes nonobvious sequence/structural determinants on both sides of the cleavage site. In vitro genetic selection experiments, and the testing of additional R1.1 sequence variants are underway to further define the specificity elements.

The molecular mechanism of processing catalysis by RNase III is also of current interest. RNase III cleaves with 5' polarity, and requires a divalent metal ion, preferably Mg^{2+}, for activity. RNase III can cleave a phosphorothioate-substituted phosphodiester bond (2), as well as substrates containing a 2'-deoxynucleotide residue at the cleavage site (3). To investigate the catalytic mechanism, we are using enzyme of high specific activity, purified to homogeneity from an overexpressing bacterial strain. RNase III obeys Michaelis-Menten steady-state kinetics: the processing of a 47 nt R1.1 RNA substrate exhibits a K_m of 0.26 mM, and k_{cat} of 7.7 min^{-1} at 37 °C in physiological salt. The specificity constant (3×10^7 M^{-1}-min^{-1}) is consistent with the efficient in vivo processing by RNase III. The enzyme can cleave substrate when Mg^{2+} is replaced by Mn^{2+} or Co^{2+}, but not with Ca^{2+} or Zn^{2+}; the latter two species moreover can competitively inhibit the Mg^{2+}-catalyzed reaction. Recent evidence indicates a specific Asp residue in catalysis (4); a preliminary model for the active site is suggested, containing a Mg^{2+}-binding site(s), with at least one acidic amino acid side chain. We note the preliminary functional similarity with the proposed two metal ion mechanism for nucleases (5); planned experiments will focus on identifying active site residues, and determining metal ion stoichiometry and placement.

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K 036 A DOMINANT NEGATIVE MUTANT OF 2-5A-DEPENDENT RNase INHIBITS ANTIVIRAL AND ANTIPROLIFERATIVE EFFECTS OF INTERFERON, Bret A. Hassel, Aimin Zhou, Carole Sotomayor, and Robert H. Silverman, Department of Cancer Biology, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio, 44195.

The 2-5A system is an RNA degradation pathway implicated in the molecular mechanism of interferon action. Interferon treatment of cells induces the production of 2-5A synthetases which in the presence of double-stranded RNA produce 5'-phosphorylated, 2',5'-linked oligoadenylates (2-5A) from ATP. RNA breakdown is then mediated by 2-5A-dependent RNase (2DR), a uniquely-regulated nuclease which it is active only when complexed with 2-5A. We have isolated a cDNA for murine 2DR by screening a cDNA expression library with a radiolabeled 2-5A analogue. The initial cDNA (2-5A-BP) expressed a 74 kDa protein *in vitro* which showed identical 2-5A binding properties to the 80 kDa 2DR present in murine L cell extract in assays containing 2-5A or 2-5A analogues as competitors. Radiolabeled 2-5A-BP cDNA hybridized to a 5.7 kb mRNA from murine L929 cells which was induced by interferon even in the presence of cycloheximide. Linkage of a ^{32}P-2-5A analogue to 2-5A-BP and 2DR followed by partial proteolysis revealed identical patterns of labeled peptides indicating that 2-5A-BP is a truncated form of 2DR. Expression *in vitro* of nested 3'-deletions of 2DR cDNA indicated that a duplicate P-loop sequence was critical for 2-5A binding function. Intriguing homologies were also found between 2DR and zinc finger proteins, protein kinases and *E. coli* RNase E. 2-5A-BP functioned as a dominant negative inhibitor of the 2-5A system when stably-expressed in murine SVT2 cells to about 20-fold excess over endogenous 2DR. Indeed, 2-5A mediated rRNA cleavage was inhibited in the 2-5A-BP producing cells. Interestingly, expression of 2-5A-BP resulted in about a three-log reduction in interferon-protection from encephalomyocarditis virus challenge as compared to interferon-treated, control cells. However, interferon protection against vesicular stomatitis virus was identical in the 2-5A-BP expressing and control cell lines. Remarkably, cells expressing 2-5A-BP were resistant to the antiproliferative activity of interferon. Currently we are characterizing human and murine genomic clones for 2DR and also we have constructed an apparent full-length cDNA for human 2DR. We are expressing complete 2DR to purify and study its biochemical activities.

K 037 CHARACTERIZATION OF 5'→3' EXONUCLEASE-1 AND YEAST CELLS LACKING THE ENZYME, Audrey Stevens, Frank W. Larimer, and Cecilia L. Hsu, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830-8077.

5'→3' Exonuclease-1 was purified from *Saccharomyces cerevisiae* as an RNase accounting for 20-30% of the Mg^{+2}-dependent RNase activity of crude extracts. Purification and characterization studies showed that it is a 160-kDa protein that hydrolyzes RNA to 5'-NMP by a 5'→3' mode. Capped mRNA was quite resistant to hydrolysis. To determine the metabolic role of this enzyme, a yeast gene designated *XRN1* was cloned, disrupted, and sequenced. The gene is not essential, but its loss causes diverse effects in yeast cells. The gene was also cloned in other laboratories on the basis of both enzymatic and functional activities, and these studies showed that at high levels it also has homologous pairing and DNA strand exchange activity and 5'→3' DNase activity[review (1)]. We are currently investigating roles of the protein in RNA metabolism.

Certain characteristics of our yeast cells lacking an active gene (*xrn1*) have been examined. Cells are larger (1.5 to 1.8 fold) and have an increased doubling time (1.9 to 2.1 fold). The protein, 25S and 18S rRNA, and mRNA synthesis rates are 80-90%, 45-50%, and 80-90%, respectively, that of wt cells. The portion of the internal transcribed spacer 1 found on 20S pre-rRNA accumulates, showing that an endonucleolytic cleavage at the 3' terminus of 18S rRNA is involved in the 20S pre-rRNA to 18S mature rRNA conversion.

Levels of protein bands of *xrn1* cells resolved by one-dimensional PAGE show substantial differences from wt. Synthesis rates observed for the same protein bands, as well as measurements of several specific mRNA levels by Northern analysis, suggest disparities in mRNA levels. Results show 2-4 times longer half-lives of specific short-lived mRNAs in the *xrn1* cells. Further analysis of the slowed turnover rates of short-lived mRNA species and the higher cellular levels of some of these mRNAs shows the accumulation of essentially full-length mRNAs that do not bind to oligo(dT)-cellulose. Examination of the cap structure status of the accumulated poly(A)-deficient mRNAs by measurements of m^7G content of total RNA and the rate of 5'→3' exoribonuclease-1 hydrolysis of specific full-length mRNA species show that approximately 50% lack the cap structure. Primer extension analysis of the 5'-terminus of ribosomal protein 51A (*RP51A*) mRNA shows that 30-40% of the oligo(dT)-unbound molecules are slightly shortened at the 5' end. Actin (*ACT1*) mRNA may also be partially degraded at the 5'-terminus. The accumulation of mRNA species modified at the 5'-termini together with the restriction of the rate of mRNA turnover in *xrn1* cells suggest a role for 5'→3' exonuclease-1 in the turnover process.

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RNA Maturation

K 038 THE CHEMICAL MECHANISM OF PRE-mRNA SPLICING, Melissa J. Moore, Charles C. Query, John D. Crispino and Phillip A. Sharp, Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139-4307

Introns are excised from eukaryotic pre-mRNA molecules by a two-step mechanism involving sequential transesterification reactions. These reactions are catalyzed within the spliceosome, a 50-60S complex composed of pre-mRNA, four small ribonucleoprotein particles (snRNPS: U1, U2, U4/6, and U5) and a host of associated protein factors. Until recently, however, little was known about either the exact chemical mechanisms of catalysis in this system, or the nature of the participating spliceosomal groups. To address these issues, an efficient method for introducing site-specific chemical modifications into long RNA molecules was developed (1). This technology is currently being used to study the chemical mechanisms of the transesterification reactions, to identify components of the spliceosome that are involved in pre-mRNA splice site recognition and/or catalysis, and to determine the chemical structural requirements for the pre-mRNA substrate.

The chemical mechanism for each step of splicing has been determined by following the stereochemical outcome of both steps. Using substrates containing chiral phosphorothioate linkages at the splice sites, it has been shown that each step results in complete inversion of stereochemistry at the reactive phosphate. This is considered strong evidence for a direct chemical mechanism involving a single S_N2 displacement reaction in each step. Interestingly, both steps are strongly inhibited by the R_p phosphorothioate diasteromer, while neither is affected by the S_p diasteromer; this indicates that the two steps cannot be forward and reverse reactions catalyzed by a single active site. To identify spliceosomal components that are involved in catalysis and/or substrate recognition, specific crosslinking groups are being introduced at the splice sites. These include both modified bases and crosslinkable groups attached to the splice site phosphates. Additionally, nucleotides containing base, sugar and phosphate modifications have been introduced at the splice sites and branch site to investigate the chemical requirements for recognition and function of these pre-mRNA positions. These experiments are ongoing.

1. Moore and Sharp, *Science* **256**, 992-997 (1992)

Ribozymes

K 039 CIS AND TRANS-ACTING FORMS OF THE HEPATITIS DELTA VIRUS RIBOZYME Michael Been, Anne Perrotta, M. Puttaraju, and Sarah Rosenstein. Department of Biochemistry, Duke University Medical Center, Durham, N.C. 27710.

The self-cleaving sequences in hepatitis delta virus (HDV) RNA fold into a structural motif distinct from other ribozymes described to date. We have taken the following approach to characterize the HDV self-cleaving ribozyme: (1) identification of a highly-active self-cleaving domain from the genomic and antigenomic sequences, (2) prediction and testing of a plausible secondary structure compatible with both sequences, (3) dissection of the self-cleaving structure into enzyme and substrate components, and (4) analysis of the *trans* form of the reaction with HDV-derived ribozyme variants. The smallest highly-active contiguous self-cleaving sequence from either the genomic or antigenomic sequence is approximately 85 nucleotides long; the cleavage site is located one nucleotide from the 5' end. Shorter forms of the ribozyme, deleted from the 3' end, cleave much slower and are less resistant to denaturants such as urea or formamide. Both sequences can be folded into similar four-stem structures (numbered I-IV, 5' to 3'). The pairing in each stem of the antigenomic sequence was tested by measuring the effect of site-specific changes on self cleavage in combination with enzymatic and chemical structure probing of the ribozyme variants. All four stems are required for optimal activity and formation of native-like structure. However, stem IV can be shortened with no decrease in the rate constant for self-cleavage or loss of the core structure. Thus, smaller active forms of the ribozyme can be generated. Mutagenesis has been used to identify bases that may form part of the active site or core of the ribozyme.

A *trans*-acting form of the ribozyme, which utilizes an intermolecular form of stem I to specify target-site selection, will cleave model substrates *in vitro* although turnover is slow. Turnover is not limited by chemistry (cleavage) and a mutation in the core structure which severely decreases the rate constant for cleavage under pre-steady state conditions shows normal turnover kinetics under steady state conditions. The small target size and low turnover would appear to place limits on the usefulness of the HDV ribozyme for *in vivo* uses. Therefore, new HDV-derived ribozymes are being designed and tested for the effects on substrate selection, specificity, enzymatic turnover, and other properties. An exonuclease-resistant circular form of a *trans*-acting HDV ribozyme has been designed and is partially characterized.

K 040 NUCLEASES WITHIN NUCLEASES: INTRON-ENCODED MOBILITY ENDONUCLEASES, Marlene Belfort, Molecular Genetics Program, Wadsworth Laboratories, New York State Dept. of Health, Albany, NY 12201-0509.

Group I introns are remarkable not only as ribozymes that catalyze their own splicing, but also as mobile genetic elements. The mobility or "homing" of group I introns, transfer from intron-containing to intronless alleles of the same gene, is dependent upon intron-encoded site-specific DNA endonucleases. Each endonuclease cleaves the corresponding intronless but not the intron-containing allele. Cleavage at or near the intron insertion site creates a double-strand break, with the DNA ends initiating recombination events that result in intron inheritance.

I-*Tev*I, the endonuclease encoded by the *td* intron of phage T4, has several very unusual properties. First, unlike its eukaryotic counterparts, I-*Tev*I cleaves at a distance from the intron insertion site. Thus, a staggered cut 23-25 nucleotides upstream of the intron insertion site leaves 2-nt 3'-extensions with 3'-OH and 5'-PO_4 termini. Second, specificity determinants reside both in the insertion site and cleavage site domains of the DNA substrate, also termed the "homing site". Third, although cleavage of mutant substrates can occur over a range of distances and at a variety of different sequences, I-*Tev*I manifests both distance and sequence preferences. Remarkably, despite these sequence preferences, the enzyme is extremely tolerant of base changes - not a single nucleotide within 48-bp encompassing the homing site is required for I-*Tev*I activity. Finally, physical analysis reveals that I-*Tev*I-DNA contacts occur over 3 turns of the helix, predominantly across the minor groove of the DNA. Minor-groove recognition would effectively extend the range of available natural substrates, many of which contain modifications in the major groove, thereby increasing the potential for dissemination of the intron.

Similar homing-type endonucleases occur in group I introns in the bacterial and eukaryotic kingdoms and in the distinctive introns of the archaeal kingdom. They also occur in protein-coding sequences in all three kingdoms. Their distribution argues strongly that endonuclease coding sequences are themselves mobile genetic elements. Whether their phylogenetic diversity is an indicator of their antiquity, or their having crossed kingdom lines more recently in evolution, remains a provocative question.

K 041 MECHANISM OF NUCLEASE ACTIVITY OF A GROUP I RIBOZYME, Thomas R. Cech[1], Daniel Herschlag[2], Timothy S. McConnell[1], and Joseph A. Piccirilli[1], [1]Howard Hughes Medical Institute, Department of Chemistry and Biochemistry, University of Colorado, Boulder, [2]Stanford University, Stanford.

In nature, the *Tetrahymena thermophila* pre-ribosomal RNA intron accomplishes a self-splicing reaction, excising itself from the precursor RNA and ligating the adjacent exons. Study of this reaction *in vitro* in the absence of protein has shown that the intron folds into a specific structure that catalyzes a series of transesterification reactions (i.e., the exchange of partners in a phosphodiester bond). A guanosine nucleoside or nucleotide bound in a specific site within the intron acts as a nucleophile, initiating the first transesterification reaction.

Shortened forms of the intron can act as artificial nucleases *in vitro*. An RNA substrate or "target RNA" is bound to the ribozyme by base pairing to the internal guide sequence (IGS). This requires Watson-Crick complementarity between the substrate and the IGS, as well as a U adjacent to the reaction site in the substrate. The substrate is then cleaved by guanosine in a reaction analogous to the first step of RNA self-splicing. Alternatively, water (or OH^-) can be used as a nucleophile, catalyzing a hydrolysis reaction.

In order to catalyze RNA chain cleavage, the ribozyme must stabilize the pentacoordinate transition state. A model for transition state interactions that have been experimentally determined includes the following features: (1) the 3' oxygen atom of the ribose of the U (the leaving group) is oriented in-line with the 3'-O of the guanosine nucleophile, as justified by the stereochemistry of the reaction. (2) An active site metal ion (normally Mg^{2+} or Mn^{2+}) is coordinated to this same leaving group 3' oxygen atom, stabilizing its developing negative charge, as indicated by a metal ion specificity switch with a 3' sulfur-substituted substrate. The possibility of a second metal ion, activating the 3'-OH of G for nucleophilic attack, is intriguing. A number of protein enzymes have two metal ion active sites. (3) General acid catalysis, a common strategy for protein enzymes, does not appear to be provided in this RNA system. Perhaps in general ribozymes will use metal ions to stabilize leaving groups and activate nucleophiles instead of functional groups of the RNA to perform general acid-base catalysis.

K 042 EVOLUTIONARY ENGINEERING OF RIBOZYMES WITH NOVEL ENDONUCLEASE ACTIVITIES

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A number of naturally-occurring ribozymes have been described that are able to cleave RNA or DNA substrates in a sequence-specific manner. In each case, the sequence specificity of the ribozyme is determined by base pairing interactions between ribozyme and substrate, and this specificity can be changed by simply changing the substrate-binding portion of the ribozyme. Other purposeful modifications of the ribozyme can be made that alter its activity, structural composition, and resistance to nuclease digestion. For those desired properties that cannot be acquired by rational design, we turn to *in vitro* evolution procedures, constructing a large population of ribozyme variants and allowing those individuals that best exhibit the desired property to emerge through a competition for survival.

We have used *in vitro* evolution to "teach" a group I ribozyme to cleave a target DNA substrate under physiologic conditions [1]. The kinetic properties of the ribozyme can be further sculpted by continuing the evolution procedure under modified selection constraints. We also have been able to change the metal dependence of a group I ribozyme, evolving it to operate in the presence of Ca^{2+}, rather than Mg^{2+} or Mn^{2+} as is required by the wild type. Ribozyme endonucleases, because of their malleable sequence specificity and evolvability, are the most versatile nucleases known. This versatility is likely to be useful in their application as research tools and as therapeutic agents.

1 Beaudry, A.A. & Joyce, G.F. *Science* **257**, 635-641, 1992.

Late Abstract

HOW DOES MAMMALIAN UV ENDONUCLEASE III/AP ENDONUCLEASE I RELATE TO XP-D, CYCLOBUTANE DIMERS, AND RIBOSOMES?

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A number of years ago our laboratory noted that a human AP endonuclease activity, AP endo I, was missing or altered specifically in non-transformed fibroblasts cultured from XP-D individuals (1). More recently we have observed that a UV endonuclease, UV endo III, is similarly affected and indeed is *inseparable* from the AP endonuclease (2).

DNA irradiated with 525 J/m^2 is nicked by the enzyme, but DNA irradiated with 46 J/m^2 does not appear to be nicked. The lightly irradiated DNA does appear to be nicked, however, when treated with UV endonuclease III, then with T4 UV endonuclease(ser), a mutant enzyme that has pyrimidine dimer glycosylase, but not AP endonuclease activity and hence does not itself nick irradiated DNA. The simplest interpretation of these results is that UV endo III cleaves a phosphodiester bond within a cyclobutane dimer.

The enzyme has been purified from mouse cells to yield a single detectable peptide of M_r = 32,000. Sequence analysis of the peptide indicates identity with ribosomal protein S3, and the activity (and peptide) cross-react with antisera directed against the S3 protein obtained from ribosomes. Both the peptide and the activity are strongly bound by concanavalin A, UEA and SBA, but not by WGA, DBA and RCA. This lectin binding pattern indicates a nuclear localization.

Using a cloned cDNA for S3 originally isolated from rat cells by Chan *et al.* (3), we prepared an *E. coli* expression vector for rat ribosomal protein S3. Upon expression, we recover activity which, although weak, appears to be the same as that of legitimate mammalian UV endonuclease III. Moreover, when that protein is exposed to antisera made against rat ribosomal protein S3, it cross-reacts with both the protein and activity from *E. coli.*

In a final study, *in situ* confocal immunolocalization revealed S3 to be associated with both ribosomes and nuclei.

We are most interested in how this protein might be recruited from ribosomes to act as DNA repair nuclease and how it may be altered in XP-D cells.

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Recombination Nucleases; Repair Nucleases

K 100 THE ACTION OF T4 ENDONUCLEASE V ON OLIGONUCLEOTIDES CONTAINING A SITE-SPECIFIC PYRIMIDINE DIMER OR ABASIC SITE, Katherine Atkins Latham[§†] and R. Stephen Lloyd[§], [§]Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, TX 77550; [†]Center for Molecular Toxicology, Department of Biochemistry, Vanderbilt University, Nashville, TN 37232.

T4 endonuclease V is a 16 kD DNA repair enzyme that incises DNA at the site of UV-induced cyclobutane pyrimidine dimers. Four sequential activities have been identified that lead to DNA strand cleavage: salt-dependent linear diffusion on non-target DNA, pyrimidine dimer-specific binding, pyrimidine dimer-specific DNA glycosylase activity, and abasic (AP) lyase activity. Studies have demonstrated the responsibility of the primary amine of the N-terminus for both catalytic activities.

The catalytic activities of T4 endonuclease V are being further investigated using defined length oligonucleotides with a single site-specific lesion: either a pyrimidine dimer or an abasic site. Experiments with the pyrimidine dimer-containing oligonucleotides (supplied by J. -S. Taylor) have resulted in the expected β-elimination product. Surprisingly, another product is also evident from these studies: a putative δ-elimination product that is formed only after virtually all of the original reactant has disappeared. The slow formation of this product leads to the proposition that the enzyme is having to undergo a separate encounter with the DNA after catalyzing the initial β-elimination. Metal-inactivated endonuclease V and other similar experimental approaches are being used to study this hypothesis.

Abasic sites are also being created as a substrate for the enzyme. The activity of endonuclease V towards both apurinic and apyrimidinic sites is being compared in order to determine the enzyme's specificity for helical and extra-helical abasic DNA structures relative to pyrimidine dimer sites. Supported by ES04091.

K 101 DELETION MUTAGENESIS OF N-TERMINAL AMINO ACID RESIDUES OF ENDONUCLEASE V - ESSENTIAL ROLE FOR THE POSITIONING OF THE CATALYTIC α NH_2 GROUP. Mary Lou Augustine and R. Stephen Lloyd, Sealy Center for Molecular Science, U. of Texas Medical Branch, Galveston, TX 77550.

Previous chemical modification studies have demonstrated a critical role for the N-terminal amine in T4 endonuclease V for both pyrimidine dimer-specific DNA glycosylase and AP lyase activities (Schrock and Lloyd, J. Biol. Chem., (1991) 266, 17631-17639). In order to determine whether the location of the N-terminus or the relative length of the N-terminal residues prior to the initiation of the first α-helix is critical for enzyme activity, site-directed deletion mutagenesis was used to individually remove codons from Thr2 through Leu6. After verifying by DNA sequence analysis that the desired changes had been made, the mutated proteins were expressed in DNA repair-proficient and -deficient *E. coli* cells. The UV survival of repair-deficient cells expressing the mutant proteins was not enhanced above that of cells containing vector alone. It was also established that there was no enhancement in the survival of UV-irradiated T4 *denV1* phage. Since all of the mutants showed an identical UV phenotype, the ΔThr2 was purified and tested for its ability to incise dimer- and apurinic-containing DNA. Using either DNA substrate, the ΔThr2 displayed ~1% specific activity. However, using a 40-mer containing 1 dimer, it was determined that the sites of DNA incision had been shifted such that breaks were made both 5' and 3' to the dimer site. Supported by ES04091.

K 102 CHARACTERIZATION OF A PUTATIVE HELIX-TURN-HELIX MOTIF PRESENT IN THE *E. coli* ALKA AND IN CERTAIN OTHER DNA REPAIR PROTEINS. Magnar Bjørås, Lars Eide, Rune Johansen and Erling Seeberg. Div. for Environm. Toxicol. Norw. Def. Res. Estab. 2007 Kjeller, Norway, and Biotech. Centre and Biology Inst., University of Oslo, 0315 Oslo, Norway.

In spite of that all DNA glycosylases hydrolyse N-glycosylic bonds in DNA no common motif has been found that is characteristic for such enzymes. However, a multiprotein alignment reveals one region present in four enzymes of this class, i.e. the alkylbase DNA glycosylases AlkA of *E. coli* and MAG of *S. cerevisiae* as well as in the redoxy enzyme Nth and the A:8-oxoG glycosylase MutY of *E. coli*. A similiar region has also been identified in other proteins involved in DNA repair and recombination, e.g.in the 5'-3' exonuclease domain of DNA polymerase I, and in the *E. coli* UvrC and the human ERCC1 proteins. It can be predicted that this motif represents a DNA binding domain of the helix-turn-helix (HTH) type. The turn is composed of four, rather than three amino-acids, and appears particularly hydrofobic and flexible. To investigate the properties of oligopeptides containing this domain we have cloned DNA fragments encoding this part of the enzymes from several different genes in E.coli expression vectors. Surprisingly, it was found that the HTH motif in AlkA, comprising only 37 out of the 272 aminoacids present in the complete protein, is able to complement the alkylation sensitivity of glycosylase defective mutants. Furthermore, purification of the oligopeptide reveals that the peptide itself possesses quite effective DNA glycosylase activity. It thus appears that we have isolated the active site of the AlkA enzyme. Site-directed mutagenesis is now being employed to identify aminoacids essential for enzyme activity. Experiments are also in progress to characterize the corresponding region from other proteins. (Supported by the Norwegian Cancer Society, grant 90048, the Norwegian Research Council for Science and Humanities, and the Royal Norwegian Council for Industrial and Scientific Research.)

K 103 CHARACTERIZATION OF TWO *DROSOPHILA* AP ENDONUCLEASES AND THEIR DIFFERING SUBCELLULAR LOCATIONS IN THE NUCLEUS AND MITOCHONDRIA.

James P. Carney, David M. Wilson III, Walter A. Deutsch and Mark R. Kelley, Depts. of Molecular & Cellular Biochemistry and Medicine, Loyola University Medical School, Maywood, IL 60153 and Louisiana State University, Baton Rouge, LA 70803.

We have been studying the subcellular location and further characterization of two previously identified AP endonucleases in *Drosophila*. API (66 kDa, Type III) AP endonuclease is predominantly found in the nucleus throughout *Drosophila* development, while APII (63 kDa, Type I) is found in mitochondria. This pattern persists throughout *Drosophila* development, including the adult stage. Both of these proteins are recognized on Western blots by antibody to the human APE isolated and cloned by Dr. Bruce Demple, Harvard and to rabbit polyclonal antibody we produced following the construction of a fusion protein to the human APE that was over-expressed in *E. coli*. Both sets of antibodies specifically recognized the 66 and 63 kDa AP endonucleases in *Drosophila*. The 66 kDa nuclear protein was also shown to be tightly associated with chromatin, even following a 2 M NaCl wash of the purified chromatin. Using Western blot analysis, we have shown that *Drosophila* carrying the *mei-9* mutation showed a 40% decrease of the 66/63 kDa AP endonuclease. *mus201*, a mutagen sensitive mutant on the second chromosome, showed no decrease in these two proteins, while *mus310*, a third chromosome mutant, showed a 166% increase in the presence of these two proteins. This data leads us to conclude that neither *mus201* nor *mei-9* are the stuctural genes for these AP endonucleases, but more likely regulate the production of the 66/63 kDa AP endonucleases. Further data on the similarity and differences between these two proteins will be presented. We are currently using our affinity purified antibody to screen exprssion libraries and purifying these two proteins to homogeneity for cloning purposes.

K 104 *In vitro* RECONSTITUTION OF THE DNA BASE EXCISION REPAIR PATHWAY, Grigory Dianov and Tomas Lindahl, Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Potters Bar, Herts, EN6 3LD, UK.
Double-stranded oligodeoxyribonucleotides containing a single uracil residue at a defined position were employed as substrates in *in vitro* DNA repair assays to estimate the size of the repair patch. The uracil-containing oligonucleotides were efficiently repaired by gently prepared cell extracts of *E. coli* NH5033 (*recB*$^-$, *sbcB*$^-$, *endA*$^-$) and HeLa cells. Restriction enzyme analysis of the corrected oligonucleotides showed that the cell extracts preferentially carry out repair by replacing a single nucleotide. This excludes the involvement of the 5'→3' exonuclease function of *E. coli* DNA polymerase I, or the analogous mammalian exonuclease, because such enzymes are unable to cleave the phosphodiester bond on the immediate 3'side of an AP site subsequent to nicking at the 5'side by an AP endonuclease. Thus, the minimal patch size would be at least two nucleotides if the excision step were catalysed by an exonuclease. The events resulting in replacement of a single damaged nucleotide residue were reconstructed *in vitro* with purified *E. coli* enzymes including uracil-DNA glycosylase, endonuclease IV, DNA deoxyribophosphodiesterase (dRpase), DNA polymerase I and DNA ligase. The dRpase was isolated from *E. coli* KL148 (del.*sbcB*) and was free from endo- or exonuclease activity. The data indicate that after excision of the uracil residue by uracil-DNA glycosylase and hydrolytic cleavage at the 5' side of the resulting AP site by an AP endonuclease, a baseless sugar-phosphate residue is hydrolytically excised by dRpase. DNA polymerase I catalyses the addition of a single nucleotide, either before or after the excision step, and DNA ligation completes the reaction.

K 105 EVIDENCE FOR AN IMINO INTERMEDIATE IN THE T4 ENDONUCLEASE V REACTION, M. L. Dodson, Robert D. Schrock, and R. Stephen Lloyd, Sealy Center for Molecular Science, The University of Texas Medical Branch, Galveston, TX 77555, and Department of Biochemistry and Biophysics, The University of California at San Francisco, San Francisco, CA 94143
Reductive methylation, NMR and site-directed mutagenesis studies have implicated the N-terminal α-amino group of T4 endonuclease V in the glycosylase and abasic lyase activities of the enzyme. A mechanism that accounted for these results predicted that an (imino) covalent substrate-enzyme intermediate was formed between the N-terminal α-amino group and C_1' of the 5'-deoxyribose of the pyrimidine dimer substrate subsequent to (or concomitantly with) the glycosylase step. Experiments to verify the existence of this intermediate indicated that enzyme inhibition by cyanide was substrate-dependent, a result classically interpreted to imply an imino reaction intermediate. In addition, sodium borohydride reduction of the intermediate to form a stable dead-end enzyme-substrate product was possible. This dead-end product was formed with both ultraviolet light-irradiated high molecular weight DNA and duplex oligonucleotides containing a defined TT cyclobutane dimer as substrate. The duplex oligonucleotide substrates demonstrated a well-defined gel shift which may facilitate high resolution footprinting of the enzyme on the DNA substrate. Rapid quench kinetic analyses of the enzyme reaction have been initiated to clarify the reaction details. Measurement of kinetic isotope effects by these fine structure kinetic methods may allow a distinction between S_N1 and S_N2 mechanisms for the initial nucleophilic attack proposed in the overall enzyme reaction scheme indicated above. Extension of these studies to determine whether other glycosylase/abasic lyase enzymes have common enzyme mechanisms are also planned.
Supported by ES04091

K 106 NUCLEASE SP: AN ENDONUCLEASE FROM SPINACH THAT RECOGNIZES STRUCTURAL DISTORTIONS IN DUPLEX DNA, Paul W. Doetsch[1], James M. Puckett[2], Jr., James A. Strickland[1], and Luigi G. Marzilli[2], [1]Department of Biochemistry, School of Medicine, and [2]Department of Chemistry, Emory University, Atlanta, GA 30322

Nuclease SP, an endonuclease functionally similar to a number of single-strand-specific nucleases, cleaves duplex DNA containing a variety of structural modifications produced by agents such as ultraviolet light and cis-diamminedichloroplatinum. We have purified this enzyme to apparent homogeneity and determined a number of its physical properties. The enzyme is quite thermostable and is stimulated by Co^{2+}. In addition, we have carried out a series of experiments designed to reveal the mode of DNA strand cleavage mediated by nuclese SP in the vicinity of defined structural distortions. Synthetic oligonucleotides containing specific UV photoproducts (either cyclobutane pyrimidine dimers or (6-4) pyrimidine-pyrimidone photoproducts) or other types of DNA modifications at single locations were utilized as nuclease SP substrates. The exact positions of nuclease SP cleavage in relationship to the location of a particular DNA adduct have been determined. These studies provide further insight into the functioning of nuclease SP and its relationship to other single-strand-specific nucleases. This work was supported by NIH Grant CA 42607.

K 107 **PURIFICATION AND CHARACTERIZATION OF AN ACID DNase (UV DNase) FROM THE RAT BRAIN**
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DNases play a major role in DNA replication, repair, recombination and restriction. Earlier this laboratory has identified two major DNases in brain, with one of them having an acidic pH optimum (acid DNase) while the other with an alkaline pH optimum (alkaline DNase). Preliminary studies also suggested a role for these two nucleases in DNA replication and DNA repair.

The acid DNase of rat brain which was found to attack the native DNA but not denatured DNA has now been purified to apparent homogeneity. The enzyme shows an approximate mol. wt. of 60 kD by gel filtration and SDS-PAGE methods. Although the enzyme exhibits an optimum pH of 5.0, it is also active at neutral pH under low ionic conditions (0.02 M NaCl). The purified enzyme is an endonuclease and attacks either native DNA or native DNA exposed to UV irradiation, but has no activity towards either depurinated DNA or single stranded DNA. Hence this enzyme is named as UV DNase. The presence of this enzyme in nuclear fraction of brain cells has been established by actually purifying the enzyme from nuclei. Divalent cations are not needed for the enzyme activity which is however, inhibited by sulphate and phosphate ions. Studies on the mechanism of action of the enzyme suggested that the enzyme does not excise pyrimidine dimers and the site of clevage appears to be locally distorted dA-dT rich regions around the pyrimidine dimers in a double helix.

K 108 *HO* ENDONUCLEASE GENERATED DOUBLE-STRAND DNA BREAKS CAN INDUCE DAMAGE-INDUCIBLE GENES AND RECIPROCAL TRANSLOCATIONS IN THE YEAST *SACCHAROMYCES CEREVISIAE*.

Michael T. Fasullo, Department of Radiotherapy, Loyola University School of Medicine, Maywood, IL 60153

DNA damage elicits cell cycle arrest, damage-inducible genes, and recombination and mutagenesis. To elucidate a genetic pathway that transmits the DNA damage signal, yeast strains were constructed in which a single DNA double-strand break *in vivo* can stimulate recombination, cell cycle arrest, and damage-inducible genes. The double-strand break can be generated by *in vivo* by a galactose-inducible *HO* endonuclease. Interchromosomal ectopic recombination is monitored using a modified version of the *his3-Δ3'* recombinational substrate containing a *HO* recognition site within the substrate on chromosome IV and an additional *his3-Δ5'* on chromosome II, recombination between the substrates generates reciprocal translocations. In the same strain, damage-inducible genes are monitored by a *din1-lacz* fusion (*DIN1* encodes the large subunit of ribonucleotide reductase). We have found that a single double-strand break present on chromosome IV in the *his3* recombinational substrate stimulates both reciprocal translocations (approximately 500 fold) and damage-inducible genes. Interestingly, a double-strand break present only at the *MAT* locus does not induce damage-inducible genes. A series of *rad*-mutations have been introduced into this strain background. In *rad52*- strains, spontaneous reciprocal recombination does not occur ($<10^{-10}$); however, reciprocal translocations can still be stimulated to a low frequency of 10^{-8}. Additional *rad* mutations, including *rad1*, are being introduced into this strain background, and these results will be discussed.

K 109 ENZYMOLOGICAL CHARACTERIZATION OF VSR DNA MISMATCH ENDONUCLEASE

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Previously, we demonstrated that the *vsr* gene product of *E. coli* K-12 is a strand- and sequence-specific DNA mismatch endonuclease. It specifically recognizes one particular base/base mismatch (T/G) within a special DNA sequence context and sets an endonucleolytic cut on the 5' side of the mismatched thymidine residue, the phosphate monoester moiety staying with the T (1).
In order to qualitatively characterize the substrate spectrum of Vsr endonuclease we have studied enzyme-catalyzed cleavage of radioactively labelled heteroduplex DNA molecules constructed from synthetic oligonucleotides and containing a T/G mismatch in a variety of different sequence environments. The prototype sequence context of the T/G mismatch, as recognized and processed by Vsr endonuclease, is defined by the cognate sequence of Dcm DNA cytosine methyltransferase ($CC^{A}/_{T}GG$, second C residue in one strand replaced by T and hence mismatched to G). The enzyme tolerates a number of deviations from this ideal sequence (1,2).
Presently, we try to assess the mechanism of Vsr-mediated DNA incision by a number of methods including quantitative analysis of the reaction kinetics. To this end, we have developed a novel and convenient assay based on chemically synthesized heteroduplex DNA carrying a fluorescent label at the 5'-terminus of the cleaved strand.

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K 110 MOLECULAR AND BIOCHEMICAL ANALYSIS OF THE DNA REPAIR/RIBOSOMAL PROTEIN AP3/PO IN *DROSOPHILA*,

David T. Grabowski, James P. Carney, David M. Wilson III, Laura Efferson, Walter A. Deutsch and Mark R. Kelley, Departments. of Biochemistry and Molecular Biology, Loyola University Medical School, Maywood, IL 60153, Louisiana State University, Baton Rouge, LA 70803 and the Sealy Center for Molecular Sciences, U. of Texas Medical Branch, Galveston TX 77555

A *Drosophila* gene (AP3), previously isolated using antibody to a human apurinic/apyrimidinic (AP) endonuclease was found to be the homologue of the human PO ribosome gene. Using the cDNA AP3 clone, we constructed a fusion protein that was used to overexpress the protein in *E. coli*, and the purified protein was used for antibody production. Using Western blot analysis, AP3 is found associated with the nuclear matrix and ribosomes in *Drosophila* and is expressed throughout development, a similar result seen following Northern analysis of the AP3 mRNA. The *Drosophila* protein contains well-defined Casein Kinase I and II phosphorylation sites which have been shown to be phosphorylated *in vitro* by Casein Kinase I and II. The AP3 gene was also over-expressed in the pET11d *E. coli* expression system and purified to homogeneity from inclusion bodies. The purified AP3 protein shows Class II AP endonuclease activity that is not altered by the presence or absence of $MgCl_2$, but is inhibited by EDTA. Activity of AP3 did not change following phosphorylation by Casein Kinase I or II. The human homologue of AP3, PO is overexpressed 30- to 50-fold constitutively in human tumor cell lines that are Mer- and is induced by DNA-damaging agents such as L-PAM, 4HC and HN2 commonly used as chemotherapeutic antitumor agents.

K 111 SYNTHESIS OF DROSOPHILA RRP1 IN AN APURINIC ENDONUCLEASE DEFICIENT STRAIN OF *E.COLI*: COMPLEMENTATION OF REPAIR DEFICIENCY

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A *Drosophila* protein Rrp1(Recombination Repair Protein 1) was previously shown to possess four tightly associated enzymatic activities: DNA strand transfer, ssDNA renaturation, dsDNA 3'-exonuclease and apurinic (AP) endonuclease. The carboxy terminal third of Rrp1 is homologous to *Escherichia coli* exonuclease III, and all members of this protein family recognize and cleave abasic sites that are caused by DNA damaging agents. In order to address the biological function of Rrp1, we recently constructed a plasmid, pRrp1-tac, from which expression of recombinant Rrp1 protein is driven by the IPTG dependent tac promoter. pRrp1-tac directs the synthesis of a protein that reacts with anti-Rrp1 antiserum and has DNA strand transfer activity in a crude *E. coli* extract. In the *E. coli* strain BL21, pRrp1-tac cannot be maintained in the absence of a high copy number plasmid that overexpresses lac repressor, while in the strain XL1-Blue a low level of lac repressor is sufficient.
Since Rrp1 is an AP endonuclease, we investigated whether Rrp1 protein can substitute for *E.coli* AP endonucleases *in vivo*. pRrp1-tac was introduced into the repair deficient strain BW528 (nfo:kan; Δxth) and the sensitivities of BW528 and BW528pRrp1-tac to DNA damaging agents were compared using survival curves and gradient plates. BW528pRrp1-tac shows a 100-fold increase in survival over BW528 when challenged with 5 mM hydrogen peroxide for 15 min. While pRrp1-tac confers resistance to several DNA damaging agents (hydrogen peroxide, t-butylhydroperoxide, mitomycin C and bleomycin), a control plasmid lacking the tac promoter does not. A similar level of complementation is observed when a plasmid directing the synthesis of only the nuclease region of Rrp1 is introduced into BW528, while a plasmid synthesizing a C-terminally-truncated exonuclease-deficient form of Rrp1 does not complement.

K 112 PURIFICATION AND CHARACTERISATION OF A DOUBLE-STRAND EXONUCLEASE FROM THE NUCLEI OF A MUTANT INDIAN MUNTJAC CELL LINE DEFECTIVE IN ITS ABILITY TO CORRECTLY PROCESS DOUBLE STRAND BREAKS, Mathew Hall and R T. Johnson, CRC Mammalian DNA Repair Group, Department of Zoology, Downing Street, Cambridge, U.K, CB2 3EJ.

Single-strand and double-strand breaks (DSBs) in the chromosomal DNA of mammalian cells can act as highly recombinogenic substrates as well as provide the sites for endo and exonuclease activity. DNA breaks arise from exposure to damaging agents, such as UV or DMS, either via lesion repair or through attempts to replicate on a damaged template.

Analysis of a DMS induced break-sealing deficiency in a SV40-transformed Indian muntjac cell line (SVM) using plasmid based assay systems reveals that this cell has a severe and constitutive deficiency in its ability faithfully to rejoin restriction endonuclease DSBs introduced into the cell on plasmid molecules compared to a spontaneously transformed counterpart, DM . Homologous recombination between co-transfected plasmids, however, was found to be largely normal in SVM. This in vivo break-joining deficiency has been faithfully reproduced in vitro using nuclear extracts from SVM and DM cell lines, and these experiments have shown that linear DNA molecules are degraded at a greatly increased rate in the SVM nuclear extract. It is thought that this effect is due to the over-expression or deregulated expression of a double strand (ds)exonuclease in the SVM cell, which acts at DS termini.

We have now purified a protein with a monomeric molecular mass of approx 40 kDa from the nucleus of the SVM cell which has considerable DS exonuclease activity and are at present characterising its properties and establishing its possible role in the processing of DNA single strand gaps and DS termini in mammalian cells.

K 113 STRUCTURE - FUNTION DYNAMICS IN THE *ESCHERICHIA COLI* uvrB PROTEIN. Eric Hildebrand and Lawrence Grossman, Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, MD 21205

The *uvr*B gene product is one of three proteins required for double endonucleolytic incisions flanking DNA damage sites in *E. coli*. It is a cryptic ATPase, turned on by interaction with uvrA dimer and DNA. This activity, essential for incision, may provide the 'power stroke' that drives translocation of the uvrA2-uvrB complex to a damage site.

To probe the mechanisms of activation and regulation of this cryptic ATPase activity, we have introduced a tryptophan residue at the ATPase site of uvrB [there are no Trp in the wild-type protein], and modified the sole cysteine residue with an extrinsic fluorescent tag. These reporter groups, which do not significantly affect *in vivo* or *in vitro* behavior of the modified proteins, are intended to permit discrimination among reaction intermediates for kinetic studies, assess conformational change, and provide dynamic assays for protein-protein or protein-DNA interactions *via* steady-state and time-resolved fluorescence techniques.

The ATPase activity of uvrB can be activated *in vitro* in the absence of uvrA protein, by proteolytic elimination of the C-terminal domain of *ca.* 43 amino acids. A conformational change at or near the ATPase site accompanies proteolytic activation, as shown by the quenching of Trp fluorescence in the 'mutant' F47W protein. Further examination of fluorescence properties of the tryptophan residue do not, however, support an initial hypothesis that a steric block to the ATP binding site has simply been removed. Modification of cysteine 303 with the fluorophore IAEDANS allows us to further characterize this conformational change, by resonance energy transfer experiments. AEDANS-labeling of uvrB also provides a unique signal by which the more complex interaction of uvrA and uvrB may be followed dynamically.

K 114 STRAND CLEAVAGE AND REUNION BY FLP RECOMBINASE: LOGIC OF THE PARTIAL ACTIVE SITE. M. Jayaram, J. Lee, J. W. Chen and S. Yang, Department of Microbiology, University of Texas, Austin, TX 78712.

Phosphoryl transfer is the general chemistry involved in DNA recombination, RNA splicing and DNA transposition. The mechanisms of phosphoryl transfer during strand cleavage and strand exchange steps of DNA recombination have been studied. The recombinase was the Flp protein from yeast (or its 'step-arrest' mutants) and the substrates were synthetic half-recombination sites. The following results were obtained: 1. A Flp monomer bound to a half-site does not cleave that half-site, but cleaves the partner half-site to which a second Flp monomer is bound. 2. A functional recombinase active site is assembled from amino acids shared by more than one Flp monomer. 3. An active site-mutant of Flp can bind the substrate and stabilize the transition state; and an appropriate exogenous non-protein nucleophile can then participate in the cleavage and exchange steps to mediate a complete recombination event, the implications of these findings in phosphoryl transfer mechanisms in nucleic acids and in the evolution of catalysis will be discussed.

K 115 STUDIES ON THE BIOCHEMISTRY OF DNA REPAIR AND RECOMBINATION IN A MAMMALIAN SYSTEM
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We have designed an *in vitro* system to study the repair of DNA double strand breaks by homologous recombination. Mammalian nuclear extract or fractions of it were used. The recombinational repair of gaps and deletions was analysed by several methods, including a direct and quantitative assay, the DNA transfer assay, DTA (MCB 11/1, 445). Cross over and non cross over products were generated *in vitro* as evidenced by PCR analysis. More recently, we have isolated an about 5 proteins containing complex of app. 600 kDa (Recombin. Complex 1, RC-1), which catalyzes the repair reactions. RC-1 has been tested for various enzymatic activities, generally involved in DNA metabolism. A DNA polymerase, a DNA ligase, and a 5'-3' exonuclease activity have been found to be associated with RC-1. The characteristics of these activities are being investigated. The 5'-3' exonuclease is most active on DNA ends containing protruding 3' sequences. In a coupled assay, a concerted reaction of the DNA polymerase and the DNA ligase could be demonstrated. No DNA helicase, topoisomerase or terminal transferase activities, and a weak DNA double-strand nicking activity have been detected in RC-1. The bovine single-strand DNA binding protein (bRP-A), but not PCNA, has been observed to stimulate the overall repair-recombination reaction as well as individual enzymatic activities of RC-1.

K 116 DISINTEGRATION: NOT JUST REVERSAL OF INTEGRATION, Colleen B. Jonsson and Monica J. Roth, Department of Biochemistry, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854

Establishment of the retroviral proviral state is accomplished by the integrase (IN) protein. After reverse transcription of the viral RNA, IN specifically recognizes the long terminal repeat at each viral DNA end, and removes the two 3' terminal nucleotides proximal to the highly conserved dinucleotide sequence, CA. The second step mediated by integrase is a concerted cleavage-ligation or strand transfer reaction. The host DNA is cleaved by integrase creating 5' overhangs that are ligated to the processed 3' ends of the viral DNA. The reverse process of the second reaction or disintegration, the excision of one viral end joined to a host target sequence, has been demonstrated *in vitro*. The molecular basis for these protein:DNA recognition processes are unknown, as well as the biochemical mechanism that mediates the reverse reactions. We have used chemical modification of integrase and structural modifications of the reverse reaction substrate to probe the biochemistry of the protein:DNA recognition and catalytic process. We found the strand transfer reaction of the forward reaction was completely inhibited by N-ethylmaleimide, while the reverse reactions were only partially sensitive. Recognition of the reverse reaction substrate was differentially influenced by the structure and environment of the DNA junction. These data suggest the mechanism in which integrase recognizes the forward reaction substrate differs from that of the reverse reaction substrate.

K 117 NUCLEASE ACTIVITY OF THE RECBCD ENZYME AND ITS SUBUNITS ON SINGLE-STRANDED DNA,
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We are investigating the nuclease activity of the RecBCD enzyme from Escherichia coli to determine which subunit or subunits are required for the nuclease reaction, determine which subunit is responsible for the ATP hydrolysis that stimulates nuclease activity, and investigate the mechanism by which ATP hydrolysis stimulates this activity. The RecBCD enzyme cleaves a 5'-end-labeled DNA oligomer (15 nucleotides) with or without ATP. A ladder of intermediates and products, differing in size by one nucleotide, can be detected on DNA sequencing gels. The distribution of products is the same whether or not ATP is present. The most prominent 5'-end-labeled products are about 6-10 nucleotides in size. The reaction rate in the absence of ATP is about $1x10^6$ M^{-1} sec^{-1}. ATP (500 μM) stimulates the reaction catalyzed by the RecBCD enzyme by about 100-fold. Neither the isolated RecB nor the RecC subunit has nuclease activity by itself. However, the RecBC enzyme, prepared by incubation of RecB and RecC together, has nuclease activity, in the absence of ATP. The rate is about 10-fold slower than that of the RecBCD enzyme, also without ATP. Unlike the RecBCD enzyme, the nuclease reaction catalyzed by RecBC on the 15-mer substrate is not significantly stimulated ATP, although the 15-mer does stimulate ATP hydrolysis by RecBC. This result suggests that ATP hydrolysis by the RecB protein is not efficiently coupled to DNA cleavage. ATP-stimulation of the nuclease activity may be due to ATP hydrolysis by RecD.

K 118 *DROSOPHILA* RIBOSOMAL PROTEIN S3 ACTS ON DNA CONTAINING AP SITES
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The cDNA of *Drosophila* S3 has been identified by screening a 5.5 to 7.5 day pupal library with a rat cDNA encoding S3. The *Drosophila* clone has a single open reading frame that predicts a protein of Mr=27,470. The S3 cDNA maps to a region on the third chromosome in the vicinity of M(3)95A, a previously identified *Minute* ribosomal gene. A fusion containing S3 has been used to overexpress the protein in *E. coli xth, nfo*, and the purified protein in turn used for the generation of specific antibodies. Western and Northern analysis show that S3 is expressed throughout the life-cycle of *Drosophila*, where the protein is found to be associated with both ribosomes, as well as the nuclear matrix and chromatin. The biochemical characterization of S3 has focused on its possible activity on apurinic/apyrimidinic (AP) DNA, in which AP lyase activity has been identified for both the *in vitro* overproduced fusion and non-fusion constructs. Notably, the AP activity is inactivated by antisera purified against the S3 protein. While specific activity on depurinated DNA is not altered by the presence or absence of $MgCl_2$, activity on non-treated DNA is significantly increased in the presence of $MgCl_2$. The AP endonuclease activity is inhibited by tRNA, a characteristic shared by *E. coli* endonuclease III. Studies are underway to determine if S3 also acts as a DNA glycosylase for thymine glycols.

K 119 REACTIONS OF HOLLIDAY-STRUCTURE RESOLVING ENDONUCLEASE VII OF PHAGE T4 CAN INITIATE MISMATCH REPAIR *IN VITRO*. Börries Kemper, Patricia Solaro and Karin Birkenkamp, Institute for Genetics, Weyertal 121, 5000 Köln 41, Germany.

The reactivity of cruciform resolving endonuclease VII (gp49 of phage T4 (1-3)) was tested *in vitro* with DNA-loops of 20, 8, 4 and 1 nucleotides and anyone of the twelve possible mismatches, all located within the same sequence context of synthetic oligonucleotides. Endonuclease VII introduces double-strand breaks by nick and counter-nick within 6nt 3' from the mispairings. The delay between nick and counter-nick was sufficient to allow T4 DNA-polymerase and T4 DNA-ligase to completely repair a C/C mismatch and the loops of 8 or 20 nts *in vitro*, thereby saving the DNA from double-strand breakage. Very short repair tracks of 3 to 4nts were mapped between the mismatch and anyone of the endonuclease VII induced nicks. The repair reaction was terminated by finally sealing the nicks with DNA-ligase in the same reaction mixture.

High relative cleavage efficiencies of endonuclease VII at mismatches in heteroduplexes correlate with relative low thermal stabilities and *vice versa* (measured by denaturing gradient gel electrophoresis). This suggests a plausible connection between local 'breathing' of DNA and recognition by the enzyme.

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K 120 THE REJOINING OF NONCOMPLEMENTARY DNA DOUBLE-STRAND BREAKS PRODUCED BY RESTRICTION ENZYMES IN MAMMALIAN AND BACTERIAL CELLS. Jeff King and William Morgan, Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, CA 94143.

In order for noncomplementary DNA ends involving protruding single strands to be joined to form complete double stranded molecules containing only Watson-Crick base pairs, modifications to the ends must occur (e.g. fill-in DNA synthesis, exonuclease degradation). The rejoining of such DNA ends has previously been studied in cultured monkey cells exposed to SV40 DNA (Roth, Porter, and Wilson, 1985, Mol. Cell Biol. **6**:4295) and in *Xenopus laevis* cell extracts exposed to linearized plasmid DNA (Pfeiffer and Vielmetter, 1988, Nucl. Acids Res. **16**:907). We have been studying the rejoining of noncomplementary ends in a system that utilizes a shuttle vector, designated pHAZE, stabily maintained in a human lymphoblastoid cell line (Lutze and Winegar, 1990, Mutat. Res. **245**:305) and, using isolated DNA fragments, in *E. coli*. In the pHAZE system, two restriction enzymes that produce noncomplementary ends and cut within the lacZ gene are electroporated into the cells. After approximately twenty-four hours, plasmid DNA is recovered from the cells and used to transform *E. coli*. Mutant plasmids are detected in a color assay that identifies colonies that lack a functional lacZ gene. Mutant plasmids are restriction mapped to identify plasmids generated by the rejoining of the ends produced by the restriction enzymes, and the pertinent mutants are sequenced. To study end joining in *E. coli* we gel purify linear segments of pBR322 produced by cutting with two unique enzymes. The linear DNA, which must be circularized *in vivo* to produce antibiotic resistant colonies, is used to transform *E. coli*. DNA sequence analysis of the junctions produced by these systems reveals that in both the human cell line and in *E. coli*, rejoining events are typically found to involve short complementary DNA sequences.

This work was supported by the Office of Health and Environmental Research U.S. Department of Energy, contract DE-AC03-76-SF01012.

K 121 Kinetics and Efficiency of DNA Unwinding by the RecBC Enzyme from E.coli. Firouzeh Korangy and Douglas A. Julin, Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742.

We have studied the properties of the RecBC enzyme reconstituted from the individually purified RecB and RecC subunits. The RecBC enzyme is an active helicase, capable of unwinding long strectches of DNA. Both the unwinding and ATPase activities of RecBC are reduced with respect to the wild-type. The ATP hydrolysis and DNA unwinding catalyzed by this enzyme are also less processive than the wild-type enzyme. The experiments with the RecBC enzyme have shown that it can efficiently couple ATP hydrolysis to DNA unwinding. We have measured the helicase and ATPase activities of the enzyme by several methods and calculated the ratio of ATP hydrolyzed per base pair unwound. The RecBC enzyme shows a ratio of 1.5 ATP/bp unwound, while the wild-type enzyme hydrolyzes about 2 ATP/bp unwound. The lower ratio of ATP/bp suggests that the RecD subunit hydrolyzes ATP in the unwinding reaction catalyzed by the RecBCD enzyme. The RecBC enzyme is quite similar to the RecBCD-K177Q mutant enzyme(conserved lysine to glutamine mutation in the ATP binding motif) in all the activities measured, with the exception that the RecBC enzyme has very low nuclease activity on double-stranded DNA.

K 122 MOLECULAR AND BIOCHEMICAL ANALYSIS OF EXONUCLEASE I MUTANTS OF *ESCHERICHIA COLI*, Sidney R. Kushner and Jose L. Navarro-Bordonaba, Department of Genetics, University of Georgia, Athens, Georgia 30602.

Exonuclease I is an ATP-independent 3' to 5' exonuclease encoded by the *sbcB* gene of *Escherichia coli*. Mutations in the *sbcB* gene were first detected by their ability to suppress the recombination and repair deficiencies associated with *recB* and *recC* mutants (RecBCD enzyme). Subsequently two types of mutations in the structural gene for exonuclease I were identified. *sbcB* mutations suppress both the DNA repair and recombination deficiencies while *xonA* mutants suppress on the DNA repair deficiencies. The *sbcB10* and *sbcB15* alleles have been cloned and sequenced. Single amino acid changes at residue 181 (*sbcB10*) and residue 183 (*sbcB15*) account for the phenotypic properties associated with these alleles. In addition, gene replacement experiments have demonstrated that the complete absence of the exonuclease I protein leads to the XonA phenotype. The *sbcB15* encoded exonuclease I protein has been purified to apparent homogeneity and its catalytic activities examined. The apparent K_m for the mutant protein was approximately ten fold higher than the wild type protein. The activity of the mutant protein on a variety of substrates has been tested. The implications of these results will be discussed.
(This work was supported in part by a grant from the National Institutes of General Medical Sciences (GM27797) to S.R. K.)

K 123 A 5'→3' EXONUCLEASE IN *XENOPUS* OOCYTE NUCLEI: ROLE IN RECOMBINATION AND EXPRESSION DURING DEVELOPMENT, Chris W. Lehman and Dana Carroll, Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT 84132

Mature *Xenopus laevis* oocytes contain large stores of a 5'→3' exonuclease. This nuclease is utilized in an initial step during homologous recombination of linear extrachromosomal substrates. This initial step generates 3' single strands that can base pair with their complements and go on to form complete recombination products through the resection-annealing pathway (as observed under some circumstances in yeast, plant, and mammalian cells). The recombination reaction requires homology, nonhomologous substrates are degraded. A nuclear preparation from *Xenopus* oocytes has been used which catalyzes this recombination reaction and is used to provide extract for the characterization of the nuclease. The nuclease is NTP independent but requires a divalent cation (Lehman & Carroll, Proc. Natl. Acad. Sci. USA (1991) **88**:10840). Additionally, we have found that early in oocyte development, the resection-annealing reaction does not occur, ends are ligated with regeneration of restriction sites at their junctions. The role of increased expression of the 5'→3' exonuclease in changing recombination capabilities during maturation of the oocytes will be presented. Also, the implications that these differences have for functional requirements at these oocyte stages will be discussed.

K 124 TOWARD THE CLONING OF THE *MICROCOCCUS LUTEUS* UV ENDONUCLEASE GENE.

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The pyrimidine dimer specific DNA repair enzyme from *Micrococcus luteus* shares a similar reaction mechanism with two other enzymes, the T4 endonuclease V and a yeast UV endonuclease. Since the *M. luteus* enzyme and endonuclease V have been reported to have similar MWs (18 and 16kd respectively), efforts were made to clone the *M. luteus* gene using a wide variety of expression strategies, DNA hybridization strategies based on the *denV* gene and the utilization of antibodies to endonuclease V. However, none of these approaches were successful. An additional strategy was adopted that relied on large scale purification of the enzyme. Six chromatographic steps were used to isolate an ~18kd protein to ~80% purity that co-purified with dimer-specific nicking activity. N-terminal sequence analysis revealed amino acids sequence with some similarity to endonuclease V. When this gene was cloned, it was found to be the *M. luteus* homolog to a ribosomal protein. Further purification revealed that the 18kd protein could be eliminated with no loss in the specific activity of the preparation. Two proteins remaining in the active fractions had, MWs of ~30 and 32kd. The N-termini of both proteins were sequenced and yielded an identical amino acid sequence. This gene was cloned and bears strong sequence homology with *E. coli* endonuclease III. Efforts are directed at determining if this protein is the dimer-specific DNA repair enzyme. Supported by ES04091.

K 125 PROPERTIES OF THE *RECJ* EXONUCLEASE OF *E. COLI*,

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The *recJ* gene of *E. coli* is necessary for recombination and UV repair via the RecE and RecF pathway of recombination. The *recJ* gene encodes a 5'-specific exonuclease with a strong preference for single-stranded DNA. We have purified the RecJ exonuclease and examined its biochemical properties on a variety of substrates and in combination with other proteins. RecJ nuclease is processive. Single-strand DNA binding protein(SSB) stimulates RecJ's nuclease activity, we presume by removing hairpin regions that impede the processivity of the nuclease. In contrast, RecA protein inhibits RecJ nuclease on single-strand DNA substrates. RecJ has a limited ability to digest double-strand DNA if a 5' single-stranded region is provided to initiate the reaction. Active RecJ nuclease is a monomer although the protein will aggregate specifically into larger multimeric, inactive complexes.

We are presently investigating the effect of RecJ nuclease on coupled strand transfer reactions with RecA protein. By co-precipitation and affinity purification we are determining if RecJ interacts with other proteins in *E. coli*. We are also characterizing genetic suppressor mutations to *recJ* in order to identify other genes that may regulate, interact with, or substitute for RecJ function in the bacterial cell.

K 126 SEQUENCE SPECIFIC INTERACTION OF UvrABC ENDONUCLEASE WITH PSORALEN INTERSTRAND CROSS-LINKS,

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The sequence specificity of a repair enzyme is a combination of the DNA sequence containing the lesion and the orientation in which the chemical can react with the DNA bases. A psoralen molecule can fit into a 5' TpA site in two orientations. Therefore this presents two different repair specificities. Using footprinting experiments, we show that the affinity of UvrAB proteins for different cross-links varied in a sequence and psoralen orientation dependent manner. Our studies reveal that a lesion specific UvrB footprint is formed only on the DNA strand, which is incised. The efficiency of UvrABC incision of various cross-links requires the formation of UvrB-lesion footprint, but does not correlate with B-DNA footprint intensity. Therefore the results suggests that in the repair of some cross-links, the UvrC mediated incision step may be the rate determining step. At low protein concentrations, the time dependent assembly of UvrAB proteins on to cross-link and its conversion to DNA-UvrB complex was demonstrated.

K 127 CHARACTERIZATION OF THE APURINIC ENDO- AND EXONUCLEASE ACTIVITIES OF DROSOPHILA RRP1

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Drosophila Rrp1 (Recombination Repair Protein 1) belongs to a family of DNA repair nucleases that includes *Escherichia coli* exonuclease III, *Streptococcus pneumoniae* exonuclease A, bovine BAP, mouse APEX endonuclease, and human APE. Within a 252 amino acid region collinear homology is shared between all members. Rrp1 is unique in that it includes a 427 amino acid N-terminal region not related sequence to any known sequence. This region is thought to interact with ssDNA and may influence the enzymatic properties of Rrp1.

Rrp1 is enzymatically similar to exonuclease III when purified from Drosophila embryos and when expressed in E. coli from its cDNA. The protein copurifies with an apurinic (AP) endonuclease and a dsDNA 3'-exonuclease. The AP endonuclease specific activity is 1.2×10^5 u/mg using partially depurinated plasmid DNA as a substrate. A 5'-end labeled dsDNA oligonucleotide substrate containing a single AP site was used to further characterize the endonuclease. Rrp1 cleaves the abasic oligonucleotide only at the abasic site (either apurinic or apyrimidinic), but does not cleave a purine containing control substrate. Cleavage of the ssDNA AP oligonucleotide is approximately 100-fold less efficient. Two experimental results demonstrate that Rrp1 cuts the phosphodiester backbone at one position 5' to the AP site and leaves a 3'-OH terminus. First, the Rrp1 cleavage product comigrates with a DNase I cleavage product. Second, the newly formed terminus can support DNA synthesis by T4 DNA polymerase. This characterizes Rrp1 as a class II AP endonuclease.

The exonuclease activity of Rrp1 requires a base paired *terminal* nucleotide and is 3'-5' specific. Surprisingly the ratio of the exonuclease to the AP endonuclease specific activity is dramatically different for Rrp1 protein and its bacterial homologue. ssDNA is a strong inhibitor of the Rrp1 exonuclease and ssDNA inhibition may explain the low exonuclease specific activity we observe. Additional characterization of the Rrp1 AP endonuclease and 3'-exonuclease activities will be presented.

K 128 THE EFFECTS OF THE 46/47 PROTEIN COMPLEX ON THE BACTERIOPHAGE T4 in vitro REPLICATION AND RECOMBINATION SYSTEM, Robert D. Schrock, III[1], Katherine Hale[2], Thomas Kodadek[2], and Bruce M. Alberts[1], [1]Department of Biochemistry and Biophysics, University of California, School of Medicine, San Francisco, CA 94143-0448, [2]Department of Chemistry, University of Texas at Austin, Austin, TX 78712.

The protein complex encoded by bacteriophage T4 genes 46 and 47 is likely to be involved in recombination-mediated DNA replication. It has been suggested that the 46/47 protein complex is an endo/exonuclease that forms substrates for subsequent recombinational events. We are currently characterizing the 46/47 protein complex in the context of *in vitro* DNA replication and recombination reactions using purified bacteriophage T4 proteins.

We have found that the 46/47 protein complex itself does not cleave single-stranded or double-stranded DNA. It is possible that the 46/47 proteins mediate the nuclease activity of another unidentified T4 protein. In addition, the 46/47 protein complex inhibited the polymerase and 3'→5' exonuclease activities of 43 protein (T4 polymerase) *in vitro*. The addition of ATP increased the inhibitory effect of the 46/47 protein complex on the 3'→5' exonuclease activity of 43 protein by ~3 fold. This same 3 fold increase in 43 protein exonuclease inhibition is also observed with the addition of ATPγS, a non-hydrolyzable analogue of ATP. The 46/47 protein complex also inhibited the 3'→5' exonuclease activity of bacteriophage T7 polymerase. Since the replication proteins from these two bacteriophages are not interchangeable, inhibition of the T7 polymerase exonuclease activity by the 46/47 protein complex indicates that the 46/47 proteins interact with DNA rather than specifically with T4 polymerase. We suggest that the 46/47 protein complex binds to the 3'-OH end of DNA and competes with polymerase (from T4 or T7) for this site. One can imagine that such an interaction might facilitate strand invasion and formation of recombination intermediates.

K 129 YEAST DNA REPAIR AND RECOMBINATION PROTEINS Rad1 AND Rad10 ARE SUBUNITS OF AN ENDONUCLEASE, Alan E. Tomkinson*, A. Jane Cooper, Lee Bardwell, Nancy J. Tappe and Errol C. Friedberg. *Institute of Biotechnology, Center for Molecular Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78245-3207 and Laboratory of Molecular Pathology, Department of Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235.

Damage-specific recognition and damage-specific incision of DNA during nucleotide excision repair (NER) in the yeast *S. cerevisiae* requires at least 7 genes, including *RAD1* and *RAD10*. These two genes are also involved in a specialized pathway of mitotic recombination. In contrast to the situation in *E. coli*, there is little biochemical information about the molecular mechanisms of NER in eukaryotes. Previous experiments with *in vitro*-translated Rad1 and Rad10 proteins have demonstrated a stable and specific interaction between them. The region of Rad10 protein required for this interaction is homologous to the human excision repair gene, *ERCC1*. We have demonstrated that the Rad1/Rad10 protein complex is an endonuclease. This endonuclease activity is consistent with the predicted role of these proteins in mitotic recombination and the specific incision of DNA during NER, although we expect that the specificity and reactivity of the Rad1/Rad10 endonuclease is modulated by additional protein-protein interactions *in vivo*. Based on the homology between the genes of the yeast *RAD3* epistasis group and several mammalian genes invoved in repair of DNA damage, we predict that the mammalian genome contains a functional homolog of the the yeast *RAD1* gene, and that the product of this gene interacts with Ercc1 protein to generate an endonuclease activity.

K 130 IMMUNOCYTOCHEMICAL METHODS FOR MEASURING HUMAN AP ENDONUCLEASE IN HEMATOPOIETIC CELLS

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AP endonuclease activity is present in extracts of mammalian cells and tissues from a variety of sources. It is highly likely that there is more than one AP endonuclease in human cells. Moreover, it is conceivable that AP endonucleases are the subject of posttranslational modification or processing. This may explain the conflicting reports regarding the number of independent AP endonuclease activities expressed by human cells.

The major human AP endonuclease (HAP1) has recenly been purified and a polyclonal antibody against it was raised in rabbits. We have used the affinity-purified polyclonal rabbit anti-HAP1 in immunocytochemical determination of HAP1 in human hematopoietic cells. The assay makes use of anti-HAP1 as the primary and FITC-conjugated swine-anti-rabbit-IgG as the second antibody. The HAP1 antigen, as assessed with this antibody, does not tolerate traditional immunocytochemical fixation methods (e.g. acetone, ethanol, methanol), but it is quite well maintained if fixed with paraformaldehyde. HAP1 antigen is expressed in several types of human leukemia cell lines as well as in normal peripheral blood mononuclear cells. We have developed both a cytochemical slide method as well as a flow cytometric version. With these methods the HAP1 expression in phenotypically defined cells is being investigated. The details of these methods will be described and the differences of HAP1 expression in different types of human hematopoietic cells will be discussed.

It can be concluded that this highly specific antibody is a helpful tool in assessing the expression of the major human AP endonuclease in individual cells.

K 131 AN ACID ENDONUCLEOLYTIC ACTIVITY IN MAMMALIAN CELL NUCLEI, Raymond L. Warters and Bradley W. Lyons, Department of Radiology, University of Utah Health Sciences Center, Salt Lake City, UT 84132

A nucleolytic activity has been detected in nuclei from human and rodent cell lines. The nuclease appears to be an endonucleolytic activity since it degrades nuclear DNA into a length distribution between 50 and 300 kbp in both G1 and S phase cell DNA with little or no acid solubilization of DNA. The endonuclease activity requires ATP and functions optimally at pH 5.0 and in hypotonic molarities of monovalent cation. Since the nucleolytic activity persists in extensively washed nuclei, the enzyme appears to be tightly bound within the cell nucleus. A similar distribution of DNA lengths is observed in rodent cells undergoing either programmed or necrotic cell death in response to environmenal trauma. We speculate that this endonuclease activity is involved in both cell death processes. (Supported by NIH grants CA 45154 and CA 25957).

Replication Nucleases; Topoisomerases

K 200 ATP INDEPENDENT TOPOISOMERASE FROM THE THERMOPHILIC BACTERIUM, FERVIDOBACTERIUM ISLANDICUM. C. Bouthier de la Tour, C. Portemer, and M. Duguet. Laboratoire d'enzymologie des acides nucléiques. Institut de Génétique et Microbiologie. Bât 400. Université Paris - Sud. 91405. Orsay Cedex. France.

F. islandicum is a thermophilic bacterium belonging to the most slowly evolving family of bacteria (eubacteria), the *thermotogales.* Two topoisomerase activities have been previously separated in *thermotogales* : a reverse gyrase and an ATP independent topoisomerase activities. To further characterize the various topoisomerases of this ancestral order and compare them to their mesophilic homologs, we have decided to study the enzymes from a *thermotogale, Fervidobacterium islandicum* (growth temperature: 50°C- 80°C). Up to date, no thermophilic eubacterial topoisomerase has been identified. Here, we report the purification and characterization of the ATP independent topoisomerase. The enzyme is a monomer of about 75 kDa; it is a type I topoisomerase sharing similar properties with the other bacterial type I topoisomerases: *F. islandicum* topoisomerase I absolutely requires Mg^{2+} for activity; it relaxes negatively but not positively supercoiled DNA and is inhibited by single stranded M13 DNA. The effect of ATP on the enzymatic activity is less clear. 2 mM ATP curtails the topoisomerisation reaction; however, the inhibition does not seem be accompanied by an hydrolysis of a high energy phosphate bond. Indeed, non hydrolysable analogs (AMPPCP, AMPPNP, ATPγS) were all as potent as ATP. Moreover, the ability of ATP to inhibit the enzyme activity decreases when Mg^{2+} concentrations increase in the reaction mixture. The same results were obtained with the *E. Coli* enzyme (protein w). Thus, it is likely that ATP acts by trapping Mg^{2+} ions, preventing the binding of the enzyme to DNA.
A specific feature of *F. islandicum* topoisomerase I is its thermophily. The optimal temperature for the enzymatic activity is 75°C. Studies of the thermal stability of the enzyme are also described. At 65°C, which is the optimum growth temperature of *F. islandicum*, more than 90% activity was retained after a 15 min preincubation of the enzyme alone.

K 201 ISOLATION AND CHARACTERIZATION OF A GENE ENCODING DNA TOPOISOMERASE I IN *DROSOPHILA MELANOGASTER*, Sheryl D. Brown, Patti C. Huang, and Tao-shih Hsieh, Department of Biochemistry, Duke University Medical Center, Durham, NC 27710

We have isolated and characterized a gene encoding DNA topoisomerase I in *Drosophila melanogaster* (*Top1*) in order to examine the structure and ultimately, the function and regulation of this gene. We have isolated and sequenced two full length cDNA clones of 5.2 and 3.8 kb which have identical protein coding regions but differ at their 3' untranslated regions. We have also isolated two overlapping λ genomic DNA clones and have shown that the entire transcribed region of the *Top1* gene is contained within this overlap. Nucleotide sequence analysis has revealed that the *Top1* gene consists of at least eight exons. The exact location of each splice junction has been determined by comparison of the nucleotide sequence of the genomic DNA to that of the cDNA. The cloned cDNA has been overexpressed in a bacterial inducible expression system, and we have shown that the extract expressing our cloned gene contains a protein that is recognized by anti-topoisomerase I antibody and also possesses the supercoil-relaxation activity characteristic of eukaryotic DNA topoisomerase I. The profile of *Top1* gene expression at the RNA and protein levels has been examined at various stages of the *Drosophila* life cycle. Multiple species of *Top1* message have been seen with a specific species corresponding to 5 kb in length and another group of species averaging approximately 4 kb in length. The 5 kb message is maximal the the time of highest mitotic activity during *Drosophila* development as are Top1 protein levels maximal at this time.

K 202 *IN VITRO* CHARACTERIZATION OF THE EXONUCLEASE FUNCTION OF WILD TYPE AND MUTANT BACTERIOPHAGE T4 DNA POLYMERASES ON ABASIC TEMPLATE LESIONS AND THE EFFECT OF SEQUENCE CONTEXT ON LESION BYPASS, Hong Cai[1], Linda B. Bloom[1], Steven Creighton[1], Ramon Erjita[2], Linda J. Reha-Krantz[3] and Myron F. Goodman[1], [1]University of Southern California, Department of Biological Sciences, Los Angeles, CA 90089-1340, [2]Center for Research and Development, Department of Molecular Genetics, Barcelona, Spain 08034, [3]University of Alberta, Department of Genetics, Edmonton, Alberta, Canada T6G 2E9.

DNA polymerase of bacteriophage T4 is the major determinant of the T4 spontaneous mutation rate and has provided an excellent model system to study the role of DNA polymerase exonucleolytic proofreading on replication fidelity. The N-terminal domain of the DNA polymerase contains the exonuclease activity. Several N-terminal mutants have been identified and extensively purified (Reha-Krantz,L. J., (1991) *Proc. Natl. Acad. Sci. USA* 88, 2417-2421). We present an *in vitro* characterization of the exonuclease activity of these mutants along with that of the wild type polymerase. We measured the efficiency of the T4 polymerases to bypass a single site-directed abasic DNA template lesion. Mutants having partial or total loss of exonuclease function exhibited efficient lesion bypass. However, the mutator polymerase, mel62, having a point mutation in the N-terminal region, outside the conserved exonuclease domain, bypassed the lesion much more efficiently than wild-type polymerase even though it had a normal exonuclease activity. We suggest that mel62 may generally inhibit or uncouple movement of primer between polymerase and exonuclease domains. We also provide data on the effect of sequence context on the extension past abasic lesions using HIV-1 reverse transcriptases. We observed that the "A-rule" favoring insertion of A opposite the lesion is observed is also observed for extension. Generally, when A is situated opposite the lesion it is extended more easily than the other bases. However, we have also observed that C situated opposite the lesion is extended very poorly by the "next correct" nucleotide, but can be extended much more efficiently than A when primer-template misalignment is permitted. These data support NMR structural studies showing that A opposite an abasic site is stacked within the helix while C is extrahelical.

K 203 REGULATION OF TOPOISOMERASE II ACTIVITY BY PHOSPHORYLATION, A.H. Corbett, R.F. DeVore, A.W. Fernald, and N. Osheroff. Dept. of Biochemistry, Vanderbilt University, Nashville, TN 37232-0146

Topoisomerase II exists in the cell as a phosphoprotein. Metabolic labeling, mapping, and genetic studies indicate that the enzyme is phosphorylated by casein kinase II in *Drosophila* and yeast cells. Other evidence suggests that the enzyme may be modified by protein kinase C in other cell types. *In vitro*, topoisomerase II is a high affinity substrate for either of these kinases and phosphorylation by either stimulates overall catalytic activity approximately 2- to 3-fold; however, the mechanism by which modification enhances enzyme activity is not known. Therefore, the effects of casein kinase II or protein kinase C modification were assessed. Results indicate that phosphorylation by either kinase stimulates overall catalytic activity by the same mechanism. Modification by either kinase did not enhance enzyme•DNA binding, pre-strand passage DNA cleavage/religation, DNA strand passage, or post-strand passage DNA cleavage/religation. In contrast, modification by either kinase led to an approximate 2- to 3-fold stimulation of ATP hydrolysis. Casein kinase II and protein kinase C, which recognize distinct amino acid sequences, were able to simultaneously phosphorylate topoisomerase II. However, no additional stimulation of catalytic activity was observed. Finally, simultaneous modification attenuated the effects of the DNA cleavage-enhancing drugs amsacrine and etoposide but had no effect on the sensitivity of the enzyme toward novobiocin. Supported by NIH Grants GM-33944, DK-43325, CA-09582, and ACS Grant FRA-370.

K 204 Reverse Gyrase: a Helicase-like Domain and a Type I Topoisomerase in the same Polypeptide

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Reverse gyrase is a type I DNA topoisomerase present only in hyperthermophilic organisms. It is widely distributed among Archaebacteria, and was also recently found in some Eubacteria. Reverse gyrase has the unique ability to catalyze *in vitro* positive supercoiling of a closed circular DNA at 75-80°C. It is the only topoisomerase I to be dependent on ATP and able to perform a reaction of DNA gyration. The enzyme from *Sulfolobus acidocaldarius* is composed of a single polypeptide of apparent molecular mass about 130,000. Mechanistic studies indicated that reverse gyrase transiently cleaves a single DNA strand, forming a covalent link with the 5' end of the broken strand, as previously described for eubacterial topoisomerase I.

We report here the cloning and sequencing of the gene encoding reverse gyrase from the archaebacterium *Sulfolobus acidocaldarius* strain DSM 639. Analysis of the 1247 aminoacid sequence suggests that reverse gyrase is constituted of two distinct domains: a C-terminal domain clearly related to eubacterial topoisomerase I family especially with Top A and a N-terminal domain without any similarity to topoisomerases I or II, but containing several helicase motifs, including an ATP-binding site. These results support our previous mechanistic studies of reverse gyrase and a model in which positive supercoiling is driven by a helicase-plus-topoisomerase process.

Reverse gyrase from *Sulfolobus acidocaldarius* is the first Archaebacterial topoisomerase I to be cloned and sequenced. The finding of a composite structure for reverse gyrase addresses a fundamental question about the emergence of such a gene.

K 205 ARCHAEBACTERIA AND EVOLUTION OF TYPE II DNA TOPOISOMERASES, Patrick Forterre, Agnès Bergerat, Danielle Gadelle, Bernard Labedan, Melissa Holmes*, Mike Dyall-Smith*, Institut de Génétique et Microbiologie, URA 1354 CNRS, Université Paris-Sud, 91405, Orsay Cedex, France, * Dept. of microbiology, University of Melbourne, Parkville, Victoria 3052, Australia.

DNA topoisomerases II (Topo II) are essential for chromosome condensation and segregation and for the control of DNA supercoiling. Interestingly, very different Topo II have been detected in eubacteria and eukaryotes. In eubacteria, one of the two Topo II, DNA gyrase, introduces negative supercoiling, whereas in eukaryotes, all Topo II relaxe both positively and negatively supercoiled DNA. Accordingly, understanding the evolution of these enzymes could give new insights into the eukaryote/prokaryote transition. With this objective in mind, we are studying Topo II in archaebacteria, a group of prokaryotic microorganisms with a phylogenetic position intermediate between eubacteria and eukaryotes. We have obtained genetic and physiological evidences for the existence of a DNA gyrase-like enzyme in halophilic (salt-loving) archaebacteria. In contrast, we have obtained recently biochemical evidences for an eukaryotic-like Topo II in the hyperthermophilic archaebacterium *Sulfolobus shibatae* (absence of gyrase activity, resistance to coumarins and sensitivity to antitumoral drugs). We also present a comprehensive phylogenetic tree of all type II DNA topoisomerases, including an archaebacterial one, suggesting a duplication of DNA topoisomerase II genes before the eukaryotic/prokaryotic divergence.

K 206 MUTATIONAL ANALYSIS OF A TYPE II TOPOISOMERASE CLEAVAGE SITE: BASE PREFERENCES FOR THE ENZYME AND FOR INHIBITORS, Catherine H. Freudenreich and Kenneth N. Kreuzer, Department of Microbiology, Duke University Medical Center, Durham, NC 27710

The bacteriophage T4 topoisomerase is inhibited by various antitumor agents (e.g. *m*-AMSA), each of which traps the enzyme in a DNA cleavage complex. We have analyzed the sequence preferences for cleavage of a strong *m*-AMSA-induced T4 topoisomerase cleavage site contained on a 30-bp oligonucleotide. Oligonucleotide substrates that contained each of the four nucleotides at 14 positions surrounding the cleavage site, including the positions within the 4-bp stagger created by cleavage, were tested for cleavage in the absence or presence of several type II topoisomerase inhibitors (*m*-AMSA, mitoxantrone diacetate, a modified ellipticine, VP-16, and oxolinic acid). In addition, cleavage was tested with an *m*-AMSA-resistant T4 topoisomerase that has altered sensitivity to several of the inhibitors (Huff and Kreuzer (1990) J. Biol. Chem. 265, 20496-20505). Strong base preferences were detected at eight positions flanking the cleavage site. At these positions, the same base preferences were found regardless of which inhibitor or which enzyme was used to induce cleavage, indicating that these positions are important in recognition of the cleavage site by the enzyme. The preferred bases showed dyad symmetry with respect to the cleavage site, indicating that the two protomers of the topoisomerase dimer interact with DNA in an analogous manner. In contrast, the preferred bases immediately adjacent to the cleaved phosphodiester bonds were highly specific to the inhibitor used, with the position to the 5' side of each cleaved bond showing the greatest specificity. These results strongly suggest that the inhibitors interact directly with the DNA bases at the cleavage site, placing the inhibitor binding site precisely at the site of DNA cleavage. The base preferences we found at these inhibitor-specific positions are similar to those found with the eukaryotic enzyme for the inhibitors which have been used in published consensus results (Fosse *et al.* (1991) Nucl. Acids Res. 19, 2861-2868; Pommier *et al.* (1991) Nucl. Acids Res. 19, 5973-5980), suggesting that the same rules of inhibitor-DNA interactions may influence cleavage complex formation by diverse type II topoisomerases.

K 207 SPONTANEOUS CLEAVAGE OF AN UNUSUAL DNA STRUCTURE BY TOPOISOMERASE II, Stacie J. Froelich-Ammon, Kevin C. Gale, Anita H. Corbett, and Neil Osheroff, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146

While the transient cleavage of double-stranded DNA by topoisomerase II has been the subject of numerous studies, little is known about the cleavage of unusual DNA structures by the enzyme. Since structures such as hairpins, cruciforms, and Holliday junctions resemble nucleic acid recombination intermediates, they may provide useful models for analyzing the potential mechanism of topoisomerase II-mediated recombinational events. Therefore, the ability of the *Drosophila* type II enzyme to cleave a 51-base oligonucleotide (bases 3941-3991 in the (+) strand of ØX174, see below) containing a hairpin loop was characterized. Topoisomerase II cleaved the 51-mer within the hairpin, one base prior to the 3' single-strand DNA tail (see arrow). In contrast to results with double-stranded DNA substrates, cleavage of the hairpin was time-dependent, was not salt-reversible, and did not depend on the presence of protein denaturants. Spontaneous cleavage of the hairpin required at least an 8-base tail on the 5' end. By comparison, high levels of DNA cleavage were observed when the 3' tail was only 4 bases long. Although topoisomerase II displayed no sequence specificity for the single-stranded tails, base pairing within the hairpin was a prerequisite for cleavage. Finally, structure rather than sequence at the point of breakage appeared to be the predominant factor for DNA cleavage. Indeed, when the sequence of the entire oligonucleotide or of the bases immediately surrounding the point of breakage were inverted, efficient DNA cleavage was still observed at the 3' base of the hairpin. These results indicate the potential for topoisomerase II mediating recombination events at unusual DNA structures.

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AATTGTATGTTTTCA
               TGCCTCCAAA
                ::::::::: T
               CGGAGGTTC
TTGCTTGGTATTTTT ↑
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K 208 ANALYSIS OF VACCINIA TOPOISOMERASE MUTANTS,
Malini Gupta, Chang-Xi Zhu and Yuk-Ching Tse-Dinh, Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, New York 10595.
Vaccinia topoisomerase I is highly homologous to other eukaryotic type I topoisomerase, but is distinct in its resistance to anti-cancer drug camptothecin. Mutation in the human topoisomerase I leading to camptothecin resistant have been reported (Tamura et al, 1990). Our hypothesis is that the vaccinia enzyme could be resistant to camptothecin because of subtle changes in amino acid when compared to human, yeast, drosophila enzymes. After comparison of the available sequences of sensitive and resistant type I topoisomerases, the aspartic acid at position 221 of vaccinia topoisomerase I is mutated to valine. The resulting mutant protein is about 25 times less active than the wild type topoisomerase I. The mutant enzyme is sensitive to camptothecin, as the relaxation of supercoiled DNA is inhibited in the presence of camptothecin. The cleavage of DNA is enhanced while the religation reaction is inhibited by camptothecin. This demonstrates that even though the size of vaccinia virus topoisomerase I is only one third of the other camptothecin sensitive topoisomerases, it has a potential binding site for camptothecin. Besides the aspartic acid at position 221, there are probably other amino acids influnencing the interaction of enzyme-DNA complex with camptothecin. This domain that influences resistance or sensitivity to camptothecin is probably important for strand breakage and rejoining reaction of topoisomerase function. This mutant when transformed into *E.coli* strain JD5, which has a dinD1::lacZ fusion, induces the SOS response. We have constructed other mutants at different amino acids position and are testing for camptothecin sensitivity. We have also isolated other mutants that also induce the SOS response in *E.coli.*

K 209 A 3' TO 5' EXONUCLEASE ACTIVITY ASSOCIATED WITH HUMAN RNA POLYMERASE II,
Diane K. Hawley, Daguang Wang, Angelina A. Platas, Debra K. Wiest, and Melissa S. Holtz, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403.
We have found that human RNA polymerase II (pol II) is associated with a 3' to 5' exonuclease activity that removes nucleoside-5'-monophosphates from the 3' end of transcripts in isolated ternary complexes. The shortened transcripts remain bound in functional elongation complexes and can be re-extended upon addition of NTPs. This exonuclease activity requires Mg^{++}, is prevented by α-amanitin, and is greatly stimulated by SII, a protein that acts as a transcription elongation factor in vitro. In addition, as has been shown for the DNA polymerase I 3' to 5' exonuclease activity, the pol II-associated exonuclease activity is competitive with pyrophosphorolysis. Purified pol II retains residual exonuclease activity under experimental conditions that inhibit SII, suggesting that the activity is intrinsic to the polymerase. Although the physiological role is currently unknown, our initial studies have suggested that the exonuclease activity can serve a proofreading function in vitro.

K 210 COUMERMYCIN A_1 RESISTANCE IN THE LYME DISEASE AGENT, *BORRELIA BURGDORFERI*,
D. Scott Samuels and Claude F. Garon, Laboratory of Vectors and Pathogens, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton MT 59840

Coumermycin A_1 is an inhibitor of DNA gyrase, an enzyme that catalyzes supercoiling of DNA and is required for bacterial DNA replication. We have previously shown that *Borrelia burgdorferi*, a spirochete and the causative agent of Lyme disease, was more sensitive than many other eubacteria to coumermycin. Coumermycin at 0.2 µg/ml inhibited growth (MIC) and the slightly inhibitory dose of 0.03 µg/ml induced the reversible relaxation of two negatively-supercoiled circular plasmids within 2 hours (15% of the doubling time). Because there are very few *B. burgdorferi* mutants of any sort derived from selection, we isolated 11 coumermycin-resistant clones from approximately 10^{10} cells. All had a MIC greater than 10 µg/ml and maintained coumermycin resistance after at least 15 generations in the absence of selection. Two variants expressed proteins not found at significant levels in parental cells. CR9B had a new protein with a molecular mass of 27 kDa and CR9C had a new protein with a molecular mass of 23 kDa. None of the variants appeared to have lost the circular plasmids. Three of the variants (CR8A, CR9C, and CR9E) maintained circular plasmid supercoiling while the others had relaxed circular plasmids (in the absence of coumermycin). We are currently determining the N-terminal sequences of the 27 kDa and 23 kDa proteins, evaluating the expression of *gyr*B in the variants, and assaying the inhibition of DNA gyrase supercoiling activity by coumermycin in crude extracts from the variants.

Restriction Nucleases; Fungal Nucleases/Mitochondrial Nucleases

K 300 DNA ACTIVATION OF ENDONUCLEASE *NaeI* INDUCES PROTEIN MULTIMERIZATION,
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NaeI is a prototype for a novel, expanding class of endonucleases whose ability to cleave DNA depends on a two-site mechanism (Yang, C. C. & Topal, Biochem. 31, 000, 1992). Reaction-rate experiments (Conrad, M. and Topal, P N A S 86, 9707, 1989) and electron microscopy studies (Topal, *et. al.* Biochem. 30, 2006, 1991) indicate that the enzyme has two DNA-binding sites that recognize the same core sequence: GCC/GGC. These sites are non-identical: they prefer different families of sequences flanking GCCGGC (Yang & Topal, 1992). Here we report that *NaeI* bound to DNA protected 24 bp from DNAaseI and did so symmetrically around its recognition palindrome, implying a bifold subunit structure. Results of increasing *NaeI* concentration on the reaction rate of DNA cleavage indicated that an *NaeI* multimer is the active species. At low *NaeI* concentrations in the presence of a constant amount of substrate and activator DNAs, one transition from an inactive conformation to a conformation able to nick but not cleave substrate was evident. This low-concentration *NaeI* conformer did not use nicked recognition sites as substrate, since it did not accumulate double-strand cleavages. At higher *NaeI* concentrations a second transition to a higher-order conformation was evident from a loss of nicking activity and a concomitant appearance of double-strand cleavage activity. Activator DNA shifted the protein concentration dependence of the equilibrium between inactive and active NaeI conformers to lower *NaeI* concentrations; the effectiveness of this shift depended on the affinity of the DNA for the *NaeI* activator site.

K 301 UNUSUAL INTERACTION OF *ECORII* ENDONUCLEASE WITH ITS SUBSTRATE DNA,
Ashok S. Bhagwat and Sam Gabbara, Department of Chemistry, Wayne State University, Detroit, MI 48202.
EcoRII is unusual among type II restriction enzymes in that, while it cleaves substrates such as pBR322 and bacteriophage lambda that contain several recognition sites for the enzyme efficiently, substrates such as the genomes of bacteriophages T3 and T7 which contain a small number of recognition sites are cut poorly by it. Interestingly, pBR322, or a short DNA duplex containing a single site for the enzyme, can activate the enzyme to cleave resistant substrates. We have found that at low concentrations, activator short duplexes are themselves cleaved poorly by the enzyme. Further, the reaction shows substrate cooperativity, and at high concentrations, the duplexes are both activators and good substrates for the enzyme. This supports the model that the activation of *EcoRII* involves binding of more than one DNA molecule and provides a simple system to study the mechanism of activation. Using a gel mobility shift assay, we have shown that the enzyme forms sequence-specific, methylation-sensitive complexes with the duplexes in the absence of activating DNA. Therefore, resistance of the short duplexes to the enzyme at low concentrations cannot be due to an inability of the enzyme to bind the duplexes. Interestingly, these complexes are stable in the presence of Mg^{++}, the cofactor for the enzyme, and the complexes obtained in the presence of Mg^{++} do not contain DNA that is cleaved by the enzyme. The inefficient step in the action of *EcoRII* on resistant substrates must occur subsequent to initial substrate-binding and it is this step that the activating DNA must regulate. We are testing various aspects of the action of this enzyme with DNA molecules containing more than one substrate site, with substrates that are resistant to cleavage and by developing a simple general assay for endonuclease and other DNA-modifying enzymes.

K 302 GENETIC ANALYSIS OF THE 145 Kd ENDODEOXYRIBONUCLEASE OF SACCHAROMYCES.
David G. Burbee, Lonnie J. O'Brien, and Barbara Armstrong. Biosciences Division, General Atomics, P.O. Box 85608, San Diego, California, 92186-9784.

We have commenced a logical exploration of the nucleases of the yeast Saccharomyces cerevisiae. We are concentrating on the two largest endodeoxyribonucleases, of approximate molecular weights of 145 and 135 Kd. Both enzymes degrade double and single stranded DNA and both require a divalent cation. The two enzymes are antigenically distinct.

Analysis of the gene for the 145 Kd endonuclease suggests that the enzyme is dimeric in structure and that the dimers (whether homodimeric or heterodimeric is unknown at this time) may be linked by an extensive leucine zipper motif. The gene for the endonuclease is located on chromosome II in a complex locus that appears to overlap with a second gene that probably encodes an RNA binding protein. We have shown previously that insertional inactivation of this region results in a double strand break repair defect. These mutations probably inactivate both genes. We are now making disruptions of each individual gene to explore this phenotype further.

K 303 DNA DETERMINANTS OF *IN VIVO* RESTRICTION: BACTERIOPHAGE T4 ENDONUCLEASE II-DEPENDENT CLEAVAGE OF PLASMID DNA.
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Endonuclease II of coliphage T4 is necessary for the *in vivo* restriction of plasmid DNA in phage-infected cells. Double-stranded restriction cleavage at 12 sites in pBR322 commenced before 10 min postinfection with T4 at 37°C, and proceeded more slowly in the presence of competing phage DNA than in its absence, utilizing the same sites in both cases; in a 200 bp segment of the plasmid single-stranded nicks also were frequent. The plasmid sites were cleaved with a speed that varied with the site, yielding frequencies of cleavage at different sites varying between 10 and 90%, at 50 min postinfection. All sites contained good matches to a consensus, 5'-GRCCGCNTYGC-3', most frequently cleaved around the variable central base pair, generating fragments with blunt ends or 1-2 base 5' overhangs. Using the frequency of cleavage to determine a weighted consensus, a larger sequence, 5'-CGRCCGCNTTGSYNGC-3', was identified. Thus, DNA sequence elements 3' of the cut site appear important for rapid cleavage. Several models describing the sequence-dependent structure of DNA suggest structural anomalies around the cleavage sites. The endonuclease II restriction system is most similar to type II systems, though it differs from known type II systems in several respects.

K 304 A SPECTROFLUOROMETRIC METHOD FOR MEASUREMENT OF RESTRICTION ENDONUCLEASE ACTIVITY BASED ON FLUORESCENCE POLARIZATION, Thomas R. Gingeras[1], Soumitra Ghosh[1], Kirsten Blumeyer[1], Peggy Eis[2] and David Millar[2], Life Sciences Research Laboratory[1], Baxter Diagnostics Inc., San Diego, CA 92121 and The Scripps Research Institute[2], La Jolla, CA 92037

A method is described for monitoring the enzymatic activity of type II restriction endonucleases using fluorophore-labeled oligonucleotide substrates. Cleavage of the duplex oligonucleotide substrates, in which one strand is covalently labeled with fluorescein, results in a time-dependent change of fluorescence polarization due to the formation of short, labeled fragments which are single-stranded at the temperature of the assay. This non-isotopic homogeneous approach is extremely sensitive and is much simpler than the current radiolabeled procedures which require fixed timepoint assays and the use of filter binding or gel electrophoresis for kinetic analysis of the cleavage reaction. The *PaeR7* endonuclease has been used as an example for the applicability of the method. The kinetic parameters for the hydrolysis of a fluorescein-modified oligonucleotide substrate of the enzyme have been determined by using this approach to follow reaction rates.

K 305 FUSARIUM SP. PRODUCE A DNASE IN RESPONSE TO PLANT CONTACT WHICH SIGNALS THE ACTIVATION OF PLANT DEFENSE GENES, Lee A. Hadwiger, David Gerhold, Ming-Mei Chang, Department of Plant Pathology, Washington State University, Pullman, WA 99164-6430

Multiple strains of *F. solani* and *F. oxysporum* isolated from infected plants, humans, and crustaceans produce and release a heat-stable DNase following contact with pea endocarp tissue. The 22 kDa nuclease from *F. solani* f. sp. *phaseoli* makes random single-stranded "nicks" in single- or double-stranded DNA, leaving a 5' phosphate group and a 3' hydroxyl group on the nicked DNA strand. *In vitro* the enzyme catalytic activity is increased ~400-fold by 10 mM Ca^{++} or Mn^{++} and is stable to 100°C for 10 min. The *Fusarium* DNase is continually released and becomes readily detectable in plant nuclei 2 h post inoculation. Pure nuclease applied to pea endocarp tissue at high concentrations prior to the inoculum renders pea tissue susceptible to *F. solani* f. sp. *phaseoli*, a bean pathogen normally resisted by peas. Pure nuclease added to pea tissue at low conc. induces the pea genes DRRG 49, DRRG 206, and β-glucanase which are associated with the defense response of peas. The active DNase quickly enters the plant nucleus and mimics the action of several DNA altering compounds in activating these defense genes. The possibility that the DNase may be capable of signalling the pea gene activation via DNA conformational changes caused by nicking will be discussed.

K 306 REPAIR OF DNA SINGLE- AND DOUBLE-STRAND BREAKS IN *ESCHERICHIA COLI*, Joseph Heitman,[1] Michel Blot,[2] John Wang,[3] and David Thaler[3], [1]Section of Genetics and Department of Pharmacology, Duke University and Howard Hughes Medical Institute, Durham, NC 27710 [2]Department of Microbiology, Biocenter, Basel CH-4056 Switzerland, [3]Rockefeller University, New York, NY 10021

We are interested in how DNA nicks and breaks are repaired and the roles of DNA lesions in recombination initiation. In the course of studies on EcoRI endonuclease substrate recognition, conditional heat sensitive alleles were isolated that permit cell growth at 42°C but become active and toxic at lower temperatures (30° to 37°C). The purified mutant enzymes fall into two classes. One class makes double-strand DNA breaks (DSB) whereas the other class predominantly nicks DNA at EcoRI sites *in vitro*. *In vivo*, under intermediate conditions, both types of mutant enzyme produce DNA lesions that induce the *E. coli* SOS DNA repair response. Enzymes that produce DNA DSBs *in vitro* are not more toxic to *recA*⁻ than to *recA*⁺ strains, suggesting neither SOS induction nor RecA mediated recombination are required for EcoRI DSB repair (Heitman et al., PNAS *86*:2281-2285, 1989). Interestingly, *recD*⁻ mutant strains, which lack one subunit of the RecBCD helicase/exonuclease, are more resistant to DSBs produced by either EcoRI or x-rays, suggesting that RecBCD action may contribute to DSB toxicity and that repair of some types of DNA lesions is not optimal in wild-type *E. coli*. In contrast to EcoRI mutants that produce DSBs, mutant enzymes that predominantly nick DNA *in vitro* are markedly more toxic in the absence of SOS induction. We suggest that some event transpires at naked DNA nicks, such as entry of helicases or recombinases, that subsequently requires SOS induced functions for resolution.

Lastly, we have studied the repair of a different type of DNA DSB, in this case generated by the radiomimetic antibiotic bleomycin, which cleaves DNA by shattering sugar residues, releasing a base at the site of cleavage and leaving 3'-phosphate or phosphoglycolate ends. We find that a gene expressed by Tn5, *ble*, confers increased resistance to bleomycin and EMS. The *aid*C gene, known to be induced by alkyl-DNA lesions, is also required for Ble action. We propose a model in which Ble induces *aid*C and the AidC protein repairs two-carbon alkyl-phosphate DNA lesions produced by EMS or bleomycin (Blot et al. Mol. Microbiol., in press).

K 307 SOLUTION STRUCTURE AND ENDONUCLEASE AFFINITY OF THE DNase INHIBITOR PROTEIN Im9, Russell Wallis, Mike Osborne, Lu.-Y. Lian⁺, Geoffrey R. Moore*, Colin Kleanthous and Richard James, School of Biological Sciences and School of Chemical Sciences*, University of East Anglia, Norwich NR4 7TJ, Biological NMR Centre⁺, University of Leicester, Leicester LE1 7EH, England

Colicin E9 is a plasmid-encoded antibacterial protein whose cytotoxic activity against *E.coli* is due to a non-specific DNase activity. Each E colicin plasmid also codes for the production of a specific immunity (Im9) protein which protects the producing cell against the cytotoxic activity of its colicin, located in the C-terminus of the protein, by binding to this domain. The Im9 protein when expressed in *E.coli* cells provides biological protection against externally added colicin E9, but not against colicin E2, E7 or E8 which are also DNases and share very considerable sequence homology with colicin E9. We are interested in determining, 1) the three dimensional structures of both the DNase domain of ColE9 and its cognate immunity protein so that the mechanism of inhibition and the specificity of their interaction can be understood and, 2) the dissociation constants for the ColE9/ Im9 complex.

To facilitate these studies the Im9 protein (9,583Da) has been overproduced using a bacterial expression system. The purified protein has been characterized extensively with respect to its anti-DNase activity *in vitro* and has been shown to bind to colicin E9 stoichiometrically.

In this work we report our current progress in the elucidation of the solution structure of this DNase inhibitor, in which we have employed ^{1}H and ^{15}N detection in combination with 2D and 3D NMR experiments. The data indicate that Im9 contains a high percentage of α-helical secondary structure and only a small amount of β-sheet secondary structure.

We also report initial experiments aimed at determining the dissociation constant for the ColE9 DNase/ Im9 heterodimer. The complex is sufficiently stable that equilibrium methods are not possible, and so the individual association and dissociation rate constants are being determined. Preliminary data on the dissociation rate constant, obtained by exchange of radiolabelled Im9 into unlabelled complex, suggest that this rate is of the order of 10^{-6} - 10^{-7} s^{-1}. The association rate of protein-protein complexes generally falls between 10^{5}-10^{6} M^{-1} s^{-1} (Janin, J & Chothia, C (1990) J. Biol. Chem. **265**, 16027). Using this value for the Col E9 complex the K_d can be estimated as 10^{-11} - 10^{-13} M. Thus, DNase inhibition by the Im9 protein appears to be of the same order of magnitude as that of protease inhibition by protein inhibitors.

K 308 IN VIVO RECONSTITUTION OF LATENT ANTICODON NUCLEASE: A tRNA RESTRICTION ENZYME MASKED BY HSD RESTRICTION-MODIFICATION PROTEINS. Gabriel Kaufmann, Michal Amitsur and Daphne Chapman-Shimshoni. Department of Biochemistry, Tel Aviv University, Israel 69978.

E. coli prr$^+$ strains encode a latent form of phage T4-induced, $tRNA^{Lys}$-specific anticodon nuclease. The latent enzyme comprises a core factor encoded by prrC and cognate masking elements encoded by flanking, hsd type Ic restriction modification genes. We have reconstituted latent anticodon nuclease from separate core and masking components in vivo by complementing a prr cosmid carrying a null prrC mutation with PrrC provided in trans. Expression of prrC from a low copy plasmid, insufficient to elicit detectable core activity by itself, yielded in the presence of the hsd masking factors a full-fledged latent anticodon nuclease phenotype. The data indicate that the Hsd proteins not only mask PrrC's activity but also stabilize it, maintaining the RNA restriction enzyme as an antiviral contingency.

K 309 I-*Tev*II, *sun*Y INTRON ENDONUCLEASE: OVEREXPRESSION, PARTIAL PURIFICATION, AND INTERACTION WITH ITS DNA SUBSTRATE, Nick Loizos[1,2], Marlene Belfort[2], [1]Department of Microbiology, Immunology and Molecular Genetics, Albany Medical College, Albany, NY 12208, [2]Molecular Genetics Program, Wadsworth Laboratories, New York State Department of Health, Albany, NY 12201

The *sun*Y gene of bacteriophage T4 is interrupted by an intron containing an open reading frame (ORF). This ORF encodes an endonuclease, I-*Tev*II, used to mobilize the intron. *In vitro*-translated I-*Tev*II preparations from rabbit reticulocyte extracts were used to map the cleavage site of an intron-minus *sun*Y allele. Cleavage takes place 15 and 13 nucleotides downstream of the intron insertion site on the sense and antisense strands, respectively. To overproduce and purify I-*Tev*II, the ORF was placed under control of a T7 promoter (1). I-*Tev*II synthesized in this expression system has been shown to cleave the target DNA similarly to *in vitro*-translated I-*Tev*II preparations. Specific I-*Tev*II-DNA complexes can be detected by gel mobility-shift assays. Such analysis provides a basis for ongoing physical studies of the I-*Tev*II-DNA interaction.

1. Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendorff, J.W. (1990) *Methods Enzymol.* 185, 60-89.

K 310 A COMPREHENSIVE CHARACTERIZATION OF INTERACTIONS BETWEEN THE td INTRON-ENCODED ENDONUCLEASE AND ITS HOMING-SITE TARGET. John E. Mueller[1], Mary Bryk[1,2], Nick Loizos[1,2], Susan M. Quirk[1] and Marlene Belfort[1], [1]Molecular Genetics Program, Wadsworth Laboratories, New York State Dept of Health, Albany, NY 12201, [2]Dept. of Microbiology, Immunology and Molecular Genetics, Albany Medical College, Albany, NY 12208.

The intron in the td gene of bacteriophage T4 encodes a double-strand DNA endonuclease, I-TevI, involved in homing of the intron. DNAse I footprinting of the I-TevI-homing site complex has shown that primary binding takes place at the intron insertion site, which is 23 to 25 basepairs downstream of the endonuclease cleavage site (Bell-Pedersen et al., PNAS, 88, 7719-7723, 1991). To examine this interaction in greater detail, we have performed genetic and physical analyses on individual I-TevI-DNA complexes. Genetic experiments, involving a binding-enrichment scheme with randomly-mutagenized substrates, indicate the presence of two distinct hypomutable domains (DI and DII) within the td homing site and also show that I-TevI is surprisingly tolerant of base substitutions. No single base within a 48-bp region of the homing site is absolutely necessary for I-TevI binding. The physical studies, including hydroxyl-radical protection, ethylation and methylation interference analyses, reveal two areas of I-TevI contact including DI and DII: one flanking the intron insertion site and the other approaching the endonuclease cleavage site. Both minor groove and DNA backbone interactions are inferred from the interference analyses. Like other minor-groove binding proteins, electrophoretic studies have shown that I-TevI bends its DNA substrate. Furthermore, the studies indicate that I-TevI-DNA contacts are not at the single-nucleotide level, but rather that they are either redundant or at the level of DNA structure, and that non-contacted bases within the td homing site are involved in I-TevI recognition. Together these properties suggest how I-TevI recognizes T-even phage DNA, which is heavily modified in the major groove.

K 311 A NOVEL MEIOSIS-INDUCED SITE-SPECIFIC ENDONUCLEASE ACTIVITY IN YEAST MITOCHONDRIA, **Kunihiro Ohta, David Keszenman-Pereyra, [1]Alain Nicolas, & Takehiko Shibata,** Laboratory of Microbiology, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan, [1]Institut de Génétique at Microbiologie, Bât.400, Université Paris-Sud 91405, Orsay Cedex, France.

Sequence-specific endonucleases in eukaryotic cells have been shown to play a crucial role in the initiation of genetic recombination. We have found a sequence-specific DNA endonuclease activity that cuts DNAs of phage ϕ105c and phage ϕx174 in a cell-free extracts of a synchronously-sporulating strain (SK1) of *S. cerevisiae.* The preparation from SK1 cells in early meiotic phase had 40-fold higher activity than that in exponential growing phase. Subcellular fractionation revealed that this activity is condensed in the crude mitochondrial fraction. In addition, the activity to cut ϕ105c DNA was not detected in the extract from ρ^- mutant (large deletion mutations that block all mitochondrial protein synthesis) of the SK1 strain. Furthermore, the ability to produce the endonuclease was able to be transferred into a ρ^0 strain by mitochondrial transfer through cytoduction. Comparison of nucleotide sequences around the cutting sites in ϕ105c and ϕx174 DNA suggests that the endonuclease recognizes a ~20-base pair sequence and generates ends with 4 base-3' overhang. The sequences around the cutting sites are different from those by any other yeast mitochondrial endonucleases. As well as other yeast mitochondrial sequence-specific endonucleases, the endonuclease in SK1 strain is speculated to initiate gene conversion in mitochondrial genome.

K 312 CLONING OF BOVINE ENDONUCLEASE G cDNA. A STEP CLOSER TOWARDS UNDERSTANDING ITS FUNCTION, A. Ruiz-Carrillo and J. Côté, Department of Biochemistry School of Medicine, and Cancer Research Center of Laval University. Québec, Canada G1R 2J6.

Endonuclease G is a low abundance but highly active DNase present in higher eukaryotic cells that selectively cleaves double stranded DNA at $(dG)_n.(dC)_n$ tracts (1) and single stranded DNA at $(dC)_n$ runs (2). Endonuclease G is also able to recognize both the triple [GGC]- and double-stranded conformations of the homopolymer. The calf thymus enzyme is composed of two presumably identical subunits of ca. 26 kD each (2). The N-terminal sequence of the bovine enzyme has been determined and used to isolate several full-length cDNA clones from an enriched calf liver library. Genomic analysis has indicated that the structural gene is single copy and contains at least two introns. The deduced protein sequence predicts that endonuclease G is synthesized as a 32 kD precursor carrying a 49 amino acid-long leader sequence at the N-terminus, a prediction born out by *in vitro* translation of mRNA. Current evidence suggests that the precursor species is short-lived and does not accumulate in the cell. Sequence analysis has revealed that endonuclease G shares significant homology with yeast mitochondrial NUC1 (3), a nuclease that does not specifically recognize $(dG)_n.(dC)_n$ sequences. On the other hand, antibodies against the endo-exonuclease of *Neurospora crassa* do not react with endonuclease G.

Since endonuclease G activity was also found in fractionated mitochondria (2), our results suggest that the enzyme is likely targeted to the mitochondrion shortly after its synthesis. Nevertheless, it is not yet unambiguously confirmed whether endonuclease G is also present in the nucleus. This question is currently under investigation.

(1) A. Ruiz-Carrillo and J. Renaud (1987). EMBO J. **6**, 401-407.
(2) J. Côté, J. Renaud and A. Ruiz-Carrillo (1989). J.Biol.Chem. **264**, 3301-3310.
(3) R.D. Vincent et al. (1988). Nucleic Acids Res. **16**, 3297-3312.

K 313 AFFINITY PURIFICATION AND ACTIVE SITE CHARACTERIZATION OF S1 NUCLEASE, V. Shankar and S. Gite, Division of Biochemical Sciences, National Chemical Laboratory, Pune, 411008, India.

A simple procedure, involving heat treatment, DEAE Sephadex, AMP-Sepharose and Bio-Gel P-60 chromatography, was developed for the purification of S1 nuclease to homogeneity. Chemical modification of the purified enzyme revealed the involvement of lysine and histidine residues in the catalytic activity of the enzyme. However, the lysine modified enzyme having very little catalytic activity showed a 70% decrease in its ability to bind 5'AMP, a competitive inhibitor of S1 nuclease, whereas the histidine modified enzyme (having very little catalytic activity) could effectively bind 5'AMP, suggesting that lysine is involved in substrate binding and histidine in catalysis. Moreover, lysine and histidine modification was accompanied by a concommitant loss of ss DNase, RNase and phosphomonoesterase activities of the enzyme indicating a common catalytic site for the hydrolysis of monomeric and polymeric substrates. Although carboxylic acid modification resulted in the loss of catalytic activity of the enzyme, atomic adsorption and CD studies revealed that it is involved in metal binding. Out of the 3 Zn atoms present in S1 nuclease, Zn I is probably involved in catalysis whereas Zn II and Zn III are required for structural stability.

K 314 CLONING, SEQUENCING, AND OVEREXPRESSING THE *NaeI* RESTRICTION ENDONUCLEASE GENE FROM *NOCARDIA AEROCOLONIGENES*, C.H. Taron, E.M. Van Cott, G.G. Wilson, L.S. Moran, B.E. Slatko, L.J. Hornstra, J.S. Benner, R.B. Kucera and E.P. Guthrie, New England Biolabs Inc. 32 Tozer Road, Beverly, MA 01915

NaeI is a type II restriction modification system from *Nocardia aerocolonigenes*. The endonuclease recognizes the palindromic hexanuclotide sequence 5' GCC↓GGC 3' and cleaves to produce a blunt end. The *NaeI* methylase gene (*naeIM*) was cloned previously by Van Cott and Wilson (Gene 74: 55-59, 1988). This clone exbited methylase activity but no levels endonuclease activity were detected, suggesting the endouclease gene (*naeIR*) was either not present intact on the clone, or was present but not expressed. Subcloning the methylase clone into *Streptomyces lividans*, an organism more closely related to *Nocardia*, confirmed the absence of *naeIR*. To clone *naeIR*, a *SacI* genomic DNA library was made using the original methylase clone in pBR322 as a cloning vector. This library was selected for *naeIM* by digestion with *NaeI*, transformed into *E. coli* AP1-200 and positive clones appeared as blue colonies on LB agar plates containing X-gal. Nucleotide sequencing of *naeIM* and *naeIR* yielded two large open reading frames (ORFs). The *naeIR* ORF was identified by correlation of its translated peptide sequence to the amino terminal peptide sequence of the *NaeI* endonuclease. The endonuclease gene was amplified by PCR and cloned behind the strong promoter P_{tac}. This clone was transformed into *E. coli* K802 containing the *MspI* methylase gene. The *MspI* recognition sequence is 5' CCGG 3' (the internal tetramer of the *NaeI* recognition site) with *MspI* modification fully protecting against *NaeI* digestion. This construct yielded approximately 750 fold overexpression of *NaeI*.

K 315 MOLECULAR ANALYSIS OF THE INTERACTION BETWEEN THE DNase COLICIN E9 AND ITS INHIBITOR PROTEIN Im9, Richard James, Russell Wallis, Geoffrey R. Moore* and Colin Kleanthous, School of Biological Sciences and School of Chemical Sciences*, University of East Anglia, Norwich NR4 7TJ, England

E colicins are plasmid-encoded antibacterial proteins. Each E colicin plasmid codes for the production of a specific immunity protein which protects the producing cell against the cytotoxic activity of its colicin, located in the C-terminus of the protein, by binding to this domain.Colicins E2, E8 and E9 are non-specific DNases. The E9 immunity protein (Im9) only protects *E.coli* cells against externally added colicin E9, even though there is >65% sequence homology between the Im2, Im8 and Im9 immunity proteins. We are interested in the sequence information within both the immunity protein and the colicin that determines this level of specificity.

The specificity-determining region of the Im9 protein for its interaction with colicin E9 has been localized to residues 16-43 of the 86 amino-acid protein by the use of gene fusions. A comparison of the alignment of residues in the corresponding region of the Im2, Im8 and Im9 proteins have identified 9 candidate specificity-determining residues. Using site-directed mutagenesis, we have changed each of these residues in the Im9 protein to the residue found in the same position in the Im8 protein. The immunity phenotype conferred by the "mutant" immunity protein was then tested. Of the nine residues, only one (Val-34 to Asp-34) showed any evidence of conferring immunity to colicin E8. Changing other residues in the specificity-determining region to the equivalent Im8 residue did not affect the phenotype conferred by the "mutant" protein, with the exception of the change of Val-37 to Glu-37, which resulted in low-level E8 immunity. While the substitutions at positions 34 and 37 of the Im9 protein introduced immunity towards ColE8 they did not diminish the immunity towards ColE9, suggesting that the two DNase inhibitor proteins may have a common specificity framework which can be modified by single mutations.

We have also used chemical modification of the unique cysteine residue of Im9 (Cys-23) in order to probe further this specificity-determining region. Cys-23 in the purified Im9 protein is accessible to modification with the thiol-specific reagent DTNB and the stoichiometry of labelling is close to 1:1. This residue, however, cannot be labelled by DTNB when the Im9 protein is complexed to colicin E9. This result is consistent with the Cys-23 residue being buried in the complex. On the other hand, when the purified Im9 protein modified at Cys-23 with a variety of reagents was used in DNase inhibition assays with colicin E9, the modified Im9 proteins still possessed anti-DNase activity but only upto a certain derivative molecular weight.These results are discussed in terms of the proximity of this residue to the DNase combining site.

K 316 SENSITIVE CHEMILUMINESCENT DETECTION OF DIGOXIGENIN-LABELED NUCLEIC ACIDS

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We have optimized methods for the nonradioactive labeling of nucleic acids with the steroid hapten digoxigenin (DIG). DIG is an artificial hapten which is not present in most relevant tissues and therefore does not lead to unspecific signals, which can occur with natural labels like biotin. DIG is bound via a spacer arm to uridin-nucleotides and incorporated enzymatically at a defined density into nucleic acids probes by random primed DNA labeling, polymerase chain reaction, *in vitro* RNA transcription or 3´-end labeling / tailing. Alternatively, amino-groups of nucleic acids and proteins can be chemically labeled with a DIG-N-hydroxysuccinimide ester. All these labeling methods provide high yields of stable DIG-labeled probes.

K 400 PURIFICATION AND CHARACTERIZATION OF E. coli RNase E,

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Messenger RNA processing and degradation are important steps in the regulation of gene expression. Mutations in the *E. coli rne* gene affect the processing and degradation of many transcripts. The *rne* temperature-sensitive mutation was originally isolated from an *E. coli* strain defective in the maturation of 5S rRNA. The mutation affects the activity of an essential *E. coli* ribo-endonuclease, RNase E. *Rne* may be the structural gene for this enzyme. The complete *rne* gene has been cloned and sequenced (Casarégola *et al.*, *J. Mol. Biol.*, in press). Antibodies against its product are now available. We have purified RNase E about 200-fold. Western blot analysis shows that the *rne* gene product co-purifies with RNase E activity. Further fractionation on glycerol gradients shows that six polypeptides co-sediment with RNase E activity. Their sizes are 180, 170, 85, 50, 45 and 43kD by SDS-PAGE. The 180kD polypeptide is the product of the *rne* gene. The sedimentation analysis indicates that RNase E could be a multisubunit enzyme containing more that one type of polypeptide. Cell lysis in the presence of detergent and subsequent treatment with high salt are necessary for efficient solubilization of RNase E. The presence of detergent throughout the preparation is essential for maintaining activity. It is also important to use a cocktail of protease inhibitors to prevent degradation of the *rne* gene product. Many of our earlier preparations, which contained proteolytic fragments of the *rne* gene product, had high levels of RNase E activity. A careful comparison of preparations with degraded *rne* gene product to preparations with intact *rne* gene product shows that the specificity of RNase E cleavage is the same. We favor the notion that proteolysis of the *rne* gene product liberates a core polypeptide which has RNase E activity. We have analyzed, *in vitro*, the processing of 5S rRNA, *ompA* mRNA and bacteriophage T4 gene 32 mRNA. These transcripts are accurately processed by RNase E. Heat-inactivation of enzyme from the temperature-sensitive mutant abolishes processing. Similar heat-treatment of wild-type RNase E has no effect on activity. Our RNase E preparations appear to be free of other endo- and exonucleases. During our purification, we found no evidence that a related activity called RNase K (Lundberg *et al.*, *EMBO J.* 9, 2731, 1990) could be separated from RNase E. We suspect that RNase K is a proteolysis product of RNase E that retains nucleolytic activity.

K 401 HAMMERHEAD RIBOZYMES AND MRNA ARE SENSITIVE TO DIFFERENT POPULATIONS OF NUCLEASE IN CELLULAR EXTRACTS.

Brent Edington and Kenneth Draper, Dept. of Cell Biology, Ribozyme Pharmaceuticals Inc., Boulder,CO 80301

The stability of hammerhead ribozymes was compared in both Vero and HeLa cell cytoplasmic, membranous and nuclear fractions. Vero cytoplasmic and nuclear fractions were found to require Mg^{+2} for optimal nuclease activity, while nuclease action in the membranous fraction was not affected by the addition of divalent cations. HeLa membranous and nuclear fractions were also activated by Mg^{+2}, while the cytoplasmic samples required Zn^{+2} for nuclease activation. Relative stabilities of ribozymes and mRNAs were compared in Vero and HeLa cytoplasmic fractions. In the absence of appropriate divalent cations,ribozyme digestion was considerably slower than the digestion of mRNA. The addition of Mg^{+2} to Vero cytoplasmic extracts and Zn^{+2} to the HeLa cytoplasmic extracts stimulated ribozyme degradation and enhanced mRNA digestion to even greater rates. These data show that the nuclease sensitivity of ribozymes is cell-type specific, varies with the intracellular compartment studied and cannot be predicted from studies with mRNA.

K 402 CHARACTERIZATION OF I-Ppo I, AN INTRON-ENCODED ENDONUCLEASE FROM PHYSARUM POLYCEPHALUM, Eldora L. Ellison, Donna E. Muscarella and Volker M. Vogt, Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853
The genetic mobility of group I introns is dependent upon the site-specific endonucleases which they encode. We have characterized several properties of I-Ppo I, the endonuclease that mediates "homing" of intron 3 (PpLSU3) in the nuclear ribosomal DNA (rDNA) of the slime mold *Physarum polycephalum*. From deletion analysis, we conclude that the minimum recognition site is a sequence of 15 bp, which is partially symmetric. The purified enzyme behaves as a dimer in gel filtration and sedimentation. We have studied the interaction of I-Ppo with DNA by bandshift analysis, MPE footprinting and DMS protection. The enzyme, which binds in the major groove, protects ca. 21bp of DNA surrounding the cleavage site.
The I-Ppo recognition site is present in the nuclear rDNA of all eucaryotes. In yeast, expression of the enzyme is lethal, presumably because of cleavage of rDNA repeats on chromosome XII. By pulse field gel analysis, this is the only chromosome cut in vitro. Yeast mutants that survive the lethal effects of the endonuclease occur at surprisingly high frequencies, and are of at least two classes. The first consists of cells carrying point mutations in the recognition sequence. All ca. 150 copies of the rDNA are mutant, as evidenced by the inability of I-Ppo to cleave the rDNA in vitro. The second class consists of cells in which the intron has become inserted into all copies of the rDNA. These cell express active endonuclease.

K 403 THE *pac1+* GENE OF *SCHIZOSACCHAROMYCES POMBE* ENCODES A POSSIBLE HOMOLOG OF *ESCHERICHIA COLI* RIBONUCLEASE III, David Frendewey, Marc Gillespie, and Jennifer Tarnok, Department of Microbiology, New York University Medical School, New York, NY 10016
We isolated a temperature sensitive *S. pombe* mutant (*snm1*) that maintains reduced steady state levels of the U1, U2, U4, U5 and U6 snRNAs and the RNA subunit of RNase P and accumulates 3'-extended U2 and U4 RNAs at the restrictive temperature (37°C). We have cloned a gene that, when present on a multi-copy plasmid, allows *snm1* to grow rapidly at 37°C and restores normal snRNA content. The gene encodes a protein of 363 amino acids that over its carboxy-terminal two thirds shares 25% amino acid identity with *E. coli* ribonuclease (RNase) III, which participates in the processing of both rRNA and mRNA precursors. Surprisingly, the gene we identified had previously been cloned in two other laboratories by completely different strategies. In one case (Iino et al., 1991, *EMBO J.* **10**, 221-226) the gene was isolated as a multi-copy suppressor of *pat1*, a mutant that exhibits uncontrolled meiosis, and was given the name *pac1+*. In the second case (Xu et al., 1990, *Nucleic Acids Res.* **18**, 5304) the *pac1+* gene was cloned by virtue of its ability to cause sterility when overexpressed in a wild type strain. We have begun a mutational analysis of the *pac1+* gene. An in-frame deletion that removes 64 amino acids from the amino-terminal one third, which is not related to RNase III, abolishes the ability to cure the *snm1* mutant. A second in-frame deletion of 137 amino acids from the region of similarity between *pac1+* and RNase III also inactivates the gene. This deleted sequence includes a stretch of 11 perfectly conserved amino acids. A mutation in one of these residues in the *E. coli* gene abolishes RNase III activity. We are constructing the corresponding mutation in the *pac1+* gene, and we are also testing other deletion and point mutations for their effects on *pac1+* activity. The fact that overexpression of *pac1+*, which encodes a putative RNase, can both cause sterility and cure a defect in snRNA synthesis suggests that the developmental switch from vegetative growth to the mating and sporulation pathway might be regulated by RNA processing.

K 404 A SITE- AND STRAND- SPECIFIC INTRON-ENDONUCLEASE IS REQUIRED FOR MARKER EXCLUSION, Heidi Goodrich-Blair and David A. Shub, Biological Sciences and Center for Molecular Genetics, University at Albany, SUNY, Albany, NY 12222.
The virulent *Bacillus subtilis* bacteriophage SPO1 and SP82 belong to a closely related family that contain hydroxymethyl-uracil (HMU) in place of thymine in their DNA. The DNA polymerase gene of these phage is interrupted by a self-splicing group I intron. We have characterized two endonucleases, I-Hmu I (encoded by SPO1) and I-Hmu II (encoded by SP82) that are encoded entirely within the DNA polymerase intron. Other intron-endonucleases are initiators of "intron homing". They introduce a double-strand cleavage specifically on intron-less alleles and repair of this cleavage, using the intron-plus allele as a template, results in two intron-plus copies of DNA via unidirectional gene conversion. The HMU-phage intron-endonucleases have several unique features. First, they are strand- as well as site-specific, introducing a nick in the noncoding strand 4 nucleotides (nt) (I-Hmu I) or 54 nt (I-Hmu II) downstream of the 3' splice site of the intron, within exon II of the DNA polymerase gene. Second, the endonucleases cleave intron-plus as well as intron-less DNA. Third, the endonucleases show a distinct preference, both in vitro and in vivo, for the DNA of the heterologous phage. These differences in the HMU intron-endonucleases could reflect a difference in function. Previous reports indicated that in mixed infections genetic markers of SP82 are more likely to be carried by progeny than those of SPO1. This exclusion occurs over 10 kilobases of DNA surrounding the DNA polymerase gene. [1] We have found that expression of I-Hmu II by SP82 is required for this exclusion process. We hypothesize that the intron homing mechanism of I-Hmu II has been expanded to confer a selective advantage on its host in competition with close relatives.

1. Stewart, C. and Franck, M., *J. Virology* 38 1081-1083 (1981).

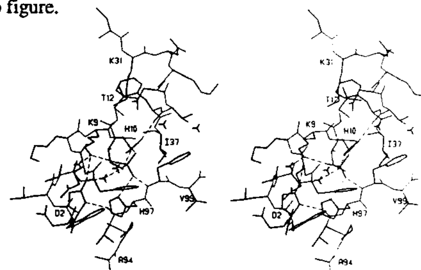
K 405 MOLECULAR CLONING OF 2-5A-DEPENDENT RNase: A TOOL FOR THE FUNCTIONAL ANALYSIS OF THE 2-5A SYSTEM IN INTERFERON-INDUCED ACTIVITIES, Bret A. Hassel, Aimin Zhou, Carole F. Sotomayor and Robert H. Silverman, Department of Cancer Biology, Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland Ohio 44195.
Interferons (IFNs) are potent cytokines which exert a pleiotropic set of activities on responsive cells including the inhibition of cellular proliferation and protection from viral infection. The 2-5A system is an interferon-regulated RNA degradation pathway whose activity is mediated through the 2-5A-dependent RNase (RNase F, RNase L, 2DR). In order to evaluate the role of this pathway in the molecular mechanism of IFN action, we have isolated cDNAs encoding the murine and human forms of 2DR. To inhibit the 2-5A pathway, we expressed in cells a truncated clone of 2DR which retained wild type 2-5A binding activity, but which lacked RNase activity. When expressed to high levels in stably-transfected cells, the mutant protein inhibited activation of the endogenous enzyme by 2-5A, thus acting as a trans-dominant inhibitor. 2-5A mediated rRNA cleavage was inhibited in the mutant cell line, but not in a cell line transfected with vector lacking cDNA. Remarkably, the mutant protein resulted in a 1000-fold reduction in IFN-protection from EMCV challenge as compared to IFN-treated, control cells. The mutant cell line also exhibits a decreased sensitivity to the antiproliferative activity of IFN. We are currently using this mutant construct in other cell types in order to examine the potential role of the 2-5A system in other cellular processes relating to the control of cell growth and differentiation.

K 406 A NOVEL CATALYTIC RNA FROM A MOBILE INTRON IN THE RIBOSOMAL DNA OF *DIDYMIUM* HAS A PROVOCATIVE SIMILARITY TO THE U1 snRNA, Steinar Johansen[1,2] and Volker M. Vogt [2], Department of Cell Biology, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway [1] and Department of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14835 [2].

We have discovered a new mobile intron in the small subunit ribosomal RNA gene in the nucleus of the myxomycete *Didymium iridis*. The intron (DiSSU1), which is 1,43 kb in length and contains an open reading frame (ORF), has several structural and functional similarities to the group I intron family. Intron RNA self-splices *in vitro* in the presence of high salts and a guanosine cofactor. The self-splicing reaction shows a complicated pattern of products, due to partial cleavage at both exon-intron junctions as well as two internal processing sites. Self splicing is unaffected by deletion of ORF. Based on an extensive deletion analysis of the intron, we conclude that the self splicing is carried out by two distinct catalytic RNA elements, one upstream and one downstream the ORF. The upstream element can be folded into the typical group I intron secondary structure of nine paired segments, and includes the highly conserved G-binding site and G-addition site. The downstream element, which comprises about 250 nt, shows no obvious structural homology with other catalytic RNAs. This novel element can efficiently cleave the 3' splice site in complete absence of upstream sequences. The sequence that spans the 3'splice site shows an almost perfect match with the 5' splice consensus sequence of nuclear mRNA introns. Futhermore, the novel catalytic RNA element contains a sequence of 10 nt similar to the part of U1 snRNA that is known to base pair with sequences at the 5'splice site of pre-mRNA. DiSSU1 appears to be the first example for the cooperative interaction of distinct catalytic RNAs in RNA splicing. Based on the sequence similarities above, we speculate that DiSSU1 splicing may embody features of ancestral mRNA splicing. Experiments to test these ideas are in progress.

K 407 THE REFINED 1.7 Å X-RAY CRYSTALLOGRAPHIC STRUCTURE OF P-30, AN AMPHIBIAN RNase WITH AN ANTI-TUMOR ACTIVITY, Steve Mosimann, Kathy Johns and Michael N.G. James, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada; W. Ardelt, S. Mikulski and K. Shogen, Alfacell Corporation, Bloomfield, New Jersey 07003.

The P-30 protein is a developmentally, regulated, amphibian ribonuclease of 12 kDa. It displays an anti-tumor activity *in vivo* and *in vitro*. It also belongs to the pancreatic RNase superfamily that includes RNase A and the angiogenins. In this work, the refined crystal structure of P-30 is presented at 1.7 Å resolution to an R-factor of 0.170. The structure is predominantly antiparallel β and it contains all 104 amino acids and a sulfate anion bound to the active site. There are four disulfide bridges in P-30 and three are conserved throughout the superfamily. Surprisingly, the N-terminal pyroglutamyl residue is found at the active site where it either forms part of the active site or it occupies the active site. Since functional differences between P-30 and RNase A should be explainable in structural terms, the structural consequences of several sequence variations have been considered. On a global level, there is hinge motion that relates the relative orientations of the antiparallel β-sheets in P-30 and RNase A. In more detail, the P-30 loop, K49-S54, bears no resemblance to the equivalent loop of RNase A (T59-H70). This is significant as this loop in RNase A is known to contribute several residues to substrate binding. Finally, P-30's preference for uridine as the 3' nucleotide is rationalized in terms of a non-conservative amino acid replacement (V101 in P-30 and S123 in RNase A) and the potential participation of K33 in substrate binding. The active site of P-30 is shown in the accompanying stereo figure.



K 408 RNA PROCESSING ENZYME RNase E
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RNase E is a processing endoribonuclease that catalyzes the maturation of 5S rRNA from its precusors, it also cleaves RNA I, a small RNA that controls the replication of ColE 1 plasmid DNA and affects the processing and turnover of many *E. coli* mRNAs as well as T4 mRNAs. There are also some indirect evidence of RNase E being involved in maturation of the 3'-end of some tRNAs. The *rne* gene specifying RNase E has been cloned by complementation of temperature sensitive mutant *rne-3071* (Dallmann *et al., Mol Gen (Life Sci Adv)* **6**: 99-107, 1987) and subsequently mapped using lambda clones from the genomic library of Kohara at 23.5 min position on the *E. coli* chromosome. We have shown that another *E. coli* mutation *ams* (altered mRNA stability) is allelic with *rne* (Taraseviciene *et al., Mol Microbiol* **5**: 851-855, 1991). A number of deletions were constructed from a 3.2 kb clone in order to define the coding region of *rne* in the cloned DNA. The smallest DNA fragment complementing the *ts* mutation in a *recA*+ background was 1.2 kb DNA, while in a *recA*- background the larger 2.6 kb fragment was required to complement the mutation and to restore the enzymatic activity. The 3.2 kb fragment was sequenced and the expression of it resulted in the synthesis of a 110,000 Da polypeptide (Chauhan *et al., Nucl Acids Res* **19**: 125-129, 1991). Rabbit antiserum was raised against this polypeptide. The Western blot analysis revealed that antibodies recognized all the truncated polypeptides derived from the deletion constructs as well as a polypeptide migrating as a 180,000 Da protein from wild type cells extracts. The results suggested that the cloned 3.2 kb fragment contained a truncated gene. The antibodies raised against the 110,000 Da polypeptide (N-terminal part of RNase E) cross-reacted with similar molecular weight proteins (~180 kDa) from a number of different bacteria. This observation suggested that RNase E is evolutionary conserved in the bacterial world. A clone encoding the 180,000 Da polypeptide was utilized in preliminary efforts to purify and characterize 180,000 Da RNase E protein. With a T7 promoter expression vector system several interesting observations have been made: the intact 180,000 Da protein was stable at least for 2 hours after the induction of synthesis; the truncated polypeptides were unstable and were degraded to a number of smaller peptides recognized by the antibodies; the protein was associated with the membranes as it co-sedimented with the membrane fraction.

K 409 MOLECULAR MECHANISM OF INHIBITION OF RNASE H ACTIVITY OF HIV-1 REVERSE TRANSCRIPTASE BY AZTMP-Xinyi Zhan, Cheat -K. Tan, Katheleen M. Downey, & Antero G. So University of Miami, Department of Medicine(R-99), Miami, FL 33136

We have recently found that the RNase H activity of HIV-1 can be selectively inhibited by AZTMP and that the sensitivity of the enzyme to AZTMP is influenced by the nature of the substrate and the divalent cation used in the reaction mixture. In the presence of Mg^{2+}, the IC_{50} for AZTMP with poly(dT)/[^{3}H]poly(A) as substrate is 5 mM, whereas with poly(dC)/[^{3}H]poly(G) as substrate IC_{50} is 250 uM. The latter concentration is approximately 4-5 fold lower than that attainable intracellularly. With both substrates, substitution of Mn^{2+} for Mg^{2+} markedly increases the sensitivity of RNase H to inhibition by AZTMP and decreases the IC_{50} of AZTMP with both substrates to about 50 uM. The mechanism of AZTMP inhibition of the RNase H activity has been determined by kinetic analysis under steady-state conditions. With poly(dT)/[^{3}H]poly(A) as substrate and Mn^{2+} as divalent cation activator, AZTMP was found to be a competitive inhibitor with a Ki of 35 uM, suggesting that AZTMP binds at the active site of the enzyme. The mechanism by which Mn^{2+} increases the the sensitivity of the enzyme to AZTMP has been determined by analyzing the products of hydrolysis by RNase H with poly(dT)/[^{32}P]poly(A) as substrate. With Mg^{2+} as divalent cation activator, oligonucleotide products are produced with little or no mononucleotides produced except after long incubation times, suggesting that the RNase H is acting primarily as an endonuclease. In contrast, with Mn^{2+} as divalent cation activator, both oligonucleotide and mononucleotide products are produced very early in the reaction, suggesting that both endonuclease and exonuclease activities are active in the presence of Mn^{2+}. It appears that endonuclease and 3' to 5' exonuclease activities of HIV-1 RT are equally sensitive to inhibition by AZTMP, since the generation of both products is inhibited by this nucleotide analogue.

Late Abstract

DNA BINDING PROTEINS AND DNA BENDING AFFECT RETROVIRAL INTEGRATION SITE SELECTION

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Retroviral integration can be reproduced in vitro, using either nucleoprotein complexes isolated from infected cells or purified, virus-encoded integrase. With a recently developed PCR assay which measures the frequency of integration events at individual positions in the target DNA, it was shown that integration in vitro occurs into naked DNA in a non-random fashion (Pryciak and Varmus, Cell 69, 769-780). Furthermore integration occurs into nucleosomal DNA more efficiently than into nucleosome-free regions, and the choice of target sites is altered, resulting in a 10bp periodic spacing of highly preferred sites. A direct comparison with DNaseI cut sites in rotationally phased mononucleosomes assembled in vitro showed that integration occurs into the major groove on the exposed face of the nucleosomal DNA.

Several features of nucleosomal DNA could be responsible for the characteristic distribution of integration sites, e.g. DNA bending, attachment of the DNA to a surface, or specific interaction of the integration machinery with histone proteins. To address this problem, we are studying integration into model targets that mimic one or several properties of nucleosomal DNA. In a first approach, we ask whether exceptionally reactive sites can be created by bending the DNA. We inserted target DNA between two lac operators, which enabled us to bend the intervening sequence upon addition of *lac* repressor. Integration into such targets using purified MLV or HIV integrase showed that DNA bending affects distribution of target sites in a manner similar to DNA wrapping around a nucleosome. Moreover lac repressor itself - unlike nucleosomes, but like the yeast $\alpha 2$ repressor - blocked integration into its binding site. We have also asked whether other DNA/protein complexes, particularly those with known structures, might affect integration target choice. The bacterial DNA binding protein CAP (catabolite gene activator protein) bends its DNA binding site to a similar extent as nucleosomal DNA; and HIV and MLV integrases indeed produce integration patterns in CAP-bound DNA reminiscent of those in nucleosomal DNA. This suggests that histones present DNA to the integration machinery in a favorable way, but might not interact directly and specifically with the machinery.