STRUCTURE AND DNA-PROTEIN INTERACTIONS OF REPLICATION ORIGINS

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Bacterial Replication Origins

876 THE *ORI* REGION OF THE ENTEROBACTERIACEAE, Judith W. Zyskind, Nancy E. Harding, Yutaka Takeda, Joseph M. Cleary, and Douglas W. Smith, Department of Biology, C-016, University of California, San Diego, La Jolla, CA 92093.

It has been shown that the functional origin of DNA replication in *Escherichia coli* and *Salmonella typhimurium* is contained within a 296 bp region located between *asn* and *uncB* on the bacterial chromosome (1,2,3). A comparison of the two *ori* sequences provided a pattern of conserved and nonconserved bases from which a secondary structure was drawn (3). To further elucidate what bases within *ori* may be changed and still retain *ori* function in *E. coli*, we have cloned the *ori* regions of *Enterobacter aerogenes*, *Erwinia carotovora*, *Klebsiella pneumoniae*, and *Beneckea harveyi*. A *Sal*I digest of chromosomal DNA from these organisms was ligated to the plasmid pMK2004 also digested with *Sal*I, then transformed into an *E. coli* K12 *polA1* mutant. The *ori* region from the above organisms was recovered on plasmids as well as was the *polA* gene from *E. aerogenes* and *E. carotovora*. All of the *ori* containing plasmids were highly unstable in cells grown in the absence of selection. The size of ori^+ inserts ranged from 6.4 Kb in the case of *B. harveyi* to 18 Kb for *E. aerogenes*. The plasmids derived from *E. carotovora* were the only ori^+ plasmids that could be transformed into a $polA^+$ *E. coli* strain. This may be due to the absence of the *uncB* gene on the *E. carotovora* ori^+ plasmids, which do carry the *asn* gene. Genetic and restriction maps of these *ori* regions as well as the sequences will be presented, and comparisons made between these *ori* regions from members of the Enterobacteriaceae.

We have studied promoters within the origin region of *S. typhimurium* using a plasmid, pMC489. This plasmid carries a promoter deficient *lac* $Z(\alpha)$ and a single *Bam*HI site for detecting promoters in inserted *Bam*HI or *Sau*3A fragments. The only *Bam*HI or *Sau*3A fragment near or within the *ori* region of *S. typhimurium* that contained promoter activity was the 106 bp *Bam*HI fragment from 0 to -106 and then only in one orientation, so that the direction of transcription initiated within this fragment was away from the origin.

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877 DISSECTION OF ESCHERICHIA COLI ORIGIN OF DNA REPLICATION AND THE FRAME MODEL. Y. Hirota*, A. Oka+, K. Sugimoto+ and M. Takanami+, * National Institute of Genetics, Mishima, Shizuoka-ken, Japan 411. + Institute for Chemical Research, Kyoto University, Uji, Kyoto-fu, Japan 611.

The precise location and size of the E. coli replication origin at the nucleotide level has been determined: the left hand end of the origin is between positions 24(A) and 37(A) and right hand end is between positions 267(C) and 268(A), and the maximum size of the ori-segment is 245 base pairs (M.G.G., 178, 9-20, 1980, Oka et al.).

A series of mutations having short sequences inserted or deleted in the vicinity of restriction sites within the 245 base pairs were introduced: BglII-2 (Ori^+ phenotype: +4, Ori^- phenotype: -16), BamHI-3 (Ori^-: +4, -4, -15), AvaII-1 (Ori^-: +3, +2, -3, -4, -5, -6, -7, -8), or HindIII-1 (Ori^-: +4, -5, -7, -8; Ori^+: -12). All the above mutants carrying insertion or deletion mutation exhibited Ori^- phenotype with two exceptions: a Ori^+ mutant inserted GATC sequence at the BglII-3 site between positions 42(C) and 43(T) created at the same sequence at positions 22(A) to 32(A) as the wild type sequence, A·AT·TATTTA, and a deletion mutant deleted 12 base pairs at the HindIII-1 site between 244 (A) and 257(G) created the same sequence, ACAG·····CAC, at positions 254(A) to 267(C) as in the wild type. All the base substitution mutations of G to A or C to T at the following sites of restriction enzymes within the 245 base pairs (marked with *), introduced by in vitro mutagenesis, exhibited Ori^+ phenotype: BamHI-3 (GGATCC), AvaII-1 (GGACC), and HindIII-1 (AAGCTT).

Based on these results, we proposed "frame model" for DNA replication origin (Nucl. Acid. Res. & Molec. Biol. 1980, Hirota et al.): the specific nucleotide sequences that determine the sites where DNA initiation proteins function, the recognition sequence, and the sequences that determine the recognition sites for the initiation proteins are separated by nucleotides at a precise distance, the distance sequences, are connected with each others in the chimeric fashion. Based on the frame model, the striking differences among the bases at the positions, as the result of insertions or the deletions within the 245 base pair segment, could be demonstrated by the frame shifts.

Plasmid Replication Origins

878 STRUCTURE-FUNCTION RELATIONSHIPS IN ESSENTIAL REGIONS FOR PLASMID REPLICATION. Donald R. Helinski, David Stalker, Avigdor Shafferman, Roberto Kolter and Aslihan Tolun, University of California, San Diego, La Jolla, CA 92093.

The essential regions of replication for a variety of plasmids have been isolated using recombinant DNA techniques. These regions are responsible not only for the replication properties of the parent plasmid but also specify plasmid incompatibility (inability of similar plasmids to co-exist in a bacterial host). We have been concerned with the structural features of the replication-incompatibility region of three plasmid elements R6K (38Kb), RK2 (56Kb) and F (94.5Kb). Plasmid R6K exhibits three origins of replication both in vivo (J. Crosa, J. Biol. Chem., in press) and in an in vitro replication system (N. Inuzuka, M. Inuzuka and D. Helinski, J. Biol. Chem., in press) that are designated α, β and γ. The R6K γ-origin has been localized to a 240 bp region that contains seven 22 bp direct repeats. Deletion of one or two of these repeats results in an unstable replicon while deletion of four or more of the repeats leads to a non-functional γ-origin. A plasmid-specified protein, designated π, which is autoregulated in its expression, is required for the initiation of replication from the γ-origin. The complete nucleotide sequence has been obtained for the R6K γ-replicon consisting of the γ-origin and the π gene. The π protein also is required for the initiation of replication at the R6K α and β origins. The π protein, however, does not appear to be a regulatory element for the initiation of replication in that its concentration can be varied over a 100-fold range without a substantial change in plasmid copy number.

The replication origin of plasmid RK2 has been localized on a 393 bp Hpa II restriction fragment that contains five 17 bp direct repeats. An adjacent region of 140 bp, that contains an additional three copies of the 17 bp direct repeats, contributes to the incompabitility properties of plasmid RK2. Deletion of one or more of these three direct repeats reduces the level of expression of incompatibility (C. Thomas, D. Stalker and D. Helinski, Molec. gen. Genet., in press). Similarly, the deletion of one or more of the 22 bp repeats of plasmid R6K substantially reduces the level of R6K incompatibility expressed by the direct repeat region of R6K (S. Yang, unpublished observations). Direct repeats also have been identified in an incompatibility region (designated incB) of the F plasmid. Five 22 bp direct repeats are located within a 251 bp segment of incB. A 58 bp segment containing two of the 22 bp repeats has been cloned onto the plasmid pACYC184 which is normally compatible with the F plasmid. The resulting hybrid plasmid is incompatible with F plasmid derivatives. Thus, in the case of three different plasmids a striking feature of the essential region of replication is the presence of direct repeats. In the case of plasmid R6K the repeat region is essential for the activity of three different origins of replications. In addition, for all three plasmid systems, direct repeats play a role in plasmid incompatibility.

879 DISSECTION OF THE REPLICATION REGION CONTROLLING INCOMPATIBILITY, COPY NUMBER AND INITIATION OF DNA SYNTHESIS IN THE RESISTANCE PLASMIDS R100 AND R1. E. Ohtsubo, J. Rosen, T. Ryder, and H. Ohtsubo. Dept. of Microbiology, School of Medicine, SUNY at Stony Brook, NY 11794.

pSM1 and pTR1 are high copy number derivatives of the related drug resistance plasmids, R100 and R1, respectively. EM and cloning techniques have shown that all information required for autonomous replication can be restricted to a 2.5 Kb region which includes the origin of replication. Cloning work by several laboratories shows that this region contains sequences which express incompatibility and copy number control as well as sequences required for replication. We have compared the DNA sequence in this region of the evolutionary divergents, pSM1 and pTR1. Several interesting features result from the comparison and these allow us to propose a map of the replication region. The existence of a 33K protein, which we call RepA1, and which may be positively required for replication is supported by several lines of evidence. Two protein coding frames, which we call RepA2(11K) and RepA3(7K), can be found in the region known by cloning to include incompatibility and copy number control. A region of complete non homology between mutually incompatible R1 and R100 exists within the RepA2 coding frame. Though the protein we call RepA2 can be demonstrated in pSM1, it is unlikely to play a major role in incompatibility. Elsewhere in this control region, two base pair differences can be found between pSM and pTR suggesting that 1 bp change from the wild type sequence may correspond to the higher copy number of pSM and the other to the higher copy number of pTR. These bp changes would both cause amino acid changes in the RepA3 coding frame. This is consistent with the notion that each amino acid change relaxes the function of a repressor protein; however, no 7K protein has ever been demonstrated to be produced by these plasmids.

In vitro transcription studies may shed some light on the nature of replication control. The sequence of a 91 nucleotide transcript has recently been obtained. It is known to be encoded completely within the region known to express incompatibility and is transcribed in a direction opposite to that of replication. 13 of the first 14 nucleotides of this small RNA, RNAI, are homologous to a DNA sequence near the origin. A large stable secondary structure can be predicted to form in this RNA and both the bp changes hypothesized to cause their respective copy number change occur at the top of this stem. Another transcript, RNAII, has been identified which is transcribed in a direction opposite to that of RNAI and completely

overlaps it. The 3' end of RNAII is presently unknown but it is likely to be the message for RepA1 and perhaps RepA3.

The relation between this map to the genetics and biochemistry of this plasmid's replicating system and the possible involvement of an RNA species in replication control will be discussed.

Bacteriophage Replication Origins I

880 DNA INITIATION DETERMINANTS OF BACTERIOPHAGE M13 AND OF CHIMERIC DERIVATIVES CARRYING FOREIGN REPLICATION DETERMINANTS, Dan S. Ray, Joseph M. Cleary, Jane C. Hines, Myoung Hee Kim, Mike Strathearn, Nobuo Nomura, Jon Kaguni, Laurie S. Kaguni and Margaret Roark, Molecular Biology Institute and Department of Biology, UCLA, Los Angeles, CA 90024.

Specific DNA sequences located in a 507 base pair intergenic space in the genome of bacteriophage M13 specify the initiation of each of the two strands of the duplex replicative form (RF). Initiation of the viral strand of the RF involves nicking of the DNA at a specific site within the intergenic space. The complementary strand is initiated by RNA polymerase-dependent priming at a site 24 nucleotides away from the viral strand origin. This region of the viral DNA has the potential for forming secondary structures, and specific potenital hair pins have been postulated to be involved in origin function.

Fragments of the M13 RF have been cloned into the plasmid pBR322 and found to be capable of directing M13-dependent replication of the chimeric plasmid in polA hosts. A 142 base pair Hae III fragment of M13 RF is sufficient to direct M13-dependent plasmid replication although transformation is very inefficient. Clones carrying an additional 40 base pairs of M13 DNA show a 10^4-fold increase in transformation frequency. These chimeric plasmids give rise to filamentous transducing phages carrying single stranded plasmid DNA.

Deletions have been introduced into the origin region of M13 by in vitro techniques. Plaque-forming phages have been produced which contain deletions of both the DNA sequence specifying the RNA primer for the complementary strand and the hair pins postulated to constitute part of the complementary strand origin. Models of M13 replication will be discussed in light of the replication properties of these mutants.

Replication determinants of the bacteriophages G4 and ØX174, the plasmid ColE1, the E. coli replication origin and the Salmonella typhimurium his operon have been cloned into M13 and derivative cloning vectors and found to be capable of substituting for the M13 complementary strand origin in the presence of rifampicin, a specific inhibitor of complementary strand initiation. This technique provides a means of identifying the determinants that specify a single DNA strand initiation either within a replication origin or along the length of the chromosomal DNA (i.e., determinants for the initiation of discontinuous synthesis).

Bacteriophage Replication Origins II

881 PRIMARY AND SECONDARY REPLICATION SIGNALS IN BACTERIOPHAGE λ AND IS5 INSERTION ELEMENT INITIATION SYSTEMS, Gerd Hobom, Pavel Fuchs, Manfred Kröger, Bodo Rak, Institut für Biologie III, Universität Freiburg, 7800 Freiburg i. Br., FR Germany, and Monika Lusky, Department of Molecular Biology, University of California, Berkeley, CA 94720

A comparison of the available lambdoid origin sequences allows to point out several structural features in common with the phage G4 DNA F-G intercistronic region, which can be interpreted as recognition elements of a primase promoter sequence. These putative primase promoter sequences cover most of the ori central sections and are located in leftward orientation on the A-rich strand of all lambdoid origins, in agreement with the available experimental evidence. An analysis of several new ori-mutations which alter or move apart the flanking ori sections supports the view that these are cooperatively involved in stabilization of strand separation at ori; which is necessary to expose the primase promoter site as a single stranded sequence. Transcriptional activation as a preceding step to bring about initial strand separation at ori has been studied in both the p_R promoted wild type situation and in a λric5b equivalent lac promoter model system.

Further analysis of the absolute requirement in λ initiation for the secondary replication signal (ice) which is located in the λcII gene has led us to a new and different interpretation of its activity during initiation of replication in both the λmaxi and λmini systems, which will be presented.

A replication system which has many features in common with the λmini system has been detected involving both ends of the E.coli IS5 insertion element. According to a DNA sequence and genetic analysis this 1195 bp element codes for two proteins in opposite, overlapping directions, with correlated, internal transcription signals attached to them in both orientations. Only if attached to an additional external promoter and transcribed in outside - in direction is either of the ends able to support hybrid plasmid replication, in an ori-independent mini system mode of initiation. An interpretation covering all three, the λmaxi, λ mini, and IS5 mini initiation systems will be discussed.

SV40 Replication Origins

882 MUTATIONAL ANALYSIS OF THE SV40 REPLICON, Robert F. Margolskee, Daniel DiMaio, and Daniel Nathans, Department of Microbiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205

The genome of SV40 is a double-stranded, circular DNA molecule that replicates in the nucleus of infected monkey cells. DNA replication begins at a unique site (ori), and requires the activity of the SV40 T antigen. T antigen binds to the ori region in vitro and is involved in repressing transcription of the early (T antigen) genes. We have undertaken an analysis of these regulatory interactions by constructing and characterizing SV40 mutants with deletions or base pair substitutions in and around the replication origin, at each of the three T antigen binding sites described by Tjian (Cold Spring Harbor Symposium 63 655, 1978). Mutations in site 1 generally lead to cold-sensitive plaque formation, a decrease in binding of T antigen to an ori-containing restriction fragment, a slight decrease in SV40 DNA replication, overproduction of early RNA and T antigen, and decreased synthesis of late (virion) proteins. Thus these mutants appear to have defects in autoregulation of early transcription and production of late gene products, presumably related to reduced binding of T antigen to site 1. The properties of mutants with single base pair changes in site 2 depend on the particular base substitution. The most defective mutant produces very small plaques and shows a marked decrease in DNA replication, little change in T antigen production, and approximately normal binding of T antigen to an ori-containing fragment. (Deletions in site 2 described by Gluzman et al, Cold Spring Harbor Symposium 44, 293, 1980, appear to be completely defective in DNA replication.) Site 2 thus seems more directly involved in initiation of DNA replication than site 1. Deletions in site 3 have no effect on plaque size; they have not yet been further analysed.

We have also isolated second-site revertants of a number of the defective binding site mutants. Several of the suppressing mutations have been mapped to a limited segment of the T antigen gene. The pseudorevertant T antigens that correct origin defects fall into two functional classes: those that function well with many different mutant origins and those that function well with only a limited number of ori sequences. Pseudorevertants of the first type have allowed the isolation of a new set of ori mutants. We are now testing the hypothesis that the altered function of mutant T antigens is related to a change in their binding properties, i.e., that the mutations are in a functional domain of T antigen that interacts with the ori and/or autoregulatory binding sites.

883 FUNCTIONAL ANALYSIS OF THE THREE SV40 T ANTIGEN BINDING SITES, Robert Tjian and Richard M. Myers, Department of Biochemistry, University of California, Berkeley, CA 94720

We have cloned a 311 base pair restriction fragment containing the SV40 origin of DNA replication, the early promoter, and the three T antigen binding sites into pBR322. This recombinant plasmid, pSV01, replicates efficiently in monkey cells when provided with large T antigen. Deletion mutations in site I, the strongest T antigen binding site, and in site II were generated by *in vitro* mutagenesis in order to define the origin functionally. Sequences in site II, near the Bgl I cleavage site, are required for DNA replication. We found that sequences in site I are also required for replication. Plasmids lacking site III sequences have been generated and are being analyzed for their ability to replicate in monkey cells.

A rapid filter binding assay and methylation protection experiments have been used to measure the binding of purified T antigen and D2 protein to wild-type and mutant origin sequences. In all cases examined, mutant DNAs deficient in binding are also unable to replicate. These results strongly suggest that the mechanism for the initiation of viral DNA synthesis involves a direct interaction of T antigen with SV40 origin sequences. DNAse protection studies with wild-type and mutant origins indicate that the binding of T antigen and D2 protein to sites II and III is diminished when site I is absent; thus, binding to sites II and III is cooperative. The reduced binding of T antigen to sites II and III in mutant DNAs lacking site I may be responsible for the inability of these DNAs to replicate in monkey cells.

Electron microscopic data indicate that the predominant form of the D2 protein, both in free form and bound to the origin sequences, is a tetramer. When the protein is negatively stained and examined by microscopy, additional higher-order structures composed of three tetramers are seen. The kind of protein:protein interactions that form these dodecamers may be responsible for the cooperative binding of the protein to the three sites at the SV40 origin of replication.

Initiation Genes in E. Coli

884 INVOLVEMENT OF recA GENE IN THE REPLICATION PROCESS, Karl G. Lark and Cynthia A. Lark, University of Utah, Salt Lake City, UT 84112

Treatments which induce excessive production of recA protein (mutagenesis, thymine starvation, etc.) induce a form of DNA replication which continues for many cycles even in the absence of protein synthesis. This recA dependent DNA replication is a necessary component of error prone repair and requires continued recA activity. It is abolished at 42° in strains which carry a $recA_{ts}$ allele. A dominant mutation, dnaT, which prevents this replication and concomitantly reduces mutant production by MMS has been described.

Mutagenesis of the region of the chromosome in which dnaT is located, has produced mutants in which DNA replication can proceed in the absence of protein synthesis and in which all replication appears to be recA dependent. Lysates, from such cells in which DNA replication has ceased at 42° in the absence of recA function ($recA_{ts}$), cannot synthesize DNA in vitro. However, in vitro DNA synthesis can be restored by addition of recA protein.

Mutagenesis of the recA region of the chromosome also has produced mutants in which replication proceeds without protein synthesis. Neither of these mutants are constitutive overproducers of recA protein. Moreover, overproducers of recA protein do not manifest this type of DNA replication. The properties of these mutants will be discussed from the viewpoint of a model of recA-dependent DNA replication.

885 INITIATION OF CHROMOSOME REPLICATION IN Escherichia coli: THE dnaA GENE AND oriC, Kaspar von Meyenburg, Flemming G. Hansen and Egon Bech Hansen, Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby-Copenhagen, Denmark

The dnaA gene had been identified through the isolation of temperature sensitive initiation mutants (1,2); it has now been mapped precisely through the cloning on specialized transducing phages λtna (3); it is located between the dnaN and the rimA gene at 82,5 min (4, 5, 6); it codes for a slightly basic polypeptide with an apparent molecular weight of 54000. The dnaA protein is required for initiation of replication at oriC as demonstrated by the cessation of replication of a minichromosome (pOC2) at 42°C in a dnaA-Ts host (7). Maintenance of minichromosomes pOC24 and pOC34 (8) which carry considerably less chromosomal DNA from the oriC region also appear to be dependent on an active dnaA protein. Growth of integratively suppressed dnaA-Ts strains carrying these minichromosomes (which carry the ampR determinant) is totally restricted above 37°C on ampicillin containing medium. Different dnaA alleles exhibited strong differences with respect to maintenance of the minichromosomes at the permissive temperature, the dnaA167 allele allowing good, dnaA508 very poor maintenance only. Thus, the effect of the dnaA protein on initiation of replication is exerted within the 422 base pair oriC containing segment carried on pOC34 (8). Inactivation of the dnaA gene through insertion of the transposon Tn10 into the dnaA gene on λtna406 (3) now allows for a rigid test of the possible essentiality of the dnaA gene for processes other than initiation of chromosome replication.

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Enzymology of Initiation in E. Coli

886 PRIMOSOMES IN DNA REPLICATION, Robert Low, Ken-ichi Arai, Naoko Arai, Joan Kobori, Peter Burgers, Ulrich Hübscher, LeRoy Bertsch and Arthur Kornberg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305

Synthesis of a complementary strand to match the single-stranded, circular, viral (+) DNA strand of phage ϕX174 creates a parental duplex circle (RF). This synthesis is initiated by the assembly and action of a priming system, called the primosome. Of the seven proteins that participate in the assembly and function of the primosome, most all of the components remain even after the DNA duplex is completed and covalently sealed. Remarkably, the primosome in the isolated RF obviates the need for supercoiling of RF by DNA gyrase, an action previously considered essential for the site-specific cleavage by gene A protein starting viral strand synthesis in the second stage of ϕX DNA replication. Finally, priming of the synthesis of complementary strands on the nascent viral strands to produce many copies of progeny RF utilizes the same primosome, requiring the addition only of prepriming protein i. Thus a single primosome, that becomes associated with the incoming viral DNA in the initial stage of replication, may function repeatedly in the initiation of complementary strands at the subsequent stage of RF multiplication. These patterns of ϕX DNA replication suggest that a conserved primosome also functions in the progress of the replicating fork of the E. coli chromosome, particularly in initiating the synthesis of nascent (Okazaki) fragments.

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887 INITIATION OF BACTERIOPHAGE T7 DNA REPLICATION, Stanley Tabor, Michael J. Engler, Carl W. Fuller, Robert L. Lechner, Steven W. Matson, Louis J. Romano, Haruo Saito, Fuyuhiko Tamanoi, and Charles C. Richardson, Dept. Biological Chemistry, Harvard Medical School, Boston, MA 02115

We have used phage T7 as a system for studying the initiation of lagging strand synthesis at the replication fork and the initiation of DNA replication at the T7 origin. RNA primers synthesized by the T7 gene 4 protein initiate lagging strand synthesis. The gene 4 protein binds to single-stranded DNA and translocates along the DNA in a 5' to 3' direction in search of specific recognition sites. The gene 4 protein then catalyzes the synthesis of tetraribonucleotide primers complementary to the template. Using DNAs of known sequence we have shown the predominant recognition sequence for the gene 4 protein to be 3'-CTGGG-5' or 3'-CTGGT-5'; the products of synthesis at these sites are RNA primers having the sequence, pppACCC or pppACCA. The sequences, 3'-CTGGA/C-5' and 3'-CTGTN-5', are used less frequently. These primers are extended by T7 DNA polymerase, thus accounting for lagging strand synthesis.

Using a set of deletion mutants we have shown that the primary origin of T7 DNA replication in vivo is located within a 100 base-pair region 15% of the distance from the left end of the T7 DNA molecule (position 15); a secondary origin is present at approximately position 4. We have determined the nucleotide sequence of the primary origin region; this intergenic segment is AT-rich, contains a single gene 4 protein recognition site, and is immediately preceded by two tandem T7 RNA polymerase promoters.

Using purified proteins we have obtained initiation of DNA replication at the primary origin of T7 DNA as measured by the formation of replication bubbles. In addition to T7 DNA polymerase and gene 4 protein, T7 RNA polymerase and 4 rNTPs are required for initiation; no initiation occurs on T7 LG37 DNA which lacks the primary origin. We have also studied initiation of T7 DNA replication in vitro using a plasmid, pBR322, into which the primary origin region has been cloned. DNA synthesis on this plasmid is also dependent on T7 RNA polymerase and rNTPs. DNA synthesis is specific for plasmids containing the primary origin region provided the plasmids are first converted to linear forms. We have also cloned the primary origin into phage M13. Using this clone we have used in vitro site-specific mutagenesis to alter the AT-rich region, the RNA polymerase promoters and the gene 4 protein recognition site in order to determine the role of these elements in initiation both in vivo and in vitro.

888 STUDIES ON FORK INITIATION IN THE T4 BACTERIOPHAGE DNA REPLICATION SYSTEM,
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The elongation phase of bacteriophage T4 DNA replication has been duplicated *in vitro* with 7 highly purified, phage-induced proteins (1). With the goal of also reconstituting replication fork initiation, we have focused attention on the T4-induced ATP-dependent topoisomerase, the product of genes 39, 52 and 60 (2,3). Topoisomerase-deficient mutants exhibit greatly reduced rates of initiation, but normal rates of chain-elongation *in vivo* (4). A simple model for its involvement in initiation is that the enzyme facilitates local helix denaturation at a replication origin, perhaps by site-specific gyration (2). The region of single-stranded DNA thus created should be a competent substrate for assembly of the RNA priming complex (gene 41 and 61 proteins) and DNA polymerase (gene 43 protein) and its accessory proteins (gene 44/62 and 45 proteins). Replication fork movement would then proceed beyond the origin region with helix unwinding facilitated by the gene 32 protein and the presumptive helicase activity of the gene 41 protein.

The sites of topoisomerase interaction with DNA have been examined using filter-binding assays for protein-DNA complexes and by mapping the sites of double-stranded DNA cleavage induced by the enzyme during aborted topoisomerase reactions. The enzyme acts at many sites on all duplex DNA substrates tested, but the strength of interaction at these sites varies widely. The mapping of several very strong cleavage sites on the T4 genome may help identify replication origins.

The basic enzymology of the topoisomerase is also being pursued. Topoisomerization by the T4 enzyme has been shown to proceed via a reversible double-strand break mechanism (5). However, we have found that the enzyme preferentially binds to single-stranded DNA and cleaves the DNA at specific sites with concomitant attachment of protein to the DNA. These sites have been preliminarily located at or near hairpins. These newly-discovered single-strand reactions are not observed with DNA gyrase, and they could be important for the initiation of bacteriophage T4 DNA replication.

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Replication Origins in Eukaryotic DNA I

889 ISOLATION AND CHARACTERIZATION OF FUNCTIONAL CENTROMERE DNA FROM YEAST,
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We have cloned yeast DNA segments containing centromere-linked genetic loci from chromosomes III (CDC10, LEU2, & PGK) and IX (MET14). In some cases, flanking regions surrounding these loci have been obtained by overlap hybridization screening. Biochemical and genetic evidence indicates that functional centromeric DNA sequences from chromosomes III and IX are present on plasmids pYe(CDC10)1 and pYe(MET14)2, respectively. When present on a plasmid containing a yeast DNA replicator such as ars1 or ars2, a cloned centromere DNA sequence (either CEN3 or CEN11) enables the plasmid to function as a chromosome both mitotically and meiotically. Minichromosomes containing the centromere DNA are mitotically quite stable and segregate as ordinary yeast chromosomes in the first and second meiotic divisions. Genetic markers on the minichromosomes segregate in meiosis as centromere-linked genes and are unlinked to genetic markers on other chromosomes, as would be predicted for functionally independent chromosomes. No homology can be detected by hybridization between the CEN3 and CEN11 DNAs, and these DNA sequences are not repeated in the yeast genome (as assayed by Southern blot hybridizations). Either the various yeast centromeres are unique sequences or the homology between centromeres is too small to be detected by hybridization. The functional CEN3 element can be recloned as a 550 bp fragment, and the nucleotide sequence of this fragment is being determined. Direct selection of yeast transformants containing mitotically stable plasmids from a yeast "library" constructed in vector YRp7 yields plasmids of two classes; those containing fragments of 2 micron DNA (which segregate 4+:0- through meiosis), and several others containing centromeric DNA (these segregate 2+:2- through meiosis). By using this direct selection technique, we have reisolated plasmids containing CEN3 and CEN11 and, in addition, have obtained several new centromeric DNA sequences.

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890 DNA SEQUENCES THAT ALLOW THE REPLICATION AND SEGREGATION OF YEAST CHROMOSOMES.
Dan T. Stinchcomb, Carl Mann, Marjorie Thomas, and Ronald W. Davis, Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

By introducing clonally isolated DNA molecules into the yeast Saccharomyces cerevisiae, we have identified sequences that control DNA replication and segregation. YIp5, a vector that contains the yeast gene URA3, does not transform a strain bearing a rearrangement of ura3 to Ura+, presumably because of insufficient DNA homology to allow homologous recombination and integration. One segment of chromosomal DNA when inserted into YIp5, transforms the deletion mutant at high frequency (5,000 - 20,000 colonies per µg DNA). This DNA fragment (termed arsl for autonomously replicating sequence) allows all colinear DNA to be maintained in a yeast cell in an autonomously replicating state. arsl is cis-acting, it can integrate into other chromosomal replication units and it can function in multimeric form; arsl behaves as an origin of replication (1).

We searched for other arses using a selection scheme based upon the ability of ars-containing hybrids to transform the strain containing the ura3 rearrangement. Hybrid YIp5-E. coli DNA molecules failed to produce transformants. However, collections of molecular hybrids between YIp5 and six eukaryotes (yeast, N. crassa, D. discoideum, C. elegans, D. melanogaster, and Z. mays) do transform the deletion mutant. The transformants behave similarly to strains transformed by molecules containing a yeast ars. These arses may allow autonomous replication in their homologous organisms (2). The sequences responsible for the replication of two arses have been studied in detail. ars-containing hybrid molecules are mitotically unstable; transformants continually produce progeny that lack the autonomously replicating molecules. A DNA fragment (CEN4) near the centromere-linked trpl gene, alleviates this instability. Circular molecules of 10-50 kilobase pairs carrying arsl and CEN4 replicate and segregate properly during mitosis. In meiosis, the transformed phenotype primarily segregates $2^+:2^0$. The data indicate that the single copy of the autonomous plasmid replicates exactly once during premeiotic S phase, the two copies move to the same pole during the first reductional division and subsequently segregate to opposite poles in the second equational division. Therefore, CEN4, like its predecessor CEN3 (3), behaves as a centromere upon yeast transformation. The yeast transformation system provides an in vivo assay that functionally defines the DNA sequences at which DNA replication (ars) and segregation (CEN) may be initiated.

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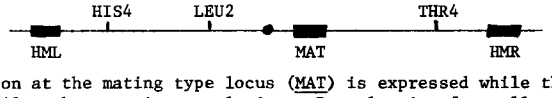
Replication Origins in Eukaryotic DNA II

891 REPLICATION OF A CIRCULAR YEAST CHROMOSOME, Carol S. Newlon and Rodney J. Devenish, Department of Zoology, University of Iowa, Iowa City, IA. 52242.

Yeast chromosome III contains three loci which specify information for mating type:



The information at the mating type locus (MAT) is expressed while the information at HML and HMR is kept silent by negative regulation. By selecting for cells which have changed mating type, chromosome III alterations can be isolated which lead simultaneously to the loss of the allele at MAT and the activation of the allele at HML or HMR. Strains in which the allele at HML is activated contain a circular chromosome which results from an intramolecular recombination between HML and MAT(1,2).

This circular chromosome is 62.6 ± 5.7 µm (∿ 190kb) in length and can be isolated as covalently closed circular DNA supercoils in CsCl-ethidium bromide gradients or on agarose gels. Based on the average spacing between replication origins in yeast DNA of 36kb (3), the ring chromosome should contain five replication origins.

We are using two approaches to study the replication of the ring chromosome. Replication origins used in vivo are being mapped by isolating replicating molecules and examining them in the electron microscope. Fragments of the chromosome capable of promoting autonomous replication of cloning vector, YIp5, not capable of replicating in yeast, are being identified and mapped on the chromosome. Progress in these experiments will be discussed.

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(3) Newlon, C.S. and W.G. Burke (1980) In ICN-UCLA Symposium on Molecular and Cellular Biology, Vol. XIX, eds. B. Alberts and C. F. Fox.

892 REPLICATION PROPERTIES OF TRP1-R1-CIRCLE: A HIGH COPY NUMBER YEAST CHROMOSOMAL DNA PLASMID, John F. Scott, Molecular Biology Institute, UCLA, Los Angeles, CA 90024

A 1.4 kb Saccharomyces cerevisiae chromosomal DNA EcoR1 restriction fragment containing the TRP1 gene and an autonomously replicating sequence (ARS1) was excised from yeast vector YRp7 (1) and ligated under dilute conditions to favor intramolecular circularization. It is likely that the ARS element represents or includes a chromosomal origin of DNA replication, although the possibility exists that it is an element required for plasmid maintenance or transmission in some other way. When yeast cells were transformed with the ligation mixture selecting for trp1 complementation colonies were obtained at high frequency (about 1000 per microgram of fragment ligated). The transformants were found to contain a 1.4 kb circular plasmid (TRP1-R1-Circle) in about 300 copies per cell. The TRP1-R1-Circle was purified from yeast and re-transformed into trp1 yeast strains containing or not containing normal amounts of the endogenous 2 μm DNA plasmid. Transformants were obtained at a frequency of 8×10^3 per microgram of DNA in an experiment where YRp7 transformants were obtained at 2×10^3 per microgram. Mitotic stabilities were compared for the plasmids in each host. The TRP1-R1-Circle was found in 85-95% of the cells after 15 generations of unselected growth in comparison with 5-15% for YRp7. Diploids homozygous for the trp1 mutation and containing the TRP1-R1-Circle were formed by mating haploid strains and subsequently selecting Trp+. They were then sporulated and tetrads dissected under non-selective conditions. The TRP1 plasmid was found to be maintained with high stability through meiosis and frequently segregated 4 Trp+ : 0 Trp- to the haploid progeny. The TRP1-R1-Circle is therefore not under the segregation control of a centromere, but is maintained with high stability mitotically and meiotically, probably due simply to its high copy number.

Further experiments will be reported which address the remarkable fact that a segment of chromosomal DNA, in effect simply excised from the chromosome and ligated into a circular molecule, becomes a high-copy-number plasmid, and the question of what features of the plasmid contribute to that property.

This work was supported in part by funds from the MBI Parvin Core Grant (USPHS CA 16163), the ACS UCLA Institutional Grant (IN-131) and an NIH Research Grant (USPHS GM 27000).

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893 REPETITIVE REPLICATION ORIGINS IN SACCHAROMYCES CEREVISIAE, Bik-Kwoon Tye, Clarence S. M. Chan and Gregory Maine, Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

Eukaryotes have complex genomes with multiple chromosomes. DNA replication is initiated at multiple sites on each chromosome. The replication of this large body of DNA is regulated so that it is completed within a well-defined period of time (S phase) in the cell cycle. It is believed that different regions of the chromosomes replicate at particular times during every S phase of the cell cycle and that there is a temporal control in the initiation of these multiple sites. Due to the limitation of conventional techniques, little is known about the nature of these initiation sites and the mechanisms which control their initiation. We have developed a method which allows us to isolate individual replication initiation sites from Saccharomyces cerevisiae.* Our approach towards understanding the control mechanism that regulates eukaryotic chromosomal replication is to examine the structure, complexity and organization of these initiation sites on the chromosomes and to study the signals which trigger their initiation.

We have isolated from yeast, a number of autonomously replicating sequences. These autonomously replicating sequences seem to fall into two categories; those that are unique, and those that are repeated in the yeast genome. So far, two classes of repetitive sequences have been isolated. The first class hybridizes to the known repetitive sequence TY1. We have shown that of all the TY1 sequences isolated, only a small fraction of them are capable of autonomous replication. Those which are capable of autonomous replication contain a replication origin within the TY1 sequence. The second class does not hybridize to TY1. In contrast to the TY1 sequences, all members of this class are capable of autonomous replication. We are presently trying to determine if this family of repetitive replication origins are co-regulated.

*Chan, C. S. M. and Tye, B.-K., Proc. Natl. Acad. Sci., 77, (1980) in press.

Procaryotic Replicons

894 NUCLEOTIDE SPECIFICITY OF THE ORIGIN OF ØX RF DNA REPLICATION, P.D. Baas, F. Heidekamp, A.D.M. van Mansfeld and H.S. Jansz, Institute of Molecular Biology and Laboratory for Physiological Chemistry, State University of Utrecht, Utrecht, The Netherlands, and G.A. van der Marel, G.H. Veeneman and J.H. van Boom, Department of Organic Chemistry, State University of Leiden, Leiden, The Netherlands

Bacteriophage ØX RF DNA replication is initiated by the introduction of a nick in the viral strand by the phage coded gene A protein. In order to study which nucleotide sequences in bacteriophage ØX174 are essential for origin function in the process of DNA replication, the following approaches were chosen:

1. Comparison of the DNA sequences around the cleavage sites of ØX gene A protein produced in superhelical covalently closed double stranded circular DNAs of other organisms;
2. Analysis of the nicking activity of ØX gene A protein on single stranded synthetic and natural DNA identifies the recognition sequence within the decamer CAACTTG$^{\downarrow}$ATA;
3. Analysis of ØX mutants with preselected base changes in the origin region induced using synthetic oligodeoxyribonucleotides indicates that the recognition sequence is degenerated (CAACTPYG$^{\downarrow}$ATA or CAACTNG$^{\downarrow}$ATA);
4. Analysis of recombinant plasmid DNA containing ØX origin sequences.

The implications of the results for the initiation mechanism of ØX RF DNA replication will be discussed.

895 REPLICATION AND INCOMPATIBILITY FUNCTIONS OF PLASMIDS OF THE X-INCOMPATIBILITY GROUP. David M. Stalker and Donald Helinski, Department of Biology, University of California, San Diego, La Jolla, CA 92093.

The X-incompatibility group of plasmids includes plasmid R485 and the extensively studied plasmid R6K. Work in our laboratory has revealed the nucleotide sequence of a large part of the replication region and demonstrated the requirement for a trans-acting protein (π protein) for initiation of R6K replication. Electron microscope analysis has revealed three origins of replication (designated α, β and γ) within the R6K replication region. Plasmid R485 is being examined for its replication properties since it is a member of the same incompatibility group as R6K. R485 is 60Kb in size and specifies sulfonamide resistance, but unlike R6K is maintained at a low copy number of 3-5 copies/chromosome in Escherichia coli. The smallest self-replicating R485 derivative obtained was 5Kb in length. This R485 derivative can be further separated into two components; a 1.2Kb DNA segment containing the functional R485 origin of replication and a 3.8Kb region which provides a product(s) in vivo which functions in trans in the maintenance of small plasmids containing the R485 origin of replication. The R6K gene for the π protein cannot substitute for the 3.8Kb region of R485 in promoting replication of DNA fragments containing the R485 origin. Although there is no extensive homology between replication regions of R485 and R6K, incompatibility between these two plasmids is quite strong. However, some homology is observed between the R485 replication region and Hind III fragments of R6K containing the α or β origins. Data will be presented on the replication properties of the low molecular weight R485 derivatives and the localization of regions of this plasmid that specify incompatibility with R6K.

896 IN VIVO AND IN VITRO PROPERTIES OF THE ØX174 A AND A* PROTEINS, Peter Weisbeek, Arie van der Ende, Fons van Mansfeld and Simon Langeveld, State University of Utrecht, Utrecht, the Netherlands.

Studies with (+) strand origin DNA of ØX cloned in pAC177 show that both in vivo and in vitro the origin fragment can act as a signal for the initiation of (+) strand DNA synthesis. The A protein produced by a superinfecting phage can use this origin, resulting in synthesis of single-stranded plasmid DNA and packaging of it into phage coats. Properties of these plasmid particles will be given.

The sequence recognized and cleaved by the A* protein have been determined. Different parts of the origin sequence can each be recognized separately by the A* protein and induce cleavage. These results can explain several aspects of the nuclease and ligase activities of the A* protein and also of the larger A protein.

897 INITIATION OF P4 DNA REPLICATION IN VITRO, Mark Krevolin, Chris Corless and Richard Calendar, University of California, Berkeley, CA 94720

Coliphage P4 contains a double-stranded DNA genome of 11.4 kb pairs. Its bidirectional "theta" form replication is dependent upon an 88 Megadalton P4 early protein called alpha (α). Since P4 DNA replication requires none of the E.coli derived initiation systems such as RNA polymerase, dnaG primase or primase-associated proteins, we believe the P4 α protein may play a role in the initiation of P4 DNA replication. To further study the initiation of P4 DNA replication we have developed an in vitro DNA replication system derived from extracts of P4 infected E.coli. This system is able to initiate the replication of exogenously added P4 supertwisted, closed circular DNA. Incorporation of [^{3}H]-TMP is linear for at least 60 min., producing precipitable nucleotide equivalent to at least 10% of the input DNA. No activity is detected from uninfected cell extracts or extracts derived from infection with P4 α amber or temperature-sensetive mutants. We have used this in vitro system to develop a complementation assay for the isolation of P4 specific proteins required for replication activity. By complementation we have purified a P4 specific protein which bands on acrylamide gels in the region identical to that of labelled α protein from P4 virl-infected cell extracts. This purified α protein is able to prime the replication of phage G4 SS to RF DNA in vitro in dnaGts background extracts where the dnaG primase activity has been heat inactivated. The ability of the P4 α protein to substitute for E.coli primase in this reaction gives further indication of an initiation role for the α protein in P4 DNA replication.

898 PRIMING OF PHAGE Ø29 REPLICATION BY PROTEIN p3, COVALENTLY LINKED TO THE 5' ENDS OF THE DNA. Margarita Salas, Juan A. García, Miguel A. Peñalva, José M. Hermoso and José M. Sogo. Centro de Biología Molecular,Madrid. Spain.

Bacteriophage Ø29 has a linear, double-stranded DNA with a protein, p3, covalently linked to the two 5' termini by a phosphodiester bond to serine (1). The replication of Ø29 starts at either end of the DNA and proceeds by a mechanism of strand displacement (2). Protein p3 is involved in the initiation of Ø29 DNA replication (3) and it has been proposed that a newly synthesized molecule of the protein acts as a primer by reaction with the 5' terminal nucleotide, dATP, and formation of a covalent protein-dAMP linkage thus providing the 3'OH group required by the DNA polymerase. Support to this model has been obtained from the finding that replicative intermediates isolated without treatment with proteolytic enzymes give rise to different circular replicating molecules, consistent with the presence of protein p3 at the termini of both parental and daughter strands.

Results obtained both in vivo and in an in vitro system of Ø29 DNA synthesis will be also discussed.

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899 PHYSIOLOGY OF INITIATION AND ELONGATION OF CHROMOSOME REPLICATION IN Escherichia coli: ANALYSIS OF NUTRITIONAL SHIFT-UP, Arieh Zaritsky and Shmuel Zabrovitz, Ben-Gurion University of the Negev, Beer -Sheva, Israel.

Detailed study of bacterial DNA synthesis is hampered by the adverse effects resulting from the low intercellular pools of thymine metabolites in Thy$^-$ strains. Deoxyguanosine can be exploited, to simulate the behavior of Thy$^+$ strains in thy, drm double mutants, and thus the use of thymine as a reliable tracer is enabled.

DNA synthesis is inhibited during the first 25 min after nutritional shift-up (from doubling time τ_1=120 min to τ_2=32 min), probably due to reduction in the intracellular concentrations of the immediate DNA-precursors.

DNA/mass ratio in the new steady state is lower (by > 16%) than predicted by current models for initiation control. Because the replication rate is at least not slower in fast-growing cells, mass at initiation per chromosome origin must increase by >16% during such a transition. The small number of chromosome origins per cell introduces a stochastic element into their response to effector(s) of initiation. Physiological studies might hint at the biochemical nature of the reaction(s) involved.

900 INITIATION OF PHAGE T5 DNA REPLICATION. R. K. Fujimura and B. C. Roop, Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830.

On the basis of observations made by us and others, we have come to the following hypothesis for T5 DNA replication. T5 DNA has a primary origin of replication at about the middle of DNA during the early phase of replication, and there are several secondary initiation sites. The primary and most of the secondary initiation sites are near promoter sites for early genes. These sites appear to be more active as initiation sites when the transcription process is active. Thus transcriptional activation appears to be the primary mechanism for activation of initiation sites. It may be caused by E. coli RNA polymerase modified by the phage infection. Another possibility for which there is some evidence is a site specific endonuclease. In some other systems, topoisomerase type II appears to participate in activation, but there is no evidence for it in T5-infected cell extracts. Primer ends may be generated by the site specific endonuclease or primer synthesized by a primase. Our in vitro system does not require rNTP's. Once the initiation site is activated and a primer is created, DNA polymerase itself is capable of recognizing that site. Other auxiliary proteins involved in the elongation process are also assembled at the site. The overall elongation process is bidirectional. (Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.)

901 GENETIC ANALYSIS OF ColE1 TYPE REPLICATION, Gianni Cesareni, Rosetta La_ catena, Luisa Castagnoli, European Molecular Biology Laboratory, 6900 Heidelberg, Germany.

The ability to reversibly integrate a plasmid into a lambda phage chromosome via int mediated recombination has enabled us to carry out a detailed genetic analisys of the replication origin of a pMB1 derivative. The integration of the plasmid into the phage in fact, confers to the hybrid structure the property to grow on a lysogen harbouring a prophage of the same immunity. This was found to be due to titration of repressor because of plasmid replication. This phenotype allowed us to isolate point mutants that are either affected in plasmid replication or its control. These mutations have been mapped in the region of approximately 600 nucleotide upstream to the origin of plasmid replication.

The analisys of the DNA sequence of some of these mutants and the study of fusions of the β-Galactosidase structural gene to the promoter(s) in the region necessary for plasmid replication allow us to present a tentative model of the mechanism of ColE1 type replication.

902 TRANSPOSITION MUTAGENESIS OF A CLONED R100.1 REPLICATION SYSTEM, Gordon Churchward, Université de Genève, Genève, Switzerland.

A 3 kilobase region of the IncFII group plasmid R100.1, composed of two PstI fragments, has been cloned onto the vector plasmid pBR322. This region contains the replication origin, and permits the hybrid plasmid to replicate in the absence of DNA polymerase I which is essential for pBR322 replication. The hybrid plasmid also expresses strong incompatibility towards R100.1, presumably reflecting expression of gene products involved in the normal regulation of R100.1 replication. Mobilization of pBR322 by the E. coli sex factor F, which results in insertion of the $\gamma\delta$ sequence of F into pBR322, provides a very convenient system for transposition mutagenesis of cloned fragments. A direct selection for mutants which no longer display incompatibility has been developed and has been used to isolate insertions of $\gamma\delta$ into the cloned DNA which abolish incompatibility towards R100.1. Nine different insertions cluster in a 150 base pair region of the cloned DNA and thus define a gene responsible for incompatibility. The plasmids containing these insertions are no longer capable of transforming a polA$^-$ strain. The properties and DNA sequence analysis of these, and other types of insertion mutants, will be described.

903 THE NATURE OF MUTATIONS AFFECTING THE REPLICATION CONTROL OF PLASMID Clo DF13
Eduard Veltkamp, Antoine Stuitje, Kees Spelt
Vrije Universiteit, Amsterdam, the Netherlands.
By in vitro recombinant DNA techniques the nonconditional and conditional Clo DF13-cop mutations were mapped within specific regions adjacent to the replication origin. The nature and position of these mutations has been determined by DNA sequence analysis. These sequence data show that single base transitions in a region of the plasmid which is assumed to be nonessential for plasmid replication, can affect the plasmid replication control. From the position and the nature of the conditional Clo DF13-cop1 (Ts) mutation it is concluded that the initiation of replication is at least regulated at the level of transcription of the origin region towards the origin. Furthermore, the position of the nonconditional cop2 and cop3 mutations within the 100 nucleotide RNA cistron, suggests that this small RNA molecule acts negatively on the regulation of replication of small plasmids such as Clo DF13 and Col E1.

904 IN VIVO ANALYSIS OF RIFAMPICIN-RESISTANT INITIATION DETERMINANTS OF ØX174,
Michael D. Strathearn and Dan S. Ray, University of California, Los Angeles, CA 90024
The ØX174 single-stranded DNA (SS) conversion to the duplex replicative form (RF) appears to be an appropriate model for E. coli lagging strand replication, initiating at multiple and possibly random sites on the ØX genome using a "mobile replication promoter." That this type of initiation does not occur on the single-stranded phage M13 or G4 templates, but does occur on ØX, suggests the existence in ØX DNA of a nucleotide sequence necessary for utilization of this initiation mechanism.

Fragments of the ØX genome have been cloned into the duplex RF of the filamentous phage M13. Fragments containing the intergenic space between the ØX genes F and G confer upon M13 the ability to carry out the conversion of phage SS → RF in the presence of rifampicin, a drug that inhibits M13 but not ØX SS → RF replication. Our aim is to determine the minimum ØX sequence required for determining the ØX complementary strand initiation mechanism in vivo.

905 Bursts of DNA-linked RNA synthesis in synchronized B. subtilis. Possible involvement of this RNA in initiation of chromosome replication.
Simone SEROR-LAURENT, Gilles HENCKES and Françoise VANNIER.
Institut de Microbiologie, Bât. 409. Université Paris XI, 91405, Orsay, FRANCE.

After thermal denaturation, an in vivo labelled RNA is found in a temperature-sensitive initiation mutant of B. subtilis, associated with high molecular weight DNA, clearly separated from other RNA species, through different techniques of separation such as Sepharose 2 B filtration, chromatography on nitrocellulose, equilibrium centrifugation in density gradients. Such aresult is obtained even when HCHO is present during denaturation and chilling of nucleic acids, after a second denaturation as well as after an incubation with proteinase K. Properties of the complex are not modified by a previous treatment with RNAase H under conditions where RNA hybridized with DNA is hydrolyzed. Our results demonstrate that DNA and RNA in the complex are associated neither via hydrogen bonds nor via a protein. They strongly suggest a covalent linkage between DNA and RNA. During synchronous replication observed after a period at non-permissive temperature, DNA-linked RNA synthesis takes place at specific times which coincide with the appearance of rifampicine-resistance of the first and the second replication cycles. The involvement of this RNA in the initiation process will be discussed.

906 FUNCTIONAL ANALYSIS OF THE E. COLI REPLICATION ORIGIN, Walter Messer, Hans-Jörg Buhk, Trinad Chakraborty, Barbara Heimann, Heinz Lother and Giovanna Morelli, Max-Planck-Institut für molekulare Genetik, Berlin-Dahlem, W-Germany

1. Regulatory sites are arranged symmetrically within the E. coli replication origin, oriC. These include: Two promoters which transcribe outward from the center of oriC (see abstract Lother and Messer), inceptors at which these transcripts are probably terminated, and binding sites for a membrane-derived DNA-binding protein (Jacq et al., ICN-UCLA Symp. XIX, in press).
2. Proteins coded for by the DNA in the immediate vicinity of oriC have been identified as products, by mapping their promoters, and by sequence analysis of the DNA. They are apparently not involved in the gross control of initiation.
3. The replication of minichromosomes requires the products of genes dnaA, dnaB (and dnaB252), dnaC, dnaG, and dnaI.

907 PROMOTERS WITHIN THE E. COLI REPLICATION ORIGIN, Heinz Lother and Walter Messer, Max-Planck-Institut für molekulare Genetik, Berlin-Dahlem, W-Germany

Promoters within and close to the E. coli replication origin, oriC, have been identified. Various restriction fragments were transcribed in vitro by purified RNA-polymerase, and the products were analyzed by polyacrylamide gel electrophoresis. Two promoters are located within the oriC segment required for efficient bidirectional replication. The transcript of Pori-1 starts at position 175 ± 7 of the oriC sequence and runs counterclockwise. The RNA at Pori-r starts at 305±8 and is transcribed clockwise. Two promoters are located outside but in the vicinity of oriC, one located at position - 30, the other at position 765. Both promote counterclockwise transcription.

908 EXPRESSION OF DNA STRAND INITIATION SEQUENCES OF ColE1 IN AN M13 PHAGE Nobuo Nomura and Dan S. Ray, Molecular Biology Institute, University of California Los Angeles, CA 90024.

Plasmid ColE1 replication initiates at a fixed origin and proceeds unidirectionally. Although the mechanism of initiation of L-strand (leading strand) synthesis in ColE1 replication has been studied extensively, that of the H-strand (lagging strand) is still unclear. In order to investigate initiation of H-strand replication of the plasmid ColE1, the origin region fragment (HaeII E) of ColE1 was cloned into the intergenic region of filamentous DNA phage M13. A site capable of promoting DNA strand initiation on a single-stranded DNA template has been detected on the L-strand of the cloned fragment. The site, named rri-1 (rifampicin resistant initiation), directs conversion of chimeric phage single-stranded DNA to parental replicative form in the presence of rifampicin, which blocks the function of the complementary strand origin of M13. The function of rri-1 is dependent on both the dnaG and dnaB gene products. It is postulated that rri-1 might be an initiation site for synthesis of the lagging DNA strand during unidirectional replication of ColE1 DNA.

Another site, named rri-2, has been detected on the H-strand of the HaeII C fragment. The analysis of characteristics of rri-2 is in progress.

909 ANALYSIS OF THE DNA REPLICATION ORIGIN OF *ERWINIA CAROTOVORA*, Yutaka Takeda, Nancy E. Harding, Judith W. Zyskind, and Douglas W. Smith, University of California at San Diego, La Jolla, CA 92093

We have cloned and characterized the origin region of *Erwinia carotovora*. Chromosomal DNA from *E. carotovora* was digested with *Sal*I, ligated with a *Sal*I digest of the plasmid cloning vehicle pMK2004, and transformed into an *Escherichia coli* K12 *polA1* mutant. Of the twelve Km^r, Ap^r, Tc^s transformants obtained, three contain plasmids with the *E. carotovora polA* gene. Plasmids isolated from two of the nine methylmethane sulfonate-sensitive clones contained a single *Sal*I fragment, 7.8 Kb in size, inserted in opposite orientation in pMK2004. These plasmids, unlike the largest *Enterobacter aerogenes ori* containing plasmids, do transform a $polA^+$ strain, and were shown to carry the *asn* but not the *uncB* gene. Deletion plasmids were constructed in order to orient *ori* and the *asn* gene. Restriction analysis of these deletion plasmids indicates that the *E. carotovora ori* region does not contain several restriction sites that are conserved in the *ori* regions of *Salmonella typhimurium* and *Escherichia coli*. The sequence of the *E. carotovora ori* region will be presented and compared to known sequences of bacterial species in the Enterobacteriaceae family.

910 REPLICATION ORIGIN REGION OF *ENTEROBACTER AEROGENES*, Nancy E. Harding, Judith W. Zyskind and Douglas W. Smith, University of California at San Diego, La Jolla, CA 92093

As part of a comparative study of the primary and secondary structures of the replication origins of the family Enterobacteriaceae, we have cloned and partially characterized the replication origin of *Enterobacter aerogenes*. Homology between this origin and that of *Salmonella typhimurium* was demonstrated by Southern blot hybridization of restriction enzyme digests of *E. aerogenes* chromosomal DNA to a probe containing an 8.6 Kb *S. typhimurium* DNA fragment carrying *ori* and the adjacent *asn* gene. The probe hybridized to an *E. aerogenes* 18 Kb *Sal*I fragment and three *Pst*I fragments of 3.1, 2.4, and 1.2 Kb. A *Sal*I digest of *E. aerogenes* chromosomal DNA was ligated to the plasmid vehicle pMK2004 and Km^r and bacterial origin function (ori^+) were selected via transformation of an *Escherichia coli* K12 *polA1* strain. Of eleven transformants, eight harbored $polA^+$ plasmids and three harbored ori^+ plasmids. All ori^+ plasmids contain an 18 Kb insert which hybridizes to three small *Bam*HI fragments (417 bp total) from the *S. typhimurium* origin. Further analysis of one of the *E. aerogenes* ori^+ plasmids, pNH3, indicated that this plasmid is highly unstable in the absence of selection and cannot transform a $polA^+$ strain. Genetic experiments using a re-ligated *Sal*I digest of pNH3 showed that this plasmid includes the *asn* and *uncB* genes. Smaller derivative ori^+ plasmids containing either a 2.4 Kb *Pst*I or a 2.0 *Bam*HI fragment were isolated and these plasmids do transform a $polA^+$ strain. A genetic and restriction map of the 18 Kb *Sal*I fragment will be presented and the sequence of the *E. aerogenes ori* region will be compared to known sequences of bacterial species in the Enterobacteriaceae family.

911 RNA TRANSCRIPTS OF THE REPLICATOR REGION OF THE R PLASMID NR1. Padmini Sampathkumar, Alan M. Easton and Robert H. Rownd, University of Wisconsin, Madison, WI 53706.
The minimal replicator region of the R plasmid NR1 is contained within two adjacent pstI fragments. The 1.1 kb fragment carries the genes for incompatibility (inc) and copy number control (cop) and the 1.6 kb fragment contains the origin of replication (ori). Using an *in vitro* transcription system, at least five RNA transcripts have been identified from the replicator region. The largest of the RNA transcripts is greater than 1100 bases in length and hybridizes to both the 1.1 kb (inc) and 1.6 kb (ori) fragments indicating that there is transcription across the junction of these two pstI fragments. A smaller RNA transcript about 340 bases long is also made from the replicator region. This transcript is not made by plasmids from which a 330 base pair Sau 3A fragment has been deleted. This deletion is located within the 1.1 kb fragment and maps about 500 bp to the left of the inc-ori junction. Mapping of the other RNA transcripts and their direction of transcription is currently in progress.

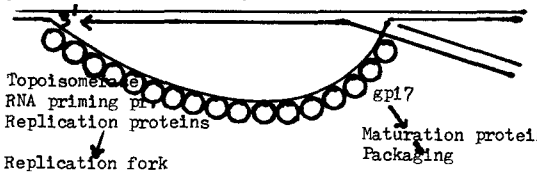
912 EFFECT OF COPY NUMBER CONTROL AND INCOMPATIBILITY ON TRANSCRIPTION OF THE REPLICATION GENES OF THE R PLASMID NR1, Robert H. Rownd, Alan M. Easton, and Verne A. Luckow, University of Wisconsin, Madison, Wisconsin 53706.
The copy number control (*cop*) and incompatibility (*inc*) gene(s) (which may be identical) of the R plasmid NR1 are located more than 1 kb from the origin (*ori*) of replication. Previous experiments have suggested that plasmid replication may be regulated by transcription starting in the *cop/inc* region which determines the frequency of initiation at the origin. We have constructed λ-*lac* phages in which the *cop/inc* region from NR1 (cop^+/inc^+), from a compatible copy number mutant (cop^-/inc^-) called pRR12, or from an incompatible copy number mutant (cop^-/inc^+) called pRR21 has been inserted adjacent to the *lacZ* gene which lacks its own promoter. In lysogens the expression of β-galactosidase is under the control of a promoter which in a plasmid would direct transcription from the *cop/inc* region toward *ori*. The β-galactosidase level in lysogens harboring a λ-*lac* without an inserted promoter or a λ-*lac* containing the *cop/inc* region from NR1, pRR12, or pRR21 was 50, 160, 400, or 1200 units, respectively, indicating an increased level of transcription from the *cop/inc* region of the copy number mutants pRR12 and pRR21. The introduction of a pBR322 vector containing a cloned *cop/inc* region into the lysogens decreased the level of expression of β-galactosidase. The decrease was larger when the λ-(*cop/inc*)-*lac* phage and the pBR322-(*cop/inc*) plasmids both had the same incompatibility phenotype (i.e. both Inc^+ or both Inc^-) irrespective of the copy number phenotype. This suggests that the *inc* gene product may act in *trans* to reduce transcription from a promoter in the *inc/cop* region which results in plasmid exclusion. The DNA base sequence of the *cop/inc* regions of NR1, pRR12, and pRR21 are presently being determined.

913 EFFECT OF TEMPERATURE AND SPECIFIC LABELLING CONDITIONS ON THE IDENTIFICATION OF T_4 BACTERIOPHAGE REPLICATION ORIGINS, Charles F. Morris and Mike Bittner, Department of Biological Chemistry, Washington University School of Medicine, St. Louis, MO 63110.

Hybridization of radioactively labelled wild type and mutant T_4 DNA isolated from T_4 infected bacterial cells to a nitrocellulose T_4 DNA restriction print sheet (Bittner *et al.*, Anal. Biochem. 102, 459-471, 1980) revealed at least four T_4 origins. Two of these origins (termed ori 3 and ori 4) appear well after ori 1 and ori 2 are clearly visible. The appearance of ori 2 is dependent upon the temperature during cell infection and the specific labelling conditions used, i.e. ^{32}P inorganic phosphate or ^{3}H thymidine. This finding is suggestive of and, in fact, consistent with previous evidence (Woucha *et al.*, J. Virol. 20, 142-156, 1976) that there are two independent, nonmixing deoxyribonucleotide pathways (a *de novo* pathway and a substrate dependent pathway) for T_4 DNA replication. [Supported by NIH grant GM26333.]

914 THE ROLE OF RECOMBINATIONAL INTERMEDIATES IN INITIATING REPLICATION FORKS IN BACTERIOPHAGE T4, Gisela Mosig, Andreas Luder and Susan Bock, Vanderbilt University Dept. of Molecular Biology, Nashville, TN 37235

To analyse interactions and competions of proteins or functional protein subcomplexes during initiation of DNA replication, we have investigated interactions between mutations in T4 "DNA" genes. We have also analysed replicative and recombinational intermediates which accumulate in these mutants.- T4 replication forks can be initiated from specific origin sequences as well as from recombinational intermediates. What kinds of interactions are involved in this second alternative mode of initiation? We propose that recombinational intermediates whose displaced single-stranded segments are covered with gene 32 protein (HDP) initiate assembly of secondary replication forks. Gps 39 and 52 (topoisomerase) and gp61(RNA priming protein) are important components of this assembly. At later times the maturation protein gp17 COMPETES in binding to such D-loops, i.e. it channels recombinational intermediates into the maturation pathway and prevents assembly of replication forks. Three lines of results led to this model: 1) certain combinations of mutations in genes 32, 39, 52 and 61 specifically prevent initiation from recombinational intermediates. 2)certain gene 32 mutations affect packaging and select for gene 17 mutations. 3)gene 17 mutations partially restore replication of topoisomerase mutants. Additional data will be presented.



915 THE MOLECULAR BASIS FOR A PLASMID COPY NUMBER MUTANT, Barry Polisky, Mark Muesing and Joseph Tamm, Department of Biology, Indiana University, Bloomington, IN 47405.

The cop$^-$ plasmid pOP1Δ6 is a recessive copy number mutant derived from ColE1. pOP1Δ6 exists at 200-300 copies per chromosome in E. coli, while ColE1 exists at 10-15 copies per chromosome. We have investigated the molecular basis for DNA overproduction by pOP1Δ6 by mapping the mutation to a restriction fragment of the plasmid genome which is about 400 bp from the origin of replication. We determined the nucleotide sequence of the appropriate mutant and cognate wild type DNA fragments. We have found that the mutation in pOP1Δ6 DNA is a single base pair alteration--a GC→TA transversion. This alteration occurs in a region of the plasmid genome which encodes a small, non-translated RNA, known as RNA1. The mutation is located in the GC rich region of dyad symmetry which precedes the stretch of U residues comprising the termination signal for RNA1 transcription. The mutation potentially destabilizes the stem preceding the terminus, allowing the prediction that the DNA overproduction phenotype of pOP1Δ6 is a consequence of readthrough transcription of RNA1. When pOP1Δ6 DNA is transcribed in vitro, RNA1 is not observed, although RNA1 is synthesized by the cop$^+$ cognate plasmid of pOP1Δ6, pNOP1. Rather, several new transcripts of larger size are observed from pOP1Δ6 DNA, which are not seen from pNOP1 DNA. In conjunction with previous genetic evidence, these results indicate that RNA1 is a negative modulator of ColE1 replication. We suggest that the secondary structure of RNA1 is a critical feature in its function as a copy number control element.

916 ORIGIN OF REPLICATION OF pT181 PLASMID IN S. aureus. Saleem A. Khan, Gail K. Adler, and Richard P. Novick, Public Health Res. Institute, New York, N.Y. 10016

pT181 is a naturally-occuring 4.4 kilobase S. aureus plasmid encoding resistance to tetracycline. The plasmid has a copy number of 20 per cell and a deletion mutant, Cop 608, has a copy number of 800 (S. Carleton, unpublished). Replication of pT181 DNA in vitro requires a plasmid coded product (RepC protein). The in vitro origin of replication has been located by using the dideoxy method and the replication proceeds unidirectionally. Studies using deletions of pT181 DNA in vitro, indicate functional origin of replication to encomprise 181 bp. Furthermore, the incompatibility region has been localized to within 105 bp and it falls within the origin. Nucleotide sequence of the origin and the surrounding area shows that the mutant, Cop 608, has a deletion of 179 bp adjacent to the origin of replication. This deletion apparently results in an increased copy number of the Cop 608 plasmid. The possible mechanisms for replication and copy number control of pT181 plasmid will be discussed.

917 INHIBITION OF COLE1 RNA PRIMER FORMATION BY A PLASMID-SPECIFIED SMALL RNA, Tateo Itoh, Gerald Selzer, Tapan Som, Hisao Masukata and Jun-ichi Tomizawa, National Institutes of Health, Bethesda, MD 20205

Transcription of ColE1 DNA by RNA polymerase in vitro initiates at two sites in a region required for maintenance of the plasmid. Certain transcripts that initiate at one of the sites can be cleaved by RNase H and then act as primers for DNA replication. Transcription from the other site produces an RNA 108 nucleotides in length (species I or RNA I). Transcripts analogous to the primer and RNA I of ColE1 are produced when p15A or small derivatives of two other ColE1-compatible plasmids, CloDF13 and RSF1030 are used as template. If purified RNA I is added to the transcription reaction containing RNase H, formation of primer is inhibited. Each RNA I can inhibit primer formation by the plasmid which specifies it, but has no effect on primer formation by heterologous templates. Thus the inhibition of primer formation by RNA I is incompatibility specific. Because RNA I does not inhibit initiation or propagation of transcription, or the processing of preformed precursors, the step that is sensitive to inhibition is probably formation of the hybrid between the primer precursor and the template that is the required substrate for RNase H. Experiments with recombinant plasmids show the region that determines the specificity of response to RNA I to be well upstream of the origin of DNA replication.

918 ϕ29 DNA-TERMINAL PROTEIN: THE STRUCTURE AND EVOLUTION, Hirofumi Yoshikawa and Junetsu Ito, Dept. of Microbiology, College of Medicine, University of Arizona, Tucson, Az. 85724

Bacillus subtilis phage ϕ29 contains a linear, double-stranded DNA molecule of approximately 18,000 base pairs. Unlike most bacteriophage genomes, ϕ29 DNA is neither circularly permuted nor terminally redundant, but it contains short inverted terminal repetitions, six nucleotides long. Furthermore it contains terminal protein covalently linked to the 5' ends. This DNA-terminal protein has been implicated as having a direct role in the initiation of ϕ29 DNA replication. We have isolated the ϕ29 DNA-terminal protein from each end separately, and compared their two-dimensional peptide maps. Our results indicated the two proteins to be identical. To understand the general significance of the DNA-terminal protein for DNA replication we have extended our study to other small Bacillus phages. Genomes of four phages examined (ϕ15, Nf, M2Y and GA-1) contained DNA terminal proteins. The apparent molecular weights of their terminal proteins are similar (29-30 K daltons), but two dimensional peptide maps of the terminal proteins have shown clear differences among these phages. These results are consistent with the notion that the terminal proteins are encoded by viral DNA rather than by the host chromosome. The DNA sequence data showed that all these phages contain short inverted terminal repeats: 5'-AAAGTA(ϕ29 and ϕ15), 5'-AAAGTAAG(Nf and M2Y) and 5'-AAATAGA(GA-1). As part of a study of the structure and function of DNA-terminal proteins we have determined the nucleotide sequence of the DNA fragment containing the structural gene of the ϕ29 DNA-terminal protein.

919 KINETIC AND CELL CYCLE ASPECTS OF oriC PLASMID REPLICATION, Alan C. Leonard, John A. Hucul and Charles E. Helmstetter, Dept. of Exp. Biology, Roswell Park Memorial Institute, Buffalo, NY 14263

Replication of plasmids containing the E. coli chromosomal origin of replication (oriC) was investigated in exponential-phase cultures, synchronously dividing cultures, and cultures of dnaA and dnaC mutants in which initiation of chromosome replication had been synchronized by temperature shifts. Plasmid replication was assayed by measurement of incorporation of tritiated thymidine into DNA isolated in alkaline sucrose gradients, cesium chloride density gradients, or by agarose gel electrophoresis. The following results were obtained: 1) All studies in both synchronous and exponential-phase cultures indicated that plasmids controlled by oriC could replicate throughout the division cycle; 2) oriC-containing plasmids appeared to terminate replication by the conversion of a replicative intermediate dimer into the CCC monomer; 3) Examination of the transfer of prelabeled plasmid DNA into and out of the replicative intermediate pool during exponential growth indicated that oriC plasmids were chosen for replication at random; and 4) The plasmids did not interfere with initiation or elongation of the bacterial chromosome during the division cycle. The timing of initiation of oriC plasmid replication during the cell cycle appears fundamentally different from that of the chromosome, but similar to that of F plasmids. The results also suggest that the chromosome-encoded gene products required for initiation of oriC plasmid replication, including the dnaA gene product, are not determinants of the timing of initiation of chromosome replication.

920 "INCOMPATIBILITY" IN PLASMIDS CARRYING THE REPLICATION ORIGIN OF THE E. COLI CHROMOSOME. K. Yamaguchi, M. Yamaguchi and *J. Tomizawa, Cancer Research Institute, Kanazawa Univ. Kanazawa, Japan and *Laboratory of Molecular Biology, NIAMDD, NIH, Bethesda, MD 20205

We have cloned the replication origin (oriC) of the E. coli chromosome with a DNA segment coding for drug resistance. An oriC plasmid consists of a HaeII segment of the oriC region (ca.-440 — +1045 in the nucleotide sequence of the oriC region (1,2)) and the Amp^r gene from plasmid pBR322 and the other the HaeII-PstI segment (ca.-440 — +488) and chloramphenicol resistance gene from phage Plclm. These plasmids are relatively stable in a recA strain (N100) since approximately 90% of cells still retain the plasmid after growth for 10 generations under an unselective condition. They are, however, rapidly eliminated in recA1 strains as other oriC plasmids (1,3). When two oriC plasmids are introduced into the same cell, they segregate into cell lines that carry only one of the plasmids. That is they are incompatible. Furthermore, a pBR322 derivative into which the oriC region is inserted excludes rapidly coexisting oriC plasmid. In order to define the incompatibility locus, various segments in the oriC region were introduced into pBR322 and it was found that two loci can express incompatibility for the oriC plasmid independently. One (incA) includes the region essential for autonomous replication of the oriC plasmid. The other (incB) is adjacent to incA (proximal to asnA) and not functional for autonomous replication.

(1) Hirota etal., Cold spring Harb. Symp., 43,129,1978. (2) Messer et al., ibid. 43,139,1978. (3) von Meyenburg et al., ibid. 43,121,1978.

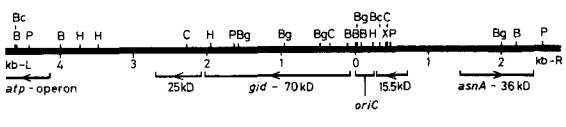
921 CONSERVATION AND DIVERGENCE IN SINGLE-STRANDED PHAGE DNA SECONDARY STRUCTURE: RELATIONS TO ORIGINS OF DNA REPLICATION, Thomas D. Edlind and Garret M. Ihler, College of Medicine, Texas A&M University, College Station, TX 77843

Using the same partial denaturing conditions employed for øX174 DNA (J. Mol. Biol. 142,131), the secondary structure of G4 DNA was analyzed by electron microscopy. Both øX174 and G4 viral DNAs were folded into characteristic three-lobed structures. The locations of stem and loop structures comprising these lobes were mapped relative to the ends of Eco RI and Pst I-cleaved G4 replicative form DNA. It was found that the secondary structure surrounding the origin of viral strand DNA replication was conserved in øX174 and G4 DNAs. This is consistent with observations that these two DNAs have similar or identical modes of viral strand replication. This conservation further supports our suggestion that secondary structure plays a role in viral strand replication, i.e., by folding the DNA to facilitate its circularization. The major difference between the secondary structures of øX174 and G4 DNAs, the presence on G4 DNA alone of a small loop within the smallest of the three lobes, was found to coincide with the G4 origin of complementary strand DNA replication. øX174 DNA lacks this unique origin, and requires several additional host functions to initiate complementary strand replication. We suggest that the structure of G4 DNA is recognized by specific host proteins and promotes initiation complex formation.

922 TRANSCRIPTION AND TRANSLATION EVENTS IN THE oriC REGION OF THE E. COLI CHROMOSOME, Flemming G. Hansen, Susanne Koefoed, Kaspar von Meyenburg and Tove Atlung, The Technical University of Denmark, DK-2800 Copenhagen, Denmark

Initiation of DNA replication at the origin of replication, oriC, requires a functional RNA polymerase, the dnaA product as well as the replication apparatus (1). We have constructed pBR322 derivatives that carry DNA segments from the oriC region (see figure). These plasmids have the DNA segments inserted in the HindIII site of pBR322, thereby destroying the normal tet gene promotor. In vivo functioning promotors have been identified by their restoration of tetracycline resistance. Active promotion was found between the genes for the 15.5 kD protein and the 36 kD asparagine synthetase. This transcription goes across the HindIII site in oriC and might contribute mRNA for the synthesis of the 70 kD and the 25 kD proteins. Promotion was also found to the left of the HindIII site in oriC; this may represent a weaker promotor for the gid gene. The analysis of the proteins expressed from these plasmids revealed that the 15.5 kD polypeptide which can be read from the known DNA sequence (2; W.Messer, pers.comm.) is made in vivo. The 36 kD (asnA), 70 kD (gid) and 25 kD proteins were located precisely by the use of Tn10 insertion mutagenesis and DNA sequencing. The relationship between these transcription events and initiation of chromosomal replication will be discussed.

1. K. von Meyenburg et al., CSH 43, 121,(1979); K.von Meyenburg and F.G.Hansen, Mechanistic Studies of DNA replication and Genetic Recombination, ICN-UCLA,(1980)
2. Y. Hirota et al. CSH 43, 129,(1979)



Restriction sites:
B: BamHI; Bc: BclI; Bg: BglII
C: ClaI; H: HindIII; P: PstI
X: XhoI

arrows: direction of transcription

923 PROPERTIES OF A REPLICATIVE ORIGIN COMPLEX ISOLATED FROM THE CHROMOSOME OF ESCHERICHIA COLI, M. Schaechter, H. Yamaki, W. Hendrickson and R. Balakrishnan, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA. 02111. We have isolated a complex of the replicative origin region of the Escherichia coli chromosome and certain proteins. The DNA found in this complex is highly enriched for a 26 Mdal region of the chromosome that spans the origin. Certain properties of this complex reside in a 5.9 Mdal EcoRl restriction fragment which is known to contain the origin. These properties include binding to nitrocellulose filters and to outer membrane. Deproteinized DNA from the complex does not bind to either, thus proteins are required. The proteins involved remain attached to the DNA after treatment with 5.5 M CsCl. The resulting highly purified complex still binds to outer membranes. This association requires magnesium or other divalent cations.

Replication Genes and Enzymes

924 A dnaA PHENOTYPIC REVERTANT WHICH APPEARS TO BE DEFECTIVE IN CONTROL OF THE dnaA PRODUCT. H. Eberle, N. Forrest and C. Stillman, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642.
An Escherichia coli $dnaA_{508}$ phenotypic revertant which appears to be defective in the control of the synthesis of the dnaA product has been isolated. This phenotypic revertant, PR1, has some of the characteristics of dnaAcos mutants which have been described by others and which are postulated to be defective in the self regulation of the dnaA product. Strain PR1 can replicate DNA at 42°C and it grows more slowly and poorly at 32°C than does the $dnaA_{508}$ parent strain. It also appears to over initiate DNA synthesis at 32°C. It has been demonstrated by T_4 and P_1 transduction that the PR1 mutation maps in the dnaA region. When the DNA-binding proteins of the PR1 strain, the $dnaA_{508}$ parent and E. coli C600 $dnaA^+$ strains were examined by DNA-cellulose and heparin-agarose chromatography several differences were noted. SDS polyacrylamide gel electrophoresis of these proteins revealed that a protein of 50-53 KD, which may be the dnaA product, is overproduced in strain PR1. The PR1 strain also showed greater amounts of 145, 105 and 87 KD proteins binding to double stranded DNA-cellulose. The 145 KD protein(s) have been shown to be the β and β' subunits of RNA polymerase by immunologic methods. Heparin-agarose binding studies reveal that less of a 60-63 KD protein and much more of a 38 KD protein from PR1 extracts bind at higher salt concentrations than is seen either with the $dnaA_{508}$ parent or the C600 $dnaA^+$ proteins. This mutant is currently being used to try to elucidate the mechanism whereby the dnaA product may be regulated. This work was supported by Contract No. DE-AC02-76EV03490 with the US/DOE.

925 EVIDENCE THAT THE ESCHERICHIA COLI DNAZ PRODUCT, A POLYMERIZATION PROTEIN, INTERACTS IN VIVO WITH THE DNAA PRODUCT, AN INITIATION PROTEIN. James R. Walker, W. G. Haldenwang, and Joyce A. Ramsey. University of Texas, Austin, Texas.

Temperature-sensitive (ts) dnaZ mutants of Escherichia coli are defective in chromosome polymerization at elevated temperatures. In an effort to identify other components of the chromosome replication mechanism, extragenic suppressors were sought. Ts^+ "revertants" were screened for the acquisition of cold-sensitivity. Approximately 2% of the "revertants" concomitantly became cold-sensitive (cs). Half the cs revertants had acquired second mutations which are tightly linked to the original dnaZts mutation and might be in the dnaZ gene (near minute 10.5). Some of the remaining suppressors were mapped near minute 82 in the sequence gyrB cs tna by P1 transduction. Transduction experiments with a set of $\lambda dnaA^+$ phages (from Kaspar von Meyenburg) and λdnaA missense and λdnaA nonsense phages (from Andrew Wright) prove that this extragenic suppressor is in dnaA. Perhaps the cs mutation results in an altered dnaA protein which stabilizes the dnaZts protein. This suggests that the initiation factor (dnaA protein) remains associated with the dnaZ protein during polymerization. This association cannot be required for $dnaZ^+$ activity in wild-type cells because dnaA mutants are not defective in polymerization.

926 DNA INITIATION: CORRECTION OF THE DEFECT IN A TS MUTANT OF HAMSTER BY NUCLEAR EXTRACT FROM WILD TYPE CELLS, R. Hand and C. Oblin, McGill Cancer Centre, Montreal, PQ H3G 1Y6

BN-2 is a temperature-sensitive mutant cell line derived from baby hamster kidney (BHK) cells. DNA synthesis is inhibited 2 to 4 h after shift to the restrictive temperature of 40°C. Fiber autoradiography studies show that initiation on replication units is the step in DNA synthesis that is inhibited (Eilen et al., J. Cell Physiol. 105:259,1980). We have now started studies to analyze the defect using an in vitro system for DNA synthesis in which cells are permeabilized by treatment with 0.01% Brij-58 so that ^{3}H-dTTP can be used to measure synthesis. Initiation on replication units is preserved in this lysed-cell system (Hand and Gautschi, J. Cell Biol. 82:485,1979). When assayed in vitro by this method, the temperature-sensitive defect in BN-2 is expressed. High salt (0.4M KCl) extracts from wild-type BHK cells that have been incubated for 3 h at 40° correct the depressed incorporation of ^{3}H-dTTP into DNA of mutant cells when added to the reaction mixture at 40°C. Extracts taken from equal numbers of cells doubles DNA synthesis in the mutant line, but produces less than a 50% increase in wild type lysed cells incubated under identical conditions. The extracts have little or no effect on mutant or wild-type cells at the permissive temperature of 33°C and extracts prepared from mutant cells and incubated for 3 h at 40° are also ineffective. High salt (0.4M LiCl) extracts taken from nuclei prepared from wild type cells ruptured with a Dounce homogenizer cause almost a two-fold increase in incorporation into mutant cells indicating that the stimulatory activity is predominantly in the nucleus. We are attempting to purify the activity.

927 GENETIC ANALYSIS OF REPLICATION DEFECTIVE MUTANTS IN SALMONELLA TYPHIMURIUM

Russell A. Maurer, Barbara C. Osmond and David Botstein, MIT, Cambridge, MA 02139

We have isolated some 60 replication defective mutants (heat- or cold-sensitive) of S. typhimurium. These mutants have been subdivided into 12 groups on the basis of transductional linkage with marker Tn10 insertions, using a spot test which allows us to score, individually, each dna mutant for linkage with each marker insertion.

For some mutants we have been able to identify recombinant λ clones carrying the corresponding dna+ gene using a plaque assay similar to that developed by J. Auerbach and P. Howard-Flanders. The plaque assay can also be used as a general method for complementation among dna- mutants.

Using these improved genetic tools we have obtained these results:

1.) Sometimes, mutants in a single cotransductional linkage group fall into more than one complementation group.

2.) We have isolated mutant λ clones carrying new negative alleles of a dna gene. In principle this method should permit recovery of absolute negative mutants which cannot be obtained in haploid strains, e.g., amber mutants. We are checking whether this is so.

3.) One of our complementation groups (which includes a mutant isolated by Rowbury, J. Gen. Microbiol. 67, 107) is the functional equivalent of the dnaC gene of E. coli.

An important goal of our experiments is to probe, in vivo, protein-protein interactions in DNA replication through the study of pseudorevertants of dna mutants. We will discuss how our new genetic tools facilitate these studies.

R.M. was supported by fellowship DRG-246FT of the Damon Runyon-Walter Winchell Cancer Fund.

928 ABSENCE OF F PLASMID REPLICATION DURING STABLE DNA REPLICATION OF THE CHROMOSOME IN AN Sdr^{C} MUTANT OF E. coli K-12, Nelda L. Subia, Ted A. Torrey and Tokio Kogoma, Department of Biology, University of New Mexico, Albuquerque, NM 87131

A mutation sdrA102 which confers upon cells the ability to replicate chromosomal DNA in the absence of protein synthesis was transferred into a plasmid-less E. coli K-12 strain. To determine whether the sdrA102 mutation relaxes the replication of stringent plasmids normally unable to replicate in the absence of concomitant protein synthesis, a Tn10-containing derivative of the F plasmid was introduced into the sdrA102 strain. The absence or presence of F plasmid replication was monitored by two methods. First, lysates from cells which had been labelled with ^{14}C thymine before and ^{3}H thymine after the addition of chloramphenicol (CM) were centrifuged in CsCl-ethidium bromide gradients to separate CCC plasmid DNA from linear DNA. The net increase in DNA synthesized during incubation with CM was determined from the ratio of ^{3}H and ^{14}C radioactivities. In the second method, F replication was monitored by measuring the copy number of the Tn10 sequence carried by the F plasmid, utilizing DNA-DNA hybridization. The results of the two types of experiments clearly indicates that F plasmid does not replicate in the sdrA mutant in the presence of CM despite the fact that bacterial DNA replicates continuously under the same conditions. The F plasmid replication ceases within a few minutes after the addition of CM. Based on these results we conclude that the effect of the sdrA102 mutation is replicon-specific.

929 SUPPRESSOR MUTATIONS (rin) THAT SPECIFICALLY SUPPRESS THE recA$^+$ DEPENDENCE OF STABLE DNA REPLICATION IN Escherichia coli, Ted A. Torrey, Gavin G. Pickett and Tokio Kogoma, Department of Biology, University of New Mexico, Albuquerque, NM 87131

The sdrA102 mutation confers upon cells the ability to replicate DNA in the absence of protein synthesis. When this mutation was combined with the recA200 which renders recA protein thermolabile, it had little effect on normal DNA replication. However, the sdrA102 recA200 double mutant exhibited temperature-sensitive stable DNA replication: it continuously replicated DNA in the presence of chloramphenicol (CM) at 30°C, whereas at 42°C DNA increased only 45-50% before stable DNA replication ceased. The amount of DNA synthesized during incubation with CM at 42°C suggested that the block might be at the initiation step. Suppressor mutants (rin-recA independence) capable of stable DNA replication at 42°C were isolated from the double mutant. In one of the suppressor mutants (rin-15), the recA200 allele was replaced by a missense mutant allele, recA56. The resultant triple mutant (sdrA102 recA56 rin-15) was found to be capable of stable DNA replication whereas the rin$^+$ counterpart was incapable due to the presence of the recA56 mutation. The sdrA102 recA56 rin-15 strain retained the characteristics of the recA56 mutation, i.e., the deficient general recombination, severe UV sensitivity and inability of prophage induction in lysogens. This indicates that the rin mutation specifically suppresses the recA$^+$ dependency of stable DNA replication. The requirement of the recA$^+$ protein for stable DNA replication may represent a third function distinguishable from the other two functions already assigned to the recA$^+$ protein, i.e. DNA strand assimilation associated with the DNA-dependent ATPase activity and the proteolytic activity in the induction of SOS functions.

930 CONTINUED DNA REPLICATION IN THE PRESENCE OF CHLORAMPHENICOL IN A dnaA rpoB DOUBLE MUTANT OF E. coli, Tokio Kogoma*, Ted A. Torrey* and Tove Atlung$^+$, Department of Biology, University of New Mexico, Albuquerque, NM 87131 and Institute of Microbiology$^+$, Øster Farimagsgade 2A, DK-1353, Copenhagen, Denmark.

Extragenic suppressors of the temperature-sensitive DNA initiation dnaA46 mutation in E. coli have been isolated and mapped at several distinct loci. One of these dnaA suppressor mutations which maps in the RNA polymerase β-subunit gene (rpoB917) not only restores DNA initiation capability at high temperatures but also renders the double mutant cold sensitive. Interestingly, DNA replication in the dnaA46 rpoB917 strain is markedly resistant to chloramphenicol (CM) although protein synthesis is abolished by the drug. This continued DNA replication in CM closely resembles the stable DNA replication observed in sdrA mutants. Both sdrA and rpoB917 dnaA46 mutants still require RNA synthesis or RNA polymerase function for reinitiation of DNA replication: the addition of rifampicin (Rif) blocks further initiation. To determine if the rpoB917 mutation alone confers upon cells the ability to reinitiate in the presence of CM, secondary mutations to Rif resistance have been isolated in the rpoB917 gene and transduced (via linkage to metB) into a dnaA$^+$ strain. The resultant Rif-resistant (dnaA$^+$ rpoB917*) transductants do not exhibit continued replication in CM, suggesting a direct involvement of the dnaA46 mutation in expression of this capacity. We have also isolated a cold-resistant derivative of the dnaA46 rpoB917 strain capable of continued DNA replication in CM or Rif. RNA synthesis in this strain is as sensitive to Rif as the dnaA46 rpoB917 parent.

931 IN VITRO COLIPHAGE N4 DNA REPLICATION, J. K. Rist,‡† A. Sugino* and L. B. Rothman-Denes,‡† †Departments of Biochemistry, and ‡Biophysics and Theoretical Biology, The University of Chicago, Chicago, IL 60637, and *Laboratory of Molecular Genetics, National Institutes of Environmental Health Sciences, Research Triangle Park, North Carolina 27709.

Coliphage N4 contains a linear double stranded DNA genome of 72 Kb pairs and a DNA dependent RNA polymerase in its virions. N4 DNA replication does not require the activity of E. coli genes dnaA, dnaB, dnaC, dnaE, dnaG and polA. Mutants in at least eight N4 cistrons affect N4 DNA replication. One cistron codes for the virion RNA polymerase required for the synthesis of N4 early RNAs. The products of three other cistrons are required for transcription of N4 middle RNAs which code for at least four functions involved in DNA synthesis (DO). Moreover, a study of the behavior of temperature sensitive mutants in the virion RNA polymerase suggests that it also plays a direct role in N4 DNA replication. We have developed an in vitro system to study the mode of initiation of N4 DNA replication. N4 infected cells are gently lysed and the soluble proteins are concentrated by ammonium sulphate precipitation. DNA synthesis in this extract is totally dependent on the addition of exogenous native N4 DNA. N4 heat denatured and other DNAs are poor templates. No activity is detected in extracts derived from N4 DO mutant infection. However, mixing of extracts from two DO mutants, in different cistrons, restores activity to nearly wild type levels. We are using this complementation assay to purify the N4 coded components required for in vitro DNA synthesis. In the course of these experiments, the cistron corresponding to the N4 coded DNA polymerase has been identified. The site of initiation of in vitro N4 DNA replication is non random. At present, we are determining the origin(s) and mode of in vivo N4 DNA replication.

932 IN VITRO STUDIES OF ØX RF → RF DNA REPLICATION. D. Reinberg, S.L. Zipursky, and J. Hurwitz. Albert Einstein College of Medicine, Bronx, New York 10461.

ØX RF → RF DNA replication involves the copying of both the (+) and (-) strands of the DNA template. It was of interest to determine whether the dnaB, dnaC and dnaG gene products play a role in the synthesis of both strands or function only to prime (-) strand DNA synthesis as they do in SS → RF. Cell-free extracts of E. coli mutants thermosensitive in dnaB, dnaC and dnaG synthesize and accumulate (+) single-stranded circular (SSC) DNA at the non-permissive temperature. Addition of purified dnaB, dnaC, or dnaG gene product to its respective inactivated extract results in the accumulation of duplex (RF molecules) rather than (+) SSC.

Our data is consistent with the hypothesis that during RF → RF replication (+) strand DNA synthesis requires only the ØX gene A protein, the rep protein, SSB and the DNA polymerase III elongation system (1) and that (-) strand DNA synthesis occurs via the same mechanism which is responsible for the initial conversion of the parental (+) strand to the parental replicative form (SS → RF).

Furthermore recombinant DNA molecules comprised of the ØX174 (+) strand origin of replication and pBR322 DNA are replicated in cell-free extracts (2). Analysis of recombinant RF → RF DNA synthesis in dnaC, dnaB, and dnaG mutant derived cell-free extracts suggests that each strand of pBR322 contains the necessary sequences to signal de novo primer formation.

1. S. Eisenberg, J. Griffith and A. Kornberg. Proc. Natl. Acad. Sci. U.S.A. (1977), 74, 3198-3202.
2. S.L. Zipursky, D. Reinberg, and J. Hurwitz. Proc. Natl. Acad. Sci. U.S.A. (1980), 77, 5182-5186.

933 EVIDENCE FOR INTERACTIONS BETWEEN PROTEINS INVOLVED IN INITIATION OF BACTERIOPHAGE T4 DNA REPLICATION, Dwight H. Hall and Paul M. Macdonald, School of Biology, Georgia Institute of Technology, Atlanta, GA 30332

We have found that mutations in genes 41 and 61, which code for proteins required for RNA priming, cause folate analog resistance. Plating techniques that eliminate T4 plaque formation on Escherichia coli by folate analog inhibition of dihydrofolate (FH_2) reductase allow the isolation of folate analog resistant (far) mutants of T4. Many of these far mutants have altered synthesis or structure of the T4-induced FH_2 reductase. In order to find new types of T4 mutants, we have isolated far mutants at 30°C and screened for those unable to grow at 43°C in the absence of folate analogs. Only one mutant, farP129, out of over 100 tested, has a single mutation causing both folate analog resistance and temperature sensitivity. This mutant induces normal levels of FH_2 reductase (coded by the frd gene) and appears to have normal expression of other T4 genes at 30°C. The results of mapping and complementation studies indicate that the farP129 mutation is in gene 41. Like other mutations in gene 41, farP129 reduces phage-induced DNA synthesis to about 15% that of wildtype T4 as measured by thymidine incorporation under restrictive conditions. Surprisingly, four other ts mutants defective in gene 41 (Caltech collection), of four tested, are also far. Three mutants defective in gene 61 have been tested and all are far whereas some other mutants defective in DNA synthesis are not far. The allele-specific suppression of phenotypes shown by double mutants carrying mutations in genes 41 and 61, 41 and frd, or 61 and frd indicates that the products of these genes interact. We suggest that abnormal interactions cause folate analog resistance by altering the structure of the FH_2 reductase. (Supported by USPHS, NIH Grant GM-24455.)

934

ISOLATION AND CHARACTERIZATION OF THE E. coli dam METHYLASE GENE. J.E. Brooks, R.M. Blumenthal, R.J. Roberts, T.R. Gingeras. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

The Escherichia coli dam (DNA adenine methylase) gene codes for an enzyme which methylates the DNA sequence GATC, and has been implicated in the postreplication correction of mismatched bases. Furthermore, the GATC sequence occurs at an unexpectedly high frequency near the E. coli origin of replication (Hirota et al., 1978. Cold Spring Harbor Symp. Quant. Biol. 43: 129-138). A 22 kb fragment containing the dam gene was previously cloned by selection for the adjacent trpS gene (G.E. Herman and P. Modrich, pers. comm.). The dam gene has been subcloned from this parent clone, using an in vitro selection with the restriction enzyme, MboI. The resulting plasmid (pdam117) contains a 1.7 kb insert which causes overexpression of the Dam+ phenotype. The nucleotide sequence of this insert has been determined which, together with in vitro transcription and Northern blot analysis, has allowed the identification of candidate sequences for the dam promoter and terminator, as well as the translational initiation and termination sites. Southern blot hybridization analysis has identified sequences homologous to the dam gene in the chromosomes of some other bacterial species. Site-specific mutants of the dam gene are currently being isolated.

935 EFFECT OF 3-DECYNOYL-N-ACETYLCYSTEAMINE ON DNA REPLICATION IN AN INITIATION MUTANT OF ESCHERICHIA COLI, Joe A. Fralick, Texas Tech University, School of Medicine, Lubbock Texas, 79430.

We have previously reported evidence that the acetylenic analogue of β,γ-decenote, 3-decynoyl-N-acetyl cysteamine (3dNAC), which blocks unsaturated fatty acid biosynthesis in Escherichia coli, preferentially affected the initiation of chromosome replication in this bacterium (Fralick, J.A. and K.G. Lark, 1973. J. Mol. Biol. 80:459). We are currently examining the effect of this analogue on chromosome replication in cells in which the dnaA gene product and hence replication complexes are limiting. Previous results have indicated that at temperatures between 30°C and 35°C the gene product of the dnaA 167 allele becomes limiting. Chromosome copy number decreases from "two" in cells growing at 30°C to "one" in cells growing at temperatures above 35°C (Fralick, J.A. 1978. J. Mol. Biol. 122:271). Over this same temperature range we find a decrease in the ability of these cells to initiate new rounds of chromosome duplication in the presence of 3dNAC. However, the temperature dependent effects of this analogue is not seen with respect to RNA or protein synthesis in this mutant. In addition no effect is observed with respect to DNA synthesis in the dnaA$^+$ parental strain or in a dnaA ts revertant strain. Similiar results have been obtained using the antibiotic cerulinin which also inhibits fatty acid biosynthesis in E. coli., indicating the effect observed is not specific for 3dNAC. These results implicate the involvement of fatty acid biosynthesis, and presumably membrane biosynthesis in the process or processes leading to the assemblage of the initiation-replication complex and/or for its activation. (i.e. initiation)

936 RNA POLYMERASE INTERACTION WITH A DNA RESTRICTION FRAGMENT CONTAINING E. coli ORIGIN OF REPLICATION, Benjamin Munson and Robert Greene, Experimental Biology Department, Roswell Park Memorial Institute, Buffalo,NY 14263

The exact role of RNA polymerase in the initiation of chromosome replication remains to be elucidated. The origin sequence has been shown to contain nucleotide sequences that are similar to those of known promoters; therefore, we have initiated a study to examine the interaction of RNA polymerase with this origin sequence using various approaches. We are using purified RNA polymerase and a 700 b.p. HaeIII restriction fragment which was obtained from the chromosomal origin containing composite plasmid pAL3. We have analyzed the binding of RNA polymerase to this fragment using the nitrocellulose binding technique and by direct gel electrophoresis of the transcription complex on agarose and polyacrylamide. Utilizing both of these procedures, we have shown specific interaction of RNA polymerase with the origin fragment similar to RNA polymerase binding to the tetracycline resistance promoter. Additionally, RNA polymerase is able to afford protection against HindIII clearage on the origin containing fragment in a manner analogous to RNA polymerase protection of HindIII site in the tetracycline resistance promoter. Results will also be presented concerning initiation of RNA synthesis on the origin fragment.

937 ISOLATION AND CHARACTERIZATION OF AMBER MUTATIONS IN THE dnaA GENE OF E. COLI, Nancy A. Schaus, Kathy O'Day, and Andrew Wright, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Mass. 02111.

Following localized mutagenesis of the tna-dnaA region of the Escherichia coli K-12 chromosome, thirty-six mutants were isolated which are defective in the initiation of DNA replication, as determined by their dependence on integrative suppression by prophage P2sig5. Through the use of a temperature sensitive amber suppressor, three of the thirty-six mutants were shown to contain amber mutations. These mutations, which are designated am311, am366, and am91, map between gyrB and tna, at 82 minutes on the E. coli chromosome. The mutations can be complemented by a λdnaA$^+$ specialized transducing phage, but not by a λdnaA46(Ts) mutant derivative. The analysis, in UV-irradiated Su$^-$ cells, of the polypeptides encoded by λ specialized transducing phage which carry each of the amber mutations indicates that, in each case, the amber mutation results in the failure to observe the dnaA polypeptide. When the UV-irradiated cells are Su$^+$, the dnaA product is restored in each case. This result, taken together with the results of the mapping and complementation studies, provide strong evidence that am311, am366 and am91 are amber mutations in dnaA.

The ability of secondary mutations in rpoB to suppress the initiation defect of the amber mutant strains was examined. Of the three amber mutations tested, only dnaA311(Am) can be suppressed by secondary mutations in rpoB under Su$^-$ conditions. In addition, rpoB mediated suppression is clearly allele specific. These results suggest that the dnaA product is required for rpoB suppression.

938 PURIFICATION OF THE β SUBUNIT OF THE DNA POLYMERASE III HOLOENZYME OF ESCHERICHIA COLI AND ANALYSIS OF ITS FUNCTION IN THE INITIATION OF DNA ELONGATION. Charles S. McHenry and Kyung Oh Johanson, Department of Biochemistry and Molecular Biology, The University of Texas Medical School, P.O. Box 20708, Houston, Texas 77025

The β subunit of the DNA polymerase III holoenzyme has been purified 11,000-fold to homogeneity from *E. coli* HMS-83. The native and denatured molecular weights of β are 72,000 and 37,000, respectively, as determined by equilibrium sedimentation. Thus, β appears to exist as a dimer when in a state free of other holoenzyme components. An antibody directed specifically against the β subunit has been prepared and used to probe the structure of the DNA polymerase III holoenzyme and the mechanism of its reaction. Immunoprecipitation studies demonstrate that the β subunit forms a complex with other holoenzyme components. This antibody has been shown to inhibit only those reactions for which holoenzyme is required and the polymerase III core alone will not suffice. Furthermore, the β antibody inhibits formation of an ATP (or dATP) requiring initiation complex with primed DNA, but not subsequent elongation. Using the β antibody to block reinitiation, we have shown that holoenzyme can replicate most of the G4 genome (> 5,000 nucleotides) without dissociating.

939 AMPLIFICATION AND STUDIES OF PROTEINS WHICH PARTICIPATE IN THE INITIATION OF BACTERIOPHAGE λ DNA REPLICATION, Roger McMacken, Johns Hopkins Univ., Baltimore, MD 21205

The bacteriophage λ O and P replication proteins act, together with numerous *E. coli* replication proteins, to establish the initiation of bidirectional DNA replication at the λ origin. In order to amplify the intracellular levels of these λ replication proteins, we cloned the λ *O* and *P* genes into several types of thermoinducible, high copy number plasmids. The level of λ P protein present in each plasmid carrier was measured, using as an assay the capacity of the P protein to complex the *E. coli dnaB* protein and block its function in the *in vitro* replication of ØX174 viral DNA. Certain of the recombinant plasmids yielded 30 fold elevated levels of λ P protein relative to the level obtained after induction of a λ lysogen. With the aid of such plasmids, we have developed a purification scheme that has enabled us to isolate homogeneous P protein. In related studies we have isolated the *dnaB, dnaG* and *ssb* genes of *E. coli* and cloned them into thermoinducible and/or high copy number plasmids. As a consequence, intracellular levels of the *dnaB,* primase and single-stranded DNA binding proteins were amplified 10 to 150 fold.

Isolation of the λ *O* gene replication protein has been hampered by its extreme vulnerability to proteolytic attack *in vivo* (half-life = 1.5 minutes). We have examined various agents known to inhibit intracellular proteolysis for their effect on the chemical stability of the λ O polypeptide. We have discovered that those metabolic poisons which cause cellular high energy phosphate pools to be rapidly depleted, such as cyanide, arsenate and azide, also induce a complete stabilization of the λ O gene polypeptide.

940 THE REACTIONS OF RecA PROTEIN. Paul Howard-Flanders, Era Cassuto & Steve West. Yale University, New Haven, CT 06511.

Work in many laboratories has led to the purification of the recA protein of E. coli and the demonstration that it promotes hydrogen bonding between single DNA strands and also between one strand and a duplex molecule (McEntee et al. 1979; Shibata et al., 1979). Because postreplication gaps in UV-irradiated bacteria induce sister exchanges with unit efficiency, we have investigated the reactions promoted by recA protein with duplex DNA containing single stranded gaps (gapped DNA). Gapped DNA is nearly as effective per nucleotide as single stranded DNA in stimulating the single strand-dependent ATPase activity of RecA protein. Moreover, in the presence of the non-hydrolysable analog .-S-ATP, RecA protein forms thick rod-like structures on gapped or tailed DNA that are readily seen by electron microscopy. If complexes are formed with ATP, they may be short lived but might participate in the search for homology. RecA protein promotes homologous pairing between gapped and intact DNA duplexes which is stable after protein removal. This binding may involve four-strand hydrogen bonding (McGavin, 1971) between the edges of the base pairs in the wide grove of the Watson Crick helix (face to face pairing). While a gap is needed for pairing, it is not necessary for the gap to be in homologous DNA for joint molecules to be formed. Moreover, joint molecules are formed normally even when unwinding is impaired by interstrand crosslinks, indicating that extensive unwinding of the free ends at the gap is not needed (West et al., 1980, Cassuto et al, 1980).

941 ENZYMATIC INITIATION OF IN VITRO DNA SYNTHESIS BY VACCINIA VIRUS INDUCED PROTEINS. Samuel H. Wilson and Kazushi Tanabe, NCI, NIH, Bethesda, Md. 20034.

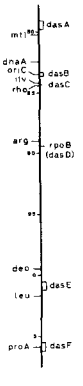
Extracts from vaccinia virus infected Hela cells were examined for an enzymatic activity capable of 1) initiating replication of denatured vaccinia virus DNA by purified vaccinia virus induced DNA polymerase, and 2) forming a heteropolymer containing both dNMP and rNMP. An extract was prepared and chromatographed on DEAE Sephacel and then on phosphocellulose (PC) essentially according to the method described by Nevins and Joklik; In fractions from the DEAE column, activity with both assays appeared in congruent sharp peaks at the 80 mM KCl point of the linear gradient used for elution. No similar peaks were detected when extracts from uninfected cells were analyzed. Fractions were pooled and chromatographed on PC at pH 7.8. Both activities co-eluted at 50 mM KCl. This PC fraction did not contain DNA or RNA polymerase activity. The fraction stimulated replication of vaccinia virus DNA by the virus DNA polymerase 20-fold. The stimulating effect did not require the presence of the fraction during the DNA polymerase reaction but could be obtained by a preincubation; this effect of preincubation was completely dependent upon both the PC fraction and nucleotides. The effect of the fraction upon DNA polymerase activity was not limited to the homologous vaccinia virus system, as replication of fd DNA by the mouse cellular DNA polymerases was also stimulated. These results demonstrating the presence of a novel rNMP-dNMP co-polymer forming activity that co-chromatographed with a DNA polymerase stimulating (initiating) activity are consistent with the existence of a primase in vaccinia virus infected Hela cells. However, the mechanism of the DNA polymerase initiation remains to be elucidated.

942 CLONING OF THE uvrC GENE OF ESCHERICHIA COLI: THE EXPRESSION OF A DNA REPAIR GENE, Surendra Sharma, Akinori Ohta*, William Dowhan* and Robb E. Moses, Department of Cell Biology, Baylor College of Medicine and Department of Biochemistry, University of Texas Medical School*, Houston, Texas 77030.

The function(s) coded for by the uvrC gene in E. coli is required for the completion of the excision repair process. Our results show that the uvrC gene product is essential for initiation of the DNA excision repair process (J. Bact. 137:397, 1979). Using F' 150 as the DNA source to enrich the initial numbers of uvrC gene molecules, we have cloned restriction fragments carrying the complete uvrC gene and its derivatives in the plasmid vector pBR322. These hybrid plasmids complement the UV sensitivity of uvrC$^-$ cells. A 1.9 Kb E. coli DNA fragment contains the structural gene for uvrC function. Further analysis of various hybrid plasmids indicates that the uvrC gene may be a part of a complex genetic assembly with respect to its promoter and structural gene. A fine structure restriction map encompassing the uvrC gene has been prepared using endonucleases Hpa II, Hae III, Taq I and Sau 3A. We have used these hybrid plasmids to identify the protein product(s) of the cloned uvrC gene employing maxi-cells (Sancar and Rupp, J. Bact. 137:692, 1979).

943 ANALYSIS OF 6 dnaA SUPPRESSOR LOCI IN E.COLI, Tove Atlung, Institute of Microbiology, University of Copenhagen.

A genetic approach was used to study the function of the dnaA product in the initiation of DNA replication. Spontaneous suppressor mutations for the temperature-sensitivity of the dnaA46 mutant were isolated to identify proteins with which the dnaA protein interacts and possibly the site of action of the dnaA protein in the oriC region. Six loci for dnaA suppression ("das") were found by this selection. They have been mapped by Hfr and F' matings and P1 transductions. The dasB, dasC and rpoB (dasD) were mapped precisely by transduction with specialized transducing phages λasn, λilv and λrif, and by complementation with plasmids carrying DNA segments derived from these phages. The dasC and rpoB mutations have been analysed for dominance over das$^+$ ana for allele specificity of suppression using 7 different dnaA mutations. The DNA physiology the dasC and rpoB suppressed dnaA46 strains has been studied, and one of the rpoB mutations was found to confer stable DNA replication phenotype.



Eucaryotic Replicons

944 REPLICATION OF THE YEAST PLASMID, 2μ CIRCLE, James R. Broach, Department of Microbiology, SUNY, Stony Brook, NY 11794

By examining both the transformation efficiency of yeast of various plasmids containing defined regions of the 2μ circle genome and the characteristics of the resultant transformants, we have identified several regions of the 2μ circle genome which are involved in 2μ circle replication. First, by identifying those DNA fragments from the molecule which promote high frequency transformation of yeast, we have localized the origin of replication to a sequence partially within the large unique region, which, as determined by subsequent deletion analysis, extends from the middle of the inverted repeat region into the contiguous unique region. Second, by examining the relative efficiency of replication in yeast of various *in vitro* generated mutants of 2μ circle, we have determined that efficient use of the 2μ circle origin requires a protein encoded in the large unique region of the 2μ circle genome. This protein appears to act at the origin of replication to maintain a high copy level of the plasmid in the cell.

945 ORIGINS OF REPLICATION IN VERTEBRATE DNA, J. Herbert Taylor and Shinichi Watanabe, Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306

DNA fragments of Xenopus laevis produced by the endonuclease, EcoRI, were cloned in the Escherichia coli plasmid ACYC189 and tested for ability to initiate and complete replication of the recombinant plasmids when injected into unfertilized eggs of X. laevis. On the basis of the amount of ^{3}H-thymidine incorporation stimulated in comparison to the vector alone, two recombinant plasmids, pSW14 and pSW9, which contain a small segment (507 base pairs) or several kilobases, respectively, were selected for more extensive analysis. The incorporation into pSW14 was shown to be replication rather than repair synthesis on the basis of a buoyant density shift when iododeoxyuridine was incorporated along with ^{3}H-thymidine. To determine the number of replications of pSW14 a novel method was employed. Because pSW14 turned out to be a head to head dimer of the vector with a small segment of Xenopus DNA in one of the EcoRI sites, the plasmid has three EcoRI sites, the two bracketing the Xenopus fragment and one opposite on the circle. By co-transforming E. coli with pSW14 and a pBR322 containing the EcoRI methylase gene, supercoiled plasmids completely resistant to EcoRI were produced, isolated and injected into the Xenopus eggs. Supercoiled DNA recovered after 4 hours, which had incorporated ^{3}H-thymidine, was still either completely resistant or completely susceptible to EcoRI as would be expected by complete semiconservative replication of at least a large fraction of the DNA injected. About one-third of the DNA recovered as labeled supercoils had replicated two times. On the assumption that the small Xenopus fragment contains a functional origin, it was sequenced.

946 THE ORIGIN AND DIRECTION OF 2-μm YEAST DNA REPLICATION IN VITRO, Akio Sugino, Hitoshi Kojo, Kwang Chul Kim and Barry D. Greenberg, Laboratory of Molecular Genetics, NIEHS, NIH, Research Triangle Park, N. C. 27709.

Yeast plasmid 2-μm DNA is currently being used for the study of eukaryotic DNA replication and as a vector for the cloning of specific genes by transformation of yeast cells. Its replication is under strict cell cycle control, depending on the same nuclear gene products required for chromosomal DNA replication. Hence this small DNA is very useful as a model probe for the study of the chromosomal replicon.

We have developed an in vitro 2-μm DNA replication system with activity at least an order of magnitude higher than previously described. The origin and direction of replication were determined using either native 2-μm DNA or a chimeric plasmid consisting of 2-μm DNA and E. coli plasmid pMB9 DNA, with the 2-μm DNA moiety as template. Replication starts at one of the inverted repeat portions (about 140 nucleotides from the end of the inverted repeat) and proceeds bidirectionally. DNA synthesis is also initiated, albeit less efficiently, at a specific site which differs from the pMB9 replication origin, when the chimeric plasmid DNA is used.

947 POLIOVIRUS RNA-DEPENDENT RNA POLYMERASE SYNTHESIZES COMPLETE COPIES OF VIRION RNA IN VITRO. Terry A. Van Dyke, Richard J. Rickles, and James B. Flanegan, University of Florida, Gainesville, Florida 32610.

A poliovirus-specific RNA-dependent RNA polymerase was purified from infected HeLa cells and was shown to synthesize complete copies of virion RNA in vitro when supplied with an oligo(U) primer. Polymerase activity was associated with only one virus-specific polypeptide, p63. NCVP2, a precursor of p63, was not active as a polymerase in the assay, nor did it contribute to the activity of p63. The full-sized RNA synthesized by the purified enzyme was complementary to virion RNA and was linked covalently to the oligo(U) primer. Initiation and elongation rates for the in vitro reaction were found to be significantly affected by changes in the pH, temperature, and magnesium ion concentration. Under optimum conditions, a full-sized product was synthesized in about eight minutes. A poliovirus-specific enzyme which initiates RNA synthesis in the absence of oligo(U) is currently being characterized for its polypeptide composition and its ability to synthesize full-sized product RNA. The mechanism of initiation in this reaction is also being investigated.

948 CYTOSINE ARABINOSIDE LIMITS INITIATION OF DNA REPLICATION TO A FIXED NUCLEOTIDE SEQUENCE IN METHOTREXATE-RESISTANT CHO CELLS. Nicholas H. Heintz and Joyce L. Hamlin. University of Virginia School of Medicine, Charlottesville, Virginia 22908.

CHOC 400 cells developed in our laboratory are resistant to 0.8 mM methotrexate by virtue of a 500-fold amplification of the gene for dihydrofolate reductase (DHFR). The amplified sequence can be visualized on ethidium bromide-stained gels as a series of 18-25 unique restriction fragments (total length, 120±10 kb), a subset of which contain the coding sequence for DHFR. Replication of this region was studied by arresting CHOC 400 cells in G1 by isoleucine deprivation followed by release into complete medium containing ^{14}C-thymidine and varying concentrations of cytosine arabinoside (Ara C), adenine arabinoside, or dideoxythymidine (ddT) for at least 12 hr. By this time, 95% of the cells have entered S. Incorporation of ^{14}C-thymidine into high molecular weight DNA is inhibited in a dose-dependent fashion by the arabinosides, but not by ddT. Analysis of DNA on alkaline sucrose gradients or on denaturing gels showed that increasing concentrations of Ara C results in progressively shorter labelled nascent strands. DNA isolated from cells released into S in the presence of 1 ug Ara C/ml has a specific radioactivity of less than 2% of the control DNA. Restriction endonuclease analysis followed by autoradiography of the Ara C-treated DNA showed that labelling is confined to a small number of discrete fragments (3 EcoRI bands, 3 XbaI bands, and 2 from HindIII). DNA isolated from untreated cells is labelled throughout the 20 bands comprising the 120 kb amplified segment. This data indicates that as CHOC 400 cells enter S, Ara C limits initiation of replication to a fixed nucleotide sequence that may contain an origin of DNA synthesis. In addition some of these fragments are identical to those generated in similar experiments in CHO cells.

949 SEQUENCING AND SUBCLONING ANALYSIS OF AUTONOMOUSLY REPLICATING SEQUENCES FROM YEAST (SACCHAROMYCES CEREVISIAE) CHROMOSOMAL DNA, Gary Tschumper and John Carbon, Department of Biology, University of California, Santa Barbara, California 93106.

Bacterial plasmids containing certain cloned fragments of yeast chromosomal DNA are capable of autonomous replication in yeast. These fragments contain a genetic element ars (autonomous replicating sequence) which behaves as a chromosomal replicator. Two yeast replicators have been characterized: ars1, which is adjacent to TRP1 near the centromere of chromosome IV; and ars2, which is located near ARG4 on the long arm of chromosome VIII. The DNA sequence of approximately 1500 base pairs surrounding each of these replicators has been determined. The ars1 element is contained within a fragment of about 800 base pairs and the ars2 element has been subcloned on a fragment of about 200 base pairs.

The DNA sequences of these two yeast replicators show little homology, however each has distinctive features. The ars1 fragment contains a perfect 'Hogness Box' (-TATAAATA-) along with some short regions of dyad symmetry. The ars2 fragment contains an 18 base pair A+T region of perfect dyad symmetry (-AAATATAAATTTATATTT-). This dyad contains a nearly complete 'Hogness Box' and each half of the dyad has perfect alphabetic symmetry. A yeast delta (δ) sequence is located on each side of the ars2 fragment. A yeast tRNA^{Gln} gene is located near one of the delta sequences. Plasmids containing ars2 transform yeast with greater efficiency than plasmids containing ars1. Apparently these two yeast replicators differ both in structure and function.

950 HIGH FREQUENCY TRANSFORMATION OF MOUSE L-CELLS AND IN VITRO REPLICATION STUDIES WITH EUKARYOTIC ORIGIN-CONTAINING PLASMIDS, Friedrich Grummt, Waltraud Albert and Detlev Buttgereit, Institut für Biochemie, Universität Würzburg, 8700 Würzburg, Germany.
A vector consisting of pBR322-, HSV1-TK- and yeast 2 μ plasmid origin DNA (provided by Werner Goebel, Institute of Microbiology, Würzburg) transforms mouse L-TK$^-$ cells at high frequency (> 1000 transformants/10^6 cells). The transforming DNA is replicated autonomously in mouse cells without being integrated into the genome as shown by Hirt extraction, agarose gel electrophoresis and hybridisation after Southern transfer as well as by re-transformation of E. coli and/or mouse L-TK$^-$ cells. An in vitro replication system will be described similar to the transcription system of Manley et al. (Proc. Natl. Acad. Sci. USA 77, 3855-3859) which apparently is strongly dependent on eukaryotic origin sequences in the template DNA and on DNA polymerase α. Studies on the functional topography of DNA polymerase α are described concerning the location of the template-, the substrate- and the Ap_4A-binding site on this heterooligomeric enzyme. DNA polymerase α from calf thymus was isolated by chromatography on P-cellulose, Sephacryl, blue-, Ap_4A- and aphidicolin-sepharose. The binding sites are characterized by one and two dimensional separation of the seven subunits of this enzyme under denaturing and isofocusing conditions, subsequent protein blotting and binding of radiolabeled DNA, dCTP or Ap_4A.

951 REGULATION OF REPLICON INITIATION AND CELL SURVIVAL IN EUCARYOTIC CELLS CONTAINING DNA DAMAGE, John P. Murnane, John E. Byfield, Chao-Tung Chen and Cheng-Hsia Wang, University of California, San Diego La Jolla, Calif. 92037
Inhibition of replicon initiation has been clearly demonstrated to occur in eucaryotic cells containing DNA damage. Our laboratory recently reported that caffeine is capable of reversing this effect. Evidence is now presented which demonstrates a close correlation between the ability of caffeine analogs to reverse the inhibitory effects of nitrogen mustard on DNA synthesis and their ability to enhance lethality in these cells. These effects are unrelated to caffein's inhibitory effects on purine biosynthesis which inhibits DNA synthesis at higher concentrations, and are similarly unrelated to inhibition of post-replication repair or removal of DNA damage. We therefore propose that regulation of replicon initiation in damaged DNA may be a cellular survival mechanism for avoiding additional complications resulting from this damage. The method by which this regulation is achieved and the nature of the effects of caffeine on this process are currently under investigation.

952 MONKEY GENOMIC SEQUENCES THAT ARE HOMOLOGOUS TO THE REGION AROUND THE SV40 ORIGIN OF REPLICATION. Susan T. Lord, Cary Queen, Maxine F. Singer and Thomas F. McCutchan, National Cancer Institute, Bethesda, MD 20205

Three different African Green monkey DNA segments were cloned from a genomic library in λ phage on the basis of their hybridization with the HindIII C fragment of SV40. This fragment contains sequences corresponding to the origin of DNA replication and the 5' ends of both early and late mRNAs. From each phage a segment which contains the SV40 homology was subcloned in pBR322 and the SV40-like region sequenced. A detailed description of one of these, pCaOri9.32, is reported elsewhere (T. McCutchan and M. Singer, PNAS, in press); the structures of the other two, pCaOri5.01 and pCaOri7.01, and a comparison of all three structures will be described here.

Each monkey segment contains several disconnected sequences homologous to SV40 segments. The arrangement and number of these sequences within each clone are different. All three clones contain sequences homologous to the 27 base pair region of dyad symmetry at the SV40 origin. The sequence GGGCGGA, which is present in SV40 as a set of short repeats located on the late side of the origin, is found several times within each clone. The clones also contain other repeated DNA sequences. The most striking of these is a repeat of 120 base pairs in pCaOri7.01. The clones are also similar in that each has an unusually high G plus C content (greater than 63% compared to the total monkey DNA, 40%) with the G and C residues distributed asymmetrically between the strands.

953 REGIONS OF TETRAHYMENA rDNA FUNCTIONING AS AUTONOMOUS REPLICATING SEGMENTS IN YEAST, Ronald E. Pearlman, Gyorgy B. Kiss and Anthony A. Amin, Department of Biology, York University, Toronto, Ontario, Canada, M3J 1P3
We have cloned within a number of plasmids the entire extrachromosomal rDNA of Tetrahymena thermophila. A marker (his 3^+) selectable in yeast has been included in these plasmids. Plasmids containing DNA from the center and the termini but not from the coding region of rDNA replicate autonomously in yeast. A 650 bp fragment from the rDNA terminus which functions as an ars in yeast has been sequenced. This region is 85% A/T and shares no extensive homology with the putative replication origin of yeast 2 micron circle or with any other replication origin which has been sequenced. A 650 bp region at the center of rDNA included in a plasmid specifying ars function has also been sequenced. This is also 85% A/T but the longest identical region in this and the terminal ars fragment is 12 bp. These data suggest that "foreign" DNA can be recognized by the yeast DNA replication apparatus and that yeast must recognize a number of different sequences as autonomous replicating segments; i.e. putative origins of replication.

954 PARVOVIRUS H-1 REPLICATION IN VITRO: CONVERSION OF SINGLE STRANDED VIRAL DNA INTO DOUBLE STRANDED REPLICATIVE FORM, Regine Kollek and Mehran Goulian, Department of Medicine, M-013, University of California San Diego, La Jolla, California 92093
The genome of the autonomous parvovirus H-1 consists of a linear, single stranded DNA with hairpin duplexes at both ends. H-1 replicates in the cell nucleus via a double stranded replicative form (RF). Little is known about the enzymology of the replication process, which involves the following steps: (i) conversion of the viral DNA into RF; (ii) multiplication of the progeny RF; and (iii) synthesis of the viral DNA. In order to analyze the enzymes involved in this sequence of reactions, purified viral DNA was used as a template for complementary strand synthesis mediated by cytosol fractions from human NB cells. We found that the crude cytosol, as well as a DEAE-cellulose fraction, was able to convert single stranded viral DNA into RF. Nature and characteristics of the participating factors are currently under investigation.

955 THE RELATIONSHIP BETWEEN REPLICATION AND ENCAPSIDATION OF SV40 DNA, Ann Roman and Hwa-Tang Wang, Indiana University School of Medicine, Indianapolis, IN 46223
Newly synthesized SV40 DNA can either be used as a template for further DNA synthesis (re-enter replication) or can be encapsidated. The factor(s) controlling the choice of pathways is under investigation in this laboratory. Briefly, infected cells are pulse-labeled and then incubated in the presence of bromodeoxyuridine. Re-entry kinetics are established by monitoring the accumulation of DNA labeled in one strand and containing BUdR in the other strand. These molecules represent DNA labeled during the pulse and replicated again during the BUdR chase. On the same cultures maturation kinetics are determined by quantitating the radioisotope found in different nucleoprotein complexes along the maturation pathway. The kinetics of re-entry correlate well with the kinetics of encapsidation: Cessation of re-entry of pulse-labeled DNA into replication parallels the accumulation of this DNA in previrions and virions. The effect of viral mutants on the kinetics of both pathways is now being examined.

956 CELL CYCLE CONTROL OF DNA SYNTHESIS IN EARLY XENOPUS LAEVIS EMBRYOGENESIS USING INJECTED DNA AS A PROBE, John Newport, Karen Butner, Marc Kirschner, Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143
A number of prokaryotic plasmids have been shown to replicate when injected into either 'activated' or fertilized Xenopus eggs. DNA replication of these plasmids in the egg is semi-conservative as monitored by density shifts of BrdU labeled DNA. The efficiency of replication varies depending on the plasmid used. The plasmid p(cen5)3 consisting of PBR322 with a 2 kb yeast insert containing the yeast centromeric DNA (Clarke and Carbon) replicated 2-4 times as efficiently as PBR322 alone. Synthesis of injected plasmids is periodic and occurs during the period of the cell cycle in which endogenous Xenopus genomic DNA is synthesized. This demonstrates that control of DNA replication of the injected plasmid is coupled to the cell cycle of the egg. When fertilized eggs are injected with cytoplasm from unfertilized eggs (CSF) the cell cycle of the fertilized egg is arrested in metaphase and no further DNA replication occurs (Masui). Injection of PBR322 into a fertilized egg which has been treated with CSF one hour earlier undergoes a single round of replication. This suggests that 'naive' injected DNA is converted during replication into a form which responds to the molecular signals maintaining CSF treated eggs in metaphase. Some of the properties of this replication dependent modification will be presented. The DNA synthesis response of nuclei isolated from cells in either G1 or G2 phases of the cell cycle injected into fertilized eggs at S phase will be described. Evidence will be presented on whether replication of injected plasmids in fertilized eggs originates from a specific sequence on the plasmid and whether this sequence remains a competent origin of replication at later times in Xenopus development.

957 INHIBITORY EFFECT OF SPECIFIC pBR322 DNA SEQUENCES UPON SV40 REPLICATION IN SIMIAN CELLS. Monika Lusky and Mike Botchan, University of California, Berkeley, CA. 94720. Several authors have noted that recombinant pBR322-SV40 plasmids propagated in E. coli replicate inefficiently in simian cells (see Hanahan et al., Cell 21, 1980; Benoist and Chambon, PNAS, 1980, Peden et al., Science 209, 1980); furthermore Hanahan et al. suggested that such molecules replicating in simian cells lose specific activity with respect to retransformation of E. coli cells. This phenomenon is due to pBR322 plasmid sequences, which must be Cis to the replicating viral moiety to manifest the effect; secondly, the effect is not due to poor expression of the SV40 early gene products, in the simian nucleus. In an experiment wherein multiple rounds of replication of the parental plasmids were obtained after transfection into permissive COS-7 cells (Gluzman, Cell, in press) the plasmid sequences were extracted by the method of Hirt and reintroduced into E. coli HB101 cells. Among the few isolated transformants selected most plasmids were found to have suffered deletions around the pBR322 PVUII site. These molecules must have had a strong selective advantage as this class of deletion could not be detected in the original simian cell extract. Furthermore these deleted plasmids now replicate as well as wild-type SV40 DNA in simian cells and can be returned to E. coli with specific activities equivalent to pBR322 DNA. A detailed restriction map of one such selected pBR-SV40 recombinant, taken together with the structures of other recombinant plasmids which still contain the "poison" sequences, reveals that the specific pBR322 DNA responsible for these phenomena must lie between the plasmid PVUII site and the plasmid origin of replication. We are presently testing the notion that specific eukaryotic DNA modifications in this region are responsible for the restrictions defined above.

958 PREFERENTIAL BINDING OF SIMIAN VIRUS LARGE T ANTIGEN TO SUPERCOILED DNA, Atsushi Yuki, Richard J. Hronek and Lowell P. Hager, University of Illinois, Urbana, IL 61801 Highly purified large SV40 T antigen obtained from SV80 cells, a SV40 transformed human cell line, binds preferentially to covalently closed circular (ccc) supercoiled SV40 DNA. When purified preparations of large T are first incubated with SV40 DNA mixtures containing equivalent amounts of supercoiled, open circular and linear DNA and then electrophoresed in agarose gels, all of the SV40 supercoiled DNA binds preferentially to T antigen and moves to a new band position. No binding of T antigen to open circular and linear DNA can be detected until all of the free supercoiled DNA has reacted with T antigen. Parallel experiments with pBR322 and pY182 DNA's show that the preferential binding of T to supercoiled DNA is dependent on structural considerations and apparently is independent of nucleotide sequence. Supercoiled preparations of both plasmid DNAs form exclusive complexes with T antigen. Protection experiments indicate that the nuclease S1 sensitive sites in ccc supercoiled SV40 DNA are not involved in the formation of the T antigen-supercoiled DNA complex. The effects of pH, metal ions and salt concentration on complex formation have been studied. Magnesium ions have particularly interesting effects. In the presence of Mg^{++}, the T antigen-supercoiled DNA complex forms a single band in agarose gels. In the absence of Mg^{++}, multiple complexes are formed as indicated by the formation of bands in the agarose gels having a mobility intermediate between free supercoils and the T antigen-DNA complex.

959 ISOLATION AND CHARACTERIZATION OF HUMAN DNA SEQUENCES HOMOLOGOUS TO THE SV40 ORIGIN OF REPLICATION, Susan E. Conrad and Michael R. Botchan, University of California, Berkeley, Calif. 94720

We have screened a library of human DNA cloned into the vector Charon 4A for sequences that are homolgous to the SV40 origin of replication. In a screen of 10^6 plaques, one hundred and eighty positive clones were isolated. Restriction encyme analysis has shown that for several of the "origin-like"(SVOL) clones, the homology to SV40 does in fact span the viral origin of replication. We have tested the human SVOL sequences for homology to the Alu family of repeats found in the human genome. In many of the clones examined, the Eco R1 generated fragment that hybridizes to an SV40 origin probe does not hybridize to an Alu family probe, indicating that the sequences that we have isolated are not merely a subset of the Alu family of repeats. One SVOL sequence has been subcloned onto a plasmid vector containing the chicken tk gene.The presence of the SVOL sequence increases the frequency of tk^+colonies obtained by DNA transfection 5-10 fold. Preliminary experiments indicate the presence of free plasmid DNA in the resulting tk^+ cell lines. One SVOL sequence has been subcloned, and in collaboration with R.Myers and R. Tjian, we have tested its ability to bind T antigen. Our results indicate that T antigen is able to bind to this molecule in a site-specific manner. The hybridizing region in this SVOL sequence has been sequenced, and shows structural as well as sequence homology to the SV40 origin region. We are currently attempting to map the ability to bind T antigen and to enhance the frequency of tk transformation to the region that hybridizes to the SV40 origin of replication.

960 SEQUENCES OF REPLICATION INITIATION REGIONS OF MITOCHONDRIAL DNA FROM DIFFERENT SPECIES OF PARAMECIUM AURELIA, Arthur E. Pritchard and Donald J. Cummings, University of Colorado Health Sciences Center, Denver, Colorado 80262

Fragments containing the replication initiation region of mitochondrial (mt) DNA from four species of Paramecium aurelia have been cloned and characterized. Sequences have been obtained for clones derived from mt DNA dimers which are replicative intermediates. Previous studies have shown that replication is initiated by the formation of a covalent cross-link at the initiation end of the linear molecule. As a consequence of this cross-link, the immediate product at the end of a round of replication is a dimer molecule in a head-to-head configuration, or palindrome. Sequence studies on two different species show that the bases involved in the cross-linking event at the center of the dimer molecule are nearly all A or T and are not arranged in a palindrome. In one species, this region consists of a 34 base pair sequence (all A or T) which is repeated 11 times. Outside of this central region, there is an increasing number of G and C residues and the sequence becomes palindromic. Available evidence indicates that these sequences are not due to a cloning artifact.

961 INITIATION OF POLYOMA DNA REPLICATION OCCURS IN TWO STAGES, Vincent Pigiet, Alicia Buckler-White, Marc Krauss and Robert Benbow, The Johns Hopkins University, Baltimore, Md 21218

Electron microscopic and kinetic analysis of replicating polyoma DNA molecules indicate that initiation of replication occurs as a two-step process. Replicative intermediates (RI's) extracted from either infected 3T6 cells or from nuclei incubated in vitro were digested with restriction endonuclease Bgl I, which cleaves very close to the origin of replication. Mose of the Bgl I digested molecules contained either a single replication fork (Y's) or two forks of unequal length. Although the latter could be generated from molecules replicating bidirectionally with unequal fork rates, the Y-shaped molecules (comprising 46% of the replicating molecules in vivo) indicate a substantial amount of unidirectional synthesis. Kinetic data, however, indicate that the majority of molecules terminate approximately 180° from the origin. Initiation, therefore, is asynchronous, with initiation of a single fork as the primary event. Although replication eventually becomes bidirectional in the majority of late RI's, some replication is truly unidirectional. Isolation of RI's containing only one fork (i.e., initiation intermediates) is in progress.

962 REPLICATION DIRECTED BY A CLONED ADENOVIRUS ORIGIN. Jerry A. Harpst, Kuan-chi Chow, Jeffry L. Corden*, and George D. Pearson, Dept. of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, and *Institut de Chimie Biologique, Strasbourg, France.

Replication initiates at or near either end of the linear adenovirus DNA molecule. One of us (JLC) has cloned the leftmost 1344 bp of type 2 adenovirus DNA into the EcoRI site of pBR322. We have studied the replication of this recombinant plasmid, called XD-7, in the cell-free adenovirus DNA replication system of Challberg & Kelly (PNAS 76: 655, 1979). Three lines of evidence indicated that the cloned adenovirus sequences directed the replication of XD-7 DNA. (1) XD-7 DNA incorporated [α-^{32}P]dCTP linearly for 2.5 hrs and exceeded by 3 to 6 times the incorporation directed by pBR322 DNA. (2) Hinf restriction mapping indicated that the origin was broadly located in the region of XD-7 DNA containing the adenovirus insert. (3) Electron microscopy of the XD-7 reaction showed (195 molecules scored): 82% monomer circles, 7% dimer circles, 4% rolling circles, 2% theta structures, and 5% forked linear molecules. In contrast, the pBR322 reaction showed (170 molecules scored): 98% monomer circles, 0.5% dimer circles, 1% rolling circles, and 0.5% forked linear molecules. Control reactions lacking plasmid DNA contained no circular forms, but had about the same frequency of forked linear molecules as in the pBR322 reaction. We discuss these results in the context of a new model for adenovirus DNA replication.

963 DIFFERENTIAL REACTIVATION OF UV DAMAGE TO INDUCIBLE THIONEIN GENES IN NORMAL AND REPAIR DEFICIENT HUMAN CELLS, C. E. Hildebrand and G. F. Strniste, Genetics Group, Los Alamos Scientific Laboratory, Los Alamos, NM 87545

The ubiquitous, low molecular weight, thiol-rich, metal-binding protein, metallothionein (MT) can be induced in normal human skin fibroblasts (NF) and xeroderma pigmentosum (XP) cells in culture during exposure to $ZnCl_2$ at a maximal subtoxic level of 200 μM. Both cell types show similar induction kinetics: during continuous exposure to 200 μM Zn^{2+} the cellular MT synthesis rate rises within 7 h from a low, basal rate to a maximal rate at least 50-fold > basal rate. Induction of MT synthesis is inhibited by actinomycin D (5 μg/ml). Irradiation of these cells with FUV caused inactivation of induction of MT synthesis: i.e. exposure to FUV followed by induction with Zn^{2+} resulted in a dramatic UV-dose-dependent decrease in the MT synthesis rate measured 8.5 h postinduction. The UV-sensitivity of MT induction appeared to be greater in XP cells than in NF cells. However, this apparent difference in sensitivity of MT induction could be explained (a) by the differential repair capacities of normal and XP cells and (b) by considerations of the kinetics repair of FUV-induced lesions superimposed on kinetics of MT induction. Liquid holding recovery experiments demonstrated that NF cells possess the capacity to reactivate MT gene expression completely while no reactivation is observed in XP cells. The implications of these findings are discussed with regard to (a) UV transcriptional mapping of inducible gene functions and (b) application of this inducible gene system as a new method for assessing repair capacities for various types of DNA-lesions produced by chemical and physical agents. (This work was supported by the US Department of Energy.)

964 SEQUENCE VARIABILITY IN A REGION OF THE HUMAN MT-DNA PROXIMAL TO THE ORIGIN OF DNA REPLICATION, Barry D. Greenberg, Akio Sugino, and John E. Newbold, University of North Carolina, Chapel Hill, NC 27514, and National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

Several investigations have documented inter- and intraspecies variability in the DNA sequences of mammalian mitochondrial DNA (mtDNA). In comparing sheep and goat mtDNAs, Upholt and Dawid (Cell 11, 577-585, 1977) showed that the most variable sequences flank the D-loop region. We have been interested in characterizing intraspecific variability among human placental mtDNAs. By use of a variety of restriction enzymes we have localized the most variable region to an area proximal to the origin of DNA replication (and hence the D-loop region), as seen by differences in the relative migration of restriction fragments on agarose gels. A 2.8 - 3.0 kb KpnI fragment has been identified as containing this entire variable region and has been cloned intact from each mtDNA preparation. More detailed restriction studies and sequence analyses are underway to characterize this variability.