Instituto Juan March
de Estudios e Investigaciones

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CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Initiation of Replication in Prokaryotic Extrachromosomal Elements

Organized by

M. Espinosa, R. Díaz-Orejas, D. K. Chattoraj
and E. G. H. Wagner

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INTRODUCTION

M. Espinosa and R. Díaz-Orejas

Bacteria are at the origin of life on earth. Basic mechanisms that were found and developed in microorganisms served the role of the main theme in the many variations played during cellular evolution. At the core of cellular proliferation is the copying (replication) of its genetic material. This is an essential process that requires efficiency and fidelity and that is regulated to prevent either the loss or the overproduction of the essential genetic information in the descendants. The elucidation, by Watson and Crick, of the structure of DNA, the macromolecule in which genetic information is stored, opened the way to the molecular characterization of its replication: DNA is normally formed by two strands that are complementary and that form a double helix. In the replication process each one of these complementary strands separate and serve as the template for the synthesis of the other. However, DNA replication does not occur spontaneously: it is the result of the action of DNA polymerases and other replication factors that catalize the process with high efficiency and accuracy. The functional-structural characterisation of these catalizers is a formidable task that started with the identification of DNA polymerases by A. Kornberg and that still continues. This workshop is a case in this search focusing on replication of prokaryotic extra chromosomal genetic elements: plasmids and bacteriophages (phages).

These elements are molecular "parasites" that propagate at the expense of bacteria but could also enrich the genetic contents of the host. Phages can have an extra-bacterial life because they can enclose DNA within a protein capsid. Plasmids lack this ability and they have an intracellular life in peace with the host. The greater autonomy of bacteriophages is reflected in a greater (or sometime complete) independence from the host replication machinery. The peaceful coexistence of plasmids within their hosts is the result of complex regulatory mechanisms that couple plasmid replication to the bacterial cell cycle.

These genetic elements can incorporate new genetic material by different mechanisms and can also transfer it to new hosts either by infection (phage) or by conjugative mechanisms (plasmid). Therefore, plasmids and phages serve the role of genetic vectors in bacterial populations (or in biotechnological applications) and contribute to maintain and expand new abilities in these populations.

DNA Replication has three clearly differentiated stages: initiation, elongation and termination. Initiation of DNA replication is the critical step at which the process starts, but also the stage at which the mechanism and the direction of replication as well as the regulation of the process are determined. The starting point of replication is usually determined by specific proteins (initiators) and by specific DNA sequences (origin).
Sometimes activation of the initiator or the initiation complex by chaperones is required in some plasmid and phage systems. The interaction of initiator

proteins with the origin of replication can cause a conformational change that could expose particular regions of the protein for interaction with host replication factors. In some cases, initiator interactions with spatially separate sites have been reported in phages and also in plasmid, but usually initiation starts from a fixed origin.

DNA polymerases are unable to start "de novo" synthesis of DNA. The incorporation of the first nucleotide (building block) by DNA polymerases requires a template and a primer: the latter can be provided by a protein (protein-primed replication), a DNA (rolling circle replication, initiation by DNA strand invasion) or an RNA (theta-type replication). DNA polymerases include also a read-proof exonuclease activity that allows the discrimination and eventual correction of mis-incorporated nucleotide at the end of the nascent chain. This general feature is also maintained in DNA polymerases able to use a protein primer: in these systems a slide-back mechanism makes possible the proofreading of the first nucleotide incorporated.

It is assumed that incorporation of DNA polymerases at the replication fork completes the assembly of the replication complex (replisome). The replisome could include, in addition to activities to prime and synthesize DNA (primase and DNA polymerase), other activities needed to open the DNA strands ahead of the polymerase which are provided by DNA helicases. Specific topoisomerases can solve the torsional stress produced by the unwinding of DNA strands ahead of the replication fork and single stranded DNA binding proteins can stabilize single stranded regions exposed in replicating intermediates. Removal of the RNA primers, replacement of these by DNA segments and ligation of the DNA pieces are also required during replication and these activities are catalyzed by different enzymes.

Termination of DNA replication is achieved by different strategies depending on the topology of the DNA template (lineal versus circular) and on the mechanisms of initiation (rolling circle versus theta replication in circular templates; initiation from internal sites or via terminal protein in linear templates).

Blockage of DNA replication can occur due to the action of particular loaders of replication initiation factors or by the explicit action of proteins that inactivate replication initiation factors. Re-assembling of the initiation complexes modulated by chaperones or specific neutralization of the inhibitors are required in these cases for initiation of DNA replication. Roadblocks formed by termination proteins bound to specific DNA sequences or by persistent RNA-DNA hybrids can also terminate DNA replication. In plasmids replicating by rolling circle, termination of DNA replication is catalized by the nicking-closing activity of the initiator protein at the origin of replication. At this stage a double stranded copy and a single stranded circular intermediate. The nicking-closing

activity of the initiator protein at termination can lead to the covalent link to this initiator protein of a small piece of DNA and to the inactivation of this initiator. The single stranded intermediate is converted to a double stranded copy by host replication machinery.

Coupling of DNA replication to the growth of the host cells is a basic strategy that plasmids use to maintain themselves in the hosts. This is achieved by mechanisms that count the number of origins and adjust the average initiation frequency to one per plasmid and per cell cycle. Protein, RNA, DNA and combinations of these are involved in the replication control. Contributions of protein and RNA to this control are relatively well understood. The mechanistic role of DNA sequences in this process, that are instrumental either in regulating the expression of the initiator or in forming protein-ori active/inactive complexes, is the subject of intense research. Phages also control their DNA replication but here the option is, in general, between all (lytic propagation) or nothing (switch-off of the lytic origin and activation of plasmid origin, or passive replication by integration into the chromosome).

The different stages of replication were analyzed in the Workshop using different plasmid and phage systems. Initiation of replication was a focus in these presentations. Much of the functional studies on replication and its control in extra- chromosomal genetic elements involved genetics, biochemistry and physiological analyses. A clear understanding of the macromolecular interactions and mechanisms involved, requires also structural data on initiators, host replication factors, replication control elements and on the macromolecular assemblies involved in DNA replication and its control. From the advanced resolution of some of these structures and the preliminary reports of others, it was clear that the confluence between structure and function is occurring. This will greatly contribute to our understanding of the replication process in prokaryotes. Plasmid and phages appear as a privileged window from which this important scenario could be observed and explored.

This Workshop has been probably the first in which scientists working with these extrachromosomal elements have get together. We were fortunate that the original initiative of this convergence was taken by the Juan March Foundation. On behalf of ourselves, of the two additional organizers, D. Chattoraj and G. Wagner, and of all participants we would like to thank the Juan March Foundation for making possible and very pleasant this highly stimulating Workshop.

Ramón Díaz Orejas and Manuel Espinosa Padrón.

Session I: Modes of initiation (I)

Chairperson: Kurt Nordström

PROTEIN-PRIMED REPLICATION OF PHAGE ø29 DNA. M. Salas, J. Méndez, B. Illana, P. Crucitti, A. Bravo, M. De Vega, V. Truniger, J. Saturno, A. Abril, J.M. Lázaro, A. Bonnin, J.A. Esteban, W. Meijer, V. Murthy, R. Freire, M.S. Soengas, I. Gascón, V. González-Huici, L. Villar, C. Gutiérrez, J.M. Hermoso and L. Blanco, Centro de Biología Molecular Severo Ochoa. Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain.

Bacteriophage ø29 DNA replication starts at each end of the linear genome by a protein-priming mechanism in which the viral DNA polymerase catalyzes both the covalent linkage of dAMP to the hydroxyl group of residue Ser^{232} in the primer terminal protein (TP) and the elongation of the TP-dAMP initiation complex up to unit length ø29 DNA (1). Polymerization catalyzed by the ø29 DNA polymerase is highly processive and coupled to strand displacement (2). Initiation of replication is directed by the second 3' terminal nucleotide from each DNA end (3). The first nucleotide is then recovered by a sliding-back mechanism favoured by the nucleotide reiteration at the ø29 DNA ends. This seems to be a general mechanism for maintaining the integrity of the DNA ends during protein-primed DNA replication.

The DNA polymerase-primer TP heterodimer formed for initiation does not dissociate immediately after sliding-back. There is a transition stage in which the DNA polymerase synthesizes a 5-nucleotide long DNA molecule while complexed with the primer TP, undergoes some structural change during replication of nucleotides 6-9, and dissociates from the TP when nucleotide 10 is inserted onto the nascent DNA chain (4).

Structure-function studies on the viral DNA polymerase (5) and TP will be presented, as well as the role in replication of the viral proteins p6 (DBP) and p5 (SSB) (6). The role of the viral protein p1, involved in ø29 DNA replication *in vivo*, in the assembly of a membrane-associated multiprotein complex will be described (7).

Using an *in vitro* replication system with four purified viral proteins (TP, DNA polymerase, DBP and SSB) we have obtained 1000–fold amplification of small amounts (0.5 ng) of ø29 TP-DNA. The amplified DNA was as infective as the DNA isolated from viral particles (8). In addition, the very early protein p17, essential for *in vivo* replication under conditions of low multiplicity of infection, improved the yield of *in vitro* amplified DNA. The role of the SSB in this system will be discussed, as well as the effect of mutations in the TP once it is bound to DNA (parental TP), as part of the newly generated replication origin.

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Replication of *Streptomyces* linear plasmids

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Like the linear viral replicons adenovirus and B. subtilis phage ϕ29 (for review, see Salas, 1991), *Streptomyces* linear plasmids have protein attached to their 5' DNA termini (Hirochika and Sakaguchi, 1982). However, unlike adenovirus and ϕ29, which initiate processive replication of full-length DNA strands at the telomeres by a protein-primed strand displacement mechanism (Salas, 1991), the S. rochei linear plasmid pSLA2 replicates divergently towards its telomeres from a site located near the center of the molecule. The central replication origin contains a series of iterons located within a gene encoding an essential DNA binding protein (Rep1); a second essential protein (Rep2), which resembles DNA helicases and has ATPase activity stimulated by single-strand DNA is expressed from the same transcript (Chang et al, 1996). Replication from the central origin generates 3' leading strand overhangs at the telomeres; the recessed 5' ends of lagging stands produced by the joining together of Okazaki fragments are then extended (i.e. "patched") to produce full length duplex DNA molecules (Chang and Cohen, 1994). The existence of both an internal origin of replication within *Streptomyces* linear plasmids and a mechanism for patching the ends of lagging strand DNA at the termini of these replicons allows their replication as either linear or circular DNA molecules (Shiffman and Cohen, 1992; Chang and Cohen, 1994; Chang et al, 1996).

Several possible mechanisms for the filling-in of recessed 5' ends of *Streptomyces* linear plasmids and chromosomes recently have been reviewed (Chen, 1996). In the first model, base pairing between palindomes that exist in the telomeres of *Streptomyces* linear plasmids and chromosomes enable the projecting 3' DNA end to fold back on itself, forming a duplex DNA segment that anchors the 3' terminus and generates a DNA site recognized by the terminal protein (TP); the TP then serves as a primer for attachment of the first dNMP by phophodiester bond linkage. This model predicts that palindromic sequences within the telomeres will be required for correct folding and positioning of the 3' overhang.

In a second model, synthesis of the 5' end of the lagging strand is primed by the TP on full length DNA, displacing the parental 5' end with its attached TP. The displaced DNA terminus then pairs with the 3' protruding end and homologous recombination accomplishes strand exchange and resolution of the half-Holliday junction. According to this model, homogenization of terminal sequences by gene conversion should result in the repair of damaged telomeres. In a third model, the most-distal palindrome of the 3' overhang folds back on itself and serves as a primer for patching synthesis using the 3' overhang as a template.

This event produces a terminal hairpin whose single strand loop is cleaved by the TP acting as an endonuclease. The TP remains attached to the 5' terminus that it generates at the very end of the molecule and the palindrome-primed nacent DNA is ligated to the recessed 5' end of the lagging strand. If replication at the telomeres of pSLA2 occurs by this mode, the imperfect distal palindrome of the pSLA2 telomere would "flip" during each replication cycle and consequently exist in both orientations in a population of linear pSLA2 plasmids, as has been found in replication of the telomeres of helper-dependent parvoviruses.

Here we report experiments that define the telomere sequence requirements for replication of pSLA2 in a linear form and also test the three models described above. Our results, which identify a "minimal telomere" required for linear pSLA2 replication show that neither homogenization of telomeres nor inversion of the terminal palindrome occurs during replication---eliminating the homologous recombination and terminal hairpin models as principal mechanisms for fill-in of the recessed 5' ends of pSLA2 DNA. Instead, our findings demonstrate that the pairing of distal and proximal palindromes within the 3' leading strand overhang is necessary for the production of full-length duplex pSLA2 DNA, and thus, that the initiation of TP-primed DNA synthesis at pSLA2 telomeres requires anchoring of the 3' terminus to a site near the base of the 3' overhang. We further show that circularized pSLA2 is linearized in vivo site-specifically by cleavage precisely at telomere termini. Together our results suggest that the termini of pSLA2 linear plasmids are replicated by mechanism that involves both fold-back and pairing of distant palindromes, plus DNA cleavage.

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INITIATION OF BACTERIOPHAGE T4 DNA REPLICATION FROM ORIGINS AND BY "JOIN-COPY" AND "JOIN-CUT-COPY" RECOMBINATION.

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Phage T4 has multiple pathways to initiate its DNA replication (1):

1) origin-dependent initiation uses RNA polymerase-dependent transcripts as primers for leading strand DNA synthesis and

2) recombination-dependent initiation uses 3' DNA ends as primers.

T4 primase synthesizes primers for Okazaki pieces. Moreover, there are several origins whose sequences show no similarities, and recombinational intermediates can prime DNA synthesis in at least two ways [which we have called "join-copy" and "join-cut copy" pathways; (2)]. The latter pathway can bypass the requirement for T4 primase (3).

What are the physiological roles of these different initiation modes, and how did they evolve?

We will a) compare initiation of DNA replication from two different origins, A and E of bacteriophage T4,

b) discuss requirements for two pathways of recombination-dependent DNA replication that we have called "join-copy" and "join-cut-copy" pathways

c) propose a model for the evolution of *oriA* by acquisition of foreign DNA by a modification of the join-cut-copy pathway and

d) discuss the role of T4 gp32 and its poisoning by the phage P2 Tin protein in the T4 replication pathways.

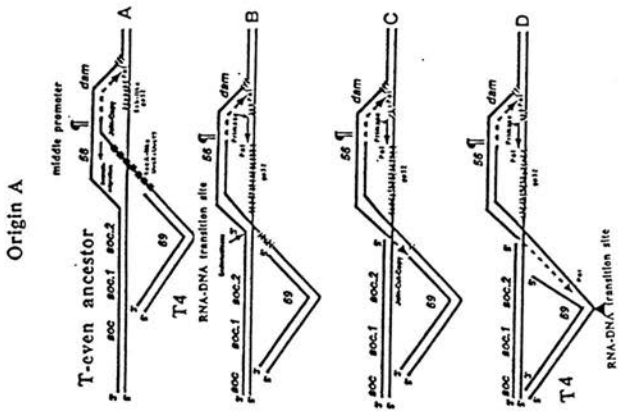
Comparing the requirements for initiation from *ori A* and *ori E*, we found that *ori A* initiation (like *oriF* initiation) depends on transcription from a T4 middle promoter (which requires activation by the T4 MotA protein). In contrast, *oriE* initiation depends on transcription from an early promoter, and it is independent of *motA*. In both origins (as well as in *oriF*) there are multiple transition points between RNA primers and nascent DNA, and they are located at least 600 nucleotides downstream of the promoters, at or near transcription termination sites.

In *oriA* (like in *oriF*) the major transition site occurs near a region, where a 5' segment of the primer transcript can base-pair with the non-transcribed DNA strand, suggesting the possibility that such base-pairing, like a wedge, may keep the RNA-DNA transition sites open. Although such a sequence is not obvious near the RNA-DNA transition sites of *oriE*, there is a countertranscript which might act similarly.

When torsional stress in the DNA is reduced, *oriE* is used almost exclusively to initiate T4 DNA replication. This may be related to some distinct properties of the *oriE* region. Although leading strand synthesis from this origin is primed by RNA polymerase-dependent transcripts, *oriE* contains iterons. These are located upstream of the primer promoter, suggesting that their role is different from iterons of origins whose primers for leading strand DNA synthesis are made by primase. The iterons are protected *in vivo* from methylation and their double-stranded can be footprinted by crude T4 extracts *in vitro*. In addition, two small single-stranded DNA binding proteins encoded by the *oriE* primer transcript mentioned above, bind preferentially to iteron single stranded DNA. We are testing the possibility that they facilitate helicase entry.

In wild type T4, origin initiation is restricted to one or at most a few rounds, related to the progression of T4 development; most replication forks are initiated from recombinational intermediates. However, origin-dependent replication generates the single-stranded regions that invade homologous double stranded DNA. From these invasions replication forks can be initiated in (at least) two ways: 1) from the 3' end of the invading strand, and 2) from a 3' end of an invaded strand, after it has been cut at or near a recombinational junction. We now know that such cuts can be made by one of several different T4 endonucleases.

This mechanism requires little homology and can be slightly modified to transpose foreign DNA without transposase. In fact, a sequence comparison of the *oriA* region of T4 with a region of the closely related T-even phage T2 suggests that the T4 *oriA* region was acquired or assembled from modular components of different ancestral replicons by such a mechanism.



The *oriE* region contains an apparently duplicated and transposed segment from another region of the T4 genome (4), which may have been transposed in a similar way.

The main T4 single-stranded DNA binding protein, gp32, plays important roles in facilitating assembly and function of protein complexes in all replication modes. T4 gp 32 is poisoned by a phage P2 protein, Tin, that is synthesized in P2 lysogens. Tin alone, when cloned in a plasmid, is sufficient to inhibit all T4 DNA replication. Gene *32* mutants that change asp163 to asn or gly (called T4 *asp* mutants) are resistant to poisoning by P2 Tin protein (5). In mixed infections of *tin*-bearing bacteria with wild type and *asp* mutant T4, wild type is co-dominant, i.e. there is severe inhibition of all DNA replication.

We are using this poisoning, among other tools, to delineate functions and functional domains of gp 32 that have different importance in origin dependent and in recombination dependent DNA replication. Gene *32* amber mutants (i.e. gp32 lacking the carboxyterminus) support origin-dependent, but not recombination-dependent replication (6.7). Their residual replication depends on T4 DNA polymerase and not on host polymerases. However, the amber peptides are defective in protecting the recombining DNA against excessive nuclease activities of recombination enzymes, regardless of the presence or absence of Tin. We found that the residual origin-dependent replication of gene *32 am* mutants is not poisoned by P2 Tin and that in mixed infections of *tin*-bearing bacteria with T4 gene *32 am* mutants and *asp* mutants both origin-dependent and recombination-dependent replication can proceed, i.e., the *am* mutants do not contribute to poisoning of *asp* by Tin.

These and other results, taken together, suggest that gp 32 can exist in different conformations, and that these different conformations facilitate different interactions with DNA, and with other proteins in origin-dependent and in recombination-dependent replication.

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Session II: Modes of initiation (II)

Chairperson: Stanley N. Cohen

Abstract: Juan March Institute Meeting: February, 1998

Molecular Events in the Inititation of Replication of the Broad Host Range Plasmid RK2

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The *Escherichia coli* DnaA protein and the plasmid encoded TrfA protein are required for the initiation of replication of the broad host range plasmid RK2. The TrfA protein binds to five 17 bp direct repeat sequences (iterons) and the DnaA protein binds to four DnaA consensus binding sequences (DnaA boxes) at the minimal replication origin of RK2 (*oriV*). Binding of the TrfA protein to the iterons results in discrete localized strand opening within the A + T rich region of *oriV* (1) The DnaA protein is required to enhance or stabilize this opening but does not itself produce an RK2 open complex. Analysis of each of the four DnaA boxes indicated that box four is strictly required in *E. coli* for origin activity but this requirement is bacterial host dependent (2). An *in vitro* replication system was constructed from purified TrfA protein and *E. coli* proteins (3). With this system a specific interaction between the DnaA and DnaB proteins was shown to be required for delivery of the helicase to the RK2 origin region. Although the DnaA protein directs the DnaB-DnaC complex to the plasmid replication origin, it cannot by itself activate the helicase. Both DnaA and TrfA proteins are required for DnaB induced template unwinding. A working model is that specific changes in the nucleoprotein structure mediated by TrfA results in a repositioning of the DnaB helicase within the open region and an activation of DnaB for template unwinding. This is supported by the results of introducing mutations that alter the sequence and/or spacing between the various motifs in the minimal origin region which indicate that correct helical phasing between the DnaA boxes, the iterons and the 13-mers as well as intrinsic DNA curvature are critical requirements for origin activity (4). The purified form of the TrfA protein exists largely as a dimer in solution, however, only the monomer form binds to the iteron sequences at the replication origin (5). The dimer form of the TrfA protein is inactive in the *in vitro* replication system reconstituted from purified proteins but it can be activated for replication in this system by the *E. coli* ClpX chaperone protein (6). This activation is the result of monomerization of the TrfA dimers by ClpX. These various properties of the TrfA and DnaA proteins as well as the replication origin undoubtedly are important in understanding the molecular basis of the broad host range replication activity of the RK2 plasmid.

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INITIATION AND REGULATION OF BACTERIOPHAGE λ DNA REPLICATION

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Reconstituted multiprotein systems that support the initiation and regulation of bacteriophage λ DNA replication have previously been established and characterized. Studies of the initiation pathway revealed that the λ O and P replication proteins orchestrate the recruitment of key host replication proteins, such as the *E. coli* DnaB helicase, as well as coordinate the assembly of an ordered series of nucleoprotein structures at the viral replication origin, *oriλ*, prior to DNA unwinding and priming of DNA replication. We have focused our recent investigations on the functional properties of the various preinitiation nucleoprotein structures formed at *oriλ* and on the critical role DNA supercoiling plays in the initiation reaction. Assembly of the first-stage *oriλ*•O preinitiation complex (i.e., the O-some) on supercoiled templates induces several base pairs in the 40 bp A/T-rich segment of the viral origin to adopt a non-B DNA conformation that has limited single-stranded character. This DNA alteration is further stabilized by formation of a second-stage *oriλ*•O•P•DnaB preinitiation complex, a key nucleoprotein structure that, depending on the superhelicity of the template DNA, exists in either of two conformational states. On supercoiled DNA templates the *oriλ*•O•P•DnaB complex undergoes an ATP-independent self-conversion into an activated replication intermediate that "traps" the energy of DNA supercoiling. Once activated, this intermediate can proceed through the remaining stages of the initiation and replication pathway in the absence of superhelical tension. We suggest that activation of the *oriλ*•O•P•DnaB nucleoprotein structure represents the capture by the complex of an "open" DNA conformation that is formed in the *oriλ* A/T-rich region via the combined influence of negative superhelicity in the template DNA and of binding and bending of origin DNA by the λ O initiator. The functional lifetime of the activated *oriλ*•O•P•DnaB replication intermediate was found to closely parallel the lifetime of the non-B DNA structure in the *oriλ* A/T-rich region over a broad range of template DNA superhelicity. The half-lives ranged from ~ 45 sec on linearized DNA to about 36 min on DNA retaining an effective superhelical density of - 0.04. These and other data strongly suggest that multiple and synergistic interactions between O, P, DnaB and supercoiled *oriλ* DNA are responsible for the unusual stability of the activated replication intermediate.

In related experiments, we have used gel-retardation and UV cross-linking studies to analyze the interaction of O, P and DnaB with single-stranded (ss) DNA oligonucleotides. We have discovered that λ P protein and the C-terminal domain of λ O protein, have cryptic ssDNA

binding activities that become manifest upon assembly of a ssDNA•O•P•DnaB complex. Surprisingly, we do not detect interaction of DnaB with ssDNA in this complex. Transfer of DnaB onto the ssDNA was found to require partial disassembly of the complex by the DnaK and DnaJ molecular chaperones; concomitantly, the O and P proteins are released from the ssDNA. Related studies with the *E. coli* DnaA and DnaC replication initiator proteins indicate that they behave in a mechanistically similar fashion to λ O and P in mediating the transfer of DnaB helicase onto ssDNA, with a cryptic ssDNA-binding activity of DnaC being primarily responsible for the initial interaction of a DnaC•DnaB complex with ssDNA. We suggest that interactions of O and P with non-B DNA structures that are formed during the initiation of λ DNA replication are responsible for nucleating and stabilizing the opening of duplex DNA in the A/T-rich region of *oriλ*. It is likely that these interactions are mediated by the cryptic ssDNA-binding activities of O and P and that these events prepare the λ chromosome for the transfer of DnaB helicase onto origin DNA.

Initiation of λ DNA replication *in vivo* is strongly dependent on transcription at or near *oriλ*. Analysis of this "transcriptional activation" phenomenon *in vitro* in a multiprotein replication system indicates that transcription counteracts the inhibitory effect of DNA relaxation caused by coating of the DNA template with proteins such as the *E. coli* histonelike protein HU. To facilitate exploration of the mechanistic basis of transcriptional activation, we have constructed a set of plasmids in which *oriλ* is imbedded in a transcriptionally silent region. Biochemical studies will be discussed which indicate that transcription downstream from *oriλ* enhances formation of an activated *oriλ*•O•P•DnaB stage-two replication intermediate on HU-coated DNA templates. This stimulation presumably results from increased negative DNA supercoiling that is transiently localized to the origin region as a consequence of downstream transcription. Once activated, the *oriλ*•O•P•DnaB nucleoprotein structure effectively blocks passage of transcription complexes entering the λ replication origin from the upstream direction.

INITIATION AND TERMINATION OF pT181 REPLICATION. Richard P. Novick, Rhuzong Jin, and Maria-Elena Fernandez-Beros. Skirball Institute, New York University Medical School, 550 First Avenue, New York, NY, 10016, USA.

pT181 is a model RC replicon of 4.4 kb and about 20-25 copies per cell. It uses a dimeric plasmid-coded initiator, RepC, whose synthesis is tightly regulated by a countertranscript-driven transcriptional attenuator [1] which ensures production of about one dimer (RepC/C) per replication event [2]. RepC/C binds strongly to a 15 nucleotide palindrome (IR III) [3] located 16 nucleotides from the nick site, which is at the tip of a strong potential hairpin (IR II). RepC/C binding enhances a natural bend near its binding site [4] and melts the DSO, which is believed then to extrude the IR II hairpin as a cruciform [5]. This melted region is then nicked by the protein, one subunit remaining covalently attached to the 5' side of the nick [3]. The nicked DSO must not be allowed to relax until the replication complex is assembled [6] and it is likely that RepC/C is instrumental in sustaining the required open configuration. Replication is known to require polymerase III and PcrA, a putative Rep-like helicase [7,8], and is assumed to require SSB. Whether these four factors are sufficient, as they are for the SS phages, has yet to be determined. RepC probably interacts directly with PcrA [9] and in a *pcrA* mutant, a RepC-containing initiation complex accumulates [7], from which we infer that RepC normally activates the helicase to initiate polymerization. At the end of a replication cycle, the new leading strand is extended for a few nucleotides past the DSO nick site, which marks the junction between new and old leading strands, displacing the latter. We believe that the replication complex is paused or halted at this point, perhaps by release of PcrA. We propose that the leading strand is then cleaved at the new-old junction by the second subunit of the bound RepC/C, followed by a transesterification in which the displaced old leading strand is circularized and the originally bound subunit is released. Cleavage of the extended new leading strand by the free RepC subunit followed by a second strand transfer would circularize the new leading strand and release the initiator. This second cleavage has two important consequences - it completes the replication cycle, preventing the re-initiation seen with ϕX174, and it leaves a short oligonucleotide tail (9 or 10 nt) attached to the active site tyrosine of one subunit, referred to as RepC*, which prevents the initiator dimer from being re-cycled[10]- a necessity for copy number control. The overall scheme is illustrated in Fig. 1.

In this presentation, we address two features of the above scheme, (i) the role of the cruciform, and (ii) the properties of the derivatized initiator. (i) We have modified the distal arm of the DSO hairpin and found that the cruciform structure is required for initiation only when the strong initiator binding site is defective - which means that superhelix-driven melting enabled by strong binding of the initiator is sufficient for nicking and for retention of the melted structure during assembly of the replisome, but that the cruciform structure is needed when initiator binding is weakened by deletion or mutation or by the substitution of the binding sequence from a related plasmid, pC221. This result is consistent with earlier findings that initiation can occur on a template with greatly reduced superhelical density, but only when initiator-DSO binding is strong [11]. This means that the initiator-DSO interaction represents a subtle interplay of superhelical tension, DNA binding and cruciform extrusion, and that it has sufficient leeway to enable initiation when one of these elements is in a suboptimal configuration. It is defeated, however, by deficiencies in any two of the three elements. (ii) The post-replicationally derivatized initiator, RepC/C*, can cleave single-stranded oligonucleotides containing its recognition site, generating doubly-derivatized dimers, and can catalyze the strand transfer and re-ligation reactions that have been described for other proteins of its class [6], as illustrated in Fig. 2. Although RepC/C* has weak nicking-relaxing activity on a supercoiled template, it cannot melt the DSO or enhance cruciform formation[12]. These results suggest that it is functionally analogous to the RepC attached during replication, which is used for termination, but is incapable of being used for initiation - which means that each molecule is used once and only once for replication.

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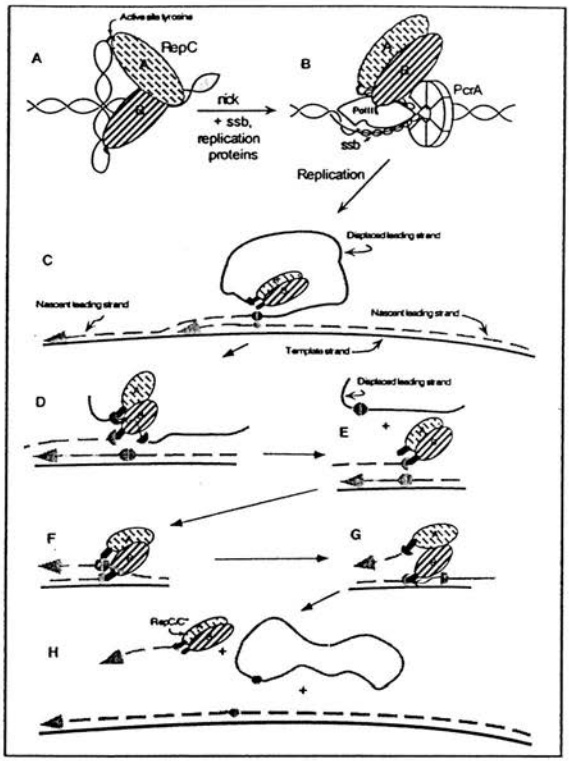

Fig. 1. Model for pT181 replication. A & B: initiation. RepC/C homodimer induces extrusion of IR II cruciform and one subunit binds to the L arm on the lagging strand while the other nicks the leading strand and becomes attached to the 5' nick terminus. The replisome is assembled concomitantly and is aassumed to include Polymerase III holoenzyme, single-strand binding protein and PcrA helicase, as well as RepC. **C-H: termination.** Only the region surrounding the DSO is shown. At the end of the replication cycle, the leading strand is extended for a short distance past the nick site, displaceing the junction between the displaced and nascent leading strands **(C)**, which is then cleaved by subunit B of the bound initiator. This is followed by a transesterification **(D)** in which the free 3' OH of the displaced leading strand attacks the protein-DNA bond between the 5' end of the same strand and subunit A of the bound initiator, releasing the old leading strand as a single-stranded circle and leaving subunit B attached to the 5' end of the nascent leading strand **(E)**. The 3' end of the leading strand is then displaced by the homologous 5' end with the attached initiator **(F)**, and the displaced leading strand is then cleaved by subunit A of the initiator, followed by a second transesterification **(G)** in which the nascent leading strand is circularized and the initiator released with the short oligonucleotide representing the 3' extension of the leading strand attached to subunit A **(H)**.

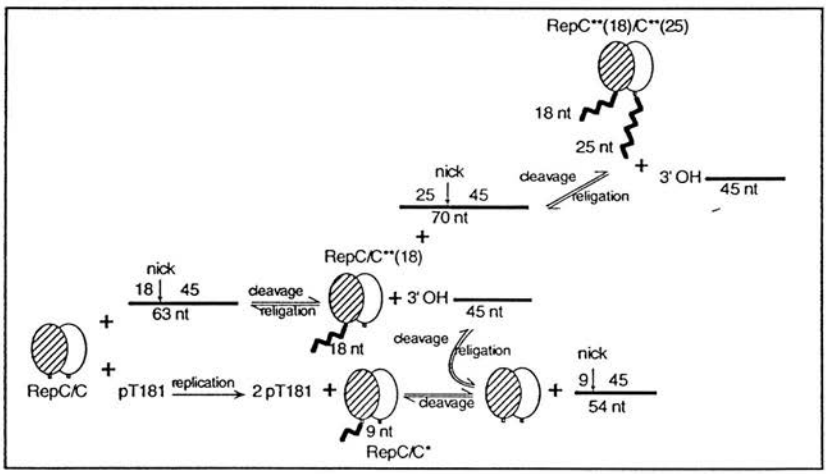

Fig. 2. Strand transfer reactions catalyzed by RepC. At bottom, replication is shown as generating RepC/C* with a 9 nt adduct on one subunit. This species can re-ligate an oligonucleotide with a free 3' end corresponding to the 3' side of the nick site (shown as a 45-mer), generating a 54-mer and releasing the protein in an active form. At top is diagrammed the possible cleavage and religation reactions, again starting with the active homodimer, RepC/C, reacting successively with oligonucleotides of 63 and 70 nt. These reversible reactions can lead to the formation of a doubly-derivatized dimer, such as the RepC**(18)/C**(25) shown.

Session III: Assembly of replication complexes (I)

Chairperson: Roger McMacken

The role of DnaK/DnaJ/GrpE molecular chaperones in λ and P1 DNA replication.

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Reconstitution of λ plasmid DNA replication system entirely composed of purified proteins allowed us to describe series of intermediate events leading to λ DNA replication. After the proper *ori*λ-λO-λP-DnaB preprimosome assembly, the DnaK/DnaJ/GrpE molecular chaperones in ATP-dependent reaction partially dissociate the λP protein from the preprimosomal complex leading to unidirectional λ DNA replication. We postulate the mechanism by which DnaJ and GrpE modulate the binding of DnaK chaperone to the λP replication protein.

The presenting data suggest that, contrary to the previous published models, the ATP hyrolysis is required for GrpE-dependent recycling of DnaK in the λP-DnaK complex and that only the DnaK which is a product of such reaction can be subsequently activated by DnaJ for binding to the λP protein substrate.

The above suggestion is not only valid for the λP protein substrate. The experiments were also performed for the P1 RepA protein. It has been previously shown by others that DnaK/DnaJ/GrpE molecular chaperones are involved in activation of RepA for binding to the *ori*P1 sequence. Here we show the role of DnaK, DnaJ and GrpE in building and dissociation of the DnaJ-RepA-DnaK complex.

The Role of the ClpX Chaperone in the Initiation of DNA Replication of the Broad-Host-Range Plasmid RK2

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The Clp family of proteins is highly conserved and has been identified in many organisms. One member of this family, the 46 kDa ClpX protein, has been shown to be involved in the initiation of replication of bacteriophage lambda and the replicative transposition of phage Mu. The ClpX protein activates the bacteriophage lambda replication initiation protein (λO) for binding to the origin sequence and also activates the bacteriophage Mu initiation protein (MuA) in a transposition complex which results in subsequent steps in the initiation of Mu DNA synthesis. It is also of interest that the ClpX protein can act not only as a molecular chaperone but also as a protease together with the proteolytic subunit ClpP in the degradation of the λO, MuA and Phd proteins. Studies on chaperone involvement in the activation of plasmid replication initiation proteins (Rep) have been limited to plasmid replicons that naturally occur in *Escherichia coli*. It was previously demonstrated that chaperone proteins are essential for the activation of the Rep protein of plasmids P1 and F for binding to nucleotide sequence repeats (iterons) at the origin of DNA replication (*ori*). We have been interested in the role of chaperones in the replication of plasmid RK2 which is a broad-host-range plasmid what can be transferred and maintained in most Gram-negative bacteria. Controlled replication of RK2 in these bacteria requires only two plasmid encoded elements: the origin of replication (*oriV*) and the Rep protein (TrfA). The purified form of the TrfA protein has been shown to exist largely as a dimer in solution, however, only the monomer form can bind to the 17-bp iteron sequences localized within the RK2 replication origin. We investigated the effect of the molecular chaperone ClpX and the ClpXP proteolytic complex on the replication initiation activity of the TrfA protein. The largely dimeric form of the TrfA wild-type protein is inactive in the initiation of replication of RK2 using an *in vitro* replication system reconstituted from purified components. However, preincubation of the TrfA protein with the ClpX molecular chaperone activates the plasmid initiator protein for replication in the purified replication system. We further observed that ClpX, in an ATP dependent reaction, greatly increases both the proportion of TrfA monomers and, therefore, the ability of a purified preparation of this protein to bind to iterons localized within RK2 origin. ELISA experiments using TrfA and ClpX proteins and a ClpX antibody confirmed a physical interaction between these two proteins. Finally, the TrfA protein was found to be a substrate for ClpXP dependent proteolytic degradation.

Session IV: Assembly of replication complexes (II)

Chairperson: Richard P. Novick

Session IV: Assembly of replication complexes (II)

Chairperson: Richard P. Novick

REMODELING OF THE PHAGE MU TRANSPOSOSOME TO A REPLISOME

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The transposition proteins of bacteriophage Mu function not only in inserting prophage DNA into the host chromosome for lysogenization but also in directing phage DNA replication by host enzymes during transposition, linking phage DNA synthesis to nonhomologous recombination. Replicative transposition of Mu is carried out in three general stages: 1) Assembly of an active oligomeric form of MuA transposase tightly bound to both Mu ends, forming a transpososome that promotes transfer of 3' hydroxyl ends to target DNA and produces a potential replication fork at each Mu end; 2) disassembly of the Mu transpososome coupled to the formation of a prereplisome composed exclusively of host proteins; and 3) assembly of the replisome promoted by PriA, PriB, and DnaT, assembly proteins of the ϕX174-type primosome. The orderly transition from transpososome to replisome prevents access of the two potential Mu replication forks to inappropriate host enzymes.

The molecular chaperone ClpX functions in the first step in the transition to a replisome, altering the tight complex (STC1) of oligomeric MuA with Mu ends in the strand transfer product. ClpX promotes conformational remodeling of MuA in STC1 to convert it to STC2. When the peptidase component ClpP is also present, ClpX can also act as part of a chaperone-linked protease to degrade the Mu repressor, which inhibits MuA transposase. While ClpXP readily degrades the Mu repressor (Vir) locked into the ClpXP-sensitive state, it does not degrade MuA in the transpososome needed to promote transition to a prereplisome. The presence of excess ClpP can stimulate ClpX-promoted transition to transpososome STC2, leaving intact MuA that holds together the two ends in a synaptic complex. Thus, ClpXP can discriminate between the repressor and MuA in the transpososome as substrates of the chaperone-linked protease or the chaperone alone, degrading the repressor while remodeling MuA and leaving it undegraded to promote the next step in replicative transposition.

Once STC1 has been remodeled to STC2, host factor(s) called MRFα_2 displace the MuA oligomer to form the prereplisome STC3. This nucleoprotein complex permits initiation of Mu DNA replication only in the presence of the primosome assembly proteins PriA, PriB, DnaT, DnaB, and DnaC. These proteins promote the engagement of DNA polymerase III holoenzyme and primase to assemble a replisome, initiating semi-discontinuous DNA replication from either one Mu end or the other. A time course of leading strand synthesis initiating at the two ends indicates that the replisome is more readily assembled at the Mu left end than the right, consistent with the preferential initiation of Mu DNA replication at the left end *in vivo*.

Proteins present in STC3 prevent the extension of the leading strand primers at each Mu end unless PriA is present, and therefore, limited strand displacement DNA synthesis by DNA polymerase I cannot be the mechanism for producing a single-stranded segment to which PriA binds, a mechanism observed for initiation of colE1 plasmid DNA synthesis. In fact, no primosome assembly sites (PAS) analogous to the sites present on phage ϕX174 DNA and at the colE1 origin can be found at the Mu ends by functional assay. The results suggest that PriA can bind at the branched junctions of the Mu strand transfer product without a single-stranded segment or a traditional PAS on the lagging strand side of the fork, promoting replisome assembly without the host initiator protein

DnaA. This mechanism is consistent with Tokio Kogoma's hypothesis (Asai and Kogoma, 1994; Kogoma, 1996) that PriA and other constituents of the ϕX-type primosome play a critical role in assembling a replisome at the sites of strand exchange during homologous recombination. Phage Mu may have evolved to exploit the host system for linking recombination with DNA replication.

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The *Bacillus subtilis* bacteriophage SPP1 G39P is a bridging molecule assembling the G40P replicative DNA helicase at the replication origin upon interacting with the replisome organizer G38P. Silvia Ayora and Juan C. Alonso. Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, E-28049 Madrid, Spain.

Initiation of DNA replication in bacteriophage SPP1 requires the products of genes *38*, *39* and *40* (G38P, G39P and G40P) as well as the host DNA primase (DnaG) and DNA polymerase III. G38P specifically binds two discrete regions (*ori*L and *ori*R), which are 32-kb apart in a linear map of the SPP1 genome (1). The *ori*L site maps within gene *38*. Both sites contain two types of repeated elements (termed Box AB and AT-rich region). Plasmid-borne gene *38*, i.e. gene *38* and its cognate *ori*L site, is able to drive theta replication in *B. subtilis*, provided that genes *39* and *40* are present in the background.

The replisome organizer, G38P, interacts with the Box AB, but not with the AT-rich region (1). G38P, which forms a higher-order nucleoprotein structure with the SPP1 *ori*L and *ori*R sites through protein-protein interactions, does not seem to modify the length of the DNA, but to bend it.

G40P is the SPP1 hexameric replication fork helicase. Only one or at most two subunits of the hexameric enzyme binds ssDNA in the presence of ATP or ATPγS. G40P binds dsDNA with a 20-fold lower affinity than ssDNA and such binding is independent of any cofactor. G40P unwinds DNA with 5' to 3' polarity in a substrate resembling a preformed fork. Monomeric DnaG primase of *B. subtilis*, which physically interacts with G40P, increases the helicase, but not the ATPase activity of G40P by stabilising the binding of G40P to ssDNA (2).

G39P, which does not bind DNA, interacts with G38P and G40P. The ATPase activity of G40P is inhibited by G39P, but increasing amounts of G38P reversed such an inhibitory effect. Unwinding of a forked substrate by G40P is enhanced more than 10-fold by the addition of G38P and G39P to the reaction mixture. *In vitro* assembly of G40P onto dsDNA is enhanced by G39P and G38P and the DNA-binding complex on dsDNA requires the concerted action of the three proteins. These data demonstrate one function for G39P and establish a function for it as an obligatory component of an oligomeric, DNA-binding complex which plays a role in initiation of phage replication.

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OLIGOMERIZATION OF PHAGE Ø29 PROTEIN p6: Analytical Ultracentrifugation and Electron Microscopy studies

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Protein p6 from *Bacillus subtilis* phage Ø29 is required *in vivo* for viral DNA synthesis and transcription repression of an early promoter. *In vitro*, protein p6 activates the initiation of Ø29 DNA replication by forming a nucleoprotein complex at the origins of replication. The path followed by the DNA in the complex is strongly bent and compacted adopting a right-handed toroidal conformation that wraps around a multimeric protein core. Protein p6 binds to DNA through the minor groove, with low sequence specificity; the N-terminal region is involved in this binding. Fluorescence studies have shown that this binding is cooperative.

Protein p6 is one of the most abundant proteins in Ø29-infected *B. subtilis* cells and binds *in vitro* to the whole viral DNA forming multiple complexes, suggesting that it could play a role in viral genome organization. In agreement with this, we have estimated a protein p6 concentration of at least 1 mM so that the number of protein p6 molecules per cell is sufficient to saturate all the *in vivo* Ø29 DNA molecules.

We have determined by sedimentation equilibrium analysis that, in the absence of DNA, protein p6 associates in higher order oligomers from preformed dimers at the concentrations estimated *in vivo*. Sedimentation velocity results indicate that oligomers of protein p6 have a more elongated shape than dimers. By electron microscopy we have obtained images of curved rod and doughnut-shaped aggregates of protein p6; computed image analysis of the latter suggests that the oligomers could correspond to the protein core predicted in the protein p6-DNA complex model (1). From these results we speculate that protein p6 could behave as a scaffolding protein on which the DNA folds.

We have also detected formation of protein p6 dimers and oligomers with crosslinking reagents. We have used this approach to study protein-protein interactions in deletion mutants. A C-terminal deletion mutant of 37 amino acids forms dimers but not oligomers. The aggregates observed by electron microscopy in the wild type protein p6 were not found in this mutant. This result is compatible with the existence of a dimerization domain different from an oligomerization one.

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Study of substrates affecting replication slippage *in vitro*

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Replication slippage is a particular type of error caused by DNA polymerases, suspected to occur in both prokaryotic and eukaryotic cells. Previous *in vivo* (1) and *in vitro* (2) studies have demonstrated that a deletion event can occur by replication slippage between short duplications (27 bp) brought together by a hairpin structure. The main polymerase of the *E. coli* cell, DNA polymerase holoenzyme III (polIII HE), DNA Polymerase I and DNA polymerase II as well as the DNA polymerases from phages T4 and T7 were able to promote slippage provided that polymerase and single stranded DNA binding protein (SSB) concentrations are optimized (3).

The Pol III HE-mediated replication slippage model proposed consists basically in (a) copy of the first duplication by the replication machinery, (b) replication pausing and dissociation of the polymerase, (c) unpairing of the newly synthesized strand followed by pairing with the second duplication and (d) polymerase loading and replication restart. The two limiting factors in this process are the presence of a duplication and a precise pause of the polymerase. We have studied the effect of the length of the direct repeats necessary to get slippage and the effect of other polymerase fork pausing sites like protein bound to DNA.

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Session V: Replication proteins: structure-function relationships

Chairperson: Saleem A. Khan

Protein-Protein interaction that potentiate host and Plasmid replication

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We have identified the replisomal proteins that interact with the RepA and π initiator proteins encoded in the plasmids pSC101 and R6K with host replisomal proteins and we find that the two initiators interact with DnaA, DnaB, DnaG and the τ subunit of DNA pol III. We have also discovered that DnaA specifically interacts with DnaG and τ. In many cases we have identified and mapped the interaction surfaces and have isolated mutants that significantly reduce the protein-protein interactions.

The π (RepA)-DnaA interaction is critical for initiation from ori γ of R6K and from pSC101 ori. Unwinding at ori γ requires π, DnaA and IHF. The mutant of π that reduces DnaA-π interaction also significantly impairs ori γ unwinding. Interestingly, the π mutant is fully competent to initiate replication from ori β, that is known to be independent of DnaA for replication.

We have identified mutations in DnaB that disrupts DnaB-RepA interaction withoutaffecting DnaA-DnaB interaction. We have also isolated RepA mutants that disruptRepA-DnaB interaction. The latter mutant abolishes pSC101 replication in vivo.
Helicase loading at the ori of pSC101 requires RepA, DnaA, IHF, DnaC and DnaB. The mutant of DnaB That disrupts DnaB-RepA interaction without affecting DnaB-DnaA interaction is unable to load helicase to the pSC101 ori. Thus DnaB isrecruited to the ori primarily by its interaction with RepA. The role of DnaA seems to be at the level of ori melting by its interaction with RepA. IHF facilitates this interaction by bending DNA.

DnaG interacts with RepA and p at its N-terminus whereas the C-terminus of the primase interacts with DnaB. The physiological roles of initiator-primase and initiator-t interactions are currently being investigated.

Structural Analysis of the Bacteriophage T7 Replication Fork

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Replication of the bacteriophage T7 chromosome is dependent on the phage encoded DNA polymerase, the product of gene 5. Processive synthesis requires a complex of the T7 DNA polymerase and a processivity factor, *Escherichia coli* thioredoxin. We have recently solved a 2.2 Å crystal structure of the T7 DNA polymerase complexed with a primer-template and a nucleoside triphosphate in the polymerase active site. The fingers, palm, and thumb of the polymerase impose an S-shaped bend in the primer-template, and the processivity factor thioredoxin lies near the DNA exiting the polymerase where it could prevent dissociation of the DNA. The incoming nucleotide is sandwiched between the 3'-terminus of the primer and the fingers subdomain, which has closed around the active site. Numerous interactions of the bound nucleotide with the template base, the polymerase, and two metals specify the correct base-pair and align the α-phosphate for attack by the 3'-hydroxyl of the primer strand. This structure illustrates how nucleotides are selected in a template-directed manner, and it provides a structural basis for a metal-assisted mechanism of phosphoryl transfer by a large group of related polymerases.

Initiation of lagging strand DNA synthesis in the T7 system is catalyzed by the gene 4 protein, which is both a primase and a helicase. The gene 4 protein is a hexamer which forms a tight complex with the T7 DNA polymerase. As a primase, it synthesizes tetraribonucleotides at specific sequences and the primers are used by T7 DNA polymerase to initiate lagging strand DNA synthesis. In order to understand the interactions of T7 DNA polymerase with the T7 gene 4 protein and the T7 gene 2.5 protein (single-stranded DNA binding protein) we are carrying out site-directed mutagenesis of the T7 DNA polymerase based on unique features in its structure not found in the structures of other Pol I-type DNA polymerases. For example, T7 DNA polymerase contains a unique, four residue loop in its thumb that contacts the template DNA four bases from the 3' end of the primer. This small loop is located at the site where the 5' end of a tetranucleotide RNA primer must be bound for use by the polymerase. Deletion of this loop has no noticeable effect on DNA synthesis catalyzed by T7 DNA polymerase but it dramatically reduces its ability to extend primers synthesized by the gene 4 protein. We believe that this loop is an important contact for either the primase, the RNA primer, or both.

We are extending the crystal structural studies of T7 DNA polymerase to include crystal complexes with the helicase, primase and single-stranded DNA binding protein. The ultimate goal is to obtain a high resolution picture of the T7 replisome.

Nicking-closing enzymes encoded by plasmid pC221

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Plasmid pC221[1] confers resistance to chloramphenicol on its staphylococcal host. In addition to the CAT gene, this ~4½kb plasmid also carries reading frames for the replication protein[2] RepD and the mobilisation protein[3] MobA. The latter two products represent two families of rolling circle replication (RCR) initiators: RepD for vegetative and MobA for conjugative processes.

RepD

RepD serves to initiate replication of pC221 by site-specific cleavage within the (+) strand at the double-stranded replication origin (dso), *oriD*. The resultant 3'OH serves to prime DNA synthesis; RepD becomes covalently attached to the 5' phosphoryl via Tyr191, conserved in all members of this family[4]. Discrimination between *oriD* and related sequences in plasmids such as pT181 is achieved by sequence-specific interactions between DNA sequences downstream of the nick site and the carboxyl terminus of the protein[5].

The nicking-closing activity of RepD can be studied i*n vitro* by a topoisomerase assay using pC221 as substrate. The nicking process serves as a model for initiation of replication; turnover of the enzyme allows study of the religation process implicit in termination. Through such means we have identified[6] residues critical for the catalytic activity of RepD. At this stage it appears that RepD represents a novel class of nicking-closing enzymes, which function without need for a consensus 'His-hydrophobic-His' motif[7]. Current work also suggests that the rate-determining step is the association between Rep protein and the downstream motif described above.

The covalent protein-DNA intermediate which exists following initiation can also be modelled using adducts generated using synthetic oligonucleotides. The intermediate has different properties to the initiator, in both activity and specificity[8]. Crucially, the covalently attached DNA exerts a level of specificity upon the complex. This suggests a model for the events of termination[9], supported by the isolation of adducts compatible with this mechanism. These intermediates also mimic the end-product of the replication process *in vivo*[10], RepD*.

In these two events, initiation and termination, there are implications for different topologies of the replication origin itself. Although specificity is conferred by a divergent downstream sequence, the cleavage site is conserved across members of this family. The sequence requirements for termination may ensure the perpetuation of this sequence even in hybrid plasmids.

The biochemical advances of RepD and its target DNAs are supported by efforts to obtain structural data. Hydrodynamic data for a variety of isoforms has been used to assist enzymological analysis. Crystals of RepD, complexes between RepD and the downstream sequence, and the covalent RepD-DNA adduct have all been obtained. To date the most advanced progress is with the protein alone, where diffraction up to 3Å has been obtained; large crystals are now routinely grown for all forms.

MobA

Study of MobA is in progress to parallel the work of RepD: mechanistically the two proteins may have much in common, but achieved through biochemically distinct means - for example, MobA possesses the Histidine motif lacking in RepD. Like RepD, the protein is being expressed in *E. coli* for study of the nicking-closing process *in vitro*.

Related RCR processes

Replication of certain bacteriophages is held as the classic example of the RCR process. The organisation and sequence of the dso of bacteriophage fd has many striking similarities with that of pC221, yet the replication initiator (Gene II) protein shows little sequence similarity to RepD (or MobA). Consequently study of the Gene II protein is also in hand as an example of an 'alternate' solution to the initiation of replication from an origin structure which, although not in the same immediate family as that of pC221, may be viewed as a 'cousin', with implications for the evolution of bacteriophages and RCR plasmids.

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Characterization of protein domains and conformational changes in a DNA replication initiator (RepA)

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RepA is the DNA replication initiator protein of the *Pseudomonas* plasmid pPS10 (1). We have previously shown that RepA, as a dimer, represses its own synthesis at the transcriptional level by binding to an inversely-repeated sequence (IR) that acts as operator (2). When monomeric, RepA initiates plasmid DNA replication by binding to four identical and contiguous 22 bp directly-repeated sequences (DRs or iterons) (3). We have also demonstrated that the major determinants of protein dimerization and DNA binding are, respectively, LZ (4,3) and HTH motifs (5).

In this communication we discuss the discovery, by means of a combined approach of biochemical (limited proteolysis, crosslinking, gel filtration and HO-footprinting) and biophysical (analitical ultracentrifugation, CD and fluorescence spectroscopies) *in vitro* techniques, the existence of two globular protein domains in RepA, C-terminus to the LZ motif. Furthermore, we show that RepA dimerization implies a compact conformation of the protein domains that makes the C-terminal one suitable for IR recognition, whereas monomerization results in the acquisition of and extended conformation of both protein domains in which each one binds differentially to the iteron sequence.

Our results provide the structural bases for the dual function (repressor vs. initiator) in Rep-type proteins. The emerging model fits well with the proposal by others of the activation by DnaK/J/GrpE or ClpA chaperones of the P1 plasmid initiator protein *in vivo* (6,7).

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Session VI: Helicases and inhibitors

Chairperson: Deepak Bastia

THE SOLVED STRUCTURE OF DnaB AND DnaBC IN CRYO-ELECTRON MICROSCOPY

DNA helicases are ubiquitous enzymes with fundamental roles in all aspects of nucleic acid metabolism (1). Their activity leads to disruption of hydrogen bonds between the two strands of duplex DNA, triggered by the energy of nucleoside 5'-triphosphate (NTP) hydrolysis. The unwinding reaction exhibits a specific polarity, which is expressed with respect to the strand of DNA on which the helicase is bound. Most DNA helicases carry out their functions as multimeric complexes. Recent observations point to the existence of a subclass of DNA helicases with a common hexameric structure (2), which act in most cases as components of large protein assemblies. In spite of their critical role and the amount of biochemical knowledge in the field, little is known about their structures. The first X-ray structure of a (monomeric) helicase has just been reported (3).
DnaB is the primary replicative helicase in Escherichia coli (4). The native protein is a hexamer of identical 52,260-Da subunits that displays a 5' to 3' polarity in DNA unwinding (4). Recent work has yielded interesting data about structural features of the protein hexamer at low resolution, based on electron microscopy studies of negatively-stained specimens (5, 6).
In solution, the hexameric DnaB forms a complex with six molecules of DnaC (27,935 Da/monomer). DnaC is essential for replication in vitro and in vivo, and its role seems to be the formation of the DnaB.DnaC complex from which the helicase is delivered to its site of action on the DNA template. Upon delivery of DnaB, the DnaC protein is released from the protein.DNA complex (7, 8).
We have studied the structure of both the DnaB hexamer and the DnaB.DnaC complex by three-dimensional reconstruction of macromolecular aggregates from cryo-electron microscopy images of frozen-hydrated specimens. This is the first report of the 3D structure of a helicase, and its complex with a loading protein, obtained from cryo-electron microscopy data. To avoid a possible source of heterogeneity in the images, both samples were prepared in a buffer containing ADP and Mg2+. Under these conditions, the tendency of the proteins to coexist in two conformational states due to partial ATP hydrolysis is greatly reduced. (The reduced stability of the DnaB.DnaC complex under these conditions was overcome during the image processing by strict image classification of the data into homogeneous image data sets).

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MECHANISTIC CHARACTERISTICS OF THE REPLICATION AND TRANSCRIPTION BLOCKAGE BY THE TERMINATOR PROTEINS

The replication forks initiated at the origins of bacterial chromosomes and of some plasmids like R6K, R1 and R100 move bidirectionally or unidirectionally and terminate at sequence specific termination sites. The termination sites are unidirectional replication fork traps comprising a specific sequence and a protein called terminator protein. The protein is called Tus in *E. coli* and binds a 22bp sequence called *Ter* in *E. coli* chromosome and plasmids. The protein *in B. subtilis* is called replication termination protein (RTP) that binds an ~30bp sequence called *Ter* (BS3 or IR). The protein-DNA complex in both *E. coli* and *B. subtilis* block replication by impeding DNA unwinding by replicative helicases. We have recently shown that the replication terminator proteins of both organisms can block transcription in the same polar fashion (EMBO J., 1996; 14:2530-9).

In the present work we have studied some mechanistic characteristics of the replication and transcription blockage by the terminator proteins. We have shown that RNA polymerase blocked by the terminator protein is arrested, not terminated by the two proteins. We have also observed that when the RNA polymerase transcribes through the terminus it dislodges the terminator protein. We shall also present data on the fate of helicase DnaB blocked by the terminator protein as well as the fate of the terminator protein when DnaB goes through the terminus. Similarly we shall present data that show the differences in sites of blockage of replication and transcription.

Protein-protein interfaces involved in the neutralization of a protein that inhibitis initiation of DNA replication in *Escherichia coli*: a genetic analysis

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The Kid protein of the conditional killer system of plasmid R1 called *parD*, inhibits initiation of DNA replication dependent on DnaB (5 and de la Cueva unpublished results). DnaB is the major replicative helicase of *E. coli*: DNA helicases are a group of motor proteins that unwind duplex DNA yielding single stranded DNA intermediates that play an essential role in DNA replication, recombination or repair (3). The inhibitory activity of the Kid protein, that acts as a bacterial poison, is modulated by the Kis protein, the second component of the *parD* system. These two proteins interact forming an antidote-poison complex that, in addition to neutralize the inhibitory activity of Kid on DNA replication, represses the *parD* system (1,2,5).

In this communication we present a genetic analysis devised to identify putative protein-protein interfaces involved in antidote-poison interactions. The approach used is based in the isolation and characterization of mutations that improve the weak ability of the antidotes of two *parD* homologous systems of chromosomal origin, *chpA* and *chpB* (4), to neutralize the toxic effect of the *parD* poison. The mutations found (ChpAI: G10R; S12L; A14V; R16Q; ChpBI: G9R, G9E, G9S, S11N, V15I, P17L; ChpBK: G62E, Q26Ochre, Q42Ochre), indicates that the amino end of the antidotes and the middle region of the killer components are involved in such interactions. According to structural predictions (PHD algorithm), the regions affected should be located in loops exposed to the solvent. We proposed that these loops are protein-protein interfaces required to form a functional antidote-poison complex (6 and Santos-Sierra, unpublished results).

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Session: VII: Replication and its control (I)

Chairperson: Donald R. Helinski

INITIATION OF REPLICATION OF PLASMID R1 IN *ESCHERICHIA COLI*

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Plasmid R1 is a low-copy-number plasmid. It replicates unidirectionally by the theta mode of replication. Its replication is controlled by an antisense RNA that post-transcriptionally controls the formation of the RepA protein that binds to and opens the origin of replication (*oriR1*) to initiate replication and is rate-limiting for initiation of replication. The key elements of *oriR1* are shown in the figure.

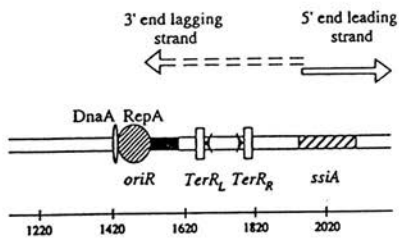

Fig. The main features of the replication origin region of plasmid R1. The bp coordinates are given at the bottom of the figure.

We have mapped the 5' end of the leading strand *in vivo* to a position about 380 bp to the right of *oriR1* (1) and the 3' end of the lagging strand to a position within *oriR1*, at the binding site for the RepA protein. The former end point is located within a site for initiation of single-strand replication, *ssiA*. In these studies, we also employed so-called *intR1* strains (2); in these a 16 bp deletion inactivates *oriC* and plasmid R1 was inserted into the deletion. In this way, chromosome replication becomes controlled by the inserted plasmid. In one of the orientations, chromosome replication is unidirectional and the dangling 3' and 5' ends in the *oriR1* region become more long-lived than in the free plasmid that is replicated in just a few seconds.

The *oriR1* of plasmid R1 contains one DnaA box. Replication of plasmid R1 does not require DnaA for replication, but the efficiency of replication is enhanced by DnaA. This was shown to be due to an increased affinity of RepA to *oriR1* in the presence of DnaA; the absence of DnaA can be overcome by overproduction of RepA (3) and foot-printing studies showed that the 3' end of the lagging strand went further to the left in the absence of DnaA.

Between *oriR1* and the 5' end of the leading strand there is a complete functional *ter* region, *i. e.* sites that have the potential to inhibit the progression of a replisome in both directions (se figure above). A functioning termination function requires the Tus (terminus utilising protein) protein. We therefore inactivated the *tus* gene and studied replication of the plasmid in such a host. The stability of maintenance was greatly impaired in spite of the fact that the plasmid used contained a functional *par* region. Deletion of the left *ter* site did not have any effect on the stability of maintenance, whereas deletion or mutation of the right *ter* site caused the same effect as inactivation of the *tus* gene. The instability of maintenance was paralleled by a significant increase in the occurrence of plasmid oligomers. The formation of these multimers did not seem to be caused by the RecA system, since plasmid maintenance did not increase when the *recA* gene was inactivated. Electron-microscopic studies revealed the presence not only of circular dimers, trimers, etc., but also of rolling-circle molecules in *tus* hosts or when the right *ter* site had been inactivated in the plasmid (4). Thus, the right *ter* site appears to

stabilise the maintenance of the plasmid by preventing both multimerisation and shifts from theta to rolling-circle replication. The right *ter* site may then be used as a signal for telling when the total plasmid genome has been replicated.

The function of the left *ter* site is obscure. There were no 3' ends at this position, but replication of the lagging strand passed the left *ter* site. Furthermore, during initiation of replication, the origin region will be single-stranded and the *ter* sites presumably nonfunctional.

The instability of maintenance caused by a nonfunctional *ter/tus* system might be explained in different ways:

i) If replication is shifted from rolling-circle to theta replication there presumably will not be any control of replication, since the control is exerted at initiation of replication. Furthermore, there is no obvious way by which a rolling-circle molecule could form a normal covalently-closed R1 circle. However, the *copA* gene most likely is active also in the rolling-circle molecules. Hence, cells with only one rolling-circle molecule are likely to be formed and these cells will divide to give plasmid-free cells until the plasmid-containing cells cease to grow due to runaway replication. This scenario is difficult to study.

ii) A plasmid dimer will have twice the probability of being replicated compared to a monomer. This might result in cells with dimers only. Depending upon the properties of the replication control system, the number of dimer molecules in cells with dimers only will be the same as the number of monomers in cells with monomers only or the number of dimers will be half that of the monomers (the latter is the case for ColE1 according to experiments performed by both Hans Bremer and David Summers). We have not been able to isolate subclones with dimers only, but we have constructed dimeric R1 plasmids and are now studying their copy number and stability of inheritance. Furthermore, a dimer of a Par$^+$ plasmid will have two *par* regions and it is not known if this inactivates the Par system by the formation of intramolecular pairs. This aspect is at present under investigation in our laboratory.

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ABSTRACT

Initiation, pausing and termination of DNA replication at ColE1 origins.

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A series of plasmids were constructed containing two unidirectional ColE1 replication origins in either the same or opposite orientations. Two dimensional (2D) agarose gel electrophoresis was used to investigate their replication mode. The results obtained demonstrated that: a) Initiation of DNA replication occurs at only one of the two potential origins per replication round regardless of their orientation; b) In opposite orientations, the silent origin acts as a pausing site for the replication fork initiated at the other origin; c) The accumulation of specific replication intermediates (Ris) with an internal bubble spanning the distance between origins leads to the formation of knotted bubbles; d) The distance between origins, up to 5.8 kb, affects neither the interference between them to initiate replication nor the pausing faculty of the silent origin; e) A complete origin is more effective to initiate replication than an origin lacking either the *pas* sites or the RNAI and II promoters, in the absence of read-through transcription; f) A complete origin and an origin lacking the *pas* sites are equally effective as pausing sites, whereas an origin lacking the RNA promoters is significantly less effective. Altogether, the results obtained strongly suggest that formation and stabilization of an RNA/DNA heteroduplex at the origin region is sufficient and enough to initiate replication as well as to account for the polar pausing faculty of silent ColE1 origins. Preliminary results suggest that the dnaB helicase leading the replication fork is not able to disrupt RNA-DNA heteroduplexes and could be responsible for the polar pausing of replication forks at the silent ColE1 origins.

Session VIII. Replication and its control (II)

Chairperson: E. Gerhart H. Wagner

Regulation of Origin Opening in Plasmid P1 by Initiator Titration and not by Origin "Handcuffing"

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Copy numbers of several bacterial plasmids seem to be regulated by multiple short repeating sequences called iterons (1,2). There are two models of how the iterons could be involved in the negative regulation of plasmid copy number. In the first model, called the initiator titration model, the concentration of plasmid-encoded initiator protein that binds to the iterons, is assumed to be rate-limiting. With the increase of copy number, the limiting amount of initiator is believed to distribute to daughter origins preventing saturation of any one origin. Thus, the increase of origin (or more precisely the iteron) concentration provides the negative feedback control of initiation. The model was questioned when it was found that an increase of initiator concentration did not increase copy number in all cases. A second model was proposed to account for copy number control when initiator concentration was apparently not rate-limiting. This model, called the handcuffing model, was based on the finding that the initiators could pair iterons in trans. It was assumed that the pairing causes steric hindrance to origin activity and the negative feedback control was due to increase in pairing opportunities with the increase of origin (iteron) concentration. We have reinvestigated the applicability of the models using reactivity to $KMnO_4$ as an assay for strand opening (initiation) in vivo. A similar assay has been used to study opening of *oriC* plasmids (3).

Opening of the origin strands is an essential early step in the theta mode of DNA replication and usually requires binding of initiator proteins to the origin (4). We find that P1 plasmid origin opening requires the host initiators DnaA and HU, and the plasmid-encoded initiator RepA to open. These three proteins were known to be essential for the plasmid replication, validating the utility of the assay to study initiation control.

The opening was prevented when the P1 origin or RepA was present in excess with respect to each other. When both the origin and RepA were present at high concentrations, the opening was efficient. The ability to open (cloned) origins at concentrations that exceed the normal copy number of the plasmid by supplying excess RepA, contradicts the currently favored view that origin concentration limits plasmid copy number by promoting handcuffing. Apparently, when the origin concentration was high, RepA binding became limiting due to titration. The prevention of opening at excess RepA appears to be due to an inhibitor co-produced with RepA. We propose that RepA titration and an inhibitor, effective under conditions of RepA overproduction, control P1 copy number.

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INITIATOR PROTEIN-ORIGIN INTERACTIONS DURING THE INITIATION AND TERMINATION OF ROLLING-CIRCLE REPLICATION OF PLASMID pT181

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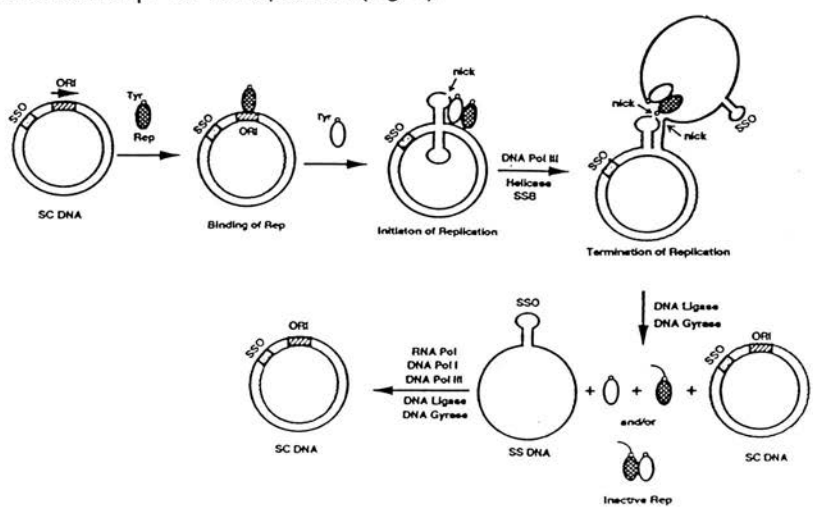
Most small multicopy plasmids of Gram-positive bacteria replicate by a rolling-circle (RC) mechanism (1-3). Replication initiator proteins encoded by RC plasmids have origin-specific nicking-closing activities that are required for the initiation and termination of replication (4). Since the initiators of many RC plasmids are rate-limiting for replication, these proteins are usually inactivated after supporting one round of replication (5). In the case of the pT181 plasmid, inactivation of the initiator RepC protein occurs by the attachment of an oligonucleotide to its active tyrosine residue. We have generated the inactivated form of RepC, termed RepC*, *in vitro* and investigated the effects of attachment of the oligonucleotide on its various biochemical activities (6). Our results demonstrate that while RepC* is inactive in nicking-closing and replication activities due to the blockage of its active tyrosine residue, it is competent in origin DNA binding and DNA religation activities. We have also investigated the oligomeric state of RepC and RepC* and found that while RepC exists as a monomer in solution, it dimerizes on the DNA. Using gel mobility-shift assays, we have found that a DNA binding mutant of RepC can be targeted to the origin in the presence of the wild-type protein primarily through a protein-protein interaction. Interestingly, RepC* is defective in its ability to dimerize on the DNA. RepC* inhibited the DNA binding and replication activity of wild-type RepC to only a very limited extent, suggesting that it is unlikely to have a significant regulatory role in replication *in vivo*. Based on these and earlier results, we propose a model for the role of RepC during the initiation and termination of pT181 RC replication (Fig. 1).



We have also studied RepC-origin interactions during the termination of plasmid pT181 replication. The origin of replication of pT181 contains three sets of inverted repeat (IR) elements, namely IRI, IRII and IRIII. Previous footprinting studies in our laboratory have shown that RepC binds to IRIII and the right arm of IRII (7). The RepC cleavage site is located within the loop of IRII between pT181 nt 70 and 71 (4). Recent studies have also shown that while both IRII and IRIII are required for the initiation of replication, IRII by itself is sufficient for the termination step (8). We have investigated the sequence requirements for termination of RC replication of plasmid pT181. By mutational analysis, we have found that several nucleotides within the stem of IRII which are critical for the initiation activity are dispensable for termination of replication (9). We also demonstrate that nucleotides in the right arm of IRII, but not the left arm, are absolutely required for termination of RC replication. The sequence of the right arm of the hairpin must be located downstream of the initiator nick site for termination, suggesting that termination requires a specific orientation of the initiator protein at the origin. To study the role of RepC in termination of replication, we have studied the single-stranded DNA cleavage and religation activity of RepC and RepC*. Using ss oligonucleotides representing IRII and its mutants, we have found that although cleavage by RepC is critical for termination at the IRII sequence, the efficiency of cleavage is not directly related to the termination activity. Results of experiments involving the religation activity of RepC* suggest that the extent of ligation of the displaced leading strand of the DNA may determine the efficiency of termination. Based on the above results, we propose a model for termination of replication of RC plasmids in which the replication termination complex is trapped after proceeding beyond the regenerated RepC nick site. This probably involves recognition of the right arm of IRII by the initiator protein (possibly in collaboration with a host termination protein). This results in pausing of the replication fork followed by cleavage and ligation of the displaced single-stranded DNA by the Rep protein.

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ROLLING-CIRCLE REPLICATION OF BACTERIAL PLASMIDS: INITIATION OF LAGGING-STRAND SYNTHESIS

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Most small multicopy plasmids of Gram-positive bacteria and many in Gram-negative bacteria replicate by a rolling-circle (RC) mechanism. This mode of replication involves the generation of a site-specific nick by the plasmid-encoded initiator proteins at the plasmid leading-strand origin, followed by covalent extension of the 3' hydroxyl end by the host DNA polymerases. Single-stranded DNA (ssDNA) released after leading strand synthesis is converted to a double-stranded form utilizing solely the host proteins. Most plasmids that replicate by the RC mode have been found to contain palindromic sequences that act as the single strand origin, *sso*. One of such sequences, the *ssoA*, is present in many plasmids from Gram-positive bacteria. We have investigated the host requirements for the functionality of the *ssoA* from the streptococcal plasmid pMV158 and the staphylococcal plasmid pE194. We have also used a newly developed *in vitro* replication system using pneumococcal cell-free extracts to investigate the requirement of the host DNA polymerase I for lagging strand synthesis. Whereas extracts from DNA polymerase I-deficient cells failed to replicate, this defect was corrected by addition of purified DNA polymerase I. Efficient DNA synthesis from the pMV158-*ssoA* required the entire DNA polymerase I (polymerase and 5'→3' exonuclease activities). In addition, ssDNA containing the pMV158- and pE194-*ssoA* was a substrate for specific RNA polymerase binding, and was a template for RNA polymerase-directed synthesis of 20 and 17 nucleotides-long RNA primers, respectively. We have also constructed mutations in two highly conserved regions present within the *ssoA*, namely a 6 nucleotide conserved sequence (CS-6) and the recombination site B (RS_B) to identify sequences important for plasmid lagging strand replication. *Streptococcus pneumoniae* harboring plasmids with mutations in the RS_B accumulated 30-fold more ssDNA than cells containing plasmids with mutations in the CS-6 sequence. Our results demonstrate that whereas the CS-6 sequence seems to function as a terminator for primer RNA synthesis, RS_B may correspond to the binding site for RNA polymerase (RNAP). Although the *ssoA* of various RC plasmids have conserved CS-6 and RS_B regions and similar folded structures, they have considerable sequence variation. Also, the *ssoA* sequences function efficiently only in their native host. We have found that the RNAP from *Staphylococcus aureus* binds with much greater affinity to the pE194-*ssoA* as compared to the pMV158-*ssoA*, suggesting that the strength of RNAP-*ssoA* interaction may be determined by the specific sequence of each *sso* which may determine the functionality of these lagging strand origins in various Gram-positive bacteria. We postulate that the specific *ssoA*-RNAP interaction plays an important role in lagging strand replication and is likely to be a key factor in determining the host range and horizontal spread of drug-resistance plasmids in Gram-positive hosts. Consistent with this observation we show that the RNAP from *S. aureus* and *B. subtilis* binds with similar high affinity to another type of *sso*, the *ssoU*. This origin of replication is present in plasmids such as pMV158 and pUB110 and, unlike the *ssoA*, is fully active in several Gram-positive bacteria.

Session IX: Control of replication

Chairperson: Margarita Salas

Biochemical and biophysical features of the CopG regulatory protein encoded by plasmid pMV158. Crystallisation and preliminary X-ray diffraction analysis

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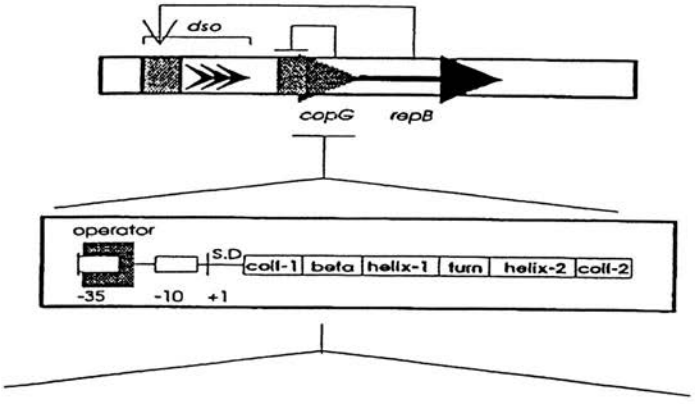
Control of replication in some plasmids is exerted by the combined action of a constitutively expressed antisense RNA and of a transcriptional repressor protein, both of them exhibiting the same hierarchical role (1-3). Such is the case of the streptococcal replicon pMV158, which is the prototype of a family of multicopy plasmids replicating by the rolling circle mechanism (4). In pMV158, the genes encoding the repressor CopG and the initiator of replication RepB proteins are co-transcribed from the single promoter P_{-} (1, 5). The *copG* gene product is a 5.1 kDa protein which binds to a DNA region that includes the promoter P_{-}, thus hindering the binding of the host RNA polymerase (6, 7). DNase I and hydroxyl radical footprinting experiments performed with purified CopG protein showed that its DNA target spans about 50 bp, the protein binding to five successive helix turns by the same face of the DNA helix. The centre of the CopG binding region contains a 13-bp sequence (the CopG operator) that shows a twofold rotational symmetry.

A new plasmid vector, and a new procedure for overproduction and purification of the protein have been developed, and thus CopG has been purified to near homogeneity. Gel filtration chromatography and analytical ultracentrifugation showed that the native protein is a spherical dimer of identical subunits, with a Stokes radius of 16 Å. Initial sequence-based secondary structure analyses of CopG indicated the existence of two putative α–helices separated by a short turn. Comparison with transcriptional repressors suggested that CopG could have a putative helix-turn-helix motif (HTH), both helices spanning from segments L17 to M24 (α-helix1), and from K28 to L36 (α-helix2), out of the 45 residues of CopG. Circular dichroism measurements of CopG indicated a consensus average content of more than 50% α-helix and 10-35% β-strand and turns, which is compatible with the predicted secondary structure of the protein. Molecular modelling of CopG showed a good fitting between the HTH motifs of well-known repressor proteins and the bihelical unit of CopG. However, modelling of CopG with the ribbon-helix-helix class of DNA binding proteins (like the Arc and Mnt repressors encoded by phage P22) also exhibited an excellent fitting.

Several mutations within the *copG* gene have been constructed. Overexpression of these mutant genes showed that deletions affecting regions other than the C-terminal end of the protein strongly reduced the intracellular stability of the respective CopG variants, in contrast to the wild type CopG, which exhibited a prolonged intracellular half-life. This indicates that CopG has a compact structure, perhaps constituted by a single domain.



Up. Schematic representation of the regulation of pMV158 copy number mediated by CopG (negative effector) and by RepB (positive effector). The antisense RNA II is not shown. Centre. Scheme of the position of the promoter P_{ss}, the CopG operator, the ribosome binding site (SD), and of the predicted secondary structure of CopG. Below. Amino acid sequence of CopG as derived from amino acid sequence and from chemical synthesis.

To investigate the detailed three-dimensional structure of CopG, we have undertaken its crystallisation, and we shall present some preliminary crystallographic results. Purified CopG protein has been crystallised using the sitting-drop vapour diffusion method. The crystals belong to orthorhombic space group $C222_1$ (cell constants a= 67.2 Å, b= 102.5 Å, c= 40.2 Å). Complete diffraction data up to 1.6 Å resolution have been collected. Considerations about the Matthews-parameter account for the most likely presence of three molecules in the asymmetric unit (2.27 $Å^3$/Da).

Eleven out of the twelve replicons belonging to the pMV158 plasmid family could also encode Cop proteins, which share features with both HTH and β-sheet DNA binding proteins.

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STUDIES OF ANTISENSE RNA CONTROL IN PLASMID R1

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Naturally antisense RNAs are ubiquitous plasmid copy number regulators, but also carry out a variety of other biological control functions in systems such as transposons, phage, and some chrosomomally encoded systems. This talk will discuss two topics.

1) CopA is an antisense RNA encoded by plasmid R1. CopA is an inhibitor of replication (and the principal copy number regulator) and acts by binding to the leader region of the repA mRNA (CopT) to prevent translation of the initiator protein RepA. The binding process, its structural requirements and its kinetics, have been the subject of previous work. Recently, we have attempted to identify CopA/CopT binding intermediates using enzymatic and chemical probing strategies. Surprisingly, we found that the major product of the binding reaction is NOT a full RNA duplex, but rather a complicated complex in which an asymmetric kissing complex is stabilized by additional (distal) base-pairing. The experimental evidence for this inhibitory complex and a 3D-model will be presented. The likelihood that the absence of full duplex formation is a rule rather than an exception will be discussed.

2) Antisense RNAs are known to be inhibitors of gene expression. In the course of studying the CopA/CopT system of plasmid R1, we fortuitously discovered that antisense RNA can activate gene expression by a novel mechanism. This activation is NOT part of the normal regulatory circuit that regulates R1 copy number but illustrates an expansion in the functional repertoire of regulatory RNAs. In vitro and in vivo results indicate that antisense/target RNA interaction (occuring during transcription of target RNA) results in transcriptional readthrough.

Experiments will be presented and discussed with respect to possible models.

TWO REPLICONS COEXIST IN PHAGE-PLASMID P4: DIFFERENTIAL ROLE OF THE NEGATIVE REGULATOR CNR IN THE TWO REPLICONS.

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P4 is a satellite bacteriophage that depends on a helper phage, such as P2, for morphogenetic and cell lysis functions. In the absence of P2, P4 can propagate in its host *Escherichia coli* as a multicopy plasmid. DNA replication of phage-plasmid P4 depends on the product of its α gene, a multifunctional protein with primase, helicase and specific DNA binding activity. Two regions in the P4 genome, both bound by the α protein, are required in *cis* for replication: *ori1*, the starting point of bidirectional P4 DNA replication, and *crr*, a region located about 4500 bp from *ori1*. A negative regulator of P4 DNA replication, the P4 Cnr protein, is required to control the copy number when P4 propagates as a plasmid. In the absence of Cnr, the P4 genomes overreplicate in the host cell, which eventually dies (1).

Over-expression of the *cnr* gene inhibits P4 lytic growth. P4 mutants insensitive to Cnr map in the α gene, suggesting a possible interaction between the two proteins (2). Using a two-hybrid system in yeast, we demonstrated that the wild type α protein interacts with Cnr in *vivo*, whereas cnr-resistant α proteins are unable to interact.

In *vitro*, Cnr stimulates the specific binding of the α protein to *ori1* and *crr* (2). Thus, complexes formed between α-Cnr and DNA are probably unable to allow initiation of replication.

By the use of a plasmid complementation test for replication in *vivo*, we delimited the P4 minimal region essential for replication. We found that two replicons coexist in P4. The first replicon comprises the *cnr* and α genes and the *ori1* and *crr* sites. The second is limited to the α-*crr* region. This indicates that the *ori1* region is dispensable and that replication can initiate at a different site. By deletion mapping, a *cis*-acting region (*ori2*) essential for replication of the α-*crr* replicon was mapped within a 270 bp fragment in the first half of the α gene. *ori2* is not essential for replication of a plasmid that contains *ori1*.

A construct that besides *crr* and α carries also the *cnr* gene was unable to replicate. This suggests that Cnr not only controls replication from *ori*, but also silences *ori2*. A possible role of Cnr in controlling initiation of P4 DNA replication will be discussed.

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Characterization of the replication regions of highly related coexisting plasmids in the phytopathogen *Pseudomonas syringae*

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Strain PT23 of the phytopathogen *P. syringae* pv. tomato contains four native plasmids: pPT23A (100 kb) and pPT23B (83 kb) are required for the production of full disease symptoms in tomato plants; pPT23C (65 kb) is cryptic and pPT23D (36 kb) is involved in copper resistance. Up to 74% of pPT23B is repeated in pPT23A, including replication sequences, which suggest that they probably originated from a duplication event (1). Using the defined minimal replicating fragment from pPT23A (1.6 kb; $oriV_{pPT23A}$) as a probe, it was shown that it is highly conserved in different pathovars of *P. syringae* (1), and that as many as six different native plasmids with closely related origins of replication coexist in the same cell. The nucleotide sequence of $oriV_{pPT23A}$ and two other related replicons was obtained. They contain a single ORF that could code for a putative replicase that is highly homologous to the RepA proteins of pTiK12 from *Thiobacillus intermedius* and ColE2-like plasmids. Besides a perfect inverted repeat (13 bp stem, 7-8 bp loop) located in the putative *repA* promoter, no other features typical of some replicons were found. At least three different regions of incompatibility were defined in the proximity of $oriV_{pPT23A}$. We have evidence that one of them (IncA) is involved in copy number control, while a second one could be part of a *par* system. Hybridization experiments using three of the Inc regions plus additional DNA adjacent to $oriV_{pPT23A}$, showed that plasmids related to pPT23A display a mosaic organization of their replication regions.

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POSTERS

LOCALIZATION OF *NIC-SITE* IN DOUBLE-STRANDED ORIGIN OF REPLICATION ON PLASMID pGA1 FROM *CORYNEBACTERIUM GLUTAMICUM.*

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Plasmid pGA1 is 4.8-kb cryptic plasmid isolated from *Corynebacterium glutamicum* LP-6[1]. Its minimal replicon (1.7 kb) carries the *rep* gene coding for the protein highly homologous to the Rep proteins of plasmids pSR1 (from *C. glutamicum*) and pNG2 (from *C. diphteriae*, replicating by rolling circle (RC) mechanism)[2]. As no homology of these Rep proteins with those of RC plasmids belonging to four known families was found, the related plasmids pGA1, pSR1, and pNG2 seem to be representatives of a new group of RC plasmids. Using the runoff DNA synthesis assay[3] we localized precisely the site- and strand-specific breakage on the pGA1 minimal replicon. This *nic*-site of double-stranded origin (*dso*) was determined in the sequence ATCCTGG A (roughly corresponding to the consensus sequence of *nic*-site of RC plasmids from pC194 family) located in the distal part of the pGA1 *rep* gene. This location of *dso* corresponds to that of plasmid pSR1, but differs from *dso* locations on other RC plasmids. Our result further supports classification of plasmids pGA1 and pSR1 into a new group of RC plasmids.

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Exonuclease Activity of the DNA Polymerase I of *Streptococcus pneumoniae*

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The DNA polymerase I of *Streptococcus pneumoniae* (Spn PolI) encoded by its *polA* gene is a member of the type I-like bacterial polymerases. This family of proteins is represented by the *Escherichia coli* DNA polymerase I (Eco PolI) and includes, among other members, the *Thermus aquaticus* polymerase (Taq Pol). Spn PolI posesses two enzymatic activities in the same polypeptide: (i) 5'→3' exonuclease activity, located at the N-terminal region of the protein, and (ii) DNA polymerase activity, located at the C-terminal region. Both enzymatic activities are involved in chromosomal replication and DNA repair processes, and are able to substitute *in vivo* for their counterparts in *E. coli*. Moreover, the polymerase activity of Spn PolI functions in the establishment and replication by the rolling circle mechanism of the promiscuous plasmid pLS1.

The pneumococcal *polA* gene was cloned and its DNA sequence determined. Aligment of six 5'→3' exonuclease N-terminal regions from bacterial polymerases and four related bacteriophage exonucleases, indicate the possible existence of six highly conserved sequence motifs containing invariant amino acids. Ten of these residues are acidic, suggesting that this activity requires the coordination of divalent metal ions at the catalytic site. Nine out of the ten carboxylate residues appear to be important for the exonuclease reaction, since inroduction of mutations at these residues in *E. coli* DNA polymerase I and DNA polymerase I from *Mycobacterium tuberculosis* resulted in a decrease of the 5'→3' exonuclease activity of these enzymes. However, the nature of the reduced activity of the mutants was not further investigated.

To get more insight in the 5'→3' exonuclease activity of the Spn PolI, two mutations, D10A and D190A, have been introduced at two of the invariant residues. Both mutations resulted in a decrease of the 5'→3' exonuclease activity. The two altered polypeptides (Spn PolID10A and Spn PolID190A) have been overexpressed in *E. coli* and purified to homogeneity. Characterization of the D190A mutant revealed that it retains the polymerase activity of the wild type enzyme, and displayed the strand displacement activity of its polymerase domain. However, the introduction of the D190A mutation resulted in a 2500 fold reduction of the 5'→3' exonuclease catalytic rate present in the wild type enzyme. The mutation at the D190 residue of the pneumococcal polymerase, affected the dependency on metal activation of its exonuclease activity. These results provide experimental support for a direct involvement of this aspartic residue in a metal-assisted 5'→3' exonucleolytic reaction. The role of the D10 residue on the 5'→3' exonuclease activity of Spn PolI is currently under investigation.

The *gene 40* protein of the *Bacillus subtilis* bacteriophage SPP1 is a DnaG associated hexameric helicase

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The *Bacillus subtilis* bacteriophage SPP1 gene *40* product (G*40*P) is essential for SPP1 replication. We have purified this protein to homogeneity in three steps and showed that the NH_2-terminal sequence is consistent with the deduced amino acid sequence of gene *40*. G*40*P (predicted molecular mass 49.8 kDa) has a native molecular mass of 300 kDa (G*40*P_6). G*40*P_6 binds DNA in a sequence-independent manner with a 20-fold higher efficiency for ssDNA. G*40*P_6 binds ssDNA under the allosteric influence of ATP or ATPγS, while it binds dsDNA in the absence of any cofactor. G*40*P_6 contains ATPase activity that is enhanced in the presence of ssDNA and, to a minor extent, in presence of dsDNA and RNA. The K_m value of G*40*P_6 ATP hydrolysis is 440 μM in presence of ssDNA. ATP hydrolysis occurs in the presence of Mg^{2+}, to less extent in presence of Mn^{2+} and Ca^{2+}, but not in the presence of Ni^{2+} or Zn^{2+}. G*40*P_6 is a DNA helicase capable of unwinding DNA with a 5' to 3' polarity in a concentration dependent manner, being the hexameric form of the protein the active species. We have also purified the DnaG primase of *B. subtilis*. NH_2-terminal sequence of the purified protein was consistent with the deduced sequence of the *dna*G gene. DnaG, which behaves as a monomer in solution, physically interacts with G*40*P_6 of SPP1. The presence of DnaG increased about 4-fold the helicase activity, but not the ATPase activity, of G*40*P_6.

The mode of replication of an *Erwinia citreus* plasmid pPZG500

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A multiple-copy plasmid pPZG500 (3661 bp) was isolated from a phytophatogenic bacterium *Erwinia citreus* ATCC 31623. This is the smallest plasmid so far isolated from the genus *Erwinia*. Since the curing of pPZG500 did not cause any phenotypic change, this plasmid is not essential for the bacterial survival.

Minimal regions required for replication (*ori*) and plasmid segregation stability (*par*), which had been defined in our previous work, were sequenced by the fluorescent dideoxy method on the apparatus ABI PRISM 377. Sequencing of the 1256 bp *Eco*RV/*Bgl*II DNA fragment responsible for the plasmid replication, identified -10 and -35 promoter regions for RNAI (115 bp) and RNAII (ca 700 bp) molecules which are necessary for the initiation and regulation of the replication. In addition, an AAAAAT *ori*V oligonucleotide was observed at the position of 177 bp, near the end of the RNAII transcription. Secondary structures of RNAI and RNAII molecules were proposed by the software RnaDraw (O. Matyura and A. Wennborg, 1995), on the basis of their ΔG values at 28 °C - the optimal growth temperature for *Erwinia citreus*. In the RNAII sequence there were 17 bp α (loop III) and β (loop V) imperfect indirect repeats whose pairing might form another hairpin loop which unables RNAI to pair with RNAII and inhibits plasmid replication. This α-β pairing allows elongation of RNAII through the *ori*V and initiation of the leading strand synthesis. Downstream from the *ori*V, 126 bp apart, were identified three short indirect repeats that could also bend the plasmid DNA. In the middle of these indirect repeats there is a putative DnaA binding box (TTTTAACA), indicating the possibility that plasmid pPZG500 has ΦX174-type of primosome assembly that could be replaced with ABC-type if necessary. According to these data we propose θ type of replication for the plasmid pPZG500.

Sequencing of the 653 bp *Bgl*II/*Eco*RI DNA fragment that is responsable for the plasmid segregation stability, identified an *orf* of 629 bp (208 aa). This amino acid sequence was compared to the SwissProt protein data bank and the results indicated significant homology to the λ-integrase family. These proteins have highly conserved region with tyrosine residue within the active site of the molecule. They are capable to resolve the catanene plasmid dimmers, which are consequence of θ type replication, prior to plasmid segregation to the daughter cells, therefore ensuring the correct plasmid inheritance.

Replication of the pPZG500 replicon is dependent upon DNA polymerase I. This is an additional supporting evidence that the predicted θ type of replication is correct for that plasmid.

BACTERIOPHAGE Ø29 EARLY PROTEIN p17 IS REQUIRED FOR THE FIRST ROUNDS OF VIRAL DNA REPLICATION.

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Gene 17 of the *Bacillus subtilis* phage ø29 is known to be involved in viral DNA replication *in vivo* (Moreno *et al.*, 1974; Carrascosa *et al.*, 1976). The nonsense ø29 *sus*17(112) mutant, when infecting a non suppressor *B. subtilis* strain, shows a "leaky" phenotype, characterized by the late appearence of very tiny lysis plaques, suggesting that protein p17 can be partially dispensable (Mellado *et al.*, 1976). In our study, analysis of both phage production and DNA synthesis indicated that the presence of protein p17 is required when phage infection occurs at low multiplicity of infection (moi), which are probably the natural conditions for infection, but is dispensable at high moi. Analysis of protein p17 accumulation during ø29 infection indicates that gene 17 is rapidly expressed, yielding its maximal level (30000 molecules *per* infected cell) 10 min post-infection, when the other replicative proteins have not yet reached their optimal concentration. This suggests that protein p17 plays its role at an early stage of viral DNA amplification. Gene 17 was cloned and protein p17 was overproduced in the *E. coli* BL21 (DE3) pLysS strain upon IPTG induction, representing 15% of the total protein. Pure protein p17 was able to stimulate four-fold the *in vitro* ø29 DNA amplification, when using a limiting amount of viral template DNA, but had a null effect under conditions of DNA excess. We propose that protein p17 is required at the very beginning of the phage DNA amplification, in conditions of low number of viral DNA molecules entering the host cell, possibly by recruiting the limiting initiation proteins at the replication origins. Once the infection process is established and the other replication proteins reach optimal concentration, the presence of protein p17 would become dispensable.

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Kid, the killer protein of the parD stability system of R1, interacts with the DnaB protein and inhibits its helicase activity

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ParD is a killer stability system of the antibiotyc resistance factor R1, that is compose by two genes, *kis* and *kid*, codifying for the Kis (Killing supressor) and Kid (Killing determinant) proteins (1). The coordinate action of both proteins allows the efficient and specific counterselection of plasmid-free segregants (2). This toxic effect is exerted at the level of DNA replication and it has been shown both 'in vivo" and "in vitro" that, if it is not neutralized by Kis, a much more unstable protein than Kid, the later inhibits DnaB dependent replication (3). We have now demonstrated that Kid inhibits DnaB helicase activity "in vitro"., and that this inhibition is also neutralize in presence of Kis. In contrast, this protein is not able to inhibit the activity of other *Escherichia coli* helicases (Helicases I and II), implicated in DNA repair. Several experiments are now in course to characterize the mechanism of action that account for this inhibition. DnaB, but not Helicase I or Helicase II, interacts with Kid, and this interaction is not possible in presence of Kis, a result that suggests that physical interaction between Kid and DnaB may be responsible for inhibitionof the helicase activity of the later. Current work is focused in the possible inhibition of the other replicative helicase of E. coli, PriA, in the possible existence of an specific sequence at the origin of replication of E. coli to which Kid could be bound to increase its contrahelicase activity and in the identification of the interaction regions between Kid and DnaB.

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Structure-function analysis of the ø29 terminal protein

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Bacteriophage ø29 DNA replication starts at both DNA ends by a specific protein-priming mechanism in which ø29 DNA polymerase forms a heterodimeric complex with a molecule of the viral terminal protein (TP) that recognizes the replication origin, probably through the interaction between the TP in the complex and the parental TP attached to the DNA. Once the complex is located at the origin, the DNA polymerase catalyzes the linkage of dAMP to the TP, which acts as a primer, in a reaction directed by the second (3'-terminal) template nucleotide (Méndez *et al.*, 1992). After initiation, the same DNA polymerase completes replication of each parental strand.

Deletion mutagenesis studies in ø29 TP allowed to identify a DNA binding domain at the N-terminus (positions 13-71) and two DNA polymerase domains, one at the C-terminus (positions 242-262) and the other at an internal region (positions 72-80) near the N-terminus (Zaballos and Salas, 1989). We have shown that deletions from 10 to 54 amino acids at the amino end of the TP decreased the capacity to interact with the DNA polymerase. The presence of the viral protein p6 (origin-binding protein) partially restored the activity of these deletion mutants, suggesting that protein p6 mediates or favours the formation of the TP/DNA polymerase complex.

The RGD (Arg-Gly-Asp) motif is known to be responsible for protein-protein interaction in important biological reactions. A RGD sequence is present at the C-terminal region of ø29 TP and in the TP of other *B. subtilis* phages. It has been proposed that the primer TP takes the DNA polymerase to the initiation site by interacting with the parental TP (bound to DNA) through its RGD sequence (Kobayashi *et al.*, 1989). To elucidate the role of the RGD sequence of the ø29 TP, seven modified TPs were generated by site-directed mutagenesis. Ours results demonstrate that the RGD sequence of ø29 TP is involved in the interaction with the ø29 DNA polymerase, but an additional role in primer-parental TP recognition cannot be ruled out.

Several TPs of *B. subtilis* and *E. coli* bacteriophages were aligned to identify TP residues as relevant candidates for its priming (and/or parental) function. Based on this alignment, we have constructed eight point mutants in five residues highly conserved in a region near the N-terminus of the ø29 TP. Biochemical analysis of these mutant TPs in an *in vitro* replication system showed that these residues are not critical for the priming activity of TP. However, when the mutants were analyzed in an amplification assay, in which the mutant TPs must act also as parental TPs in the next replication round, two residues, Asn80 and Tyr82, were shown to be needed for parental TP recognition function.

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Requirements for Staphylococcal pT181 Replication. Ruzhong Jin, and Richard Novick. Skirball Institute of Biomolecular Medicine, NYU Medical Center, 540 First Avenue, NY, NY 10016

Small staphylococcal plasmids replicate *via* an asymmetric rolling circle mechanism, initiated by a plasmid encoded protein that introduces a site specific nick in its double-strand replication origin (DSO). DSOs of the plasmids in pT181 family consist two adjacent inverted repeat elements (IRII and IRIII), which are involved in origin recognition by the initiator (Rep) proteins. The conserved core element, IRII, can form a cruciform which contains the nick site at the tip of the structure. The divergent element, IRIII constitutes the determinant of origin recognition specificity. It has been shown in pT181 that the distal arm of the IRIII is not required for sequence-specific recognition, whereas the proximal arm and the central region of IRIII are absolutely required (Wang, *et al.* 1993). pT181 initiator RepC/C binds to IRIII and induces the melting and subsequently cruciform extrusion of the IRII (Noirot, *et al.*, 1990; Jin, *et al.*, 1996), which exposes the nick site for RepC/C to attack and initiate replication. Although initiators are specific for their binding sites on the plasmids, cross complementation between different initiators and DSOs in pT181 family has been observed both *in vitro* (Thomas, *et al.*, 1989) and *in vivo*, when the initiator proteins were present at a highly elevated level. Superhelical tension of the plasmids has also been demonstrated to be essential for the initiators to melt the DNA and enhance the cruciform formation of IRII. No DNA melting at the DSO could be detected upon RepC/C binding on linear pT181 plasmid DNA (Jin, *et al.*, 1996). Cross complementation was also eliminated on topoisomerase relaxed plasmid DNA (Thomas, *et al.*, 1989). In this report, we show that the cruciform structure of IRII in DSO is not absolutely required for the leading strand replication. Eliminating the potential cruciform structure of IRII by substituting its left arm with other sequences non-complementary to its right arm did not show any detectable defect in pT181 normal replication, namely, it did not change the copy number of the plasmids, neither did it affact the competitivity of the mutant plasmids with the wild type pT181. However, the IRII defective DSO lost its complementarity with RepD, the initiator of plasmid pC221 in the same family. Furthermore, the cruciform structure became indispensible when the RepC/C binding site (IRIII) on plasmid is suboptimal. Based on the above observation, we conclude that there is a very subtle interaction between initiator protein and its plasmid DSO. DNA superhelical tension, IRII cruciform structure, and optimal IRIII initiator binding site are the three factors on DNA that are directly involved in the initiator induced replication initiation. The plasmid has a fail-safe system to ensure the inititaiton of replication when one of these factors is defective. The system is defeated when two of the three factors are defective.

The Initiation of Replication of the Broad Host Range Plasmid RK2: Requirement of DnaA boxes

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Many prokaryotic replication origins contain one or more essential binding sites (DnaA boxes) for the DnaA protein. During the initiation of replication of the chromosome of *Escherichia coli* the DnaA protein binds to DnaA boxes and this results in the destabilization of duplex DNA at the A+T rich region and open complex formation. During the initiation of the broad host range plasmid RK2, it was previously demonstrated that the *E. coli* DnaA protein binds to four DnaA boxes localized upstream of the iteron sequences within the RK2 origin. Although the binding of the DnaA protein does not by itself produce an RK2 open complex, it does enhance and/or stabilize the initiation protein (TrfA) mediated strand opening. It has also been demonstrated that the DnaA protein directs the DnaB-DnaC complex to the RK2 plasmid replication origin. In this study we investigated the structural requirement for DnaA protein binding and the effects of this binding on the initiation of RK2 replication, specifically strand opening and helicase unwinding. Using PCR, mutations were introduced in the minimal origin to eliminate DnaA binding to each of the four DnaA boxes. A mutation in DnaA box 4 which is located immediately adjacent to the iteron sequences, completely abolished RK2 replication in *E. coli in vivo* and *in vitro*. Surprisingly, a plasmid with this mutant origin was functional in *Pseudomonas aeruginosa*. A plasmid with the deletion of all four DnaA boxes within the origin sequence was also unable to replicate in *E. coli*, and *Pseudomonas putida*, but was able to replicate in *Pseudomonas aeruginosa* and to some extent in *Azotobacter vinelandii*. These results suggest that RK2 exhibits different structural requirements for the initiation of replication in different bacterial hosts. Interestingly, replication of RK2 plasmid derivatives which contain a defective DnaA box or a deletion of all four boxes can be restored for activity *in vitro* by higher concentrations of the *E.coli* DnaA protein. It is possible that the mode of replication initiation is somewhat dependent on the concentration of DnaA protein in the host cell and the specific affinity of the host DnaA protein to a particular DnaA box localized within the RK2 origin. These results are consistent with a degree of plasticity and adaptability of the RK2 origin with respect to structural requirements for replication in different bacterial hosts.

Replication initiators involved in plasmid host range.

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Efficient communication between plasmid and bacteria replication systems is at the basis of plasmid host range. In the initial stages of the replication process molecular interactions between plasmid and bacterial proteins should lead to the formation of a productive initiation complex. This most probably determines the ability of a plasmid to colonize a bacterium.

We are studying the molecular interactions which determine the host range of pPS10, a plasmid isolated from *Pseudomonas syringae* pv.*savastanoi* which minimal replicon has been characterized (Nieto et al., 1992). This 1823 bp replicon possesses one origin of replication, *oriV*, and a sequence, *rep*A, that codes for the plasmid specific replication protein, RepA. In the origin of replication there are four 22 bp-long direct repeats (iterons) flanked by a *dnaA* box and an A+T-rich region. This *oriV* structure is typical of iteron-containing plasmids. Upstream of *repA* gene there are sequences with homology to the -35 and -10 boxes of the σ^{70} promotors and an overlapping region with sequences that show partial homology to the iterons. RepA shows, in its C-terminus, an HTH DNA binding motif and, in its N-terminal sequence, a leucine zipper motif which is involved in protein dimerization. The interaction of the protein monomers with the origin iterons leads to initiation of the plasmid replication, whereas the binding of dimers to the promotor regulates transcription of the *rep*A gene (Garcia de Viedma et al., 1996).

pPS10 can be established efficiently in *Pseudomonas aeruginosa* and *Pseudomonas putida*. However this plasmid shows an inefficient establishment in *Escherichia coli* at 30°C which becomes undetectable at 37°C and temperatures above (Fernández-Tresguerres et al., 1995). Thermosensitivity of pPS10 in *E. coli* provides a direct selection method to isolate mutants with increased host range. After hydroxylamine treatment of wild type pPS10, plasmid mutants able to transform *E. coli* at 37°C were selected. Mapping of the mutations showed that they belong to five groups all located within the *repA* gene. This identifies RepA protein as a plasmidic host range factor.

The phenotype of all isolated mutants is very similar: their copy number in *Pseudomonas* and *E. coli* is similar, they display an equivalent transformation efficiency and are equally stable in both strains. Moreover, in electroporation experiments all the mutants show the same ability to transform different Proteobacteria.

Circular dichroism studies of the wild-type and mutant RepA proteins show that the mutations do not induce significant conformational changes in the secondary structure or in the stability of the proteins.

These data suggest that the mutations affect to quaternary-type interactions of RepA, either with the DNA or with host factors.

We have studied the interactions of the RepA protein with the DNA sequences corresponding to the origin of pPS10 and to the promoter of the *repA* gene. The bandshift experiments demonstrate that some of the RepA mutants show a better interaction both with the origin and the promoter sequences, whereas in others the interactions are similar to that showed by wild type protein. This indicates that an increase in the efficiency of binding of RepA to the origin of replication is not sufficient to allow pPS10 replication in *E. coli*.

The results from the CD and bandshift experiments suggest that the increase in host range of our pPS10 mutants could be due to modifications in quaternary type interactions of RepA with other host replication proteins. We favour DnaA and/or DnaB protein/s as presumptive partner/s of RepA in host-plasmid interactions modulated by the mutation, since all of them act in the initial stages of DNA replication which must be crucial to define the establishment of the plasmid. To confirm this hypothesis, we are presently trying to isolate chromosomal mutants that allow replication of wt pPS10 in *E. coli* at 37°C.

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IHF INHIBITS DNA RELAXATION DURING R388 CONJUGATION

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E. coli integration host factor (IHF) is a dimeric protein that binds to specific sites in DNA resulting in bending. It plays roles in several biological processes, like site specific recombination, DNA replication and regulation of gene expression (Friedman, 1988). Of particular interest to our work, IHF binding to the origin of transfer (*oriT*) of F-like plasmids stimulates the relaxase catalyzed nicking at *oriT* (Nelson *et al.*, 1995).

We have analyzed the role of IHF in R388 conjugation. In R388, three plasmid-encoded proteins (TrwA, TrwB and TrwC) are involved in DNA processing for conjugal transfer (Llosa *et al.*, 1994). TrwC is the relaxase protein (Llosa *et al.*, 1995) and it has also DNA helicase activity (Grandoso *el al.*, 1994). TrwA stimulates TrwC nicking activity and represses the transcription of *trwABC* operon (Moncalián *et al.*, 1997).

DNAse I footprinting analysis identified two IHF binding sites within R388 *oriT* DNA. TrwC was found to bind to scDNA containing *oriT* but not linear DNA. IHF was found to inhibit directly TrwC relaxase activity *in vitro*, although it did not act by inhibiting TrwC binding. TrwC alone did not nick linearized DNA and IHF with or without TrwA did not help it (as in F plasmid do). However, no differences in the frequency of conjugation were found in between IHF^- and IHF^+ strains. In spite of the lack of an effect on conjugative rates an *in vivo* effect could be observed: R388 relaxed forms were more abundant in IHF^- strains. Thus, IHF could have an inhibitory role in the nicking reaction only in "resting" cells. When conjugation is activated via the mating signal this inhibition should be released.

A model will be proposed to explain the different role IHF seems to have in R388 conjugation with regard to F conjugation.

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Regulation of initiation of "theta" and "sigma" mode of λ DNA replication in *Escherichia coli*

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There are two modes of replication of λ phage DNA upon infection of *Escherichia coli* cells. The circle-to-circle ("theta") mode is called the early replication, and the rolling-circle ("sigma") mode occurs later during the infection cycle. It is not know what triggers a switch between early and late replication of λ DNA. We investigated λ DNA replication in density shift experiments, after infection of *E. coli* cells growing in a heavy medium with [^{3}H]thymidine-labelled light phages. In λ-infected wild-type cells, several rounds of "theta" replication proceeds leading to appearance of many copies of phage λ genome. Among them only a few switches to the "sigma" mode of replication. Therefore, monitoring the fate of infecting phage DNA we observed only a shift from "light-light" to "heavy-light" position. In the case of λ-infected *dnaA46* mutant, we observed significant shift of radioactivity in the direction of "heavy-heavy" fractions which indicates that parental phage DNA molecules, most probably after one round of "theta" replication (perhaps unidirectional), starts replication according to the "sigma" mode. The same results were obtained using otherwise isogenic *dnaA*$^+$ and *dnaA*-null strains. We demonstrated previously that *dnaA* gene product stimulates phage p_R promoter which is responsible for transcription activating *oriλ* region. This process, called transcriptional activation of *origin*, is necessary for transient denaturation of DNA at *oriλ* and proper installation of the replication complexes. Therefore, we propose that inefficient transcriptional activation of *oriλ* in *dnaA* mutants (due to decreased activity of the p_R promoter) allows for installation of only one replication complex which can carry out only unidirectional "theta" replication. Such unidirectional "theta" replication may switch to the "sigma" replication. Thus, in wild-type cells infected with λ phage, switch from "theta" to "sigma" replication may be caused by consumption (titration) of DnaA function by extensively replicating viral DNA molecules. In such conditions, a few of viral DNA molecules might start "sigma" mode of replication at the late stage of infection. To verify the above mentioned hypothesis, we investigated directionality of DNA replication by two-dimensional gel electrophoresis and using electron microscopy. As a model we used a plasmid derived from bacteriophage λ. In accordance with our hypothesis, we found that in the *dnaA* mutant λ plasmid DNA replicates exclusively unidirectionally, contrary to the wild-type host in which bidirectional and unidirectional replication may be observed. There are two pathways of λ plasmid replication in *E. coli*. One is based on the activity of the replication complex inherited by one of two daughter plasmid circles after each replication round, and the second is carried out by the newly assembled complexes. In certain conditions, i.e. during the relaxed response (i.e. in amino acid-starved *relA* mutants) and in the case of λ*cro*(ts)*P*ts1 plasmid mutant at 43°C, only the first pathway can be observed. We found that in these conditions λ plasmid DNA replicates unidirectionally. Therefore, we propose that λ plasmid replication carried out by the heritable replication complex proceeds unidirectionally from *oriλ*, and replication based on the newly assembled replication complexes is bidirectional.

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