The bacteriophage λ O and P protein initiators promote the replication of single-stranded DNA

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ABSTRACT

A soluble enzyme system that specifically initiates λ *dv* plasmid DNA replication at a bacteriophage λ replication origin [Wold *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79, 6176-6180] is also capable of replicating the single-stranded circular chromosomes of phages M13 and ϕ X174 to a duplex form. This chain initiation on single-stranded templates is novel in that it is absolutely dependent on the λ O and P protein chromosomal initiators and on several *Escherichia coli* proteins that are known to function in the replication of the λ chromosome *in vivo*, including the host dnaB, dnaG (primase), dnaJ and dnaK replication proteins. Strand initiation occurs at multiple sites following an O and P protein-dependent pre-priming step in which the DNA is converted into an activated nucleoprotein complex containing the bacterial dnaB protein. We propose a scheme for the initiation of DNA synthesis on single-stranded templates in this enzyme system that may be relevant to strand initiation events that occur during replication of phage λ *in vivo*.

INTRODUCTION

Replication of the bacteriophage λ chromosome is initiated on super-helical DNA at a unique origin, from which replication forks are propagated in a bidirectional fashion (see references 1 and 2 for recent reviews of λ DNA replication). This complex process is carried out through the concerted action of many viral and bacterial replication proteins (2). Only two phage λ -encoded proteins, the O and P gene products, are included among the required proteins, the remainder being *Escherichia coli* proteins such as primase (dnaG protein), DNA polymerase III holoenzyme, single-stranded DNA binding protein (SSB), RNA polymerase, and DNA gyrase, as well as the host dnaB, dnaJ and dnaK proteins (unpublished data; refs. 2-8). Although the precise molecular roles of the two λ initiators in λ DNA replication remain to be determined, the λ O protein does bind specifically to a quartet of repeating 19 bp sequences present in the λ replication origin (*ori* λ) (9, 10). In contrast, the λ P protein does not bind to DNA. Instead, it forms a complex with the *E. coli* dnaB protein (11-14) and it apparently also inter-

acts with the host dnaK protein (15) and with the λ O protein (2).

The functional roles of many *E. coli* replication proteins have been established through studies of soluble enzyme systems capable of replicating the single-stranded (SS) circular DNA chromosomes of phages M13, G4, and ϕ X174 (16, 17). Much of the complexity and variety in these DNA replication systems was found to reside in the mechanisms employed to prime DNA synthesis. Related studies of the *in vitro* replication of superhelical ϕ X174 replicative form DNA and of ColE1 plasmid DNA revealed in each case that the leading and lagging strands are initiated by separate mechanisms (16-18). The common rifampicin-resistant mechanism used for lagging strand synthesis on these duplex DNAs may be particularly relevant to studies of λ DNA replication, since two proteins required for λ replication, the *E. coli* dnaB protein and primase, are also participants in these lagging strand reactions. The nascent lagging strand fragments are primed by a mobile multiprotein complex (a primosome), which apparently contains *E. coli* proteins n', dnaB and primase, and perhaps others (18). Primosome assembly is believed to occur on the ϕ X174 and ColE1 chromosomes at discrete sites that are recognized by protein n' (19-21).

Soluble *in vitro* systems that support the initiation of bidirectional DNA replication from a λ origin have been independently developed in several laboratories (6-8, 14). A relatively crude *E. coli* fraction capable of initiating DNA replication at a cloned *E. coli* replication origin (22) can also replicate λ dv plasmid DNA if it is supplemented with purified preparations of the λ O and P protein initiators (6, 8).

In order to simplify the analysis of bidirectional λ DNA replication, we attempted to dissect this complex reaction into discrete leading strand and lagging strand initiation events. We followed the approach pioneered by Ray and coworkers for isolation and analysis of single-stranded initiation determinants (23). Fragments from the λ origin region were inserted into modified phage M13 single-stranded cloning vectors. When the resulting recombinant chromosomes were screened for their capacity to serve as templates in the *in vitro* λ dv plasmid replication system (6), we discovered a novel DNA strand initiation mechanism that depends on the action of the λ O and P initiators and on the function of key elements of the *E. coli* replication machinery. In this paper we describe the properties of this unique strand initiation system. A preliminary account of a portion of this work was recently published (14).

MATERIALS AND METHODS

Bacterial Strains, Phage Strains and Plasmids

The *E. coli* K-12 strains and their sources were: C600 from Dr. A. Kornberg; K37 (Hfr, *supD*) and RL108 (*F*⁺, *recA*⁻) from Dr. D. Ray; 71-18 [Δ (*lac-proA,B*), *supE*/ *F'* *lacI*^q*lacZ*AM15] from Dr. R. Wu; C600*dnaJ*259 from Dr. C. Georgopoulos. The M13 phage strains and their sources were: M13 from Dr. A. Kornberg; M13mp7 (24), M13mp8 and M13mp9 (25) from Dr. J. Messing; M13ori λ R, M13ori λ L, M13mp8ori λ L, M13*l*iceR and M13*l*iceL are described in this paper. Plasmids pRLM4 and pHL34 are described elsewhere (6, 26). ³H-labeled, supercoiled, phage fd replicative form (RF I and RF II) DNA was generously provided by Dr. A. Yeung of this department.

Materials

Creatine phosphate and nicotinamide mononucleotide (NMN) were from Boehringer Mannheim; rifampicin, coumermycin, polyvinyl alcohol type II, isopropylthiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (XGal) were from Sigma; spermidine was from Ames Laboratories (Milford, Connecticut); unlabeled ribonucleotides and deoxyribonucleotides were from P-L Biochemicals; [methyl-³H]dTTP (40-70 Ci/mole) was from ICN; [α -³²P]dCTP (400-450 Ci/mole) was from Amersham.

Enzymes, Proteins and Antibodies

Proteinase K, creatine kinase and *Cla* I were from Boehringer Mannheim; RNase A and RNase T1 were from Worthington; *Bam*HI, *Bgl* II, *Hinc*II, *Sal* I and T4 DNA ligase were from New England BioLabs; *Alu* I was from BRL. *E. coli* *dnaB* protein, primase, and SSB were each purified to greater than 90% homogeneity as described (6). DNA polymerase III holoenzyme ($1-5 \times 10^5$ units/mg) was purified by a modification of a published procedure (27) or was provided by Dr. C. McHenry. Bacteriophage λ O protein was purified to homogeneity as described previously (10). Homogeneous λ P replication protein was isolated using a published protocol (14, 28). *E. coli* *dnaJ* and *dnaK* (15) proteins (each > 90% pure) and anti-*dnaK* protein gamma globulin (IgG) (15) were kindly provided by Drs. M. Zylicz and C. Georgopoulos. Antibodies specifically directed against *E. coli* *dnaB* protein, *dnaC* protein, primase, or protein i (6) or against λ P replication protein (28) have been previously described.

Growth and Isolation of M13 Phage

M13 phage strains were grown on K37 or RL108 and were isolated essentially as described (29), except that as a final purification step the phage were banded in a CsCl step-gradient (30).

Isolation of Plasmid and Phage

Supercoiled phage RF DNA, present in M13-infected RL108 (29), and plasmid DNAs were isolated by the method of Norgard (31). M13 viral DNA was isolated from purified phage that had been suspended in TE buffer (10 mM Tris/HCl, pH 8.0, 1mM EDTA). The phage suspension was incubated for 1 hr at 37°C after adding RNase A and RNase T1 to final concentrations of 50 µg/ml and 25 units/ml, respectively. Proteinase K (self-digested) was added to 0.5 mg/ml and the mixture was again incubated at 37°C for 1 hr. The phage suspension was extracted twice with phenol (equilibrated with TE buffer) and several times with diethyl ether. The viral DNA was concentrated by isopropanol precipitation, suspended in TE buffer and stored at 4°C.

Construction of M13-λ Single-Stranded Viral DNAs

DNA fragments containing the λ replication origin and surrounding regions were obtained from plasmid pRLM4 and were isolated by agarose gel electrophoresis, followed by chromatography on DEAE-cellulose as described previously (6). M13mp7, M13mp8, or M13mp9 RF DNA was digested to completion with appropriate restriction endonucleases and ligated to a purified fragment with T4 DNA ligase. The ligation mixture was used to transform Ca²⁺-treated *E. coli* 71-18 (29). Recombinant phage, detected as colorless plaques on XGal plates (32), included the following hybrid phage: M13ori_R and M13ori_L carry the R and L strands, respectively, of a 1.5 kb λ *Alu* I fragment [base pairs 38453-39956 of the Sanger sequence of λ (33), containing both the phage replication origin and the λ *ice* site (26)] that was inserted into the *Hinc*II site of M13mp7; M13mp8ori_L contains the L strand of the λ replication origin on a 1.06 kb *Hinc*II fragment (base pairs 38548-39608 of the λ sequence) that was inserted into the *Hinc*II site of M13mp8; M13*ice*_R and M13*ice*_L carry the R and L strands, respectively, of the λ *ice* site on a 0.45 kb *Bam*HI - *Sal* I fragment (derived from plasmid pHL34 and containing base pairs 38453-38664 of λ) that was cloned between the *Bam*HI and *Sal* I sites of M13mp8 and M13mp9.

Partial Fractionation of *E. coli* Replication Proteins

E. coli replication proteins were partially purified by ammonium sulfate fractionation [Fraction II of Fuller *et al.* (22)]. For use in some experiments, Fraction II protein was further purified to obtain protein that was soluble in the presence of 36% saturated ammonium sulfate. This fraction, designated the "36-48% ammonium sulfate fraction", was prepared as follows. A Fraction II ammonium sulfate precipitate (22), prepared from 52 g of C600 cell paste, was suspended in 35 ml of buffer B (22) containing ammonium sul-

fate at approximately 36% saturation (0.20 g of ammonium sulfate was added to each ml of buffer B). The suspension was centrifuged at 40,000 x g for 20 min at 4°C and the protein in the supernatant fraction was concentrated by ammonium sulfate precipitation (50% saturation). The precipitate was collected by centrifugation, resuspended in 10 ml of buffer B, and chromatographed on Sephadex G-25 equilibrated in buffer B + 25 mM NaCl to remove nucleotides. The filtered protein was concentrated by ammonium sulfate precipitation as described above, redissolved in a minimal volume of buffer B, dialyzed against buffer B until its conductivity was equivalent to that of 0.4 M KCl, frozen in liquid nitrogen and stored at -70°C.

Assays for DNA Replication

The conditions used for replication of single-stranded DNA were a modification of those used for replication of λ dv plasmid DNA *in vitro* (6). The standard reaction mixture, 30 μ l, contained the following components: Hepes/KOH (pH 7.6), 33.3 mM; magnesium acetate, 9.2 mM; bovine serum albumin, 42 μ g/ml; ATP, 1.7 mM; CTP, GTP, and UTP, each 420 μ M; creatine phosphate, 36 mM; creatine kinase, 83 μ g/ml; polyvinyl alcohol 24000, 5% (wt/vol); rifampicin, 17 μ g/ml; *E. coli* SSB, 63 μ g/ml; M13mp7 viral DNA, 5 μ g/ml (450 pmol of nucleotide equivalent); *E. coli* Fraction II, 6-9 mg/ml; λ O protein, 10 μ g/ml; λ P protein, 1.5 μ g/ml; dATP, dCTP, and dGTP, each 160 μ M; dTTP, 60 μ M with [methyl-³H]dTTP at 100 cpm per pmol of total deoxynucleotide. Mixtures were assembled at 0°C. Reaction mixtures were incubated for 20 min at 30°C. DNA synthesis was measured as previously described (6).

Agarose Gel Electrophoresis

The conditions used for electrophoresis of duplex DNA in neutral agarose gels have been described previously (34). Single-stranded DNA was electrophoresed in 1% alkaline agarose gels as described (35), except that the gel was pre-electrophoresed for 1.5 hr at 4 V/cm.

RESULTS

Single-stranded Circular DNA Templates Are Replicated by an *in Vitro* λ DNA Replication System

The discovery of primosome assembly sites in ColE1 and pBR322 and the potential role of these sites as origins of lagging strand synthesis during replication of these plasmid chromosomes prompted us to search for similar rifampicin-resistant strand initiation determinants located near the bacteriophage λ replication origin. M13 was chosen as the vector to be used in this search for λ strand initiation determinants, because chain initiation

at the M13 complementary strand origin depends on *E. coli* RNA polymerase and, thus, can be specifically inhibited by the addition of rifampicin. A 1.5 kb Alu I fragment carrying both the λ origin and the λ ice site [a possible lagging strand origin for λ (36)] was inserted into M13mp7 RF DNA. Circular SS viral DNAs bearing either the L strand or the R strand of the λ Alu I fragment were isolated from preparations of recombinant phage.

When M13mp7 viral DNA was used as a template in the soluble *E. coli* replication system of Fuller *et al.* (22), we detected significant levels of DNA replication, even in the presence of rifampicin (Table 1, line 1). The properties of the observed DNA synthesis indicated that the RNA polymerase-independent strand initiations were most likely being primed by the *E. coli* general priming reaction (37), in which dnaB protein and primase synthesize short RNA transcripts on any SSB-uncoated single-stranded DNA. This nonspecific replication was suppressed both by additional SSB (Table 1, line 2) and by anti-dnaB protein IgG (data not shown). In all further reactions sufficient SSB was added to preclude initiation by the general priming reaction.

Addition of purified λ O and P replication proteins to the SSB-fortified *E. coli* protein fraction greatly stimulated the replication of

Table 1. Replication of single-stranded circular DNA templates *in vitro* promoted by the λ O and P proteins.^a

Template	Addition or omission	DNA synthesis, pmol
1. M13mp7	-SSB	80
2. M13mp7	none	10
3. M13ori λ R	none	8
4. M13ori λ R	+ λ O & P proteins	191
5. M13ori λ L	none	7
6. M13ori λ L	+ λ O & P proteins	170
7. M13mp7	+ λ O & P proteins	178
8. M13	+ λ O & P proteins	128
9. M13mp7 RF I	+ λ O & P proteins	9
10. ϕ X174	none	59
11. ϕ X174	+anti-dnaC protein IgG	0
12. ϕ X174	{+ anti-dnaC protein IgG & λ O & P proteins	29

^a Replication reactions were performed as described in Materials and Methods, except that the template DNA was varied and the λ O and P protein initiators were present only where indicated. Anti-dnaC protein IgG, when used, was added in sufficient quantity to completely block the ϕ X174 SS to RF conversion that occurs in the presence of *E. coli* Fraction II. The antibody fraction was preincubated for 10 min at 0°C with *E. coli* Fraction II prior to the initiation of DNA synthesis.

recombinant M13 chromosomes carrying either strand of the λ replication origin (Table 1, lines 3-6). Surprisingly, this stimulation did not depend on the presence of λ DNA sequences in the M13 template; however, it did require SS DNA (Table 1, lines 7-9). Moreover, the phage λ initiators also stimulated ϕ X174 viral strand replication *in vitro*. This enhancement was most apparent when the ϕ X174 complementary strand synthesis pathway (16) was blocked by the presence of antibody directed against the *E. coli* dnaC protein (Table 1, lines 10-12). For ease of discussion, we have named the λ O and P protein-dependent replication of single-stranded, circular DNA the " λ SS replication reaction".

Requirements for λ O and P Protein-Dependent Replication of M13 Viral DNA

Although the λ O and P protein-stimulated replication of SS DNA clearly does not require any λ DNA sequences, the involvement of the λ chromosomal initiators in this strand initiation reaction prompted us to examine the possible physiological relevance of the observed DNA synthesis. The requirements for the λ SS replication reaction with M13mp7 DNA as a template are listed in Table 2. Each of the three protein fractions and the SS DNA were essential, as was the presence of an ATP regeneration system. A long chain hydrophilic polymer, polyvinyl alcohol, stimulated DNA replication about four fold, although significant DNA synthesis was obtained in its absence. DNA replication depended slightly on spermidine but not at all on ribonucleoside triphosphates other than ATP.

Specific inhibitors were used to attempt to identify some of the bacterial proteins present in the relatively crude *E. coli* fraction that participate in the λ SS replication reaction (Table 3). Antibodies specifically

Table 2. Requirements for rifampicin-resistant replication of M13mp7 DNA.

Omitted component	DNA synthesis pmol
None	250
λ O protein	9
λ P protein	9
<i>E. coli</i> protein fraction	0
M13mp7 DNA	4
Creatine kinase	13
Creatine phosphate	9
ATP, c.k. & c.p. ^a	4
Polyvinyl alcohol	62
Spermidine	177
CTP, GTP, and UTP	266

^a c.k., creatine kinase; c.p., creatine phosphate.

Table 3. Effect of inhibitors on the *in vitro* replication of single-stranded viral DNA.^a

Inhibitor	Template			
	M13	M13 (+ antigen)	M13 (- rif)	ΦX174
None	100	100	100	100
Anti-dnaB protein IgG	3	63	120	13
Anti-primase	2	170	61	3
Anti-dnaK protein IgG	10	69	106	-
Anti-dnaC protein IgG	110	-	-	0
Anti-protein i IgG	83	-	80	1
Coumermycin	120	-	109	-

^a Replication reactions, containing 450 pmol of M13mp7 or ΦX174 DNA, were performed as described in Materials and Methods, except for the following modifications. Prior to its addition to the reaction mixture, the *E. coli* protein fraction (200 µg of Fraction II) was incubated for 15 min at 0°C with 80 µg of anti-dnaK protein IgG or with one of the other antibody preparations (present in amounts sufficient to block the replication of ΦX174 viral DNA). Where designated, additional amounts of purified antigen (dnaB protein, 0.5 µg; primase, 0.16 µg; or dnaK protein, 2 µg) were added to the *E. coli* protein-antibody mixture just prior to addition of the latter to the reaction mixture. Coumermycin, where indicated, was present at 0.54 µM. Rifampicin was omitted from all replication reactions in the column labeled "-rif". The λ O and P proteins were not added to reaction using ΦX174 DNA as the template. Incorporation values are given as the percentage of the incorporation obtained under standard reaction conditions with the same template (in the absence of added inhibitor). The 100% values were 140, 245, and 70 pmol of dNMP incorporated for M13mp7 (+ rifampicin), M13mp7 (- rifampicin), and ΦX174 DNA templates, respectively.

directed against dnaB protein or primase, two key components of the primosome, strongly inhibited the λ O and P protein-dependent replication of M13 DNA. Nevertheless, the strand initiation mechanism in the λ SS replication reaction is not closely related to the primosome-dependent pathway utilized for ΦX174 and ColE1 replication. This is indicated by the resistance of the reaction to antibodies which block the function of dnaC protein and protein i, both of which are required for the replication of ΦX174 viral DNA. Furthermore, the sensitivity of the λ O and P protein-promoted initiation reaction to anti-dnaK protein antibody (Table 3) (15) suggests strongly that this SS DNA replication system is physiologically relevant to replication of the λ chromosome *in vivo*, which is known to depend on the function of the host dnaK and dnaJ proteins (5). The participation of the bacterial dnaJ protein in the λ SS replication reaction is indicated (Table 4) both by (i) the inability of an *E. coli* protein fraction to support the reaction when it was prepared from a strain with a temperature-sensitive dnaJ mutation and (ii) the capacity of partially purified dnaJ protein to complement the crude

Table 4. Requirement for dnaJ protein in the λ SS replication reaction.^a

dnaJ protein	DNA synthesis, pmol
-	6
+	370

^a Replication of M13mp7 DNA was performed as described in Materials and Methods except that the *E. coli* protein fraction was prepared from strain C600dnaJ259. Partially purified dnaJ protein (500 ng) was added to the reaction mixture, as indicated.

protein fraction from the dnaJ mutant and restore DNA replication.

Control experiments (Table 3) showed that all antibody preparations were active and specific. Also, each antibody which blocked the λ SS replication reaction was neutralized by addition of excess levels of highly purified target antigen. Moreover, none of the antibodies effectively inhibited M13 DNA replication when rifampicin was omitted from the soluble enzyme system. This result is consistent with the fact that the physiological RNA polymerase-dependent replication reaction, which can proceed when rifampicin is absent, does not depend on any of the antibody targeted proteins. Finally, it is evident (Table 3) that the λ SS replication reaction, in contrast to λ dy plasmid replication in this *in vitro* system, is unaffected by coumermycin, a potent inhibitor of the gyrB subunit of DNA gyrase.

Products of the λ SS Replication Reaction

Radiolabeled products of the λ O and P protein-dependent replication of M13mp7 DNA were analyzed by equilibrium banding in CsCl/ethidium bromide (EtBr) and by neutral agarose gel electrophoresis. Approximately 70% of the ³²P-labeled product banded at the same position in a CsCl/EtBr gradient as marker supercoiled phage fd RF I DNA (Fig. 1A). The remaining product co-banded with nicked, circular, double-stranded fd RF II DNA. Electrophoretic analysis (Fig. 1B) indicated that the supercoiled product was the size expected for a supercoiled monomer of M13mp7 DNA and that the nonsupercoiled products comigrated with nicked, circular M13mp7 RF II DNA. Furthermore, electrophoresis of the nonsupercoiled DNA in an alkaline agarose gel indicated that the vast majority of the labeled product consisted of full-length, linear complementary strands (see Fig. 3, lane A). Precisely the same types of products were made in this *in vitro* system when M13mp7 DNA was replicated by the physiological RNA polymerase-dependent pathway (data not shown). We presume, then, that the elongation and termination stages of the λ SS replication reaction are similar, if not identical, to those previously described

Figure 1. (A) Analysis of the products of the λ SS replication reaction in a CsCl-ethidium bromide gradient. M13mp7 DNA was replicated in a ten times standard replication reaction as described in Materials and Methods, except that $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (at 500 cpm per pmol of total deoxynucleotide) was the labeled dNTP. The reaction was terminated by the addition of EDTA to 25 mM and proteinase K (self-digested) to 0.5 mg/ml. The mixture was incubated for 1 hr at 37°C , extracted twice with phenol and twice more with diethyl ether. To the aqueous phase was added CsCl (final density = 1.57 g/ml), ethidium bromide (0.5 mg/ml), and ^3H -labeled phage fd RF I and RF II DNA. This mixture was centrifuged to equilibrium at 15°C in a Beckman VTi65 rotor (20 hr at 45,000 rpm). Fractions (0.11 ml) were collected from the tube bottom and aliquots of each fraction (10 μl) were mixed with 10 μg of calf thymus carrier DNA and assayed for the presence of acid-insoluble radioactivity. The arrows mark the positions of the ^3H -labeled RF I and RF II DNA peaks. (B) Analysis of the products of the λ SS replication reaction by neutral agarose gel electrophoresis. The products of the λ SS replication reaction were banded in a CsCl/EtBr gradient (see above), concentrated by isopropanol precipitation, and electrophoresed in a neutral 1% agarose gel as described (34). Lane A, radioactive product (2000 cpm) from fractions 20-27 (Fig 1A); lane B, product (1500 cpm) from fractions 32-36. The positions of unlabeled M13mp7 supercoiled (SC) and nicked-circular (NC) duplex DNAs, electrophoresed in a parallel lane, are denoted. Radiolabeled bands were visualized by autoradiography of the dried gel at -70°C using Kodak X-AR5 film.

(16-18) for the replication of M13, ϕX174 and G4 viral chromosomes in extracts of *E. coli*. On the other hand, it is evident from the results reported in this paper that the strand initiation mechanism used in the λ SS replication reaction must be distinct from any of the three different single-strand initiation mechanisms previously identified as being active in extracts of *E. coli* (16, 17, 38).

Initiation of the λ SS Replication Reaction Occurs at Multiple Sites on M13mp7 DNA

The RNA polymerase-dependent initiation of M13 complementary strand synthesis takes place at a unique site located in the intergenic region of the

Figure 2. Structure of M13mp7 RF II DNA produced by replication of M13mp7 DNA via the physiological RNA polymerase-dependent pathway. The positions of the Bgl II and Cla I recognition sites are shown relative to the origin-terminus gap that is present in the complementary product strand.

M13 chromosome (39). In M13mp7 DNA this origin is located about 1.2 kb to the 5' side of Cla I and unique Bgl II restriction endonuclease recognition sequences (Fig. 2). We sought to determine if strand initiation occurs at unique or discrete sites in the λ SS replication reaction. Our experimental approach was based on the premise that the nick or gap present in nicked, circular, duplex RF product is located in close proximity to the site of strand initiation (Fig. 2), such that localization of the site of the strand interruption would approximately locate the complementary strand origin.

M13mp7 DNA was replicated in the *in vitro* system in the presence and absence of rifampicin and the λ O and P initiator proteins. A high concentration of nicotinamide mononucleotide (NMN) was present during these reactions to block DNA ligation. Labeled products, containing linear complementary strands, were isolated and subjected, before and after digestion with Bgl II and Cla I, to electrophoresis under denaturing conditions in alkaline agarose. Full-length complementary strands were the predominant products of both the λ O and P protein-dependent reaction (Fig. 3, lane A) and the RNA polymerase-dependent pathway (Fig. 3, lane C). The continuum of fragment sizes observed when the λ SS replication reaction products were cleaved with Bgl II (Fig. 3, lanes E and L) suggests that strand initiation in the O and P protein-dependent reaction occurs at multiple, if not random, sites. Consistent with this interpretation is the finding that cleavage of the λ SS reaction products with Cla I generated, in addition to the two expected Cla I fragments, a mixture of new single-stranded fragments of widely varying lengths (Fig 3, lanes D and K). Our results indicated a tendency of the SS reaction product to contain the complementary strand origin-terminus interruption on the longer of the two Cla I fragments. In related experi-

Figure 3. Analysis of the products of the *in vitro* replication of M13mp7 DNA by electrophoresis in alkaline agarose gels. Replication of M13mp7 DNA by the λ SS reaction was performed as described in the legend to Fig. 1. For replication by the RNA polymerase-dependent pathway, rifampicin and the λ O and P proteins were omitted from the reaction mixture. NMN was present in both reaction mixtures at a final concentration of 1 mg/ml. RF II reaction products (which made up > 90% of the labeled product) were isolated from CsCl-ethidium bromide gradients (*cf.* Fig 1) and portions were digested with restriction endonucleases as indicated below. The labeled product SS DNA fragments were sized by electrophoresis in a 1% alkaline agarose gel (pH 12.5) and detected by autoradiography (*cf.* Fig 1). The complementary strand origin of M13mp7 DNA (7238 bases) is located at position 5740; the single *Bgl* II site (Fig. 2) is at position 6923; the two *Cla* I sites are at positions 2527 and 6870. Lane A, RF II from the O and P protein-dependent reaction; lanes B and C, RF II from the RNA polymerase-dependent reaction carried out in the absence or presence of NMN, respectively; lanes D and E, RF II from the O and P protein-dependent reaction digested with *Cla* I or *Bgl* II, respectively; lanes F, G, and H, supercoiled M13mp7 RF I, undigested, and digested with *Cla* I or *Bgl* II, respectively; lanes I and J, RF II from the RNA polymerase-dependent reaction digested with *Cla* I or *Bgl* II, respectively; lanes K-N, longer autoradiographic exposure of lanes D, E, I and J, respectively. The arrows labeled i, m, and n locate fragments that arise as a consequence of the presence of a unique origin-terminus gap in the complementary strand product of the RNA polymerase-dependent reaction.

ments in which various M13ori λ SS DNAs were used as the template in the λ SS replication reaction, we still found no indication of preferred sites for strand initiation (data not shown). Control experiments demonstrated that the RNA polymerase-dependent M13mp7 SS to RF conversion was initiated in this soluble enzyme system almost exclusively at the known M13 complementary strand origin. Digestion of the labeled product with *Bgl* II or *Cla* I revealed in each case two new bands of the expected size (Fig. 3, lanes I, J, M and N) that were not present in control digests of labeled M13mp7 supercoiled DNA (Fig. 3, lanes F-H).

A Pre-priming Complex Is Formed in the λ SS Replication Reaction

A possible explanation for the multiplicity of initiation sites observed in the λ SS replication reaction comes from studies of the conversion of

ϕ X174 viral DNA to the duplex replicative form by *E. coli* proteins (16-18). An early step in the replication of the ϕ X174 chromosome involves the assembly of a multiprotein complex (a primosome) on SSB-coated ϕ X174 DNA. The capacity of the primosome to migrate along the coated DNA and synthesize multiple primers, causes ϕ X174 complementary strand synthesis to be initiated at numerous sites (40, 41). The participation of *E. coli* dnaB protein and primase, two constituents of the primosome, in the λ SS replication reaction makes it plausible that a primosome-like complex acts in this system too.

One diagnostic feature of the involvement of the primosome in ϕ X174 replication is the rate-limiting nature of the assembly on the viral strand of the preprimosome, a primase deficient multiprotein complex (41, 42). To determine if a similar pre-priming step was rate-limiting for the λ SS replication reaction, we examined the effect of preincubating the M13mp7 template DNA with λ and *E. coli* replication proteins prior to the initiation of DNA synthesis. The *E. coli* protein fraction used in this experiment had been fractionated further in order to reduce the levels of nucleotides as well as to lower the concentrations of primase and DNA polymerase III holoenzyme. DNA synthesis was initiated, following the addition of these limiting components, in a second-stage incubation. Indeed, the initial rate of M13mp7 complementary strand synthesis in the first minute of the second-stage incubation was elevated more than 20 fold if the template was first preincubated at 30°C with required proteins (Fig. 4A). The kinetics of template activation promoted by this λ SS replication system (Fig. 4B) are nearly identical to those reported for the assembly of the preprimosome on ϕ X174 DNA (42). Since rapid DNA synthesis required the addition of primase to the second-stage incubation mixture (data not shown), the rate-limiting step in the λ SS replication reaction appears to occur prior to primer synthesis.

An investigation of the requirements for formation of the activated replication intermediate indicated that the λ O and P initiators, the *E. coli* dnaB protein, and additional bacterial proteins were necessary (Table 5). Moreover, ATP and the ATP regenerating system (Table 5) as well as M13mp7 DNA (unpublished data) were also required during the preincubation.

Antibody preparations specifically directed against the λ P protein or the *E. coli* dnaB protein were used to test for the involvement of these proteins in the priming and DNA synthesis steps that follow formation of the activated replication intermediate. The addition of anti-dnaB protein IgG to the reaction mixture, following the first-stage incubation of replication proteins and M13mp7 DNA, completely blocked rapid DNA synthesis in the

Figure 4. Formation of an activated M13mp7 DNA replication intermediate dependent on the λ O and P initiator proteins. A) Time course of DNA synthesis in the O and P protein-dependent replication of M13mp7 DNA. The standard λ SS replication reaction was divided into two stages. The 4 times stage 1 reaction mixture, 83 μ l, contained the following components: Hepes/KOH (pH 7.6), 48 mM; magnesium acetate, 13.2 mM; bovine serum albumin, 60 μ g/ml; ATP 2.4 mM; creatine phosphate, 104 mM; creatine kinase, 120 μ g/ml; polyvinyl alcohol, 7.2% (w/v); spermidine/HCl, 2.4 mM; rifampicin, 24 μ g/ml; SSB, 6.4 μ g; M13mp7 DNA, 1.8 nmol; O protein, 2 μ g; P protein, 172 ng; dnaB protein, 180 units; and *E. coli* 36-48% ammonium sulfate fraction, 670 μ g (this fraction contains no detectable dnaB protein). This mixture was preincubated 30 min at 30°C (closed circles) or kept at 0°C (open circles). Replication was initiated at 30°C following supplementation of the first-stage reaction mixture with stage 2 components [primase (400 units), DNA polymerase III holoenzyme (288 units), rNTPs, and dNTPs] as described in Materials and Methods (final volume, 130 μ l). Aliquots (15 μ l) were removed from the second-stage reaction mixture at the times indicated and analyzed for acid insoluble [^{3} H]dTTP. When first-stage reactions were supplemented with [^{3} H]dTTP (81 μ M), just 0.5 pmol of TMP was incorporated during the 20 min preincubation period. B) Time course of activation of M13mp7 DNA during the first-stage incubation. A series of 4 times stage 1 reaction mixtures were prepared as described above and incubated at 30°C. At the times indicated, each mixture was supplemented with stage 2 components (Fig. 4A) and incubated for one minute at 30°C to permit rapid replication. Aliquots (15 μ l) were analyzed for acid insoluble radioactivity.

second-stage incubation (Table 6). On the other hand, addition of anti-P protein IgG had no effect on the activity of the preformed replication intermediate. These results indicate that the dnaB protein participates in the λ SS replication reaction in a step that follows formation of the activated intermediate, most probably RNA priming. The λ P protein in contrast, is apparently essential only for production of the intermediate and plays no critical role in subsequent steps required for DNA synthesis. We can not rule out, however, that P protein antigenic determinants become inaccessible to anti-P protein IgG following formation of the replication intermediate.

Table 5. Requirements for formation of an activated replication intermediate.^a

Component omitted in preincubation	DNA synthesis pmol
None	58
λ O protein	3
λ P protein	5
dnaB protein	4
<i>E. coli</i> protein fraction	7
ATP	6

^a Stage 1 reaction mixtures were assembled as described in the legend to Fig. 4, except that individual components were omitted as indicated. After a 20 min incubation at 30°C, each mixture was supplemented with stage 2 components (Fig. 4 legend) and with the component omitted from the stage 1 preincubation. Acid insoluble radioactivity was measured after a second-stage replication reaction of 1 min at 30°C.

DISCUSSION

Although the novel DNA initiation system reported here, the λ SS replication reaction, is specific for SS DNA, it nevertheless appears to be physiologically relevant to the process of initiation of bacteriophage λ chromosomal DNA replication *in vivo*. Not only does the λ SS replication reaction

Table 6. Effect of anti-P protein and anti-dnaB protein immunoglobulins on the activity of the activated replication intermediate.

Expt.	Antibody	DNA synthesis, pmol	
		Not preactivated ^a	Preactivated ^b (1 min synthesis)
1	None	135	30
1	Anti-dnaB protein IgG	3	1
2	None	131	19
2	Anti-P protein IgG	15	23

^a One stage replication reactions were carried out as described in Materials and Methods using 450 pmol of M13mp7 DNA as template, except that the reaction mixtures were preincubated for 10 min at 0°C with either anti-dnaB protein IgG (20 μ g) or anti-P protein IgG (21 μ g), as indicated. DNA replication was for 10 min at 30°C.

^b One times "stage 1" reaction mixtures were prepared as described in the legend to Fig. 4 and the M13mp7 DNA was activated by a 20 min preincubation at 30°C. The first-stage mixtures were incubated 10 min at 0°C in the presence of anti-dnaB protein IgG (20 μ g) or anti-P protein IgG (21 μ g), as indicated. Second-stage replication components (Fig. 4 legend) were added in appropriate amounts. The amount of rapid DNA synthesis was determined after a 1 min incubation at 30°C.

require the participation of the specific phage-coded initiators, the λ O and P proteins, but DNA strand initiation also depends on the action of four host replication proteins previously identified as being necessary for replication of the λ chromosome, the *E. coli* dnaB, dnaG (primase), dnaJ and dnaK (15) proteins. The striking similarity of the proteins required for the λ SS replication reaction to those needed for λ DNA replication *in vivo* encouraged us to document the properties of the *in vitro* system, even though specific λ DNA sequences were clearly not required in the SS DNA template.

A plausible scheme for the λ SS replication reaction is presented in Fig. 5. Initially the SS DNA circle is stoichiometrically coated with the single-stranded DNA binding protein. In the next step of the reaction the λ O and P initiators, together with *E. coli* proteins, promote the ATP-dependent transfer of the bacterial dnaB protein (and perhaps other proteins) onto the SSB-coated DNA. This creates an activated replication intermediate. Additional evidence supporting this conclusion comes from preliminary experiments which indicate that the activity of the replication intermediate, even when isolated free of unassociated protein by agarose gel filtration (42), was sensitive to antibody directed against the dnaB protein (unpublished data). The nature and stoichiometry of the polypeptides present on the activated template remain to be determined. It is also uncertain at this time if the dnaB protein present on the activated template is a constituent of a preprimosome-like (41,42) complex that is capable of movement along the SSB-coated strand. In any event, primase presumably interacts with the bound dnaB protein and synthesizes an RNA transcript that is utilized as a primer for chain elongation by DNA polymerase III holoenzyme.

Figure 5. Hypothetical scheme for the λ O and P protein-dependent replication of single-stranded, circular DNA. See text for details.

Among the various single-strand initiation mechanisms known to be operative in extracts of *E. coli*, the multienzyme system responsible for the conversion of ϕ X174 SS DNA to the duplex replicative form (16-18) most closely resembles the λ SS replication reaction. In both pathways the rate-limiting step of the overall reaction is the assembly on SSB-coated DNA of a protein complex containing the bacterial dnaB protein. Once converted to this activated replication intermediate, each type of template is rapidly replicated by the combined action of primase and the DNA polymerase III holoenzyme. Although the two enzyme systems replicate circular SS DNA by analogous pathways, each has distinctive protein requirements for the transfer of dnaB protein onto SSB-coated DNA. For ϕ X174 replication, this step also requires *E. coli* proteins dnaC, i, n, n', and n'' (11, 19, 38, 40, 43). In contrast, the λ O and P initiators and the *E. coli* dnaJ and dnaK proteins participate in the dnaB protein transfer step in the λ SS replication reaction. Moreover, our studies indicate that the dnaC protein and protein i are not necessary for the λ SS replication reaction (Table 3).

Assembly of the primosomal complex during ϕ X174 replication is believed to take place at a unique intergenic sequence to which protein n' binds (19, 41). In the case of the λ SS replication reaction, it is likely that the λ O protein initiator functions as the DNA binding protein that potentiates the transfer of dnaB protein onto the circular SS DNA template. The O protein specifically binds to repeating sequences present at the λ replication origin (9, 10), but it also binds, perhaps randomly, to multiple sites on SSB-coated single-stranded DNA. In fact, a stable single-stranded DNA-SSB-O protein complex, isolated by agarose gel chromatography, serves as an effective template in the λ SS replication system, even without further addition of O protein (unpublished data). From the biochemical properties of the proteins involved, we surmise that the *E. coli* dnaB protein is transferred from a P protein-dnaB protein complex (12, 14, 28) to the SS DNA template via interactions of the complex with *E. coli* dnaK and dnaJ proteins (5, 15) and with the carboxyl-terminal domain of the DNA-bound O protein (44).

As discussed above, several features of the λ SS replication reaction suggest that studies of this multienzyme system may provide clues about the nature of the biochemical events involved in initiation of DNA replication at the bacteriophage λ chromosomal origin. Nevertheless, the sequence independence of DNA chain initiation in the λ SS replication reaction clearly distinguishes this multienzyme system from the closely related system (6) that specifically initiates DNA synthesis at the duplex λ DNA replication origin.

This difference, however, may simply reflect the DNA-binding specificity of the O initiator, which binds with high specificity to ori λ sequences present in duplex DNA, yet binds to multiple sites on single-stranded DNA. Our studies of the λ SS replication reaction suggest that during initiation of λ DNA replication in vivo the bacterial dnaB protein (and perhaps other proteins) is transferred onto the λ chromosome at or near O protein bound to ori λ . Furthermore, this protein transfer event most likely occurs on single-stranded regions and requires neither a particular DNA sequence (Table 1) nor the direct participation of RNA polymerase or DNA gyrase (Table 3). The latter two *E. coli* proteins presumably function in some other aspect of duplex λ DNA replication, perhaps in the creation of single-stranded regions at ori λ that may be required for protein transfer events.

It has been suggested that the λ ice site, situated about 500 bp from ori λ , is a lagging strand initiation determinant (36). Other studies have identified the presence of primosome assembly sites adjacent to the replication origins of *E. coli* plasmids F and ColE1 (20, 21, 45). Accordingly, we searched for λ DNA sequences from the ori λ region that function as rifampicin-resistant strand initiation determinants. None of the M13- λ recombinant chromosomes we constructed (see Materials and Methods), whether they contained the λ ori or ice sites, or both, were replicated in vitro by the *E. coli* enzyme mixture in the presence of rifampicin. Moreover, chain initiation on these SS templates was not stimulated when the *E. coli* enzyme system was fortified with either the λ O protein or the λ P protein initiator (unpublished data). Only when all three protein fractions were present, was replication of the hybrid chromosomes observed.

The soluble enzyme system used here to replicate SS DNA is capable of initiating the synthesis of both the leading and lagging strands during replication of plasmid λ dv (6-8). Although the *E. coli* protein fraction alone supports initiation of lagging strand synthesis on such diverse chromosomes as *E. coli*, ColE1, ϕ X174, and G4 (6, 8, 20-22), in the absence of the λ O and P proteins it is incapable of initiating DNA chains on either duplex or SS λ DNA from the ori λ region. Yet, electron microscopic analysis of λ dv theta-form replication intermediates generated in vitro (6) confirms that both leading and lagging strand synthesis is usually initiated in the ori λ region (M. Wold and R. McMacken, unpublished data). We conclude that both phage-specified initiators function during λ DNA replication to promote not only leading strand synthesis, but initiation of lagging strands as well. A simple initiation mechanism for bidirectional DNA replication (40), involving

the assembly of a mobile, processive, priming (or prepriming) protein complex on each strand at ori λ , could account for our observations. In such a mechanism the initiation of lagging strand DNA synthesis in one direction is coupled to, and dependent on, initiation of leading strand DNA synthesis in the opposite direction. Nevertheless, it is not possible to exclude an alternative mechanism for the role of the λ O and P proteins in the initiation of lagging strand nascent fragments. In this scheme protein transfer reactions similar to those described in Figure 5 would be required at a λ DNA replication fork for each of the multiple lagging strand initiation events. Since the assembly of the activated nucleoprotein complex in vitro is much slower than the known rate of nascent fragment synthesis in vivo, this latter mechanism appears unlikely.

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