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Initiation of bacteriophage λ DNA replication *in vitro* with purified λ replication proteins

(purified λ O and P proteins/*Escherichia coli* replication proteins/λ*dv* plasmids/transcriptional activation)

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ABSTRACT **We have developed a soluble enzyme system that replicates exogenously added plasmid DNA (λ*dv*) bearing the replication origin of the bacteriophage λ chromosome. The system contains pure phage λ O and P replication proteins and a partially purified mixture of *Escherichia coli* replication proteins [the enzyme system of Fuller, R. S., Kaguni, J. M. & Kornberg, A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 7370–7374). The features of λ*dv* replication in this system closely resemble the known characteristics of phage λ DNA replication *in vivo*. The system (*i*) depends completely on exogenously supplied DNA, (*ii*) specifically replicates supercoiled plasmid DNA that contains a λ replication origin, (*iii*) depends on both the λ O protein and the λ P protein, (*iv*) depends on RNA polymerase, (*v*) depends on host replication proteins (e.g., primase, dnaB protein, and several others that function in the priming of DNA synthesis in *E. coli*) as judged by antibody inhibitions, and (*vi*) replicates as much as 32% of added λ*dv* plasmid DNA through a single complete round to generate catenated daughter molecules. Furthermore, replication of λ*dv* DNA *in vitro* requires DNA gyrase and an ATP-regenerating system. It is notable that addition of λ O and P proteins to the mixture of *E. coli* replication proteins inhibits replication of plasmids bearing the origin of the *E. coli* chromosome. Exploitation of this enzyme system should allow a detailed investigation of the biochemical mechanisms involved in bacteriophage λ DNA replication and its regulation.**

An essential feature of the life cycle of both prokaryotic and eukaryotic cells is a strict regulation of the initiation of a new round of chromosome replication. This replication proceeds bidirectionally from unique chromosomal origins (1). Because of the complexity of the process, the biochemical mechanisms involved in the initiation of bidirectional DNA replication and its regulation have not been elucidated for any organism.

The chromosome of coliphage λ is ideally suited for model studies on the initiation and regulation of duplex DNA replication. The results of more than 25 years of intensive research on the genetics and physiology of phage λ gene regulation and DNA replication can be brought to bear on this problem (2). Replication is initiated on supercoiled λ chromosomes (3) and proceeds bidirectionally from a single fixed origin (4). At early times after infection, λ DNA molecules are replicated in a circular mode via θ structure intermediates (3–5). This early replication generates catenated circular monomers, nicked circles (a circular DNA molecule containing an interruption in one or both DNA strands), and covalently closed circles as products (3). Both viral and host proteins participate in λ DNA replication. Genetic studies indicate that the products of λ genes *O* and *P* (6, 7) and the *Escherichia coli* dnaB, dnaJ, dnaK, primase, and DNA polymerase III holoenzyme replication proteins (unpublished data; refs. 8 and 9) are all essential for viral DNA replication. Finally, initiation of λ DNA replication *in vivo* requires RNA synthesis at or near the λ replication origin (10, 11), a process termed transcriptional activation.

The development of a soluble enzyme system that is capable of supporting the specific initiation of phage λ DNA replication *in vitro* would be a critical step toward defining the molecular events that occur in the initiation process. In this report we describe the properties of such a system.

MATERIALS AND METHODS

Reagents, Enzymes, and Antibodies. Sources were: creatine phosphate and creatine kinase, Boehringer Mannheim; polyvinyl alcohol type II, rifampicin, chloramphenicol, nalidixic acid, and coumermycin A_1, Sigma; [*methyl*-^{3}H]dTTP (40–50 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [α-^{32}P]dCTP (400–450 Ci/mmol), Amersham; 5-bromo-2′-deoxyuridine 5′-triphosphate, P-L Biochemicals; *Hin*dIII, *Bam*HI, and *Sma* I restriction endonucleases and T4 DNA ligase, New England BioLabs. T4 lysozyme and antibodies against primase, dnaC protein, n protein, and n′ protein were generously provided by A. Kornberg and coworkers. Antibodies against dnaB protein, single-stranded binding protein (SSB), and protein i were as described (12–14).

***E. coli* Strains, Plasmids, and Phage.** The *E. coli* strains and sources were: C600 from A. Kornberg and N100-(*recA*) from B. Weiss. The plasmids and sources were: pKC7 from S. Rogers (15), pKN402 from B. Uhlin, and pRLM4 and pRLM5 (this work). The phage and sources were: λ*cI*am34*cro*tn from K. Matsubara (16), λ*imm*434*cII*2002*rep*82:λ from W. Dove (17), λ*cI*90 from H. Echols, and M13*oriC*26 from D. Ray (18). Plasmid DNAs were isolated from *E. coli* N100 as described (19).

Phage λ and *E. coli* Replication Proteins. Phage λ replication proteins O and P were purified to >98% purity from *E. coli* cells carrying amplified levels of these proteins. Analysis of the λ O and P protein preparations used in this study by high resolution NaDodSO$_4$/polyacrylamide gel electrophoresis revealed the presence of a single polypeptide in each sample (Fig. 1). The isolation and characterization of the λ O and P proteins will be published elsewhere. *E. coli* dnaB protein, primase, and SSB were prepared by modifications of published procedures (13, 20, 21).

Assay of DNA Replication. The conditions used for *in vitro* DNA synthesis were a modification of those used by Fuller *et al.* (22) for replication of plasmids containing the *E. coli* replication origin. The standard reaction mixture, 25 μl, contained: Hepes/KOH (pH 7.6), 40 mM; magnesium acetate, 11 mM;

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Abbreviations: SSB, *E. coli* single-stranded DNA binding protein; RF, replicative form.

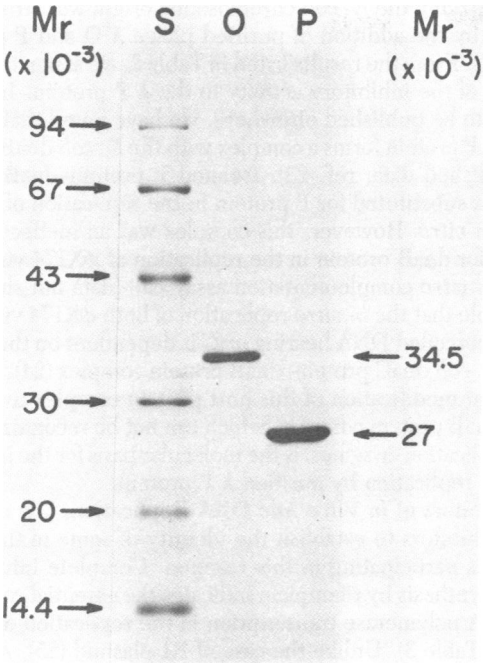

FIG. 1. Polyacrylamide gel analysis of purified λ O and P replication proteins. Proteins were denatured, reduced, and electrophoresed in a 10–20% gradient polyacrylamide gel containing 0.1% $NaDodSO_4$. Protein bands were stained with Coomassie brilliant blue R-250. Lane S, protein size standards; in order of decreasing molecular weight, they are: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin. Lane O, 10 μg of purified λ O protein. Lane P, 10 μg of purified λ P protein. The hydrophobic λ P protein binds more Coomassie blue dye on a weight basis than does the λ O protein.

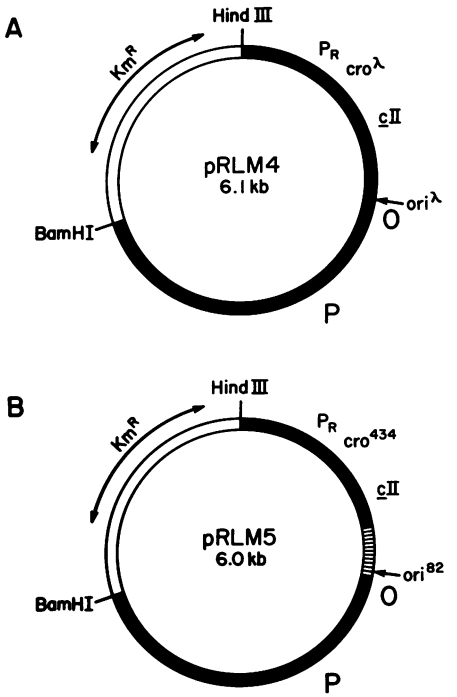

FIG. 2. Genetic maps of plasmids pRLM4 and pRLM5. pRLM4 (*A*) was constructed in the following manner. λ*cI*am34*cro*tn DNA and pKC7 DNA (15) (a pBR322 derivative specifying both kanamycin resistance, designated Km^R, and ampicillin resistance, designated Ap^R) were each digested to completion with *Hin*dIII and *Bam*HI restriction endonucleases. The two digested DNAs were mixed, ligated together with T4 DNA ligase, and used to transform N100. Km^R clones were selected at 30°C and screened both for ampicillin sensitivity, designated Ap^S, and for resistance to killing by λ*cI*90 infection, designated λ^R (16). Several clones with the phenotype expected for a carrier of pRLM4 (i.e., Km^R, Ap^S, and λ^R) were tested for the presence of plasmid. One such clone yielded plasmid pRLM4. Plasmid pRLM5 (*B*) was constructed and isolated in an identical fashion except that λ*imm*434*cII*2002*rep*82:λ DNA was used in place of λ*cI*am34*cro*tn DNA in the initial *Hin*dIII and *Bam*HI digestion. kb, Kilobases.

ATP, 2 mM; CTP, GTP, and UTP, each 500 μM; dATP, dCTP, and dGTP, each 100 μM; dTTP, 34 μM with [*methyl*-^{3}H]dTTP at 124 cpm per pmol of total deoxynucleotide; creatine phosphate, 43.2 mM; creatine kinase, 100 μg/ml; bovine serum albumin, 50 μg/ml; polyvinyl alcohol, 6% (wt/vol); DNA template, 650 pmol (deoxynucleotide equivalent); and *E. coli* enzyme fraction from strain C600 [fraction II of Fuller *et al.* (22)], 180–270 μg. λ O protein (1.43 μg) or λ P protein (65 ng), or both, were added to the reaction mixture as indicated in the individual experiments. Mixtures were assembled at 0°C. Reaction mixtures were incubated for 15 min at 30°C unless indicated otherwise in figure and table legends. DNA synthesis was measured by determining incorporation of labeled deoxynucleotide into acid-insoluble material.

RESULTS

Construction of a Supercoiled Template Containing *ori*λ. The available experimental evidence (reviewed in ref. 2) suggests that initiation of phage λ DNA replication *in vivo* occurs only on chromosomes that have been converted to a supercoiled form. The large size of the λ chromosome, 49 kilobases, complicates both isolation of supercoiled DNA and analysis of *in vitro* replication studies. λ*dv* plasmids are small, supercoiled, autonomously replicating DNA fragments that arise spontaneously from the λ genome and that contain all of the genes and sites necessary for λ DNA replication and its regulation (16). We decided to enzymatically construct related but more readily selectable plasmids that contained both *ori*λ and an antibiotic resistance gene. pRLM4, a high-copy-number λ*dv*-like plasmid bearing *ori*λ, is depicted in Fig. 2*A*. This chimeric plasmid contains the region of the λ chromosome from P_R (the rightward promoter-operator region) through gene *P* and a segment, originally from transposon Tn5, that specifies resistance to kanamycin. Plasmid pRLM4 carries a thermosensitive mutation in its *cro* gene, causing it to be maintained at an increased copy number (16) of ≈200 molecules per cell at 30°C (unpublished data). Several mg of supercoiled pRLM4 DNA can be readily isolated from a liter of cell culture. A closely related plasmid carrying the replication origin of lambdoid phage 82, pRLM5 (Fig. 2*B*), was constructed to serve as a control template in tests of the specificity of initiation of DNA replication *in vitro*.

Requirements for *in Vitro* Phage λ DNA Replication. A partially purified enzyme fraction from *E. coli* prepared by the method of Fuller *et al.* (22) supported the replication of plasmids bearing the origin of the *E. coli* chromosome (ref. 22; see also Table 2). This *E. coli* protein fraction, on the other hand, was incapable of supporting the replication of a plasmid bearing the λ replication origin, even when supplemented with either λ O protein or with λ P protein (Table 1). However, when both λ O and P replication proteins were added to the reaction mixture, extensive DNA synthesis was obtained with a template bearing *ori*λ (Table 1, line 4). In some experiments, synthesis to the extent of 32% of the input DNA was observed. DNA synthesis was linear for 10–15 min and was complete by 20 min (data not shown). Activity was maximal at O protein and P protein concentrations of 34–57 and 0.9–2.6 μg per ml, respectively.

The requirements for the *in vitro* replication of *ori*λ plasmid

Table 1. Requirements for λ*dv* replication *in vitro*

Exp.	Omission	O protein	P protein	DNA synthesis, pmol
1	None	−	−	0
2	None	+	−	0
3	None	−	+	0
4	None	+	+	161
5	None*	+	+	0.5
6	*E. coli* enzymes	+	+	0
7	PVA	+	+	0
8	ATP and CP	+	+	0

Reaction mixtures were incubated 15 min under standard conditions, except that individual components were omitted as indicated. Purified λ O and P proteins were added as indicated. pRLM4 DNA was used as the template in all reactions. In the absence of added pRLM4 DNA, synthesis on residual endogenous DNA (present in the *E. coli* protein fraction) amounted to 2–9 pmol of dNMP incorporation. The values reported here have been corrected for this background. A value of zero was assigned if the incorporation in an individual experiment was equal to or less than the incorporation measured in the absence of added template. CP, creatine phosphate; PVA, polyvinyl alcohol.

* Linearized pRLM4 DNA was utilized as the template. pRLM4 DNA was cleaved with *Sma* I restriction endonuclease at a site in the Km^R gene and a site in the λ *P* gene. After phenol extraction and concentration by isopropanol precipitation, the digested pRLM4 DNA was added to the reaction mixture.

DNA were investigated. Initiation of DNA synthesis was dependent upon the addition of supercoiled pRLM4 DNA (Table 1, line 4). Linear pRLM4 DNA was inert in this enzyme system (Table 1, line 5). In addition to a dependence on both λ O and P proteins, the observed replication was found to be absolutely dependent upon the presence of (*i*) the *E. coli* protein fraction, (*ii*) 6% polyvinyl alcohol, and (*iii*) ATP and an ATP-regenerating system.

Specificity of Replication to *oriλ* Templates. The specificity of this soluble enzyme system for *oriλ* sequences is demonstrated by the failure of supercoiled pRLM5 DNA to serve as a template (Table 2). pRLM5 differs from pRLM4 in that it contains 435 base pairs of phage 82 DNA, including the phage 82 replication origin, which replaces structurally similar *oriλ* specific sequences present in pRLM4. The inability of the λ O and P proteins to stimulate replication of pRLM5 DNA in this *in vitro* system is consistent with genetic studies that demonstrate that DNA replication is not initiated at a phage 82 origin *in vivo* in the presence of active λ O and P proteins (17). Supercoiled pKN402 DNA, a plasmid carrying the replication origin of *E. coli* plasmid R1 (which codes for its own initiators), was also inert as a template.

Replication of supercoiled M13*oriC*26 replicative form (RF) DNA carrying the *E. coli* chromosomal origin was strongly inhibited by the addition of purified phage λ O and P proteins (Table 2). From the results listed in Table 2, we assign the major portion of the inhibitory activity to the λ P protein. In experiments to be published elsewhere, we have found that the purified λ P protein forms a complex with the *E. coli* dnaB protein (unpublished data; ref. 23). Isolated P protein–dnaB protein complex substituted for P protein in the replication of pRLM4 DNA *in vitro*. However, this complex was an ineffectual substitute for dnaB protein in the replication of φX174 viral DNA in an *in vitro* complementation assay (20) (data not shown). It is notable that the *in vitro* replication of both φX174 viral DNA and supercoiled DNA bearing *oriC* is dependent on the activity of an *E. coli* dnaC protein–dnaB protein complex (24). We suggest that modification of this host protein complex to a P protein–dnaB protein complex (which can not be recognized by *E. coli* replication enzymes) is the molecular basis for the inhibition of *oriC* replication by purified λ P protein.

Table 2. Template specificity of *in vitro* replication

Template	O protein	P protein	DNA synthesis, pmol
None	+	+	4
pRLM4 (*oriλ*)	+	+	148
pRLM5 (*ori*82)	+	+	7
pKN402 (*ori*R1)	+	+	7
M13*oriC*26 RF	−	−	161
M13*oriC*26 RF	+	+	46
M13*oriC*26 RF	+	−	98
M13*oriC*26 RF	−	+	44

Supercoiled template DNAs (650 pmol) were added to standard reaction mixtures. Purified λ O protein and purified λ P protein were added as indicated.

Inhibitors of *in Vitro* λ*dv* DNA Replication. We used specific inhibitors to establish the identity of some of the *E. coli* proteins participating in this reaction. Complete inhibition of DNA synthesis by rifampicin indicates the essential role played by RNA polymerase transcription in the replication of pRLM4 DNA (Table 3). Unlike the case of R1 plasmid (25), λ*dv* replication *in vitro* was not dependent upon simultaneous synthesis of essential plasmid-encoded proteins, as judged by its resistance to chloramphenicol. The sensitivity of λ*dv* replication to nalidixic acid and coumermycin at concentrations known to block the activity of DNA gyrase (26) implicates this bacterial replication protein as a participant in the *in vitro* reaction (Table 3).

Extensive studies on the conversion of φX174 viral DNA to the duplex RF *in vitro* (1) have led to the isolation of antibodies specifically directed against *E. coli* replication proteins that function in this synthesis. Several of these antibodies were tested for their capacity to block λ*dv* replication (Table 4). Addition of antibodies directed against primase, dnaB protein, SSB, n protein, and n′ protein to concentrations sufficient to strongly inhibit φX174 replication in the soluble enzyme system severely restrict both *oriλ* and *oriC* specific replication (Table 4). Thus, each of these *E. coli* replication proteins appears to participate in the replication of λ*dv* DNA. The evidence for an essential role for protein i in λ*dv* replication is less convincing. Anti-protein i antibody inhibited pRLM4 replication considerably less well than it did φX174 viral DNA replication, which is known to depend on protein i (27). The authenticity of this *in vitro* system for phage λ DNA replication is underscored by the capacity of anti-dnaC protein antibody to inhibit *oriC*-dependent but not *oriλ*-dependent replication—precisely the response predicted from genetic studies (unpublished data; ref. 28). The specificity of the various antibody fractions used was demonstrated (*i*) by their inability to inhibit G4 viral DNA replication (which requires only primase and SSB of the proteins tested) or (*ii*) by the restoration of pRLM4 replication by addition of purified antigen, or both.

Products of λ*dv* DNA Replication. Equilibrium CsCl density gradient analysis was performed on the products of pRLM4 DNA replicated in reaction mixtures in which 5-bromo-2′-deoxyuridine 5′-triphosphate replaced dTTP. Essentially all of the product DNA banded at the hybrid density position, indicating that a single complete round of pRLM4 replication had taken place (data not shown).

The products of pRLM4 replication in standard reaction mixtures were examined by equilibrium centrifugation in CsCl/ethidium bromide. The product made early in the reaction banded as a single species near the density of the linear standard

Table 3. Sensitivity of λ*dv* replication *in vitro* to inhibitors of transcription, translation, and replication

Inhibitor	Concentration, μg/ml	DNA synthesis, pmol
None	—	146
Rifampicin	20	4
Chloramphenicol	200	137
Nalidixic acid	60	74
	230	26
	700	4
Coumermycin	0.024	95
	0.080	66
	0.320	17

(Fig. 3*A*, peak I). Three major species of pRLM4 replication products were present in the reaction mixture after DNA synthesis had ceased (Fig. 3*B*, peaks I, II, and III). No additional change in the banding pattern was obtained after prolonged incubation. Product material in each peak was further analyzed by velocity sedimentation in neutral and alkaline sucrose gradients (data not shown). This characterization suggests that (*i*) peak I material consisted of catenated (interlocked), nicked, pRLM4 plasmid DNA circles; (*ii*) peak II material was composed of catenated plasmid circles in which half of the DNA circles were nicked and half were relaxed (nonsupercoiled), covalently closed circles; (*iii*) peak III contained catenated covalently closed circles that had little or no negative supertwists; (*iv*) the catenated structures in each peak contained at least two monomeric circles; and (*v*) all circles, regardless of type, contained a full length, newly synthesized daughter strand. Unreplicated pRLM4 template DNA was neither nicked nor catenated during incubation under standard reaction conditions (data not shown).

DISCUSSION

All of the features of λ*dv* replication *in vitro* reported here correspond closely to those described for phage λ DNA replication *in vivo*. Replication of λ DNA both *in vivo* and *in vitro* (*i*) is dependent upon the presence of a unique origin DNA sequence (*oriλ*) (4, 17, 29), (*ii*) is dependent on both λ O and λ P replication proteins (5, 6), (*iii*) is dependent on RNA polymerase action (i.e., "transcriptional activation") (10), (*iv*) is dependent on *E. coli* replication proteins dnaB and primase (dnaG protein) (8) but does not require the bacterial dnaC replication protein (28), (*v*) is initiated on supercoiled DNA templates (3), and (*vi*) produces catenated nicked circles and catenated covalently closed circles as replication products (3). Moreover, our studies of λ*dv* replication in the soluble system suggest the involvement of several additional *E. coli* proteins in λ DNA replication (i.e., DNA gyrase, SSB, protein n, protein n′, and possibly protein i).

Table 4. Antibody effects on λ*dv* replication *in vitro*

Antibody	Template				
	φX174	*oriC*	pRLM4	pRLM4 (+ antigen)	G4
None	100	100	100	—	100
Anti-primase	3	5	6	130	1
Anti-dnaB protein	0	0	0	44	126
Anti-SSB	—	2	1	30	—
Anti-dnaC protein	0	0	131	—	118
Anti-n protein	13	20	13	—	122
Anti-n′ protein	3	18	5	—	124
Anti-i protein	0	50	29	—	126

Reaction mixtures were incubated for 15 min under standard conditions with 650 pmol of DNA template. The *oriC* template was M13*oriC*26 RF DNA. Incorporation values are given as a percentage of the incorporation obtained under standard reaction conditions with the same template (in the absence of added antibody). The 100% values were 30, 36, 85, and 91 pmol of dNMP incorporated for φX174 viral DNA, M13*oriC*26 RF, pRLM4, and G4 viral DNA templates, respectively. Purified λ O and P proteins were added to all reactions utilizing pRLM4 as the template. Rifampicin was added to a final concentration of 20 μg/ml to all reactions with φX174 and G4 viral DNAs as templates. Antibody fractions were incubated for 15 min at 0°C together with the *E. coli* enzyme fraction prior to assembling the reaction mixtures. Where indicated, the specific antigens for each antibody were added, in apparent several-fold excess, to the antibody-treated mixtures just prior to the 30°C incubation.

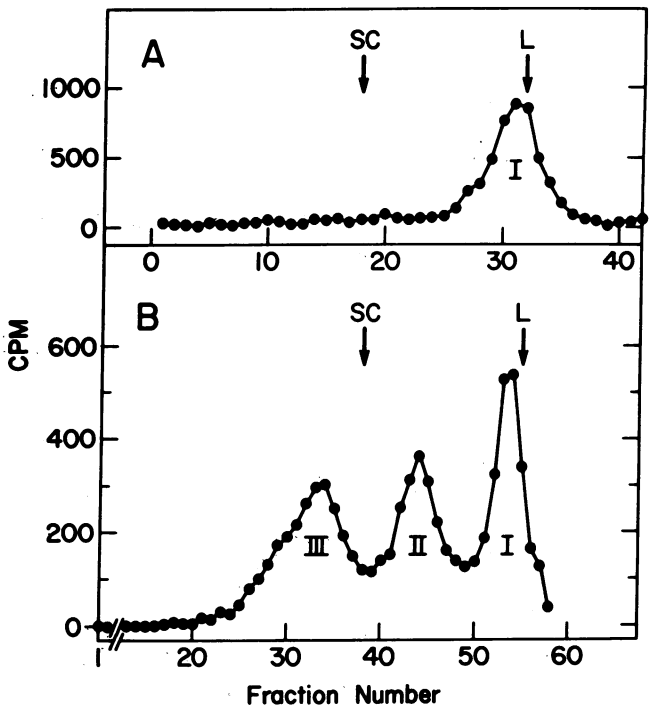

FIG. 3. Equilibrium centrifugation in ethidium bromide/CsCl of pRLM4 replication products of 5-min (*A*) and 20-min (*B*) incubations. The peak positions of ^{3}H-labeled supercoiled (SC) pRLM4 DNA and linearized (L) pRLM4 DNA are indicated by arrows. Reactions (125 μl) were under standard conditions, except that unlabeled dTTP was present at 100 μM and dCTP was at 34 μM, with [α-^{32}P]dCTP included at 530 cpm/pmol of total deoxynucleotide. Reactions were terminated by the addition of EDTA to 25 mM. Mixtures were deproteinized by extractions with phenol, followed by extractions with ether. The DNA solution (4.45 ml in 10 mM Tris chloride, pH 8.0/1 mM EDTA) was mixed with 4.32 g of CsCl and 2.5 mg of ethidium bromide and centrifuged for 18 hr at 45,000 rpm at 15°C in a Beckman VTi65 rotor. Fractions were assayed for acid-insoluble radioactivity.

The nature of transcriptional activation of phage λ DNA replication remains enigmatic. Interestingly, recent studies (11) suggest that RNA polymerase transcription directly across *oriλ* is not necessary for activation of replication; transcription of a region in the vicinity of *oriλ* (>100 base pairs distant) suffices. The enzyme system described here allows a biochemical approach to the resolution of this intriguing phenomenon.

The inertness of linear λ*dv* DNA in this system suggests that phage λ DNA replication is activated by negative supertwisting of the template DNA. A role for DNA gyrase in λ DNA replication, beyond that of producing supertwisted template, can be inferred from the inhibitory actions of nalidixate and coumermycin on the replication of λ*dv* DNA that is added to the reaction mixture in a supercoiled form. The other *E. coli* proteins shown here (Table 4) to be necessary for λ DNA replication *in*

vitro are believed to function in priming of DNA strand synthesis (1).

Of the two phage λ-coded initiators, O protein and P protein, only the former is replicon specific (17). The λ O protein binds specifically to a quartet of tandem 19-base-pair repeating sequences present in *oriλ* (30). *In vitro* binding studies indicate that a large molar excess of purified O protein is required to fill all of the available O protein binding sites (ca. 80 mol of O protein protomer per mol of *oriλ* DNA) (ref. 30; unpublished data). An even larger molar excess of O protein (450-fold) is necessary in the soluble enzyme system to generate nearly maximal *λdv* replication. We suggest two possible reasons for the high level of O protein required. Purified λ O protein readily self-aggregates into large, inactive multimeric complexes (unpublished data), and this phenomenon may also occur in the replication reaction mixture. The extreme metabolic instability of the λ O protein *in vivo* ($t_{1/2}$ = 1.5 min), caused by cellular ATP-dependent proteolysis (unpublished data), may be a factor *in vitro* as well.

Biochemical studies of purified phage λ P protein (unpublished data) lead us to conclude that it is a viral analogue of the host dnaC protein (24). The λ P protein readily forms a complex with the *E. coli* dnaB protein and presumably directs this key host replication protein to the λ chromosome, possibly through interactions with the carboxyl-terminal domain of origin-bound O protein (17). In this regard, it is interesting to note that the amount of λ P protein required to yield maximal *λdv* DNA replication *in vitro* is approximately the amount needed to titrate all of the *dnaB* protein present in the reaction mixture.

Density-labeling experiments and velocity-sedimentation analysis of the *λdv* replication products demonstrated that a single round of replication, generating full-length daughter strands, had occurred. Furthermore, essentially all of the replication product was found to consist of catenated structures containing either nicked or covalently closed circular monomers or both. The classes of catenated *λdv* replication products obtained here closely resemble the types of catenated replication intermediates found during the circle-to-circle phase of phage λ DNA replication (3). Catenation was not observed in P protein-deficient or ATP-deficient reactions (data not shown), indicating that catenation occurred during, but not necessarily as a result of, replication. The persistence of both catenated circles and relaxed covalently closed circles in the reaction mixtures suggests DNA gyrase activity was limiting.

Klein and coworkers pioneered the development of a particulate cellophane disc system for studying *in vitro* replication of endogenous phage λ DNA in a concentrated cell lysate (31). While our studies were in progress, the Klein laboratory reported the development of a crude soluble system capable of replicating exogenous *λdv* DNA (32). The properties of *λdv* replication in their system characterized thus far are consistent with those reported here.

It is clear that the soluble enzyme system we have developed can be biochemically dissected to help elucidate the molecular mechanisms of phage λ DNA replication and its regulation. We anticipate that general new insights, both about the process of initiation of duplex DNA replication and about host–virus interactions, will ultimately result.

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