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An enzyme system for replication of duplex circular DNA: The replicative form of phage φX174

(*cistron A* protein/*rep* protein)

SHLOMO EISENBERG, JOHN F. SCOTT, AND ARTHUR KORNBERG

Department of Biochemistry, Stanford University Medical Center, Stanford, California 94305

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ABSTRACT **Viral single strands (SS) are converted to the duplex form (RF) by a soluble enzyme fraction from uninfected *Escherichia coli* [Schekman *et al.* (1975) *J. Biol. Chem.* 250, 5859–5865]. When reactions were supplemented with a soluble enzyme fraction from φX174-infected cells, replication of φX174 superhelical RF I DNA was observed. The activity supplied by infected cells was absent in cells treated with chloramphenicol or in cells infected with a φX174 phage mutant in *cistron A* (*cisA*). A host function coded by the *rep* gene, essential *in vivo* for RF replication (but not for SS → RF), was supplied by enzyme fractions from either infected or uninfected cells. Based on complementation assays, the *cisA*-dependent and the *rep*-dependent proteins have each been purified about 1000-fold. The synthetic products of the enzymatic reaction were identified as RF I and RF II in which viral (+) and complementary (−) strands were newly synthesized.**

In the life cycles of phages M13 and φX174, the infecting single-stranded circles (SS) of the virus are first converted by host proteins to produce the duplex, circular, parental replicative form (RF) (1). Multiplication of RF to produce progeny RF requires participation of a phage-induced protein in addition to that of host proteins (1, 2).

When the resolution and reconstitution of the enzymatic replication of M13 and φX174 DNAs were embarked upon (3, 4), the apparent simplicity of the first stage offered an attractive, immediate goal. A soluble enzyme preparation obtained from gently lysed cells was effective in the SS to RF conversion. Such extracts from infected cells were even able to support RF multiplication (5), and it seemed then that the parts of the enzymatic machinery for the production of progeny RF from SS might be quickly identified and pieced together. However, as the considerable complexity of the SS to RF conversion began to unfold (6–8), it seemed that this system should be understood before the problem of RF multiplication was tackled.

Now that some 12 partially purified proteins, presumably nearly all that are required for the conversion of φX174 SS to RF, are in hand (9, 10), the time seems ripe to explore RF replication. Other proteins would be required in this second stage because studies *in vivo* have shown the need for the products of the host *rep* gene (11) and the phage *cistron A* gene (1). The correct state of the RF template, whether intact or modified, would also have to be determined.

In the present report we describe the enzymatic synthesis of φX174 RF I, and RF II from a superhelical RF I template. This reaction requires the soluble enzyme fraction that sustains the SS to RF conversion and, in addition, an extract from *cisA*$^+$ phage-infected cells. It is also essential that one of the extracts be derived from *rep*$^+$ cells. The extensive purification of the *cisA*-dependent and *rep*-dependent proteins will be described and the participation of proteins required for SS to RF conversion will be discussed.

Abbreviations: SS, single-stranded circles of DNA; RF, replicative form of DNA. RF I is double-stranded, covalently closed circular, and superhelical. RF II is double-stranded and circular with one or more single-strand breaks.

MATERIALS AND METHODS

Bacterial and Phage Strains. Bacterial strains were *Escherichia coli* H560 (6), HMS 83 (6), C (12), HF4720 (12), and HF4704 *rep*$_3$ (11). Phages were φX174 *am*3, a lysis-defective phage with an amber mutation in *cisE* (1), and φX174 *am*50, an amber mutant with a defect in *cisA* (obtained from Dr. R. Sinsheimer).

Preparation of Fraction II from Uninfected Cells. The soluble enzyme fraction (Fraction II) is an ammonium sulfate precipitate (obtained by adding 0.24 g to 1 ml of bacterial lysate) backwashed with an ammonium sulfate solution (0.24 g to 1 ml) (9).

Growth of φX174-Infected Cells. *E. coli* HF4720 were grown in mT3XD medium (12) at 37° with aeration in a Fermacell (New Brunswick Fermentor) to an OD_{590} of 0.5 (2×10^8 cells per ml). The cells were infected with φX174 at multiplicity of infection (MOI) of 5. At 5–10 min after infection, chloramphenicol (Calbiochem) was added (final concentration of 30 μg/ml) to inhibit single-stranded (viral) DNA synthesis and phage production (but not RF multiplication). At 30 min after infection, cells were harvested in a Sharples centrifuge at 10°, resuspended (10^{11} cells per ml) in 50 mM Tris·HCl (pH 7.5)–10% (wt/vol) sucrose, frozen in liquid nitrogen, and stored at −20°.

Fraction II from φX174-Infected Cells. Cells resuspended at 2×10^{10}/ml in 130 mM Tris·HCl (pH 7.5), 10% sucrose, 10 mM spermidine chloride, 4 mM dithiothreitol, 5 mM EDTA, 50 mM NaCl were treated with lysozyme (80 μg/ml), and incubated at 0° for 40 min, followed by either heat lysis (3) or Brij lysis (13). NaCl was added to the lysate to a concentration of 1 M. The lysate was centrifuged at 28,000 × *g* at 0° for 30–60 min (supernatant is Fraction I). Ammonium sulfate (0.226 g/ml of Fraction I) was added over a period of 10 min; the suspension, at 0°, was stirred for 15 min and centrifuged at 29,000 × *g* for 15 min. The precipitate was dissolved in Buffer A [50 mM Tris·HCl (pH 7.5), 20% (vol/vol) glycerol, 1 mM EDTA, 10 mM dithiothreitol] + 1 M NaCl.

φX RF I. [^{3}H]Thymidine-labeled φX RF I DNA was isolated from φX *am*3-infected *E. coli* C (treated with 30 μg/ml of chloramphenicol at 10 min after infection) and purified by sedimentation in neutral sucrose and ethidium bromide–CsCl gradients (12). The RF preparations were >90% RF I as judged by sedimentation velocity on alkaline sucrose gradients and by electron microscopy.

RESULTS

A soluble enzyme system for RF replication

DNA synthesis by a crude enzyme fraction (Fraction II), prepared from uninfected *E. coli*, is well supported by the addition

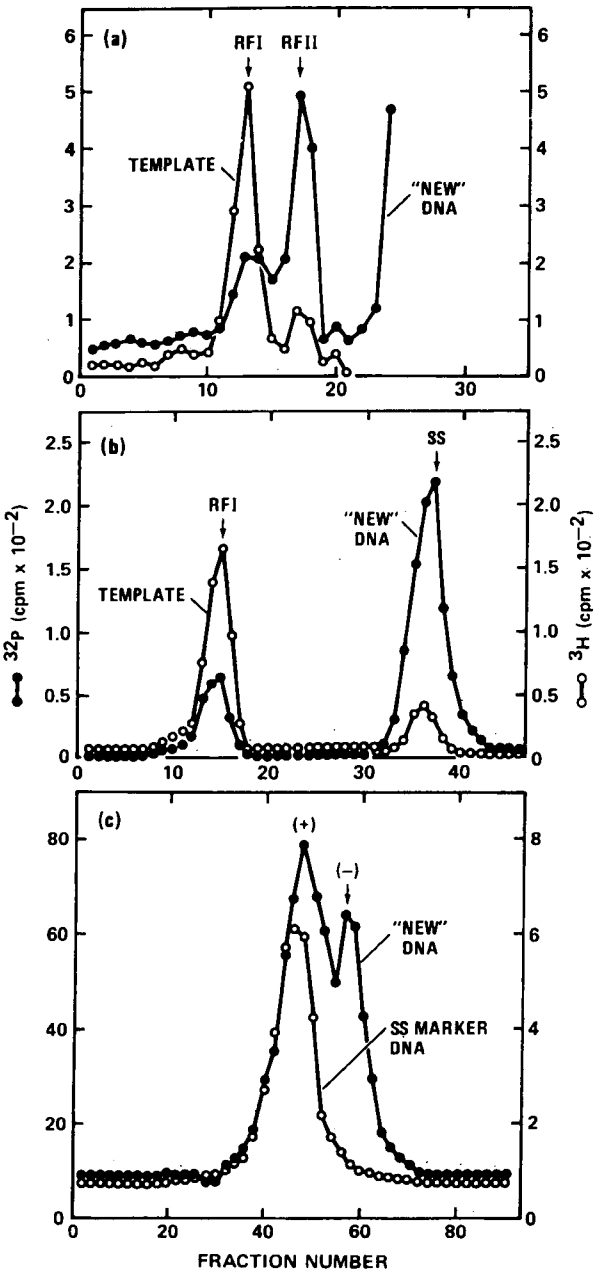

FIG. 1. The product of RF replication is RF I and RF II. The reaction mixture was as in Table 1, with [^{3}H]thymidine ϕX RF I template and [α-^{32}P]dCTP. (a) The product was purified by sedimentation velocity through a neutral sucrose gradient. (b) The RF peaks were pooled and concentrated with 2-propanol (12). The DNA was dissolved with 50 mM Tris·HCl, pH 7.5, 10 mM EDTA; denatured with alkali; and centrifuged on a linear, alkaline, 5–20% sucrose gradient (0.2 M NaOH, 0.8 M NaCl, 2 mM EDTA, 0.1% Sarkosyl). Centrifugation was performed in an SW50.1 rotor for 75 min at 50,000 rpm and 15° in a Beckman L265B centrifuge. (c) RF II DNA was obtained in a reaction as described above, except that unlabeled ϕX RF I template was used for banding on an alkaline CsCl gradient. The RF II DNA, which was purified on neutral sucrose, was concentrated, mixed with ^{3}H-labeled ϕX single-stranded viral DNA marker, denatured with alkali, and analyzed on an equilibrium density gradient (16).

of ϕX174 SS (3) but not by ϕX174 RF (Table 1). Fraction II from infected cells was inactive in DNA synthesis with either SS or RF as template. However, the combination of Fraction II from uninfected cells with one from infected cells supported DNA synthesis in response to the addition of RF I (Table 1). When RF I was replaced by RF II, synthesis was only 20–25% as great.

Table 1. A soluble enzyme system utilizing RF I as a template for replication

Template	Source of Fraction II: Uninfected	Source of Fraction II: Infected	DNA synthesis (pmol)
None	+	−	<1
	−	+	<1
	+	+	1.5
SS	+	−	100
	−	+	<1
RF I	+	−	14
	−	+	<1
	+	+	118

The reaction mixture in a 25 μl final volume contained: 50 mM Tris·HCl at pH 7.5; 6% sucrose; 10 mM dithiothreitol; 0.1 mg/ml of bovine-serum albumin; 5 mM $MgCl_2$; 50 μM each of dATP, dCTP, and dGTP, and 18 μM of [^{3}H]dTTP (specific activity 150–700 cpm/pmol), 800 μM ATP, 100 μM each of CTP, UTP, and GTP, and 2 mM spermidine chloride; occasionally [α-^{32}P]dCTP was the labeled deoxynucleoside triphosphate in place of dTTP. To this reaction mixture, 40 μg of Fraction II from uninfected *E. coli* H560, 1 μg of Fraction II from ϕX *am*3-infected *E. coli* HF4720 cells, and 1 μg of ϕX RF I DNA were added. The reaction was carried out at 30° for 20 min and stopped by the addition of 0.2 ml of 0.1 M sodium pyrophosphate and 1 ml of 10% trichloroacetic acid. The precipitate was collected on glass fiber filters (Whatman GF/C), washed three times with a 1 M HCl, 0.2 M sodium pyrophosphate solution, dried, and counted in 5 ml of a toluene-based scintillation fluid in a Nuclear Chicago liquid scintillation counter. DNA synthesis is expressed as total nucleotide incorporation.

The product of RF replication is RF I and RF II

The DNA synthesized by the crude enzyme fractions from uninfected and infected cells upon addition of RF I as template (as in Table 1) was first purified by sedimentation on a neutral sucrose gradient (Fig. 1a) and then analyzed by sedimentation velocity on alkaline sucrose (Fig. 1b). About 25% of the newly synthesized RF was in the form of RF I, indicating that a complete round of replication had been supported by the enzyme preparation. Analysis of the synthetic RF II by banding in alkaline CsCl showed that both the viral (+) and complementary (−) strands were synthesized (Fig. 1c).

Table 2. A protein dependent on ϕX174 *cistron A* required for RF replication

	μg	DNA synthesis (pmol)
Fr. II uninfected only	40.0	14
Plus Fr. II infected, wild type	0.5	51
Plus Fr. II infected, wild type	1.0	114
Plus Fr. II infected (chloramphenicol)	1.0	13
Plus Fr. II infected, *am*50 (*cisA*)	1.0	13

Fraction II from uninfected *E. coli* H560 and ϕX174-infected HF4720 were each prepared as described in *Materials and Methods*. ϕX *am*3 (*cisE*, lysis-defective) and ϕX *am*50 (*cisA*) were used for infections. Chloramphenicol treatment of ϕX *am*3-infected *E. coli* HF4720 was by addition of chloramphenicol (to a concentration of 200 μg/ml) 1 min before infection with phage. Synthesis was measured as described in Table 1.

Table 3. A protein dependent on *E. coli rep*$^+$ required for RF replication

Fraction II		Complementing	DNA synthesis
Uninfected	Infected	rep_3^+ fraction	(pmol)
rep	*rep*		0.3
rep$^+$	*rep*$^+$		24
rep	*rep*$^+$		11
rep	*rep*	FR II, 0.4 μg	3.7
rep	*rep*	FR II, 0.8 μg	7.0

Fraction II is from uninfected and infected HF4704 rep_3 and HF4704 *rep*$^+$ cells prepared as described in *Materials and Methods*. Complementing *rep*$^+$ Fraction II was prepared from HMS83. Synthesis was measured as described in Table 1, except that 10 μg of Fraction II from infected cells and 0.5 μg of DNA were used in each reaction.

A φX174-induced protein is required for RF multiplication

In the absence of *cisA* protein, *in vivo*, parental RF accumulates as RF I, but no further replication takes place (14). This condition can be achieved either by treating the cells with chloramphenicol (at 200 μg/ml) before infection or by using a phage mutant in *cistron A* (φX *am*50) (14). Fraction II prepared from infected cells treated with chloramphenicol 1 min before infection failed to complement Fraction II from uninfected cells in RF replication (Table 2). This result suggests that a protein synthesized after infection is required. That synthesis of this protein depends on an intact *cistron A* gene in the phage is indicated by the inactivity of Fraction II prepared from φX *am*50-infected cells in the complementation assay (Table 2).

The *E. coli rep* protein is required for RF replication

The infected *E. coli* rep_3 mutant, *in vivo*, sustains the synthesis of parental RF but fails to promote its replication (11). Fraction II prepared from an uninfected *rep* mutant converted SS to RF (data not shown) but failed to support RF replication when complemented with a Fraction II obtained from infected cells also mutant in the *rep* gene (Table 3). Replication of RF was observed with Fraction II prepared from either uninfected or infected cells of the *rep*$^+$ genotype (Table 3), suggesting that the product of this gene is essential.

Partial purification of the φX *cisA*- and *E. coli rep*-dependent proteins

The dependence of the RF replication system upon φX *cisA* and *E. coli rep* functions provided an assay for purification of these activities. Table 4 records current progress in purification of the φX *cisA*- and *E. coli rep*-dependent proteins; further purification is needed to obtain homogeneous preparations for characterization.

DISCUSSION

Understanding the molecular mechanisms of duplex DNA replication requires the resolution of the enzymatic machinery responsible for this operation. An excellent probe for identifying and resolving the parts of this cellular machinery is replication of the single-stranded (SS) and the duplex circular replicative form (RF) of phage φX174 in *E. coli* (7).

Table 4. Partial purification of φX174 *cisA*- and *E. coli rep*-dependent proteins

	cis A			*rep*	
	Specific activity (units/mg of protein × 10^3)	Recovery (%)		Specific activity (units/mg of protein × 10^3)	Recovery (%)
I Extract	(0.53)	(100)	I Extract	(0.032)	(100)
II Ammonium sulfate	4.8	100	II Ammonium sulfate	0.45	68
III Bio-Gel, A-0.5m	10.4	93	III Bio-Rex 70	5.0	46
IV Bio-Rex 70	67	68	IV DNA-cellulose	38	8
V DNA-cellulose	410	19			

Purification of φX *cisA*-dependent protein: Fraction I (lysate supernatant, 190 ml) and Fraction II (6.5 ml) were prepared from φX *am*3-infected *E. coli* HF4720 (see *Materials and Methods*). Fraction II was passed through a Bio-Gel A-0.5m column (150 ml) (Fraction III, 38 ml). Fraction III was dialyzed against Buffer B (50 mM imidazole·HCl at pH 6.7, 20% glycerol, 1 mM EDTA, 10 mM dithiothreitol) + 0.3 M NaCl, and applied to a Bio-Rex 70 column (18 ml) equilibrated with Buffer B + 0.3 M NaCl. The column was washed with 2 column volumes of Buffer B + 0.3 M NaCl, and the φX174 *cisA*-dependent activity was eluted with Buffer B + 1 M NaCl (Fraction IV, 11.5 ml). Fraction IV (1 ml) was diluted with Buffer B to a conductivity value of Buffer B + 0.4 M NaCl and applied to a single-stranded DNA-cellulose column (0.5 ml). The column was washed with 2 column volumes of Buffer B + 0.4 M NaCl and the activity was eluted with Buffer B + 1 M NaCl. Of the input activity, 50% was recovered in the 1 M NaCl eluate. Assays were performed as in Table 1. One unit is defined as 1 pmol of total nucleotide incorporated/min. Purification of *E. coli rep*-dependent protein: Fraction I (4400 ml) was prepared from *E. coli* HMS83 (900 g) as described previously for the purification of *dnaC* protein (9). The Fraction II is an ammonium sulfate precipitate (obtained by the addition of 0.24 g ammonium sulfate to 1 ml of Fraction I) which was backwashed first with 440 ml of an ammonium sulfate solution (0.24 g added per ml of buffer) and then 440 ml of a more dilute solution (0.20 g added per ml of buffer) and three successive times with 170 ml, 100 ml, and 90 ml of a still more dilute ammonium sulfate solution (0.18 g added to 1 ml of buffer) essentially as previously described (9). Fraction II (50 ml) in Buffer C (50 mM imidazole·HCl at pH 6.9, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol) was diluted to 350 ml and applied to a Bio-Rex 70 (200–400 mesh) column (110 ml) equilibrated with Buffer C + 0.2 M NaCl. The column was washed with 75 ml of Buffer C + 0.2 M NaCl followed by 200 ml Buffer C + 0.4 M NaCl. The *rep*-dependent activity was then eluted with Buffer C + 2.0 M NaCl (200 ml), diluted to 600 ml in Buffer C, and precipitated by ammonium sulfate (0.31 g for each ml). The precipitate was dissolved in Buffer D (50 mM Tris·HCl at pH 7.5, 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol) (Fraction III, 9.8 ml), diluted to 190 ml, and applied to a single-stranded DNA-cellulose column (25 ml) equilibrated with Buffer D + 0.1 M NaCl. The column was washed with Buffer D + 0.1 M NaCl, followed by a 0.125–0.550 M NaCl gradient. The *rep*-dependent activity was eluted with Buffer D + 2.0 M NaCl (50 ml) and precipitated by the addition of an equal volume of saturated neutralized ammonium sulfate solution. The precipitate was collected after standing overnight on ice, and was dissolved in Buffer C (Fraction IV, 1.7 ml). Calculation of yield was based on the activity recovered from Fraction I in an ammonium sulfate pellet (prepared by adding 0.24 g for each ml) and backwashed with an ammonium sulfate solution (0.24 g added to 1 ml of buffer). Assays of Fraction I were not reliable.

The present investigation is an extension from a series of earlier studies in which examination of the first stage of ϕX174 viral DNA replication, conversion of SS to RF, identified and provided some 12 cellular components. The complexity of this multienzyme system, comprising the proteins required for replication of the host chromosome, suggests that this system would, to some extent, be employed for duplex DNA replication.

Not included in the roster of proteins that serve in SS to RF conversion are two additional proteins, a need for which may be anticipated from earlier investigations (1, 11). One of these, the *rep* protein, whose function is still unknown, is furnished by the host (17). The other, the *cistron A* protein, is induced by the phage (1) and is thought to nick the RF I at a specific location (13, 14). We have purified each of these gene-dependent proteins by complementation assays. The extensive purification, approximately 1000-fold at this point, suggests that these proteins are in fact the products coded by these genes. Characterization of their functions awaits their complete purification and coordination with other proteins required in RF replication.

Among the proteins, one which is surely needed is a DNA polymerase for chain elongation. We expect that the DNA polymerase III holoenzyme would be the choice. Evidence supporting this conjecture is the specific and total inhibition of RF replication by antiserum to copolymerase III* (data not shown), one of the components of the holoenzyme (15). We may also expect that DNA polymerase I serves in gap filling and DNA ligase, in sealing of chains. The need for additional proteins, which may include some of those required for conversion of SS to RF, remains to be determined.

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