

## A mechanism of duplex DNA replication revealed by enzymatic studies of phage $\phi$ X174: Catalytic strand separation in advance of replication\*

(*rep* protein/*cis*tron A protein/RF replication/duplex DNA unwinding/DNA-dependent ATPase)

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**ABSTRACT** The enzyme system for duplicating the duplex, circular DNA of phage  $\phi$ X174 (replicative form) in stage II of the replicative life cycle was shown to proceed in two steps: synthesis of the viral (+) strand [stage II(+)], followed by synthesis of the complementary (-) strand [stage II(-)] [Eisenberg *et al.* (1976) *Proc. Natl. Acad. Sci. USA* 73, 3151-3155]. Novel features of the mechanism of the stage II(+) reaction have now been observed. The product, synthesized in extensive net quantities, is a covalently closed, circular, single-stranded DNA. The supercoiled replicative form I template and three of the four required proteins—the phage-induced *cis*tron A protein (*cisA*), the host *rep* protein (*rep*), and the DNA polymerase III holoenzyme (holoenzyme)—act catalytically; the *Escherichia coli* DNA unwinding (or binding) protein binds the product stoichiometrically. In a reaction uncoupled from replication, *cisA*, *rep*, DNA binding protein, ATP, and  $Mg^{2+}$  separate the supercoiled replicative form I into its component single strands coated with DNA binding protein. In the presence of  $Mg^{2+}$ , *cisA*, nicks the replicative form I; *rep*, ATP, and  $Mg^{2+}$  achieve strand separation with a concurrent cleavage of ATP and binding of DNA binding protein to the single strands. *rep* exhibits a single-stranded DNA-dependent ATPase activity. These observations suggest that the *rep* enzymatically melts the duplex at the replicating fork, using energy provided by ATP; this mechanism may apply to the replication of the *E. coli* chromosome as well.

Replication of phage  $\phi$ X174 DNA proceeds in three stages: stage I, conversion of single-stranded viral (+) circle to a circular, duplex, replicative form (RF); stage II, multiplication of RF; and stage III, synthesis of viral strands with the complementary (-) strand of RF used as template (1).

Crude enzyme systems are capable of sustaining the stage II reaction of RF replication (2, 3). These systems have served as assays for the purification of  $\phi$ X174 *cis*tron A protein (*cisA*) (2, 4) and the partial purification of *Escherichia coli* *rep* protein (*rep*) (2). Using these proteins, we resolved the stage II reaction into two phases: a stage II(+) reaction comprising the continuous synthesis of viral (+) strands, and a stage II(-) reaction comprising the subsequent synthesis of complementary (-) strand with the stage II(+) product as template (5).

In the present report, we describe novel features of the mechanism of the stage II(+) reaction. The reaction requires the  $\phi$ X174 *cisA*, the *E. coli* *rep*, DNA polymerase III holoenzyme, the DNA binding protein (DBP) [formerly called DNA

unwinding protein (6-8)], ATP,  $Mg^{2+}$ , and the four deoxyribonucleoside triphosphates (dNTPs). Viral (+) single-stranded circles are synthesized in extensive net quantities as the exclusive product of the reaction. In a reaction uncoupled from DNA replication, the duplex superhelical RF I template is separated into viral (+) and complementary (-) single strands. This partial reaction is absolutely dependent on *cisA*, *rep*, and DBP, ATP, and  $Mg^{2+}$  and is accompanied by the cleavage of ATP; *rep* also acts as an ATPase when supplied with single-stranded DNA regions.

These findings have important implications for the mechanism of  $\phi$ X174 DNA replication and for the general question of strand separation during fork movement in the semiconservative replication of duplex DNA.

### MATERIALS AND METHODS

**Source of Enzymes.**  $\phi$ X174 *cisA* (60,000 daltons) was prepared by an improved procedure and was more than 90% pure as judged by sodium dodecyl sulfate (NaDodSO<sub>4</sub>)/polyacrylamide gel electrophoresis ( $4.4 \times 10^6$  units/mg of protein) (Eisenberg *et al.*, unpublished data). *E. coli* *rep* was prepared from JFS-19 [*rha*, *lys*, *thy*, *polB*, *str*<sup>r</sup>/F<sup>+</sup>/ColE1 (pLC 44-7) *ilv*<sup>+</sup>, *cya*<sup>+</sup> *rho*<sup>+</sup>, *rep*<sup>+</sup>] cells, which overproduce *rep* 7- to 10-fold above wild-type levels. Plasmid pLC 44-7 was obtained by screening of the collection of ColE1/*E. coli* hybrids prepared by Clarke and Carbon (9). Details of the screening and purification, which yields *rep* (68,000 daltons) more than 95% pure as judged by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis ( $2.0 \times 10^7$  units/mg of protein), will be described elsewhere. DNA polymerase III holoenzyme was DEAE-Sephadex peak fraction V ( $5.9 \times 10^5$  units/mg of protein; approximately 60% pure) (C. S. McHenry and A. Kornberg, unpublished data). Pure DBP [the *E. coli* DNA unwinding protein of Sigal *et al.* (6)] was prepared as described previously (7).

**Stage II(+) Reaction.** The standard reaction mixture in a 25- $\mu$ l volume contained 50 mM Tris-HCl (pH 7.5), 5% sucrose, 10 mM dithiothreitol, bovine serum albumin at 0.1 mg/ml, 5 mM  $MgCl_2$ , 50  $\mu$ M each of dATP, dCTP, and dGTP, and 18  $\mu$ M [<sup>3</sup>H]dTTP (specific activity, 150 cpm/pmol total dNTP), 800  $\mu$ M ATP, 700 pmol (total nucleotide) of  $\phi$ X RF I DNA, 1000 units of *rep*, 500 units of *cisA*, 1.5  $\mu$ g of DBP, and 16 units of DNA polymerase III holoenzyme. Reaction mixtures were incubated at 30°, and aliquots were treated with 0.2 ml of 0.1 M sodium pyrophosphate, placed in ice, and treated with 1 ml of 10% trichloroacetic acid. The acid-precipitable material was collected on filters and monitored for radioactivity, as described previously (5). When the stage II(+) reaction was used as an assay for one of the required components, that component was present in a decreased amount, as indicated. One unit is defined

Abbreviations: RF, duplex 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); *cisA*, phage-induced *cis*tron A protein; *rep*, host *rep* protein; DBP, DNA binding protein; dNTPs, deoxyribonucleoside triphosphates; NaDodSO<sub>4</sub>, sodium dodecyl sulfate.

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Table 1. The stage II(+) reaction:  
Extensive viral (+) strand DNA synthesis

Limiting component	Limiting component (molecules $\times 10^{-10}$ )	Product circles (molecules $\times 10^{-10}$ )	Ratio, col 2:col 1
RF I	0.5	11.0	22.0
<i>cisA</i>	2.3	15.0	5.6
<i>rep</i>	0.25	7.2	29.0
DBP	625.0	5.0	0.0080*
DBP	1250.0	11.0	0.0088

Stage II(+) reactions were as described in *Materials and Methods* but with individual components present in a limiting quantity, where indicated. Reaction times in limiting-component reactions were as follows: RF I, 60 min; *cisA*, 160 min; *rep*, 120 min; and DBP, 60 min; with both levels of DBP, initial rates were the same.

\* Represents 42 nucleotides/tetramer.

as incorporation of 1 pmol of total deoxynucleotide per minute.

**Nonreplicative Strand Separation Reaction.** The standard reaction mixture was as described for the stage II(+) reaction except that DNA polymerase III holoenzyme and the four dNTPs were omitted; in some instances, half the level of DBP was used.

**Assay for ATPase.** The reaction mixture contained, in a total volume of 12  $\mu$ l, 30 mM Tris-HCl (pH 7.5), 6% sucrose, 12 mM dithiothreitol, bovine serum albumin at 120  $\mu$ g/ml, 5 mM  $MgCl_2$ , 40  $\mu$ M [ $\alpha$ - $^{32}P$ ]ATP (specific activity, 630 cpm/pmol), 220 pmol (total nucleotide) of  $\phi$ X174 single-stranded DNA (isolated from virus particles), and a sample to be assayed for ATPase. Reactions were incubated at 30 $^\circ$ ; 1- $\mu$ l aliquots were removed and applied to prewashed polyethyleneimine-impregnated cellulose strips, for thin-layer-chromatography (Brinkmann MN300) with nonradioactive ATP and ADP markers. The strips were developed at 23 $^\circ$  in an ascending solvent containing 1 M formic acid and 0.5 M LiCl. The ADP spot, defined under UV light, was cut out and its radioactivity was measured in a gas flow counter (Nuclear Chicago). One unit of ATPase is defined as that amount which generates 1 pmol of ADP per minute.

**Electron Microscopic Observation of Products.** Samples of purified product DNA, or aliquots removed directly from reaction mixtures, were prepared for observation essentially as described by Davis *et al.* (10). The grids were examined in a Philips electron microscope.

## RESULTS

**Product of Stage II(+) Is the Circular Viral (+) Strand.** Phage-induced *cisA* and *rep* [from uninfected cells carrying a *ColE1-E. coli rep* hybrid from the Clarke and Carbon colony bank (9)] have been purified to near homogeneity as judged by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis (Eisenberg *et al.* and Scott *et al.*, unpublished data). These two proteins, together with DBP and DNA polymerase III holoenzyme, synthesized viral (+) strand in large net quantities (Table 1). The reaction is similar to, but far more efficient than, that described previously (5) in which less highly purified proteins were used.

The product of this net synthetic reaction was sedimented preparatively in alkaline sucrose gradients (Fig. 1). The product sedimented as a sharp peak, coinciding with the  $\phi$ X174 single-stranded circular DNA (prepared from virus particles)

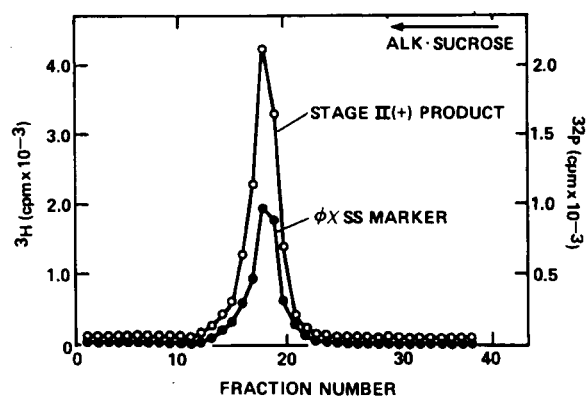


FIG. 1. Sedimentation analysis of the product synthesized in stage II(+) reaction. The reaction mixture was as described in *Materials and Methods*, except that [ $\alpha$ - $^{32}P$ ]dCTP was used instead of [ $^3H$ ]dTTP. The reaction was stopped by addition of EDTA (to 0.1 M) after incubation for 30 min. The  $^{32}P$ -labeled product, produced in 10-fold excess over template, was treated with 0.2 M NaOH and sedimented through a 5–20% alkaline sucrose gradient. The product, which sedimented as a single sharp peak, was concentrated by vacuum dialysis against 50 mM Tris-HCl at pH 7.5, 1 mM EDTA. An aliquot of the  $^{32}P$ -labeled product was treated with 0.2 M NaOH, mixed with  $^3H$ -labeled purified  $\phi$ X174 phage (pretreated with 0.2 M NaOH for 30 min at 30 $^\circ$ ), and then centrifuged through a 5–20% analytical alkaline gradient. Alkaline sucrose solutions were described previously (11). Sedimentations were performed in a SW 56 rotor at 50,000 rpm and 15 $^\circ$  for 3.5 hr in a Beckman centrifuge.

serving as a 16S marker. The peak contained more than 90% of the total product, and more than 95% of the molecules appeared as single-stranded circles in the electron microscope. The purified product did not hybridize to  $\phi$ X (+) strand DNA covalently coupled to cellulose (<1%) under conditions that trap 68% of (–) strands; we assume the product to be (+) strands as shown previously (5).

**Stage II(+) Reaction Is Limited in Extent by DBP and in Rate by *rep*, *cisA*, Holoenzyme, and RF I Template.** The rates and extent of stage II(+) reactions were examined by varying the components of the standard mixture (Fig. 2). Decreasing the level of DBP to one-half did not affect the initial rate of the reaction, but it did decrease the extent of the reaction stoichiometrically. Conversely, lowering the levels of *cisA* or *rep*

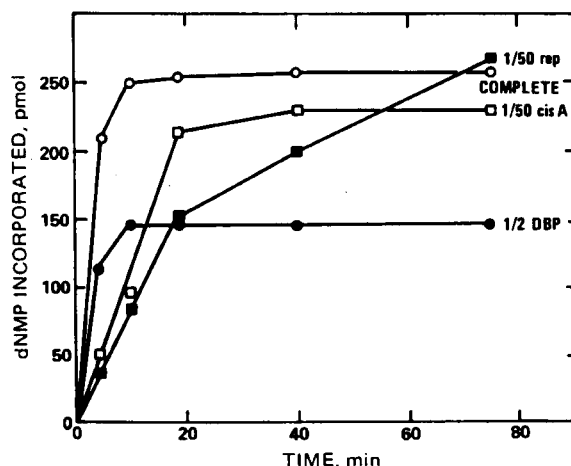


FIG. 2. Factors influencing the rate and extent of synthesis of (+) single strands. The complete stage II(+) reaction was as described in *Materials and Methods*. Aliquots were removed from individual reaction mixtures and processed as described; the mixtures were complete or contained decreased amounts of *rep*, *cisA*, or DBP.

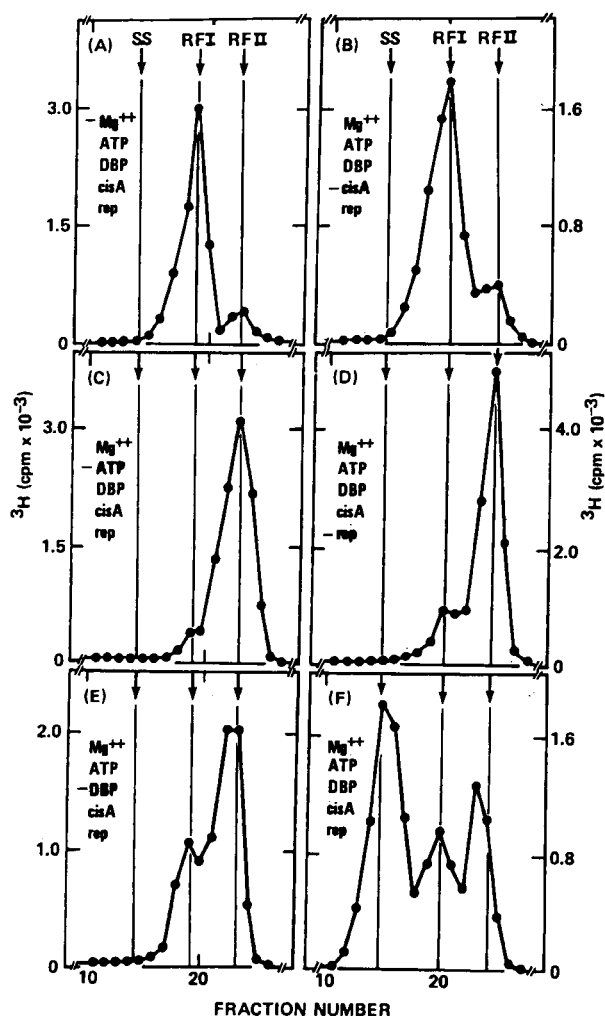


FIG. 3. Requirements for the nicking and unwinding of superhelical RF I in the absence of replication. The complete reaction mixture was as described for the nonreplicative strand-separation reaction in *Materials and Methods*, except that holoenzyme and the four dNTPs were omitted in all cases. Reaction mixtures were incubated for 20 min, stopped by addition of EDTA to 0.1 M and Na-DodSO<sub>4</sub> to 1%, and sedimented through 5–20% analytical neutral sucrose gradients with solutions described previously (11). Sedimentations were performed in a SW 56 rotor at 50,000 rpm and 20° for 2 hr in a Beckman centrifuge. The complete reaction mixture (F) contained *cisA*, *rep*, Mg<sup>2+</sup>, ATP, and DBP. Component omitted is marked by a minus sign and stippling in each of the other panels.

depressed the initial rate but not the extent. Reduction of holoenzyme level by one-half resulted in a proportional change in the initial rate of the reaction (data not shown); at RF I levels of 90 and 180 pmol (as compared to 700 pmol in the standard reaction), initial rates were decreased to 29% and 51%, respectively.

Under conditions of rate limitation by a single component, the reaction proceeded until the limit imposed by available DBP was reached (about 42 nucleotides per molecule of binding protein). The number of circles produced was in considerable excess over the number of molecules of the limiting component (e.g., *cisA* protein, *rep*, or RF I) (Table 1). In a reaction containing a rate-limiting quantity of holoenzyme, roughly 1.3 product circles were produced (7000 phosphodiester bonds formed) per molecule of holoenzyme present (data not shown).

Table 2. ATP is required for stage II(+) and nonreplicative strand-separation reactions

ATP (μM)	Stage II(+) DNA synthesis (pmol/5 min)	Strand separation (pmol/20 min)
None	11	<1
25	123	68
50	n.d.*	131
100	320	n.d.
400	635	140

Stage II(+) and nonreplicative strand-separation reactions were as described in *Materials and Methods*, except that ATP was omitted or present at concentrations indicated. Stage II(+) reactions were incubated for 5 min. Strand-separation reactions were incubated for 20 min and stopped by chilling on ice; extent of the reaction was measured by assaying the DBP remaining by using an otherwise complete stage II(+) reaction and incubating for an additional 20 min. The extent of incorporation was measured as before and subtracted from stoichiometric product production anticipated from the quantity of DBP originally added; both the strand-separation and stage II(+) reactions require stoichiometric quantities of DBP.

\* n.d., not determined.

We infer from these data that the DBP binds stoichiometrically to the displaced (+) strand product and that this binding is absolutely required for the reaction to proceed. In addition, we infer that *cisA*, *rep*, holoenzyme, and RF I act catalytically.

**Twisted Duplex Circles Are Completely Unwound by *cisA*, *rep*, DBP, ATP, and Mg<sup>2+</sup>, in the Absence of DNA Replication.** When the stage II(+) reaction mixture was incubated without holoenzyme and dNTPs, there was a marked decrease in initial rate and extent of DNA synthesis upon the subsequent addition of the holoenzyme and dNTPs. This effect, as will be seen, was due to removal of RF I and depletion of DBP, suggesting that the RF was being converted to single strands coated with binding protein.

The products of the prereplication reaction (holoenzyme and dNTPs omitted from the standard reaction mixture incubated for 20 min) observed directly in the electron microscope showed the following percentage distribution: single-stranded DNA circles, 26; single-stranded full-length linears, 40; RF I, 19; and RF II, 15. The appearance of single-stranded circles or linears required the presence of *cisA*, *rep*, ATP, DBP, and Mg<sup>2+</sup>.

The products were also analyzed by neutral sucrose gradient sedimentation (Fig. 3) and, in some instances, were assayed for utilization (depletion) of DBP (Table 2). Nicking of the RF I template was dependent only on *cisA* and Mg<sup>2+</sup> (compare panels A and B with panels C, D, and E in Fig. 3). The separation of strands was absolutely dependent on *rep*, DBP, and ATP in addition to *cisA* and Mg<sup>2+</sup> (compare panel F with the others in Fig. 3). The apparent Michaelis constant ( $K_m$ ) for ATP in the nonreplicative strand-separation reaction was found to be approximately 25 μM (Table 2).

These data indicate that, in a reaction uncoupled from DNA replication, the combined action of *cisA*, *rep*, and DBP results in the nicking of φX RF I in one strand [presumably (+)], separation of the (+) and (–) strands, and coating of the single strands by DBP. In view of the absolute requirement for ATP, and a concomitant ATP hydrolysis to ADP and Pi (see below), energy for the strand-unwinding process may be provided by ATP hydrolysis.

**Rep Is an ATPase in the Presence of Single-Stranded DNA.** In the complete stage II(+) reaction, with φX RF I as template, extensive hydrolysis of [ $\gamma$ -<sup>32</sup>P]ATP was observed. When the product of the stage II(+) reaction (single-stranded DNA) was

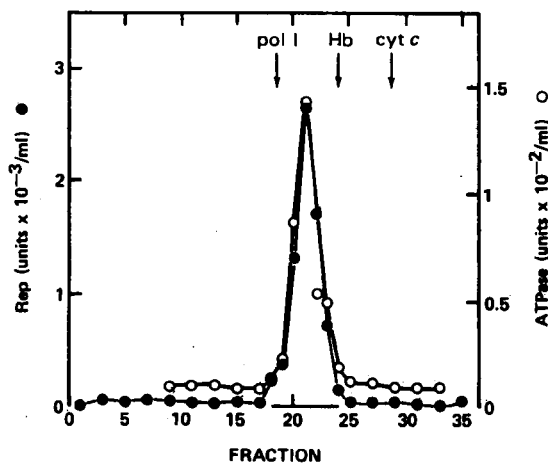


FIG. 4. Coincidence of *rep* protein activity and DNA-dependent ATPase in a glycerol-gradient sedimentation. *Rep* (1.4  $\mu$ g) was sedimented through a 20–40% glycerol gradient containing 50 mM imidazole-HCl at pH 6.8, 10 mM dithiothreitol, 1 mM EDTA, and 0.1 M ammonium sulfate. Sedimentation, in a SW 60 rotor at 58,000 rpm and 4° for 19 hr in a Beckman centrifuge, is shown from right to left. Fractions were collected from the bottom of the tube. Aliquots of fractions were assayed for *rep* activity in the stage II(+) reaction and for DNA-dependent ATPase activity (see *Materials and Methods*). Known markers sedimented in parallel gradients were DNA polymerase I (pol I), 109,000 daltons; hemoglobin (Hb), 64,000 daltons; and cytochrome *c* (cyt *c*), 12,800 daltons. Units of ATPase activity cannot be compared directly to units of *rep* activity inasmuch as ATPase assays were performed under conditions that were suboptimal and different from those for the *rep* assay.

added to the reaction mixture, with only *rep* (and  $Mg^{2+}$ ) present, hydrolysis of ATP was immediate and rapid.

Identity of the ATPase activity with the *rep* protein was indicated by their coincidence in a preparation eluted from a DEAE-cellulose column, the last step in the purification procedure. Furthermore, the ATPase activity cosedimented with the pure *rep* activity on a glycerol gradient (Fig. 4).

The ATPase turnover number of the *rep* protein (about 3500/min at 30°) is the same as that of a protein of similar molecular weight purified more than a year ago for its single-stranded DNA-dependent ATPase activity (L. Bertsch and A. Kornberg, unpublished data). A similar protein isolated by Richet and Kohiyama (12) may also be *rep*. Further characterization of the ATPase activity of *rep* will be reported elsewhere.

The dependence of *rep* on ATP for its action and its potent ATPase activity in an uncoupled reaction suggest that the energy furnished by the hydrolysis of ATP may be used in the mechanics of strand displacement.

## DISCUSSION

Insights into some fundamental features of DNA replication and movement of the replicating fork are suggested by an analysis of how the supercoiled, circular, duplex RF I of  $\phi$ X174 is multiplied by purified proteins (Fig. 5) (5).

In the first step of RF replication [stage II(+)], viral (+) strands are produced simply by: (i) nicking of the RF I (Fig. 3) in an area of the (+) strand ("the origin") by phage-induced *ctsA* to generate a 3'-hydroxyl group (4, 15–17), (ii) covalent extension of this primer point by DNA polymerase III holoenzyme to restore the origin, (iii) copying of the template (–) strand to produce a new viral (+) strand, (iv) nicking again at the origin to release a full-length linear (+) strand, and (v) closure of the (+) strand to complete a viral circle. In an alter-

native mechanism, which has not been excluded, nicking and resealing events at the origin after a short length of (+) strand had been produced would generate a D-loop type of replicating structure.

The report (4) indicating that *ctsA* protein may form a covalent bond with the 5' end of the nicked chain suggests to us that it may also function as a ligase [in the manner of the nicking-ligating action of the  $\omega$  protein (18) or the ligating-nicking action of polynucleotide ligase (19)] to seal the linear (+) strand.

The viral (+) strand product of the stage II(+) reaction serves as a template for complementary strand synthesis [stage II(–)] to produce RF I, using the constellation of host replication proteins (dna B, C, and G proteins, proteins i and n, holoenzyme, etc.). We presume that final assembly of a viral circle into a phage particle (stage III) takes place when the products of phage genes B, D, F, G, and H become available.

By analogy, replication of a large, circular chromosome (e.g., *E. coli*) may also be initiated by a nick at the origin in one strand, or in both strands if replication is bidirectional. The strand extended from the origin may be called the (+) or leading strand. From this single origin, synthesis of the (+) strand on the parental template (–) strand would be *continuous*. By contrast, synthesis of the complementary strand, called the (–) or lagging strand, would be initiated repeatedly as the parental template (+) strand is progressively exposed during fork movement; synthesis of the lagging strand would be *discontinuous*. By this scheme (20), nascent (Okazaki) fragments are initiated only in the lagging strand; short nascent fragments observed in the leading strand (21) could be the result of misincorporation and excision of uracil residues (22).

The mechanics of base-pair melting, strand separation, and movement of the replicating fork have been matters of deep interest and considerable discussion pertaining to the semi-conservative replication of DNA. That the unzipping of the helix might be catalyzed by a specific protein (23), such as *rep*, or by DNA-dependent ATPases that utilize ATP energy for the process has been proposed (12, 24).

A simple example of strand displacement concurrent with replication is the operation of *E. coli* DNA polymerase I at a nick in ColE1 or other circular duplex DNAs (unpublished observations) (25). Neither ATP nor DBP is required. The operation of the multisubunit DNA polymerase III holoenzyme is strikingly different. When supplied with the same nicked ColE1 template, the holoenzyme is active only in the presence of *ctsA*, *rep*, DBP, and ATP (unpublished observations). In the first step of replication of the supercoiled RF I [stage II(+)], progress of the replicating fork and displacement of the (+) strand depend on the presence of ATP, *ctsA*, and DBP. The discovery that *rep* is a potent DNA-dependent ATPase and that cleavage of ATP to ADP and Pi is concurrent with strand displacement (even when uncoupled from replication) suggests that ATP energy may be used by *rep* for the unzipping process. Whether ATPase action by *rep* is coupled to the strand separation or is a secondary consequence of generating single-stranded DNA, which can serve as an effector for ATPase action, has not been settled in our studies. Experimental techniques that clarify how the energy of a nucleoside triphosphate is used in the elongation of peptide chains (26, 27), muscle contraction (28, 29), and active transport (29) will be useful for further work.

Dissection of the replicative process of  $\phi$ X174 DNA into discrete enzymatic steps has afforded new insights into the molecular machinery and mechanisms of duplex DNA replication. However, future efforts to reconstitute an enzyme sys-

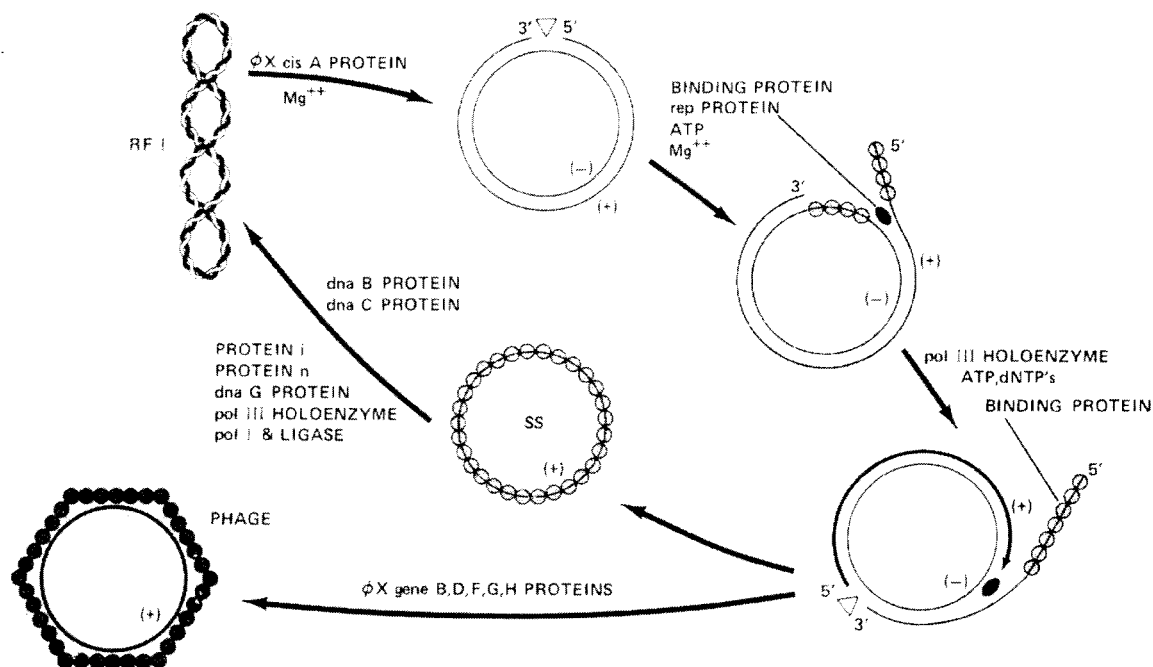


FIG. 5. Hypothetical scheme for some of the replicative events in the life cycle of  $\phi$ X174. The scheme is designed to illustrate some of the partial reactions observed in this and previous studies and to suggest that the replicative events in the life cycle of  $\phi$ X174 may be represented by two mechanisms: complementary (-) strand synthesis [SS(+)  $\rightarrow$  RF] and viral (+) strand synthesis [RF  $\rightarrow$  SS(+)]. In RF replication, (-) strand synthesis might be initiated before completion of the viral (+) circular template. The requirement for  $\phi$ X gene products for viral (+) strand assembly into phage is based on *in vivo* studies (13, 14). For further discussion see the *text*.  $\nabla$  represents the origin of replication.

tem for the concerted, rapid, and efficient replication of RF, as it occurs in the infected cell, will test current schemes and provide novel information.

**Note Added in Proof.** In stage II(+) reactions where both *rep*-dependent ATP hydrolysis and coupled DNA synthesis were measured, one ATP molecule was split per base pair melted and nucleotide residue incorporated.

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