Proc. Natl. Acad. Sci. USA
Vol. 73, No. 9, pp. 3151–3155, September 1976
Biochemistry

Enzymatic replication of viral and complementary strands of duplex DNA of phage φX174 proceeds by separate mechanisms

[*cisA* protein/*rep* protein/viral (+) strand synthesis/complementary (−) strand synthesis]

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Contributed by Arthur Kornberg, July 7, 1976

ABSTRACT **Multiplication of the duplex, circular, phage φX174DNA (replicative form, RF) in stage II of the replicative life cycle has been observed with a crude enzyme preparation [Eisenberg *et al.* (1976) *Proc. Natl. Acad. Sci. USA* 73, 1594–1597]. This stage has now been partially reconstituted with purified proteins and subdivided into two stages: II(+) and II(−). In stage II(+), viral (+) strand synthesis is carried out by four proteins: the phage-induced, *cistron A*-dependent protein, *rep*-dependent protein, DNA unwinding protein, and DNA polymerase III holoenzyme. In stage II(−), complementary (−) strand synthesis utilizes the product of stage II(+) as template and the multiprotein system previously identified in the stage I synthesis of a complementary strand on the viral DNA template to produce RF. The multiprotein system includes DNA unwinding protein, proteins i and n, *dnaB* protein, *dnaC* protein, *dnaG* protein, and DNA polymerase III holoenzyme. A discussion of these two separate mechanisms for synthesis of (+) and (−) strands suggests that they may account for essentially all the replicative stages in the life cycle of φX174.**

Three DNA replicative stages make up the life cycle of phage φX174: (I) conversion of the single-stranded, viral (+) circle (SS) to a circular, duplex, replicative form (RF); (II) multiplication of the RF; and (III) synthesis of viral strands using the complementary (−) strand of RF as template (1).

For complementary strand synthesis to form RF in stage I, the phage DNA appropriates the multiprotein system used by the host cell for replication of its own chromosome (2, 3). Stage II of RF replication was known from genetic studies also to require the action of a phage-induced protein coded by cistron *A* (1, 4) and a host protein, rep (5). Our previous study of stage II described the capacity of a crude, soluble enzyme system to sustain RF replication and to provide assays for the partial purification of the proteins dependent on *cisA* and *rep* functions (6).

In this report we describe that in the partial reconstitution with purified proteins, stage II can be subdivided into stages of (+) strand synthesis [II(+)] and (−) strand synthesis [II(−)]. Stage II(+) requires the products of the *cisA* and *rep* genes, DNA unwinding protein, and the DNA polymerase III holoenzyme. The stage II(+) product provides a template for (−) strand synthesis, which requires the multiprotein system used for synthesizing the complementary (−) strand in the stage I conversion of viral (+) strand to RF. This system includes DNA unwinding protein, proteins i and n, *dnaB* protein, *dnaC* protein, *dnaG* protein, and the DNA polymerase III holoenzyme.

Stage III, the synthesis of the viral strand *in vivo*, is coupled to its encapsidation and assembly into a virus particle. Although enzymatic reconstitution of this stage has not been described, it seems likely that the replicative mechanism will resemble that of (+) strand synthesis in stage II(+) of RF replication. A hypothetical scheme summarizing the stage II reactions and their relationship to preceding and succeeding replicative events in the φX174 life cycle is presented in Fig. 1.

Abbreviations: SS, single-stranded circle of viral DNA; RF, replicative form.

Table 1. DNA synthesis using superhelical φX174 RF I DNA as template

Additions and omissions	DNA synthesis (pmol)
Complete	292
− DNA unwinding protein	3
− *cisA* protein	4
− *rep* protein	17
− DNA polymerase III holoenzyme	1
Complete + anti-i protein	215
Complete + anti-n protein	210
Complete + anti-*dnaB* protein	280
Complete + anti-*dnaG* protein	280
− φX174 RF I, + φX174 SS DNA	3

The reaction mixture in a 25 μl volume contained: 50 mM Tris·HCl (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, dGTP, and 18 μM [^{3}H]dTTP (specific activity 150 cpm/pmol), 800 μM ATP, 100 μM each of CTP, UTP, and GTP, 2 mM spermidine chloride, 960 pmol (total nucleotide) of φX RF I DNA, 19 units of *cisA* protein, 18 units of *rep* protein, 24 units of DNA unwinding protein, and 20 units of DNA polymerase III holoenzyme. 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, 0.1 M sodium pyrophosphate solution, and dried, and its radioactivity was measured in 5 ml of a toluene-based scintillation fluid. Antibody addition, where noted, was to a reaction mixture lacking φX RF I DNA; the mixture was incubated at 0° for 10 min, before adding φX RF I DNA and starting the standard incubation. Under these conditions, each of the antibodies used inhibited the synthesis of the complementary (−) strand in the conversion of φX174 SS to RF by more than 90%.

MATERIALS AND METHODS

Source of Enzymes and Antibodies. φX174 *cisA*- and *Escherichia coli rep*-dependent proteins were Fraction V and Fraction IV, respectively (6). Sources of other purified proteins (i, n, *dnaB*, *dnaC*, *dnaG*, DNA polymerase III holoenzyme, and DNA unwinding protein) were previously described (8). S1, the single-strand-specific nuclease, was purchased from Miles Laboratories, Inc. Antibodies were prepared against each of several purified protein preparations (i, n, *dnaB*, *dnaG*, and DNA unwinding protein) and the γ-globulins were purified to homogeneity, essentially as previously described (8).

Competitive Reannealing of the Synthetic Products of Stage II(+) and II(−). The radioactively labeled product was

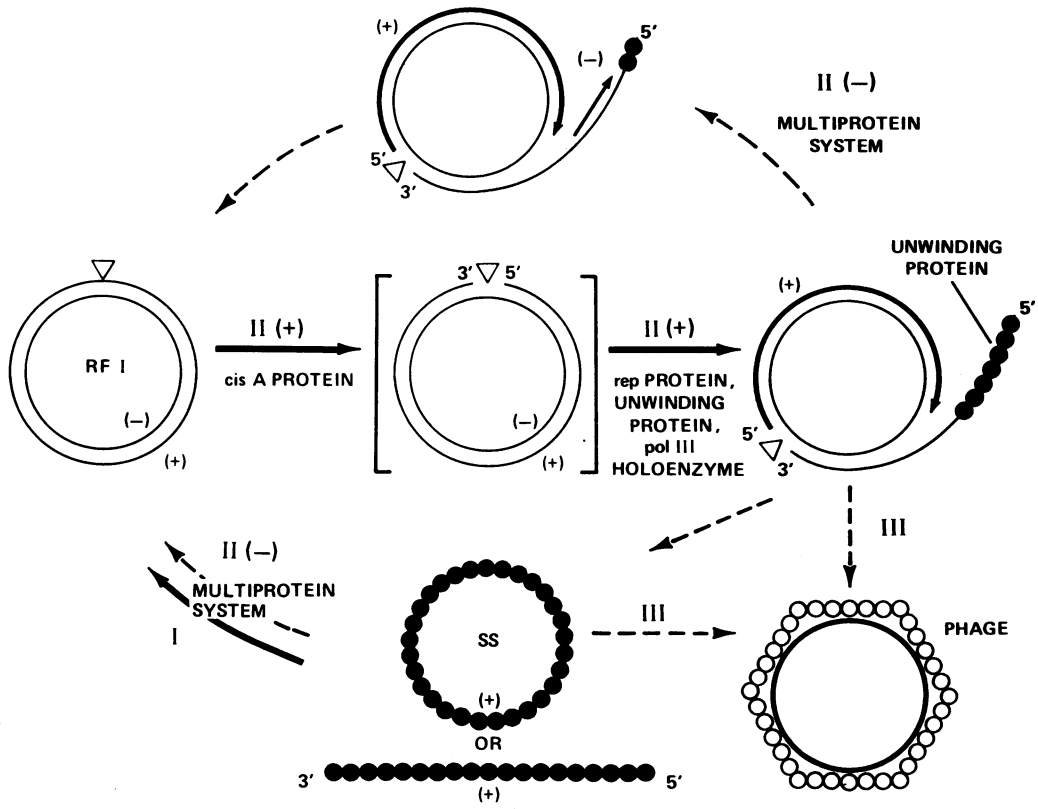

FIG. 1. Hypothetical scheme for replicative events in the life cycle of ϕX174. A round of RF replication (stage II) starts with nicking of parental viral (+) strand at the origin of replication by the *cisA* protein. The 3′ hydroxyl at the nick serves as a primer for synthesis of (+) DNA displacing the parental (+) strand to form a rolling circle structure (7). A second nicking event by *cisA* protein separates newly synthesized and parental viral DNA at their junction and generates unit length and smaller synthetic strands. DNA polymerase III holoenzyme synthesizes the (+) strand. *rep* protein and DNA unwinding protein may function by displacing and binding the (+) parental strand. The displaced viral parental (+) strand, either part of a rolling circle structure or a free single-stranded DNA, serves as a template for synthesis of the complementary (−) strand by the multiprotein system that converts ϕX SS to RF in stage I; the system includes: protein i, protein n, *dnaB* protein, *dnaC* protein, *dnaG* protein, unwinding protein, and polymerase III holoenzyme. Additional actions, including those of DNA polymerase I and ligase, are needed to complete the formation of progeny RF I in stage II. Synthesis of a viral strand, coupled to assembly into a virus particle (stage III), may use the rolling circle structure or free viral (+) strands released in stage II(+). This stage will presumably require phage-coded functions (e.g., products of genes *B, D, F, G,* and *H*). ▽ represents the origin of replication.

mixed with unlabeled ϕX RF I DNA (7–10 μg/ml), sonicated, denatured, and reannealed in the absence and presence of 300–400 μg/ml of unlabeled viral (+) strands to a C_0t [DNA concentration (moles of nucleotide/liter) × time (sec)] value of 1.5–2, essentially as previously described (9).

The amount of reannealed DNA was determined by digestion with the single-strand-specific S1 nuclease; the double-stranded DNA remaining was precipitated with trichloroacetic acid and the radioactivity was measured in a toluene-based scintillation fluid. The S1 nuclease digestion at 30° for 30 min was performed in a 250 μl reaction mixture containing 30 mM sodium acetate buffer (pH 4.5), 0.25 M NaCl, 2 mM $ZnCl_2$, 50 μg of salmon sperm DNA, an aliquot of reannealed DNA, and 4 μl of S1 nuclease (an amount sufficient to degrade 50 μg of heat-denatured salmon sperm DNA to acid-soluble form in 10 min at 30°).

Determination of Complementary (−) Strand Synthesis by Direct Hybridization. Full-length ϕX174 viral (+) DNA was covalently coupled to cellulose by the method of Noyes and Stark (10). The hybridization reaction mixture (200 μl), containing 50% vol/vol formamide, 0.1 M *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (Tes) buffer (pH 7.4), 0.1% sodium dodecyl sulfate, 1 mM EDTA, 1 mg/ml of yeast RNA carrier, an aliquot of sonicated synthetic product, and 50 μg of ϕX174 viral (+) DNA-cellulose, was heated at 80° for 1 min and incubated for 24 hr at 37° as previously described (10). After hybridization, the sample was chilled on ice, 100 μg of cellulose was added as carrier, and the cellulose was collected by centrifugation in a Beckman Microfuge for 1 min at room temperature. The cellulose was washed twice at room temperature with 0.2 ml of a buffer containing four parts of hybridization buffer and one part water, followed by two washes, each with 0.2 ml of citrate-NaCl [30 mM trisodium citrate (pH 7.0), 0.3 M NaCl]. The hybridized complementary strands were eluted by heating the DNA-cellulose at 60° for 1 min in a 99% vol/vol formamide–0.1% sodium dodecyl sulfate solution, and measured by precipitating the DNA from the eluate with 2 M HCl in the presence of 200 μg of salmon sperm DNA (serving as a carrier). The precipitate was collected on glass-fiber filters and its radioactivity was measured in toluene-based scintillation fluid.

RESULTS

Enzymatic replication of RF I initially requires only four proteins and produces only viral (+) strands

ϕX174 *cisA* protein from infected cells and the *rep* protein from uninfected cells have each been purified approximately 1000-fold (6). These two proteins, together with DNA unwinding protein and DNA polymerase III holoenzyme, were

Table 2. Analysis of DNA synthetic product by competitive reannealing

DNA analyzed	Competitive φX174 viral (+) DNA				Competition, %
	Exp. I		Exp. II		
	Not added (cpm)	Added	Not added (cpm)	Added	
^{32}P-Labeled product	1150	115	2700	240	90–91
^{3}H-Labeled RF I	2920	1600	2170	1110	45–48

To the ^{32}P-labeled product (synthesized as described in Table 1) diluted to 0.25 ml in 10 mM Tris (pH 7.5), 1 mM EDTA, 0.2 M NaCl, was added uniformly labeled φX174 RF I [^{3}H]DNA to a concentration of about 12 μg/ml. The DNA was sonicated (9) and 30 μl samples were taken for annealing reactions. To the competitive reannealing reaction, 24 μg of sonicated, unlabeled, φX174 viral (+) strand was added and the volume of each sample was adjusted to 50 μl with 10 mM Tris (pH 7.5). Annealing was performed as described in *Materials and Methods*. The reactions were performed in duplicate and the results were averaged. Reannealing in the absence of unlabeled viral strands was over 90% for both the [^{32}P]- and [^{3}H]DNA.

effective in sustaining DNA synthesis on an RF I superhelical template (Table 1). Omission of any one of these proteins caused a profound reduction in synthesis. Antibodies directed against any of the several replicative proteins (i, n, *dnaB* and *dnaG*) required for complementary (−) strand synthesis in the conversion of SS to RF had no significant effect on the reaction, suggesting that none of them was required.

Analysis of the synthetic product by competitive annealing with an excess of viral DNA showed that about 90% of the incorporated ^{32}P label was competitively replaced (Table 2). As an internal control φX RF I [^{3}H]DNA, labeled in both strands and present in the same annealing mixture, was competitively replaced to an extent near 50%. We infer from these data that the viral (+) strand is the predominant product synthesized and designate this initial event in stage II as stage II(+).

Based on nucleotide incorporation relative to the φX RF I template present in the reaction (Table 1), about 50–60% of the complementary (−) DNA template was utilized for synthesis of viral (+) strand. In an identical reaction, in which ^{3}H-labeled RF I template was used, no loss of template DNA was observed (<5%), suggesting that displacement of the parental viral (+) strand takes place simultaneously with the synthesis of viral DNA.

Sedimentation analysis of the product synthesized in stage II(+) reaction

The synthetic product of a reaction (as in Table 1) was analyzed by sedimentation through neutral and alkaline sucrose gradients (Fig. 2A, and B). Neither RF I nor RF II was formed. The rapid sedimentation of the synthetic product in neutral sucrose (Fig. 2A) suggests the presence of extensive single-stranded regions. This is supported by the banding pattern obtained in neutral CsCl (Fig. 2C), where a large proportion of the newly synthesized DNA banded at a density heavier than that of the φX RF DNA marker. Sedimentation analysis in alkaline sucrose showed that about 30% of the newly synthesized material cosedimented with a 16S φX single-stranded circular DNA marker, suggesting the formation of DNA strands longer than unit length, or circular in form (Fig. 2B). Most of the newly synthesized viral strands, however, were found to be of the size of 14 S, unit-length φX DNA.

Stage II(+) product serves as template for synthesis of (−) strand by the multiprotein system

The stage II(+) product (as in Table 1) was precipitated by 2-propanol and used as a template for further reaction with the multiprotein system that serves in conversion of φX174 SS to parental RF (Table 3). About 90% of ^{3}H-labeled DNA [stage II(+) product] was competitively removed by excess of viral (+) strand and more than 90% of that DNA failed to hybridize with the φX viral (+) DNA-cellulose. Of the ^{32}P-labeled DNA synthesized in this stage of the reaction, 80% was complementary (−) strand as shown by competitive reannealing and by hybridization to φ174 viral DNA covalently attached to cellulose (Table 3, columns 2 and 3). Omission of protein i, protein n, *dnaB* protein, or *dnaG* protein and the addition in each case of the specific antibody caused a 3- to 6-fold decrease in the synthesis of the complementary strand. A decrease of 2- to 2.5-fold was observed when *dnaC* protein was omitted. Also a decrease of 8- to 10-fold was observed by the addition of antibody against the DNA unwinding protein and complete inhibition occurred when DNA polymerase III holoenzyme was omitted (data not shown).

Thus, the product of the stage II(+) reaction can serve as a template for a multiprotein system that synthesizes the complementary strand. We designate this second phase of RF replication as stage II(−).

The synthesis of the complementary (−) strand was not extensive [only 10–20% of the viral (+) template was utilized] and neither RF I nor RF II was formed (data not shown). Additional factors are probably required in order to reconstitute a complete round of RF I replication.

DISCUSSION

Partial reconstitution of the replication of duplex, circular φX174 DNA (RF) with purified proteins has disclosed that syntheses of the viral (+) strand and the complementary (−) strand have different requirements. Synthesis of (+) strands [stage II(+)] requires only four proteins: the phage-induced *cisA* protein and three host proteins, which are the *rep* and DNA unwinding proteins and the DNA polymerase III holoenzyme. By contrast, synthesis of (−) strands [stage II(−)] requires an initiation by the multiprotein system needed for (−) strand synthesis in the first stage of the φX174 life cycle in which the infecting viral strand is converted to the parental RF.

These two separable mechanisms for (+) strand and (−) strand synthesis suggest at once that all the replicative steps in the φX174 life cycle may be accounted for by one or the other of these mechanisms (Fig. 1)*. Asymmetry in the synthesis of viral and complementary strands is not inconsistent with *in vivo* observations (12, 13). Should this generalization hold for φX174, we wonder whether the replication patterns of other circular phage DNAs, plasmids, and even the growing fork of *E. coli*

* The participation of the *rep* gene product in (+) strand synthesis in stage III *in vivo* has been recently reported (11).

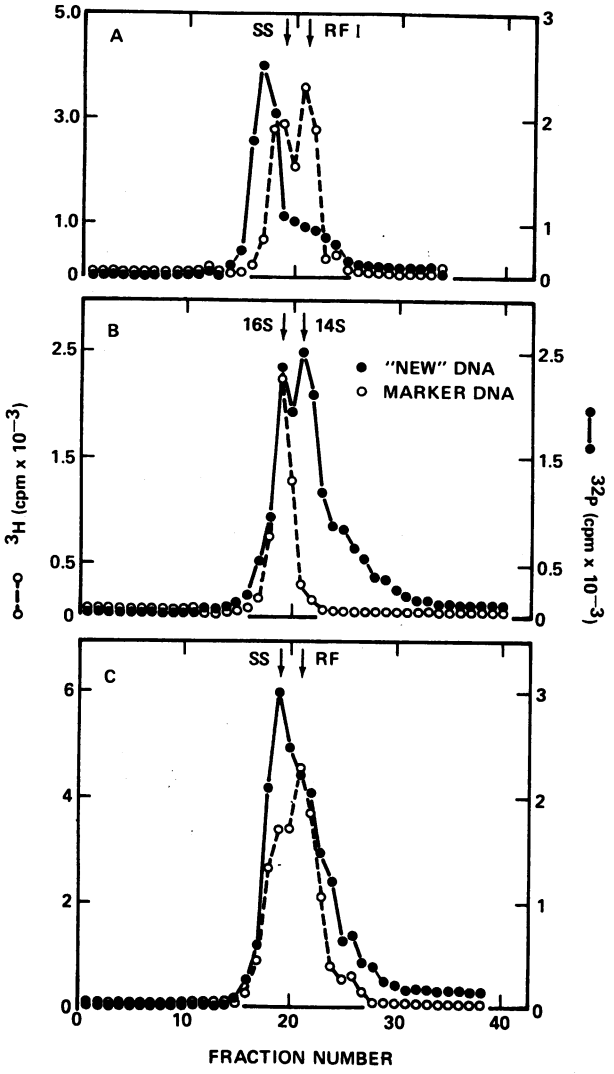

FIG. 2. Sedimentation analysis of the product synthesized in stage II(+). The reaction mixture and incubation were as in Table 1, except that [α-^{32}P]dCTP was used instead of [^{3}H]dTTP. The reaction was stopped by addition of EDTA (to 0.1 M) followed by filtration through a Bio-Gel A-0.5m column (5 ml), equilibrated with a buffer containing 50 mM Tris (pH 7.5), 0.2 mg/ml of bovine serum albumin, 50 μg/ml of heat-denatured salmon sperm DNA, 5 mM EDTA, and 1 M NaCl. The ^{32}P-labeled product was eluted in the void volume. (A) An aliquot of the ^{32}P-labeled product was mixed with ^{3}H-labeled ϕX174 single-stranded and RF I DNA markers and sedimented through a 5–20% neutral sucrose gradient. (B) An aliquot of the ^{32}P-labeled product was treated with 0.2 M NaOH, mixed with ^{3}H-labeled purified ϕX174 phage (pretreated with 0.2 M NaOH for 30 min at 30°), and then centrifuged through a 5–20% alkaline gradient. Neutral sucrose and alkaline sucrose solutions were previously described (9). The experiments with neutral and alkaline sucrose gradients were performed in a SW 50.1 rotor at 50,000 rpm and 15° for 2 and 3.5 hr, respectively. (C) A 75 μl aliquot of the ^{32}P-labeled product was diluted to 3 ml with buffer containing 50 mM Tris (pH 7.5), 0.2 mg/ml of bovine serum albumin, 5 mM EDTA, and 1 M NaCl. ^{3}H-labeled ϕX174 (single-stranded and RF I) DNA markers were added, followed by addition of 1.25 g of CsCl per g of solution. The mixture was then centrifuged in an SW 50.1 rotor for 48 hr at 48,000 rpm and 20° in a Beckman centrifuge.

chromosome are also based on two mechanisms, relatively continuous replication on the leading strand and a separate mechanism depending on initiation of DNA chains on the lagging complementary strand.

Although our results have made the distinction between (+) strand and (−) strand synthesis clear, much remains to be learned about important features of each of these mechanisms. Earlier studies suggesting that *cisA* protein nicks the (+) strand at a unique place and thereby determines the origin of replication (14–16) still require firm proof. Assuming this to be the case, covalent DNA chain growth from the 3′-hydroxyl end at the nick by the DNA polymerase III holoenzyme seems likely; displacement and binding of the 5′-end of the parental (+) strand may depend on the action of the *rep* protein and the DNA unwinding protein. These possibilities remain to be investigated. Inasmuch as the *cisA* and *rep* proteins are still impure, the presence and participation of additional proteins are still uncertain.

The actions described for these proteins in (+) strand synthesis and the characteristics of the synthetic product suggest the operation of a rolling-circle mechanism as proposed by

Table 3. Complementary (−) strand synthesis by a multiprotein system utilizing the product synthesized in stage II(+)

Additions	DNA synthesis, pmol: Acid-precipitable nucleotide	Competitive reannealing	Hybridization
Complete	31	25	24
− protein i + anti-protein i	8	5	5
− protein n + anti-protein n	6	4	5
− *dnaB* protein + anti-*dnaB* protein	10	6	7
− *dnaC* protein	19	14	10
− *dnaG* protein + anti-*dnaG* protein	6	4	4
− stage II(+) product + ϕX174 SS	153	160	170

The stage II(+) reaction product was labeled with [^{3}H]dTTP as in Table 1, except that the volume was 0.4 ml and incubation was for 40 min. The synthetic product was precipitated by the addition to the reaction mixture of 40 μl of 3 M sodium acetate buffer (pH 5.5) and 0.8 ml of 2-propanol. The precipitate was kept at −20° for 12 hr, centrifuged, and dissolved in 0.05 M Tris·HCl (pH 8.1). The reaction mixture, incubated at 30° for 30 min in a 50-μl final volume, contained: 50 mM Tris·HCl (pH 7.5), 10 mM dithiothreitol, 8 mM $MgCl_2$, 50 μM each of dATP, dCTP, dGTP, and dTTP (each α-^{32}P-labeled with a total specific activity of 3000 cpm/pmol), 800 μM ATP, 100 μM each of CTP, UTP, and GTP, 2 mM spermidine chloride, 40 units of protein i, 50 units of protein n, 30 units of *dnaB* protein, 24 units of *dnaC* protein, 30 units of *dnaG* protein, 48 units of DNA unwinding protein, 40 units of DNA polymerase III holoenzyme, and a sufficient amount of stage II(+) reaction product to provide 240 pmol of viral (+) single-stranded template [assuming that each pmol of nucleotide incorporated in stage II(+) displaces an equivalent amount of parental, viral (+) DNA]. Antibody additions were as in Table 1. An aliquot of 5 μl of chilled reaction mixture was precipitated with trichloroacetic acid to estimate the amount of DNA synthesized. The remainder was divided for competitive reannealing and hybridization analyses. Competitive reannealing was performed as described in Table 2. The amount of complementary (−) strand synthesized was estimated from the amount of ^{32}P-labeled material resistant to S1 nuclease. Hybridization was performed as described in *Materials and Methods*. The efficiency of hybridization, determined with ^{32}P-labeled complementary (−) strands obtained in a ϕX174 SS to RF reaction, was 46%; the amount of complementary (−) strand synthesized was therefore corrected by the factor of 2.17.

Gilbert and Dressler (7). The presence of newly synthesized DNA in linear strands of greater than unit length is in keeping with such a covalent extension of a nicked RF; the presence of new DNA in strands of unit length can be explained by a second nicking action by *cisA* protein at an origin restored by the covalent linkage of new DNA to old (Fig. 1). Despite the attractiveness of the rolling-circle model, an alternative such as the D-loop mechanism, assisted by nicking and ligase action, has not been excluded.

Uncertainties about the mechanism of (−) strand synthesis are even more numerous. The correct structure of the template for synthesis of complementary (−) strand is not clear. The displaced viral (+) DNA in the rolling circle structure or, alternatively, a free single-stranded viral (+) DNA released in the stage II(+) reaction could serve as template for synthesis of (−) strand. Assuming a basic similarity of (−) strand synthesis in stage II(−) to that in stage I, we propose that four of the proteins (i, n, *dnaB*, and *dnaC*) form an intermediate at a certain region of the (+) strand. This complex enables *dnaG* protein to synthesize a short transcript to prime synthesis of a DNA chain by the DNA polymerase III holoenzyme. Binding of the (+) strand by DNA unwinding protein may, as in SS → RF conversion, mask all but one or a few regions suitable for forming the pre-priming intermediate.

Subdivision of stage II into separate stages of (+) strand and (−) strand synthesis in a reconstituted enzyme system may obscure the fact that these processes are closely coordinated *in vivo*. Furthermore, other factors need to be identified, which, together with DNA polymerase I and ligase, will link the operations of stages II(+) and II(−) and utilize their products for rapid and efficient multiplication of RF I.

This work was supported in part by grants from the National Institutes of Health and the National Science Foundation. A grant from the Damon Runyon–W. A. Walter Winchell Cancer Fund provided postdoctoral fellowship support for S.E.

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