Proc. Natl. Acad. Sci. USA
Vol. 78, No. 3, pp. 1436–1440, March 1981
Biochemistry

Conservation of the primosome in successive stages of ϕX174 DNA replication

(prepriming/protein n′/*dnaB* protein/primase/supercoiling)

ROBERT L. LOW, KEN-ICHI ARAI*, AND ARTHUR KORNBERG

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Contributed by Arthur Kornberg, November 17, 1980

ABSTRACT Synthesis of a complementary strand to match the single-stranded, circular, viral (+) DNA strand of phage ϕX174 creates a parental duplex circle (replicative form, RF). This synthesis is initiated by the assembly and action of a priming system, called the primosome [Arai, K. & Kornberg, A (1981) *Proc. Natl. Acad. Sci. USA* 78, 69–73; Arai, K., Low, R. L. & Kornberg, A. (1981) *Proc. Natl. Acad. Sci. USA* 78, 707–711]. Of the seven proteins that participate in the assembly and function of the primosome, most all of the components remain even after the DNA duplex is completed and covalently sealed. Remarkably, the primosome in the isolated RF obviates the need for supercoiling of RF by DNA gyrase, an action previously considered essential for the site-specific cleavage by gene A protein that starts viral strand synthesis in the second stage of ϕX174 DNA replication. Finally, priming of the synthesis of complementary strands on the nascent viral strands to produce many copies of progeny RF utilizes the same primosome, requiring the addition only of prepriming protein i. Thus a single primosome, which becomes associated with the incoming viral DNA in the initial stage of replication, may function repeatedly in the initiation of complementary strands at the subsequent stage of RF multiplication. These patterns of ϕX174 DNA replication suggest that a conserved primosome also functions in the progress of the replicating fork of the *Escherichia coli* chromosome, particularly in initiating the synthesis of nascent (Okazaki) fragments.

The replicative cycle of phage ϕX174 (ϕX) DNA begins with conversion of viral single-stranded DNA (ssDNA) to the covalently closed duplex, parental replicative form (RF) (1, 2). This ssDNA → RF conversion has been successfully reconstituted *in vitro* and requires 11 *Escherichia coli* proteins (3). Among these, proteins n, n′, n″, i, *dnaC*, *dnaB*, and primase participate at the unique protein n′ recognition site in the assembly of a primosome (4–8). The assembled primosome, using the energy of ATP hydrolysis, travels processively in an anti-elongation direction to generate multiple primers around a viral chromosome coated with ssDNA-binding protein (SSB) (6–8).

With completion of the ssDNA → RF conversion, the parental RF is duplicated to produce multiple RF copies (RF → RF) prior to selective synthesis of viral ssDNA for packaging into progeny phage (1, 2). In the RF duplication reaction, the viral and complementary strands are replicated by distinct mechanisms. Synthesis of the viral strand begins with the ϕX-encoded gene *A* protein cleavage of the viral strand (at position 4305–4306) that enables host *rep* protein, SSB, and DNA polymerase III (pol III) holoenzyme to unwind and continuously elongate the newly created 3′-OH terminus (9–12). An apparent requirement for localized single-strandedness at the gene *A* cleavage site (13, 14), facilitated by superhelicity, has implicated gyrase in this process (15). Growth of the nascent viral strand provides the template for initiation of a complementary strand by primosome action of a ssDNA → RF system identical to that used to initiate synthesis of the parental RF (16).

In this study, we have obtained physical and functional evidence that major components of the primosome (i.e., n′ protein, *dnaB* protein, and primase) isolated by gel filtration are stably retained with the parental RF and function during RF duplication upon addition of proteins i, n″, and *dnaC*. An even more intact primosome isolated by sedimentation requires addition of only protein i. Conservation of the primosome obviates the need for gyrase action and provides a substrate for the gene *A* protein cleavage reaction far superior to the protein-free, highly supercoiled RF I isolated from infected cells. Furthermore, with the conserved primosome at the replication fork, repeated cycles of RF multiplication can take place rapidly and efficiently.

MATERIALS AND METHODS

Materials. Buffer A contained 20 mM Tris·HCl (pH 7.5), 20 mM KCl, 5% (wt/vol) sucrose, 8 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 0.2 mM ATP, and bovine serum albumin at 0.1 mg/ml. Sources of DNAs and extensively purified DNA replication proteins were as described (16–18). *E. coli* DNA polymerase I, DNA ligase, and DNA gyrase were gifts of S. Scherer (this department), I. R. Lehman (this department), and P. Brown and N. R. Cozzarelli (University of Chicago), respectively. Preparation of ^{3}H-labeled *dnaB* protein (1.5 × 10^5 cpm/μg) (17) and ^{3}H-labeled protein n′ (5 × 10^4 cpm/μg) (8) will be described elsewhere.

Preparation of Synthetic RFI by the ssDNA → RF Conversion. The reaction was carried out in three consecutive stages: (*i*) prepriming, (*ii*) priming and elongation, and (*iii*) gap filling and ligation. The 175-μl reaction mixture contained, in order of addition: 100 μl of buffer A, 100 nmol of rATP, 2.2 nmol (as nucleotide) of ϕX ssDNA, 6.6 μg of SSB, 0.7 μg of *dnaB* protein, 4.7 μg of *dnaC* protein, 0.4 μg of protein i, 0.5 μg of proteins n + n″, and 0.3 μg of protein n′. After 30 min at 30°C, 6 nmol each of dATP, dCTP, and dGTP, 2.25 nmol of [^{3}H]dTTP (1500 cpm/pmol), 12.5 nmol each of CTP, GTP, and UTP, 0.38 μg of primase, and 1 μg of pol III holoenzyme were added, and the incubation was continued for 20 min. Finally, after addition of 100 nmol of NAD, 50 ng of DNA polymerase I, and 1 μg of DNA ligase and incubation for another 40 min, the reaction mixture was filtered through a 5-ml Bio-Rad Bio-Gel A-5m column (0.5 × 26 cm) equilibrated with buffer A at room temperature. The ^{3}H-labeled RFI (^{3}H-RFI) (pooled void volume

Abbreviations: pol III holoenzyme, DNA polymerase III holoenzyme; ϕX, phage ϕX174; RF, duplex replicative form; ssDNA, single-stranded DNA; SSB, ssDNA-binding protein.

* Present address: Department of Chemistry, The Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Tokyo 108, Japan.

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fractions) was further purified on a linear sucrose gradient to remove any unreplicated, SSB-coated ϕX ssDNA. Alternatively, gel filtration was avoided and the synthetic ^{3}H-RFI was applied directly to the sucrose gradient. In both cases, the ^{3}H-RFI (1.6–2.8 nmol as nucleotide) in 250 μl was sedimented through a 5-ml linear 8–26% (wt/vol) sucrose gradient in buffer A in a Beckman SW 65 rotor for 65 min at 60,000 rpm (4°C). Peak fractions were pooled and concentrated by vacuum dialysis (Schleicher & Schuell collodion membrane) for 30 min at 4°C against buffer A.

Gyrase Supercoiling of the Synthetic RFI. Under reaction conditions as described (19), synthetic ^{3}H-RFI template (360 pmol as nucleotide) was incubated with gyrase subunits A (10 units) and B (5 units) for 40 min at 30°C and monitored for supercoiling by agarose gel electrophoresis.

Synthetic RFI as a Template for ssDNA Production by the RF → ssDNA Reaction. The following components were added at 0°C: 90 μl of Bio-Gel-purified synthetic ^{3}H-RFI or *in vivo* RFI (360 pmol as nucleotide); 80 nmol of ATP; 5.4 nmol each of dATP, dCTP, and dGTP; 1.8 nmol of [α-^{32}P]dTTP (920 cpm/pmol); 7.5 nmol each of CTP, GTP, and UTP; 20 ng of ϕX gene *A* protein; 50 ng of *rep* protein; 0.55 μg of SSB; and 0.2 μg of pol III holoenzyme. The 150-μl reaction mixture was incubated at 30°C. Aliquots (5 μl) were assayed for trichloroacetic acid-precipitable ^{32}P (20).

Synthetic RFI as a Template for Net RF Production by the RF → RF Conversion. The reaction containing either Bio-Gel or sucrose gradient-purified ^{3}H-RFI or both was identical to the RF → ssDNA reaction except that one or more of the following prepriming proteins were added: protein i (0.12 μg), *dnaC* protein (1.4 μg), and either proteins n + n″ (0.15 μg) or n″ (0.04 μg of fraction VI, 3 × 10^6 units/mg; unpublished results). Reaction mixtures using *in vivo* RFI were identical but contained in addition to proteins i, n + n″, and *dnaC*, *dnaB* protein (0.2 μg), protein n′ (0.15 μg), and primase (1.1 μg). After incubation for 40 min at 30°C, 5-μl aliquots were assayed for acid-precipitable radioactivity; the remainder was analyzed by electrophoresis on agarose gels (0.8%) at room temperature in 40 mM Tris–acetate (pH 7.8)/5 mM sodium acetate/1 mM EDTA at 1 V/cm. Autoradiograms were produced as described (5, 16, 21) and scanned by using a Quick Scan Jr. thin-layer chromatography densitometer (Helena Laboratories, Beaumont TX).

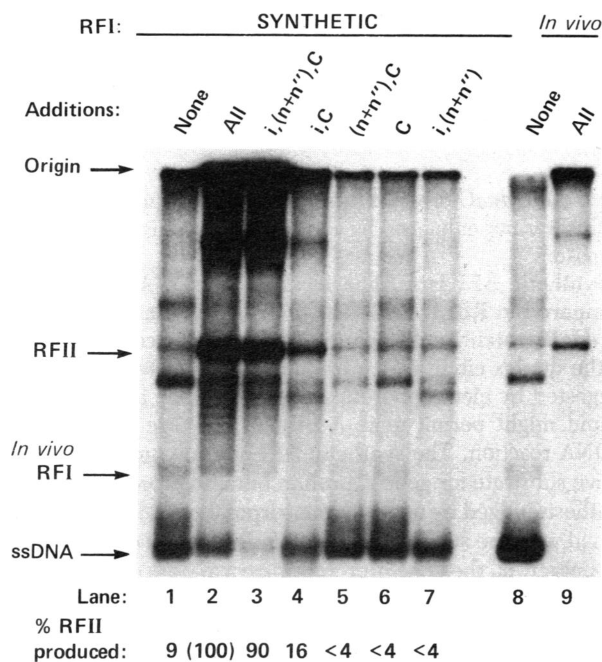

FIG. 1. Production of net RFII from Bio-Gel purified RFI. The 65-μl reaction mixture contained: 400 pmol (as nucleotide) of synthetic Bio-Gel-purified ^{3}H-RFI; 65 nmol of ATP; 0.26 μmol of spermidine·HCl, pH 6.5; and gyrase A (10 units) and B (5 units). After 40 min at 30°C, the reaction mixture was chilled and the following components were added: rNTPs, unlabeled dNTPs, [α-^{32}P]dTTP (200 cpm/pmol), gene *A* protein, *rep* protein, SSB, and pol III holoenzyme. Prepriming proteins were added as indicated (*C*, *dnaC* protein), and the mixture was incubated an additional 40 min at 30°C and processed. The autoradiogram (lanes 1–7) was scanned with a densitometer and the amount of RFII produced was compared to that in the reaction mixture supplemented with all the prepriming proteins (lane 2). Reaction mixtures with *in vivo* RFI contained 360 pmol of template (as nucleotide) in 65 μl of buffer A plus 1 mM ATP, the RF → ssDNA enzymes, and all or none of the priming proteins.

RESULTS

Retention of n′ and *dnaB* Proteins with Synthetic RFI. RFI was synthesized by stages of (*i*) prepriming, (*ii*) priming and chain elongation, and (*iii*) gap filling and ligation. The synthetic ^{3}H-RFI when filtered on Bio-Gel A-5m emerged as a single, symmetric excluded peak well separated from unincorporated ^{3}H-dTTP and free proteins. Assuming complete recovery of the DNA, 60–70% of the input ϕX ssDNA was converted to RFI. In agarose gel electrophoretic analysis, the RFI band appeared near an RFII marker, indicating it had only one or a few supertwists; extensive supercoiling like that of the *in vivo*, phenol-extracted RFI was not observed (data not shown). With the Bio-Gel filtration technique, titration of ^{3}H-labeled protein n′ and ^{3}H-labeled *dnaB* protein required for prepriming showed that one molecule of each was needed per replicated circle (8). The product after each stage of RFI synthesis retained approximately one molecule each of proteins n′ and *dnaB* (Table 1).

Functional Evidence of Retention of Priming Proteins with Synthetic RFI. Although the lack of isotopically labeled proteins n″, *dnaC*, and primase prevents a direct determination of their retention in the RFI product, functional tests can be used to determine whether these proteins and proteins n′ and *dnaB* remain associated. With protein-free RFI obtained from in-

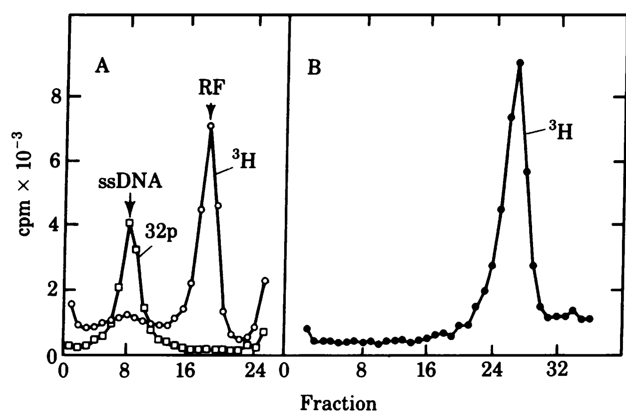

FIG. 2. Sucrose gradient centrifugation of gel-purified RF. (*A*) A mixture of Bio-Gel-purified ^{3}H-RFI (1000 pmol as nucleotide) and ϕX [^{32}P]ssDNA (200 pmol as nucleotide, produced in an RFI → ssDNA reaction and purified on Bio-Gel) in 250 μl was sedimented through a 5-ml linear 8–26% (wt/vol) sucrose gradient in buffer A. A 20-μl aliquot of each fraction (220 μl) was analyzed for ^{3}H (○) and ^{32}P (□). (*B*) Freshly prepared Bio-Gel ^{3}H-RFI (250 μl, 2.8 nmol as nucleotide) was sedimented as above. The ^{3}H-RFI peak (0.9 ml, 1400 pmol as nucleotide) was concentrated to 0.3 ml by vacuum dialysis for 30 min at 4°C against buffer A plus 1 mM ATP.

Table 1. Retention of protein n′ and *dnaB* protein with synthetic RFI

Product	Molecules per replicated circle	
	Protein n′	*dnaB* protein
Prepriming intermediate	1.0	1.2
RFII (priming, DNA synthesis)	1.0	1.1
RFI (gap-filling, ligation)	0.9	0.9

The prepriming intermediate was formed in a 150-μl reaction mixture containing: 90 μl of buffer A, 2.2 nmol (as nucleotide) of φX ssDNA, 6.6 μg of SSB, 0.15 μmol of ATP, 4.7 μg of *dnaC* protein, 0.4 μg of protein i, 0.5 μg of proteins n + n″ mixture, and either 0.7 μg of ^{3}H-labeled *dnaB* protein (1.5 × 10^5 cpm/μg) and 43 ng of protein n′ or 0.7 μg of *dnaB* protein and 43 ng of ^{3}H-labeled protein n′ (5 × 10^4 cpm/μg). After a 30-min incubation at 30°C, the intermediate was filtered through a 5-ml Bio-Gel A-5m column (0.5 × 26 cm) equilibrated at room temperature with buffer A. Void-volume fractions (25-μl aliquots) were assayed for DNA replication by using [α-^{32}P]dTTP (10 cpm/pmol). The remainder of each fraction was assayed for labeled n′ or *dnaB* protein. For synthesis of RFII, the reaction mixture was supplemented with rNTPs, unlabeled dNTPs, [α-^{32}P]dTTP (20 cpm/pmol), primase (0.3 μg), and pol III holoenzyme (0.25 μg) and incubated an additional 20 min; for RFI, DNA polymerase I (10 ng), NAD (7.5 nmol), and DNA ligase (1 μg) were also added and the incubation was extended 40 min.

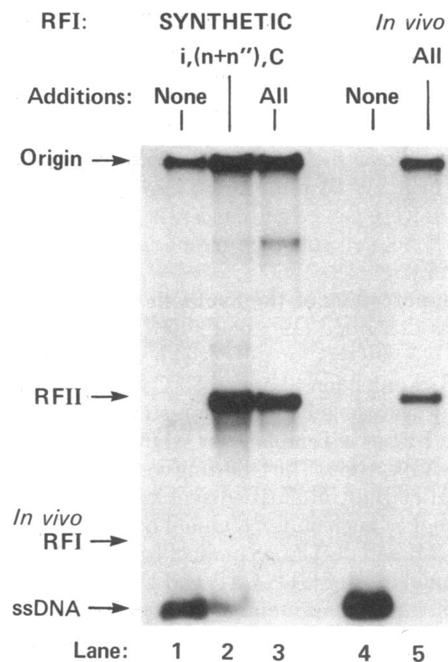

FIG. 3. Autoradiogram of RFI assay reaction products formed by using sucrose gradient-purified ^{3}H-RFI as template. Reaction mixtures contained 300 pmol (as nucleotide) of Bio-Gel-and sucrose gradient-purified ^{3}H-RFI in 75 μl. After a 40-min preincubation with gyrase at 30°C (see Fig. 1 legend), the RF → ssDNA enzymes and components described in *Materials and Methods* were added along with prepriming proteins as indicated. Reactions with *in vivo* RFI included all or none of the prepriming proteins. After 40 min at 30°C, reactions were terminated with 1% sodium dodecyl sulfate, and the products were precipitated with ethanol and processed for autoradiography.

fected cells, the entire complement of prepriming proteins is needed for RF multiplication (16). By contrast, with synthetic RFI isolated by gel filtration, the results were strikingly different (Fig. 1). Without prepriming proteins, only synthesis of ssDNA and a multigenomic (slow migrating) product was observed (lane 1). Supplementation with only proteins i, n + n″, and *dnaC* led to net RFII synthesis (lane 3); no significant enhancement was observed upon further addition of n′, *dnaB* protein, and primase (lane 2). Thus the synthetic RFI retained the functional capacities of the latter three components of the prepriming system. Omission of proteins i, n + n″, or *dnaC* markedly reduced RFII synthesis (lanes 4–7). A densitometric scan of the autoradiogram confirmed that supplementation with only i, n + n″, and *dnaC* proteins (lane 3) sustained RFII synthesis at 90% of the level of the complete prepriming system. By comparing the amount of RFII labeled in the RF → ssDNA reaction (lane 1) with that generated in the complete RF → RF reaction (lane 2), one may infer that repeated rounds of replication have taken place.

With *in vivo* RFI, the addition of the entire prepriming system (lane 9) resulted in net RFII production and a product distribution similar to that seen when synthetic RFI was supplemented with i, n + n″, and *dnaC* proteins. In subsequent assays with the synthetic RFI, protein n″ substituted for the protein n + n″ mixture, suggesting that protein n, absolutely required for the ssDNA → RF reaction and not associated with the synthetic RFI (unpublished results), may not be needed in the RF → RF reaction.

Further Purification of the Gel-Filtered Synthetic RFI by Sucrose Gradient Centrifugation. Unreplicated SSB-coated φX ssDNA, a possible site for sequestering replication proteins, was removed from the synthetic RFI in the Bio-Gel fraction by low-salt sucrose gradient centrifugation. The synthetic ^{3}H-RFI marker was well resolved from the more rapidly sedimenting SSB-coated [^{32}P]ssDNA (Fig. 2A). The synthetic ^{3}H-RFI, separated on Bio-Gel and further purified on a linear 8–26% sucrose gradient (Fig. 2B), was obtained in about 60% yield after peak fractions had been concentrated. Functional capacity in RF multiplication of this purified RFI was nearly identical to that of the Bio-Gel fraction (Fig. 3). Supplementation with i, n + n″, and *dnaC* proteins promoted net RFII production (lane 2); this was not enhanced by addition of proteins n′, *dnaB*, and primase.

Synthetic RFI Is a Superior RF → ssDNA Template as Compared to RFI from Infected Cells. Association of protein n′, *dnaB* protein, and primase with RF during covalent sealing of the duplex circle may introduce one or a few supercoils as suggested by electrophoretic gel patterns (see figure 4 of ref. 3) and might permit gene A protein cleavage in the RF → ssDNA reaction. The synthetic RFI not only proved to be an active substrate for gene *A* protein but also promoted a rate of synthesis judged by nucleotide incorporation, 28 times that obtained with the supercoiled, protein-free, *in vivo* RFI (Fig. 4). Essentially all the gene *A* protein cleavage was complete in less than a minute, inasmuch as 280 pmol of single strands (expressed as nucleotides) had been produced, starting with 180 pmol of template strand in the RF. After 60 min, the synthetic RFI supported a 5-fold production of ssDNA relative to input template and the *in vivo* RFI only a 3-fold.

Gyrase Supercoiling Is Not Required for Synthesis of an RFI Active in Duplication. Because relaxed RFI is inactive in RF replication, it was assumed that supercoiling by gyrase was essential (9). However, the purified, synthetic RFI had the same template properties and formed the same products whether or not the synthetic RF was supercoiled by gyrase (data not shown). In addition, the synthetic RFI supercoiled by gyrase (about 70% of the molecules are fully supertwisted) showed the same kinetics in the RF → ssDNA reaction as synthetic RFI not supercoiled by gyrase (Fig. 4). Furthermore, inclusion of novobiocin during RFI synthesis at 200 nM, a level that inhibits

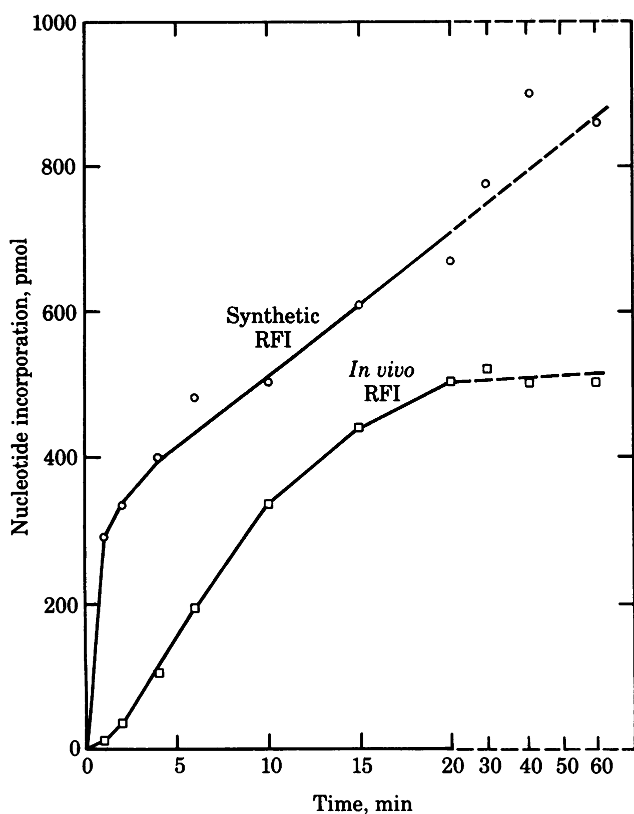

FIG. 4. Kinetics of the RF → ssDNA reaction. The reaction mixture (120 μl) contained 360 pmol (as nucleotide) of either Bio-Gel-purified synthetic ^{3}H-RFI (○) or *in vivo* RFI (□) and the RF → ssDNA enzyme system. The reaction was at 30°C; 5-μl aliquots were added to 1 ml of 10% trichloroacetic acid and 0.1 M sodium pyrophosphate. After 40 min at 0°C, precipitates were collected on GF/C filters (Whatman) and [^{32}P]dTTP incorporation into DNA was determined (20).

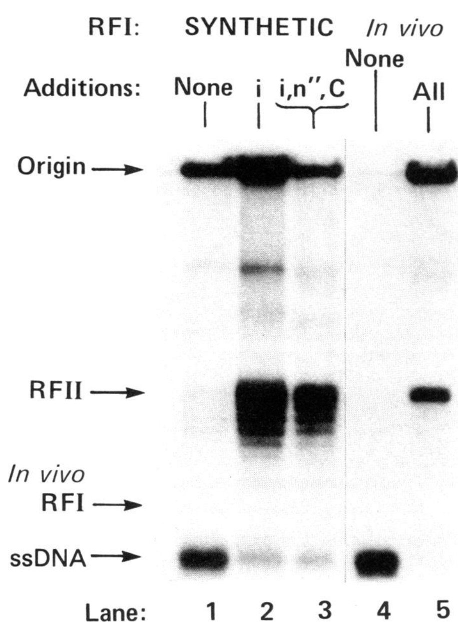

FIG. 5. Autoradiogram of products with sucrose-gradient ^{3}H-RFI as substrate. ^{3}H-RFI (1400 pmol as nucleotide) was applied to a 5-ml linear 8–26% (wt/vol) sucrose gradient in buffer A and centrifuged for 90 min at 60,000 rpm in an SW 65 rotor at 4°C. RFI peak fractions (1 ml) were pooled, concentrated to 0.3 ml by vacuum dialysis against buffer A, and assayed. Reaction mixtures (80 μl) contained: RFI (234 pmol as nucleotide), 1 mM ATP, rNTPs, unlabeled dNTPs, [α-^{32}P]dTTP (600 cpm/pmol), gene *A* and *rep* proteins, SSB, pol III holoenzyme, and prepriming proteins as indicated. Reaction mixtures with *in vivo* RFI contained 240 pmol (as nucleotide) in 80 μl of buffer A plus 1 mM ATP, the above-mentioned RF → ssDNA reaction components, and either all or none of the prepriming proteins.

gyrase action more than 70%, had no effect on the capacity of the RFI to serve in RF replication.

A More Functionally Intact Primosome Is Retained by Isolation of Synthetic RFI by Sucrose Gradient Centrifugation. As measured in the RF → RF assay, synthetic RFI purified by gel filtration requires additions of proteins i, n", and *dnaC* for restoration of primosome activity. However, RFI isolated by sucrose gradient centrifugation required supplementation with only one prepriming protein, namely protein i, to achieve net RFII synthesis (Fig. 5). The incorporation of 1120 pmol of nucleotide represents a 10-fold multiplication over the input RFI template. Additional supplementation with proteins n" and *dnaC* was not required and did not increase RFII production. Thus the sucrose gradient procedure preserves a more functionally intact primosome, one that can be restored with the addition of a single protein.

ATP is Required for Retention of *dnaB* Protein and Primase on the Synthetic RFI. When synthetic RFI, separated by the Bio-Gel procedure in the absence of ATP, was used as a template for replication (RFII synthesis), not only were proteins n", i, and *dnaC* required (as in Fig. 1), but both *dnaB* protein and primase had to be added as well; less than 10% of the single strands produced were converted to RFII in the absence of the latter two proteins, compared to 98% in their presence. Thus, in the absence of ATP, *dnaB* protein and primase are lost from the primosome. Furthermore, the template activity of the synthetic RFI isolated in the absence of ATP was reduced by 80% even when the reaction mixture was supplemented with all the replication proteins.

DISCUSSION

Conversion of φX174 viral ssDNA to RF requires 11 *E. coli* proteins (3). Seven of them constitute a priming system or primosome that moves processively around the viral chromosome to generate multiple primers (4, 6–8). The primosome, as suggested in this study, serves at the replication fork in a manner that may be responsible for initiating synthesis of nascent (Okazaki) fragments on the lagging strand of the host chromosome.

This report presents evidence that, in synthesis of the complementary strand in the ssDNA → RF conversion, a nearly intact primosome is retained with the parental RF even after ligase closure to form a covalently circular duplex. The synthetic RFI molecules, isolated by procedures that preserve protein components bound to DNA, contain one molecule each of ^{3}H-labeled protein n' and *dnaB* protein and most of the other prepriming proteins, as judged by their activity in RF multiplication. Unlike the phenol-extracted supercoiled RFI from infected cells, which requires the seven prepriming proteins, the synthetic RFI undergoes repeated rounds of RF duplication when only protein i supplements the viral strand synthesizing system. Protein i, presumably released during the ssDNA → RF conversion (22) is needed for continued primosome function. Unlike protein i, protein n is released late during parental RF synthesis but is not required in RF multiplication, having served its role in primosome assembly (unpublished results). The synthetic RFI with its conserved primosome is remarkably stable, remaining fully active after several hours of manipulations that include gel filtration at room temperature followed by sucrose gradient centrifugation and concentration by vacuum dialysis.

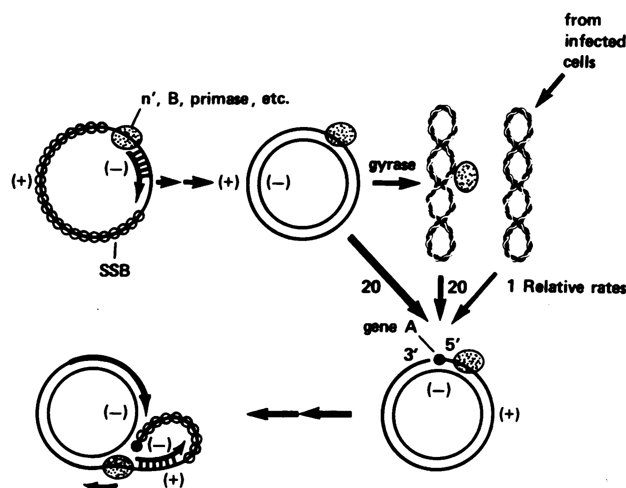

FIG. 6. Conservation of the primosome throughout the first two stages of the ϕX DNA replicative cycle. *B*, *dnaB* protein.

Two primosome components, *dnaB* protein and primase, suffice for the synthesis of multiple primers on uncoated ϕX ssDNA (17); a *dnaB* protein·ATP complex induces a DNA conformation that enables primase to act at specific sequences (8). Dissociation of *dnaB* protein from the synthetic RFI isolated in the absence of ATP (see *Results*) confirms the ATP requirement for a stable *dnaB* protein·DNA complex (8), and the concomitant loss of primase implicates a *dnaB* protein–primase interaction in the primosome. Protein n' appears to use its ATPase activity to translocate the primosome along the DNA (8). The specific functions of three other essential proteins, i, n", and *dnaC*, have yet to be determined; *dnaC* protein must have a role beyond the delivery of a *dnaB* molecule to the DNA (23).

Preservation of a more intact primosome by sucrose gradient centrifugation as compared with gel filtration suggests that some of the prepriming proteins, such as n" and *dnaC*, are less firmly associated than the others. Labeled *dnaC* and n" proteins are needed to determine how these proteins fit and function in the primosome.

During the ϕX DNA replicative cycle, conservation of the primosome has two important consequences (Fig. 6). First, retention of the primosome leads to a DNA topology that promotes efficient gene *A* protein cleavage and thereby abolishes the need for gyrase. Secondly, conservation facilitates an efficient RF duplication reaction with a nearly intact primosome established on the DNA. Traveling at the replication fork with an affinity for the viral strand, the primosome may, together with polymerase and helicase, constitute part of a larger replisome and help drive unwinding of the duplex.

These findings of primosome conservation may explain observations of Yarus and Sinsheimer (24) that during ϕX infection it is the parental RF rather than progeny RF that undergoes duplication. The viral strand, upon entering the host cell, assembles or captures a primosome, which then remains linked to the RF throughout the replicative cycle; supercoiled progeny RF serves principally in transcription.

The efficiency of conserving a primosome through successive stages of replication, exemplified in these studies of ϕX, may apply also to other single-stranded phages (polyhedral G4 and filamentous M13), and to duplex plasmids. It would be surprising if this important principle in the mechanics of DNA replication were not widely applied in bacterial and animal cells.

This work was supported in part by grants from the National Institutes of Health and the National Science Foundation. R.L.L. is a Helen Hay Whitney Foundation Fellow; K. A. was a Fellow of the American Cancer Society.

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