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A general priming system employing only *dnaB* protein and primase for DNA replication

[mobile replication promoter/DNA-dependent ATPase/single-strand binding protein/phage φX174/poly(dT)]

KEN-ICHI ARAI AND ARTHUR KORNBERG

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

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ABSTRACT **Priming of phage φX174 DNA synthesis is effected simply by *dnaB* protein and primase when the DNA is not coated by single-strand binding protein (SSB). The five prepriming proteins (n, n′, n″, i, and *dnaC* protein) required for priming a SSB-coated φX174 DNA circle are dispensable. The *dnaB* protein–primase priming system is also active on uncoated phage G4 and M13 DNAs and on poly(dT). Multiple RNA primers, 10–60 nucleotides long, are transcribed with patterns distinctive for each DNA template. Formation of a stable *dnaB* protein·DNA complex in the presence of primase and ATP supports the hypothesis that *dnaB* protein provides a mobile replication promoter signal for primase.**

Pathways for conversion of various single-stranded phage DNAs to the duplex form are distinguished by the three different mechanisms employed in priming initiation of DNA synthesis (1–3). Priming replication of M13 DNA coated with single-strand binding protein (SSB) depends on synthesis of a unique transcript by RNA polymerase (4, 5). With coated G4 DNA, primer synthesis is effected by primase (*dnaG* protein) (6–8).

By contrast, primer synthesis on SSB-coated DNA of phage φX174 (φX) demands the numerous host replication proteins presumably required for initiations in discontinuous chromosome replication (nascent, Okazaki fragments). Resolution and reconstitution of this multi-enzyme system provides insights into how these proteins participate in the priming process (9–11). DNA synthesis as well as RNA primer synthesis (12) depends on SSB coating of φX DNA. In the process that precedes primase action, φX DNA is converted to a nucleoprotein intermediate that includes *dnaB* protein and SSB (13–15). Formation of the intermediate requires participation of at least five additional proteins: proteins n, n′, n″, i, and *dnaC*. The stable replication intermediate catalyzes the synthesis of multiple primers on the φX circle when primase is added (12, 14).

Upon extensive purification of each of the replication proteins and their use in the reconstitution of the φX system, a novel feature of the priming reaction becomes apparent. In the absence of SSB, conversion of any single-stranded DNA to the duplex form can be achieved simply by the joint action of *dnaB* protein and primase to initiate synthesis by DNA polymerase III holoenzyme. Uncoupled from DNA replication, *dnaB* protein and primase, in the presence of ATP, form a complex with a single-stranded DNA template and catalyze the synthesis of short primers on the DNA. This report describes these findings and discusses their significance for the physiological role of *dnaB* protein as a mobile replication promoter.

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MATERIALS AND METHODS

Materials. Buffer A is 100 mM Tris·HCl (pH 7.5)/20% (wt/vol) sucrose/40 mM dithiothreitol/200 μg of bovine serum albumin per ml. Buffer B is 20 mM Tris·HCl (pH 7.5)/8 mM $MgCl_2$/0.1 mM EDTA/20 mM KCl/5% (wt/vol) sucrose/100 μg of bovine serum albumin per ml/5 mM dithiothreitol. ^3H- and ^{32}P-labeled rNTPs and dNTPs and sodium boro[^3H]hydride were purchased from New England Nuclear or from Amersham Corp.

Extensively purified *Escherichia coli* replication proteins were: SSB [fraction IV, 2×10^4 units/ml, 4×10^4 units/mg (16)]; *dnaB* protein [fraction V, 1.3×10^6 units/ml, 6.2×10^5 units/mg (15)]; *dnaC* protein (fraction V, 1×10^5 units/ml, 6×10^4 units/mg); protein n′ (fraction VII, 5×10^4 units/ml, 2.4×10^5 units/mg); proteins n + n″ (fraction V,* 1.5×10^5 units/ml, 5×10^5 units/mg); protein i (fraction V, 1.5×10^5 units/ml, 8×10^5 units/mg); primase [fraction VI, 5×10^5 units/ml, 1.1×10^6 units/mg (17)]; DNA polymerase III holoenzyme [fraction V, 8×10^4 units/ml, 3.8×10^5 units/mg (18)]. Procedures for purification of proteins n, n′, n″, and i and *dnaC* protein will be described elsewhere.

Assay of DNA Replication. DNA synthesis was assayed as described (16). Components were added at 0°C in the following order; 5 μl of buffer A; 0.2 μmol of $MgCl_2$; 1.2 nmol each of dATP, dCTP, and dGTP; 0.45 nmol of [^3H]dTTP (specific activity, 1500 dpm/pmol); 2.5 nmol each of GTP, CTP, and UTP; 20 nmol of ATP; 40 nmol of spermidine·HCl; 0.5 μg of SSB; 60 ng of *dnaB* protein; 50 units of *dnaC* protein; 35 ng of protein i; 40 units of proteins n + n″; 30 ng of protein n′; 60 ng of primase; 0.2 μg of DNA polymerase III holoenzyme; 250 pmol (as nucleotide) of φX DNA; and water to 25 μl. For assay of DNA synthesis catalyzed by *dnaB* protein, primase, and DNA polymerase III holoenzyme, spermidine·HCl was omitted from the reaction mixture. Incubation was at 30°C.

Assay of RNA Primer Synthesis. For RNA primer synthesis catalyzed by *dnaB* protein and primase, the reaction mixture contained in 25 μl: 5 μl of buffer A; 0.2 μmol of $MgCl_2$; 0.5–0.75 nmol (as nucleotide) of DNA as indicated; 1.25 nmol each of [^3H]GTP, CTP, and UTP (each at 4000 dpm/pmol); 20 nmol of ATP; 0.1 μg of rifampicin; 0.4 μg of *dnaB* protein; and 0.12 μg of primase. When poly(dT) (4 nmol, as nucleotide) was used as template, 20 nmol of [^3H]ATP (at 1000 dpm/pmol) was present, and GTP, CTP, and UTP were omitted. Incubation was at 30°C. The amount of RNA primer synthesized was determined by the DEAE-cellulose filter procedure (12).

Abbreviations: φX, phage φX174; SSB, single-strand binding protein.

* Protein n fraction used here was further resolved into two replication enzymes (protein n and n″) by J. Shlomai.

Formation and Isolation of φX DNA·*dnaB* Protein Complex. The reaction mixture contained in 25 μl: 5 μl of buffer A, 0.2 μmol of $MgCl_2$, 1.5 nmol (as nucleotide) of φX DNA, 20 nmol of ATP, 1.5 μg of ^{3}H-labeled *dnaB* protein (8×10^4 cpm/μg), and, when present, 0.4 μg of primase. After incubation at 30°C for 20 min, the amount of *dnaB* protein bound to φX DNA was determined by filtering the reaction mixture through Bio-Gel A-5m agarose (0.2 × 15 cm) (equilibrated with buffer B containing 0.5 mM ATP at 24°C) and collecting the excluded volume. The radioactivity in each fraction was determined in a liquid scintillation spectrometer.

Labeling of *dnaB* protein was performed according to Rice and Means (19) by using sodium boro[^{3}H]hydride (7.2 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and formaldehyde. The labeled *dnaB* protein (8×10^4 cpm/μg) retained more than 90% of its activity in DNA replication. Details of the labeling procedure will be described elsewhere.

Other Methods. Polyacrylamide gel electrophoresis in 7 M urea and autoradiography were carried out as described (20, 21).

RESULTS

The Reconstituted System for Converting φX DNA to the Duplex Form. φX viral DNA incubated with purified preparations of SSB, *dnaB* protein, *dnaC* protein, protein i, proteins n + n″, protein n′, primase, DNA polymerase III holoenzyme, and all the rNTPs and dNTPs was almost completely converted within 10 min to the duplex form. Omission of any one of several proteins (e.g., *dnaB*, *dnaC*, i, n, n′, n″, primase, or DNA polymerase III holoenzyme) abolished the activity (Table 1). The product of the reaction was the duplex replicative form II as judged by autoradiography and ethidium bromide fluorescence after separation by agarose gel electrophoresis (data not shown).

Unlike the results obtained with less purified preparations (9), the requirement for SSB was only partial and omission of spermidine had no effect on overall synthesis. Moreover, the requirement for SSB almost completely disappeared in the absence of spermidine. The possibility that SSB was supplied as a contaminant in some of the replication protein preparations was eliminated by the lack of inhibition by anti-SSB antibody, which inhibits DNA synthesis completely when SSB is present (data not shown).

***dnaB* Protein, Primase, and DNA Polymerase III Holoenzyme Suffice for DNA Replication in the Absence of SSB.** With uncoated φX DNA as template, there was no requirement for proteins i, n, n′, or n″ (Table 2); dependency on *dnaC* protein was only partial. By contrast, *dnaB* protein, primase, and DNA polymerase III holoenzyme were absolutely required. Rifampicin had no demonstrable effect. Thus we conclude that DNA synthesis is initiated by *dnaB* protein and primase when DNA is not covered with SSB. These results suggest that *dnaB* protein and primase provide primers that are elongated by DNA polymerase III holoenzyme. It would seem reasonable to expect that this system also catalyzes the conversion of other single-stranded phage DNAs to their duplex forms.

Table 1. Requirements for conversion of φX DNA to replicative form II with purified replication proteins

Condition	dNMP incorporated, pmol
Complete	210
Minus *dnaB* protein	1
Minus *dnaC* protein	2
Minus protein i	3
Minus protein n + n″	2
Minus protein n′	7
Minus primase	4
Minus DNA polymerase III holoenzyme	<1
Minus SSB	51
Minus spermidine	205
Minus SSB and spermidine	180

Incubation was 10 min.

Table 2. Requirements for conversion of φX DNA to replicative form II in the absence of SSB and spermidine

Condition	dNMP incorporated, pmol
Complete	95
Plus rifampicin (0.5 μg)	82
Plus SSB (0.5 μg)	70
Minus *dnaB* protein	6
Minus *dnaC* protein	38
Minus protein i	90
Minus protein n + n″	94
Minus protein n′	81
Minus primase	8
Minus DNA polymerase III holoenzyme	1

DNA synthesis was measured as described in *Materials and Methods* except that SSB and spermidine were not included in the complete mixture. Incubation was 10 min.

Template Specificity of a Phage DNA Replication System Requires Coating of the DNA by SSB. The combination of *dnaB* protein, primase, and DNA polymerase III holoenzyme was effective for DNA synthesis on G4 and M13 phage DNAs (Fig. 1). Again, initiation of DNA synthesis required *dnaB* protein and primase. The stimulation of DNA synthesis by *dnaB*

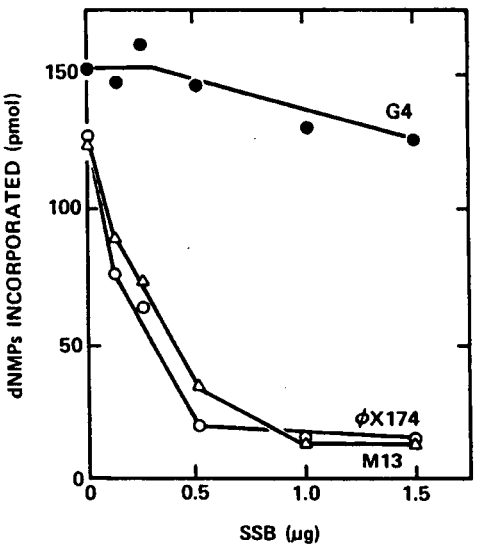

FIG. 1. Inhibition by SSB of DNA synthesis catalyzed by *dnaB* protein, primase, and DNA polymerase III holoenzyme. DNA synthesis was measured as described in *Materials and Methods* except that spermidine was omitted and 250 pmol (as nucleotide) of φX, G4, or M13 DNA was used as template. The reaction mixtures (25 μl) contained 0.1 μg of *dnaB* protein, 60 ng of primase, 0.2 μg of DNA polymerase III holoenzyme, and amounts of SSB as indicated. Incubation was 15 min.

Table 3. Template specificity of the purified replication systems

DNA template	DNA synthesis, pmol	
	G4 system*	φX system†
φX	2	140
G4	220	180
M13	1	8

DNA synthesis was measured as described in *Materials and Methods* except that φX, G4, or M13 DNA (250 pmol, as nucleotide) was used as template. Incubation was 10 min.

* No prepriming proteins; only primase, DNA polymerase III holoenzyme, and SSB were included.

† Prepriming proteins n, n′, n″, i, and *dnaC* were included, as well as *dnaB* protein, primase, DNA polymerase III holoenzyme, and SSB.

protein on G4 DNA was completely inhibited by anti-*dnaB* protein antibody but not by anti-SSB antibody. This result excludes the possibility of a SSB contaminant in the *dnaB* protein preparation. The products of the reaction were duplex replicative form II (data not shown). As anticipated, this replication system failed to discriminate among phage templates that were not covered by SSB. However, in the presence of SSB, DNA synthesis on φX and M13 circles was profoundly inhibited (Fig. 1). Almost 90% of the activity was inhibited by an amount of SSB that covered these single-stranded templates completely (16). On the other hand, DNA synthesis on G4 circles was, as expected, not significantly inhibited by any level of SSB.

These results suggest that, except for G4 DNA, SSB-coated single-stranded DNA is inert for the priming system of *dnaB* protein and primase. SSB-coated G4 DNA is unique in being primed by primase itself (refs. 8 and 17 and Table 3). As expected, SSB-coated φX DNA showed the template specificity observed in the complex replication system. In the presence of prepriming proteins (n, n′, n″, i, and *dnaC*), SSB-coated DNA was converted to the duplex form, except for SSB-coated M13 DNA, which remained inactive (Table 3). Thus, SSB determines template specificity during the priming process.

dnaB Protein and Primase Catalyze the Synthesis of Multiple Primers on φX, G4, and M13 DNAs. RNA primer formation as the basis for initiation of DNA synthesis by *dnaB* protein and primase was demonstrated directly with φX DNA. The amount of primer synthesized was surprisingly large; as much as 30% of φX DNA could be converted to the DNA-RNA hybrid form (Table 4). RNA synthesis was completely dependent on *dnaB* protein, primase, and DNA template; it was strongly inhibited by SSB. Similar results were also obtained when G4 and M13 DNA were used as templates (data not shown).

RNA primers were denatured and sized by electrophoresis in a 12% polyacrylamide gel in 7 M urea (Fig. 2). On each of the templates, multiple primers ranging in size from 10 to 60

Table 4. Requirements for RNA primer synthesis on uncoated φX DNA

Condition	RNA synthesized, pmol
Complete	189
Minus *dnaB* protein	2
Minus primase	1
Minus *dnaB* protein and primase	<1
Minus φX DNA	<1
Plus SSB	9

RNA synthesis was measured as described in *Materials and Methods* except that 625 pmol (as nucleotide) of φX DNA was used as template. Incubation was 30 min.

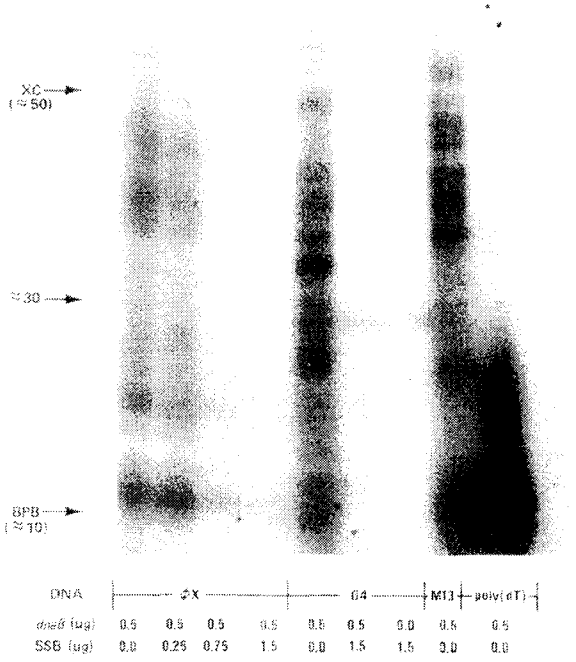

FIG. 2. Electrophoresis of RNA primer transcripts. Reaction mixtures (25 μl) contained: 5 μl of buffer A; 0.2 μmol of $MgCl_2$; 1.25 nmol each of [α-^{32}P]GTP, CTP, and UTP (each at 7000 dpm/pmol); 20 nmol of ATP; 0.1 μg of rifampicin; 500 pmol (as nucleotide) of phage DNA; 0.15 μg of primase; and additional components as indicated. The poly(dT) reaction mixture contained [α-^{32}P]ATP (1000 dpm/pmol) and 4 nmol of poly(dT). Incubation was at 30°C for 30 min. The reaction was terminated by cooling to 0°C. To each sample were added 50 μl of 95% ethanol (−20°C), 2.5 μl of 3 M sodium acetate (pH 5.5), and 30 μg (1 μl) of *E. coli* tRNA. After 4 hr at −20°C, the precipitates were collected by centrifugation and resuspended in 25 μl of deionized formamide. RNA transcripts were fractionated by electrophoresis in a 15% polyacrylamide gel containing 7 M urea and located by autoradiography (20, 21). Approximate lengths are given on the left in nucleotides. XC, xylene cyanol; BPB, bromphenol blue.

nucleotides long were synthesized (Fig. 2). However, the size distribution did not appear to be random. Unique patterns were observed with φX, G4, and M13 DNAs, suggesting that certain regions of each circle were preferentially transcribed.

SSB strongly inhibited the synthesis of primers on φX DNA (Fig. 2). On the G4 circle, synthesis of multiple primers was suppressed by SSB and a unique primer was synthesized (Fig. 2), which was indistinguishable from that synthesized by primase alone in the presence of SSB.

Prepriming Proteins Restore the Synthesis of Primers on SSB-Coated φX DNA. In the presence of prepriming proteins, SSB-coated φX DNA is active in directing the synthesis of RNA primers. However, priming activity was less than half that obtained with the simple *dnaB* protein and primase system on uncoated DNA without prepriming proteins (Table 5). In the presence of SSB, primer synthesis was dependent on proteins n, n′, n″, i, and *dnaC*, in agreement with previous observations (12). However, omission of SSB did not affect the level of RNA synthesis, consistent with results obtained in DNA synthesis (Table 1).

Table 5. Requirements for RNA primer synthesis on SSB-coated φX DNA

Condition	RNA synthesized, pmol
Complete	33.1
Minus proteins n and n″	5.0
Minus protein n′	<0.2
Minus protein i	4.4
Minus *dnaC* protein	<0.2
Minus SSB	30.3
Minus proteins n, n′, n″, i, and *dnaC*	4.7
Minus proteins n, n′, n″, i, *dnaC*, and SSB	68.8

For RNA synthesis, components were mixed at 0°C in the following order: 5 μl of buffer A; 0.2 μmol of $MgCl_2$; 500 pmol (as nucleotide) of φX DNA; 1.25 nmol each of [^{3}H]CTP, GTP, and UTP (each at 4000 dpm/pmol); 20 nmol of [^{3}H]ATP (4000 dpm/pmol); 0.1 μg of rifampicin; 0.8 μg of *dnaB* protein; 0.2 μg of primase; 80 units of protein n + n″; 80 ng of protein n′; 70 ng of protein i; 90 units of *dnaC* protein; and 1.5 μg of SSB. Final volume, 25 μl; incubation, 30 min. The amount of RNA synthesized was determined by the DEAE-cellulose filter procedure (12).

Primer Synthesis on Poly(dT). Among synthetic polynucleotides tested, poly(dT) is an active template in directing poly(dA) synthesis by *dnaB* protein, primase, and DNA polymerase III holoenzyme. *dnaB* protein and primase catalyzed a large amount of oligo(rA) synthesis on poly(dT) (data not shown); as much as 30% of the template was transcribed. RNA synthesis was completely dependent on *dnaB* protein, primase, and poly(dT); it was inhibited by SSB. Electrophoresis of RNA products indicated that primers synthesized on poly(dT) were significantly shorter than those synthesized on phage DNA (Fig. 2). Poly(rA) longer than 50 nucleotides was not detected, and oligo(rA), 10–15 nucleotides long, was the most abundant product.

Formation of φX DNA·*dnaB* Protein·Primase Complex. Interaction of *dnaB* protein with φX DNA was measured after separation of DNA·*dnaB* protein complex from unassociated *dnaB* protein by gel filtration. In the absence of primase, less than 0.3 molecule of *dnaB* protein was bound per φX DNA circle (data not shown). Addition of primase markedly stimulated formation of the *dnaB* protein·DNA complex (1.3 molecule of *dnaB* protein per circle). ATP was essential for the binding reaction. When ATP was omitted during gel filtration, a stable *dnaB* protein·DNA complex could not be observed.

Upon addition of primase and the four rNTPs, a condition that sustains the synthesis of multiple primers, the bound *dnaB* protein was not released; instead, binding of *dnaB* protein to DNA was enhanced (1.9 molecule of *dnaB* protein per circle). A similar experiment using ^{3}H-labeled primase showed that primase also remained bound to φX DNA in the presence of *dnaB* protein and rNTPs (data not shown). The binding of *dnaB* protein to DNA was abolished by SSB.

DISCUSSION

Initiation of DNA synthesis depends generally on RNA priming (3). Three mechanisms are already known in uninfected *E. coli* extracts for priming DNA synthesis on single-stranded DNA: (*i*) RNA polymerase acting on phage M13 DNA, (*ii*) primase (*dnaG* protein) acting on phage G4 DNA, and (*iii*) primase acting on a form of φX DNA produced by the action of *dnaB* protein and other replication proteins (*dnaC*, i, n, n′, n″); the φX system is thought to be the one used by the cell for initiating nascent fragments in the lagging strand at the replication fork of its chromosome. Specific and effective priming in each instance requires that the single-stranded DNA be covered with single-strand binding protein (SSB) as discussed further below.

We have now recognized another priming system, which, in its simplicity and generality, may prove to be a valuable guide to the operation of the more complex systems. *dnaB* protein forms a complex with virtually any uncoated single-stranded DNA in the presence of ATP and primase to produce primers for DNA synthesis (Fig. 3). Multiple short primers are produced throughout the length of the DNA (Fig. 2), apparently at preferred initiation points, as judged from distinctive sizes with M13, G4, and φX DNAs. When the template is covered by binding protein, the *dnaB* protein–primase system is

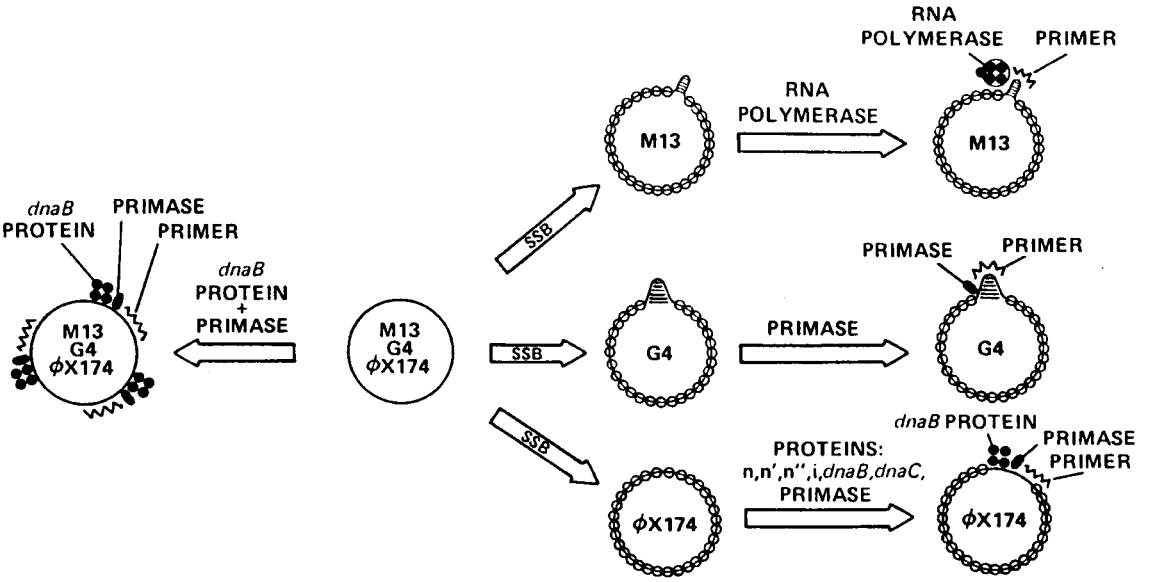

FIG. 3. Illustration of nonspecific priming by *dnaB* protein and primase on uncoated DNA and the specific priming systems for M13, G4, and φX DNAs coated with SSB.

blocked and the initiation systems specific for each template are required (Fig. 3). A unique primer is produced on M13 and on G4 DNA but, as will be described elsewhere, the size distribution of primers formed on ϕX DNA by the complex prepriming system is remarkably similar to that formed by the *dnaB* protein–primase system (data not shown).

In earlier studies, primer formation on a single-stranded DNA template absolutely required SSB; priming by only *dnaB* protein and primase was not observed in the absence of SSB. Impurities in the partially purified protein preparations might have degraded the template, the primers, or the ATP, and spermidine may have protected against this. However, the essential role of SSB is indicated by (*i*) studies with a temperature-sensitive mutant (22) that fails to sustain phage or host chromosome replication *in vivo* and (*ii*) studies *in vitro* (Fig. 3) which show that SSB is needed for specific recognition of origins of replication in M13 and G4 DNAs and for formation of an active *dnaB* protein complex with ϕX DNA (see below).

Formation of a complex of *dnaB* protein bound to ϕX DNA in the absence of SSB requires the presence of primase and ATP. Current studies show further that *dnaB* protein in this complex, while stable enough to be isolated by gel filtration, can be displaced by free *dnaB* protein in solution. However, the *dnaB* protein in the complex formed with ϕX DNA by the multiprotein prepriming system resists exchange even with a large excess of free *dnaB* protein. These findings are consistent with a proposal (14) that the unusual stability of *dnaB* protein binding to DNA, combined with a mobility on DNA engendered by its ATPase activity, enable the protein to function as a mobile promoter in the discontinuous phase of chromosome replication.

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