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Unique primed start of phage φX174 DNA replication and mobility of the primosome in a direction opposite chain synthesis

(origin of complementary strand replication/protein n′/mobile replication promotor/antielongation direction/DNA-dependent ATPase)

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ABSTRACT **A specific fragment of the φX174 viral circle sustains the primed start of complementary DNA strand synthesis *in vitro*, even though the intact circle permits primed starts at many sites. The 300-nucleotide fragment from restriction nuclease digestion contains the recognition site for protein n′, a DNA-dependent ATPase essential for priming φX174 DNA replication. This n′ recognition site contains within it a 44-nucleotide sequence with a potential hairpin structure and may be regarded as the starting signal for replication [Shlomai, J. & Kornberg, A. (1980) *Proc. Natl. Acad. Sci. USA* 77, 799–803]. After initiation on the 3′ side of this sequence, the priming system (primosome) repeatedly generates primers by moving processively on the DNA template in a direction opposite to chain elongation. This primosome mobility is an attractive model for the discontinuous phase of *Escherichia coli* chromosome replication, in which processive primosome movement with the replicating fork is proposed for repeated initiations of nascent replication fragments.**

Conversion of single-stranded DNA (ssDNA) of phage φX174 (φX) to duplex replicative form (RF) is a model system (1–3) for the discontinuous phase of *Escherichia coli* chromosomal replication in which the nascent replication (Okazaki) fragments are initiated. In this conversion, φX DNA coated by ssDNA-binding protein (SSB) must first be activated in a prepriming stage for subsequent priming by primase (2, 4–6). In this prepriming reaction, *E. coli* proteins n, n′, n″, i, *dnaC*, and *dnaB* interact to form a prepriming replication intermediate (4, 5, 7). Unlike phages M13 (8, 9) and G4 (10–12), which possess unique origins for priming complementary strand replication, φX permits multiple starts in DNA replication, as indicated by both *in vivo* (12) and *in vitro* studies (5, 7). However, the strict specificity of the prepriming system for φX DNA (7, 13) and the specific recognition by protein n′ of a 55-nucleotide sequence in φX DNA, located in the same intergenic region as the G4 origin (14), strongly suggest a unique origin for φX complementary strand replication.

This present work shows that complementary strand replication is in fact initiated at or near the protein n′ recognition locus by a multiprotein·DNA complex (a mobile replication promoter or "primosome"), which then migrates with a unique polarity on the ssDNA template in a direction opposite to primer and DNA chain synthesis. Subsequent reports will describe participation of ATP in the processivity of primosome movement and conservation of the primosome in successive stages of φX DNA replication. These studies of primosome structure and function have implications for events taking place at the *E. coli* chromosome replication fork.

MATERIALS AND METHODS

Nucleic Acids and Enzymes. DNAs, *E. coli* replication proteins, and other materials were as described (13–16). Restriction endonuclease fragments of ssDNA (17, 18) for use as templates for DNA synthesis were prepared by digestion of φX or G4 DNA (30 μg) at 37°C for 15 hr with *Hae* III (400 units) or *Hha* I endonuclease (320 units) in 400 μl of 50 mM Tris·HCl (pH 7.5)/5 mM $MgCl_2$/0.5 mM dithiothreitol. Digestion products were precipitated with ethanol from phenol-treated reaction mixtures and dissolved in 50 mM Tris·HCl (pH 7.5)/1 mM EDTA. Buffer A was 100 mM Tris·HCl (pH 7.5)/20% sucrose/40 mM dithiothreitol/200 μg of bovine serum albumin per ml.

DNA Replication Assay. Components were added in order at 0°C and incubated 20 min at 30°C: 5 μl of buffer A, 1.2 nmol each of [^{3}H] or [α-^{32}P]dCTP, dGTP, dATP, and dTTP (each at 2000 dpm/pmol), 2.5 nmol each of GTP, CTP, and UTP, 20 nmol of ATP, 0.2 μmol of $MgCl_2$, 450 pmol (as nucleotide) of φX ssDNA or its digestion products, 0.25 μg of rifampicin, SSB as indicated, 0.2 μg of DNA polymerase III holoenzyme, 0.4 μg of *dnaB* protein, 0.1 μg each of *dnaC*, i, and n′ proteins, 0.14 μg of protein mixture n + n″, 0.1 μg of primase, and water to 25 μl. With G4 DNA or its digests as templates, only SSB, DNA polymerase III holoenzyme, and primase were included.

Other Methods. Agarose gel electrophoresis of DNA was as described (16, 19). The relative amount of ^{32}P radioactivity in each band was determined by densitometric tracing of the autoradiogram with a Quick Scan Jr. TLC, Helena Laboratories (Beaumont, TX). Sizes of DNA products were determined after heat denaturation in 98% (wt/vol) formamide by electrophoresis in a 7 M urea/2.5–7.5% gradient polyacrylamide gel (5, 20). Transfer of DNA to diazobenzyloxymethyl (DBM) paper† and DNA hybridizations were as described (21, 22).

RESULTS

Unique primed start of DNA replication

Specific DNA Synthesis on a G4 DNA Fragment. Whether a unique primed start of DNA replication can be sustained by a fragment of a phage DNA circle was first determined with G4 DNA. A *Hha* I endonuclease digest (15 fragments) of G4 DNA (23) supported DNA synthesis with SSB, primase, and DNA polymerase III holoenzyme at 18% the level of untreated G4 DNA (Table 1). Omission of primase abolished almost 80% of this activity. Agarose gel electrophoresis of the G4 DNA products showed that more than 90% of the DNA was synthesized on the 1494-nucleotide *Hha* I fragment 1, which contains the origin

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Abbreviations: φX, phage φX174; ssDNA, single-stranded DNA; RF, double-stranded DNA in the circular replicative form; SSB, ssDNA-binding protein; DBM, diazobenzyloxymethyl; p[NH]ppA, 5′-adenyl-yl imidodiphosphate.

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† In some experiments, DNA fragments were transferred to aminothiophenol paper, a procedure suggested by Brian Seed of the California Institute of Technology.

Table 1. Template activity of *Hae* III and *Hha* I endonuclease digests of phage DNA

Nuclease treatment of template	Priming component(s)	DNA synthesis, pmol G4	DNA synthesis, pmol φX
None	+	290	360
	−	8	11
Hae III	+	4	39
	−	2	18
Hha I	+	51	18
	−	12	5

DNA synthesis was with 1.0 μg of SSB. The priming component for the G4 templates was only primase; the priming components for φX included the prepriming proteins n, n′, n″, i, *dnaB*, and *dnaC*, as well as primase.

of G4 complementary strand replication (10, 23) (Fig. 1). The extent of synthesis was nearly what was expected from the location of the origin within fragment 1 and its relative size.

No DNA synthesis was detected with a *Hae* III endonuclease digest (14 fragments). Although the 442-nucleotide fragment Z5a contains the G4 complementary strand origin, the cleavage site is at the stem of the "downstream" hairpin located about 60 bases downstream from the start of the primer and presumably required for recognition by primase (24). These results indicate that for primase the circular form of DNA is not essential and that primase can recognize the specific signal for the complementary strand origin of G4 DNA even in a small DNA fragment.

DNA Synthesis on a Unique Fragment of φX DNA Requiring the Prepriming Proteins. DNA synthesis with an unfractionated φX DNA *Hae* III digest (25) was 11% that with intact φX viral circle (Table 1). Omission of prepriming proteins n, n′, n″, i, and *dnaC* abolished only half the activity, suggesting some nonspecific initiation on DNA fragments. The effects of SSB on specific initiation dependent on prepriming proteins, and on nonspecific initiation obtained with only *dnaB* protein and primase (13), were examined with intact φX circular DNA and its *Hae* III fragments. Nonspecific initiation on the intact circle was inhibited almost completely by SSB (Fig. 2*A*), whereas that on the fragments was more resistant to SSB; an 80% inhibition required a 4-fold excess of SSB beyond that needed to coat the ssDNA (26) (Fig. 2*B*). At this high level of SSB, 85% of the DNA synthesis was dependent on prepriming proteins; the nonspecific DNA synthesis required only DNA polymerase III holoenzyme (data not shown).

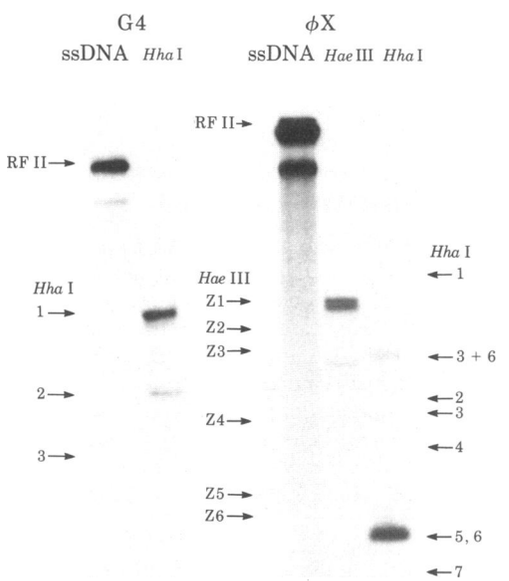

FIG. 1. Autoradiogram of DNA products synthesized on phage DNA *Hae* III or *Hha* I endonuclease fragments. DNA synthesis was with ssDNA or its *Hae* III or *Hha* I digest: G4 with 1.5 μg of SSB, φX with 4 μg of SSB. The DNA products were separated by electrophoresis in a 1.5% agarose gel and detected with ethidium bromide (arrows) and autoradiography.

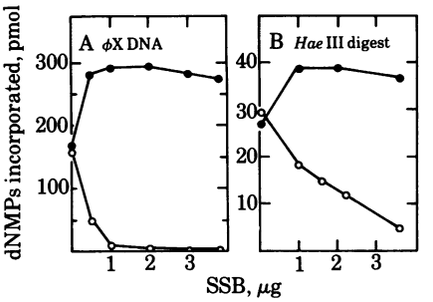

FIG. 2. Effects of SSB on specific and nonspecific DNA synthesis with φX DNA (*A*) and its *Hae* III endonuclease fragments (*B*) as templates. Reaction mixtures included only *dnaB* protein, primase, and DNA polymerase III holoenzyme (○), or also prepriming proteins n, n′, n″, i, *dnaC*, and *dnaB* (●).

Of the DNA produced with only *dnaB* protein, primase, DNA polymerase III holoenzyme, and 1.5 μg of SSB, 19%, 28%, and 53% was synthesized on the Z1, Z3, and Z4 fragments, respectively (Fig. 3, trace a). When prepriming proteins n, n′, n″, i, and *dnaC* were included, these ratios changed to 53%, 33%, and 14% (Fig. 3, trace b), suggesting that prepriming proteins stimulate specific initiation on the Z1 fragment and suppress nonspecific initiation on the Z4 fragment. With a 4-fold excess of SSB (3.6 μg) over that needed to coat the ssDNA (26), DNA synthesis on the Z3 and Z4 fragments was preferentially inhibited; 82%, 12%, and 6% of the DNA was synthesized on the Z1, Z3, and Z4 fragments, respectively (Fig. 1 and Fig. 3, trace c). Other *Hae* III fragments were completely inert. These results demonstrate that SSB inhibits nonspecific DNA synthesis and that the 1353-nucleotide Z1 fragment is specifically activated as a template for DNA replication by prepriming proteins and primase.

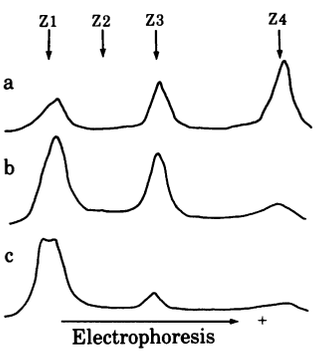

FIG. 3. [^{32}P]DNA products synthesized on *Hae* III endonuclease fragments of φX DNA. DNA synthesis was with 450 pmol (as nucleotide) of *Hae* III digests of φX ssDNA, including in trace a only *dnaB* protein, primase, DNA polymerase III holoenzyme, and 1.5 μg of SSB; trace b, prepriming proteins n, n′, n″, i, and *dnaC*, as well as the proteins in trace a; and trace c, the proteins in trace b except that 4 μg of SSB was used. DNA products were fractionated by electrophoresis in 1.5% agarose gel and quantitated by densitometric tracings of ^{32}P autoradiograms. Arrows indicate the *Hae* III fragments.

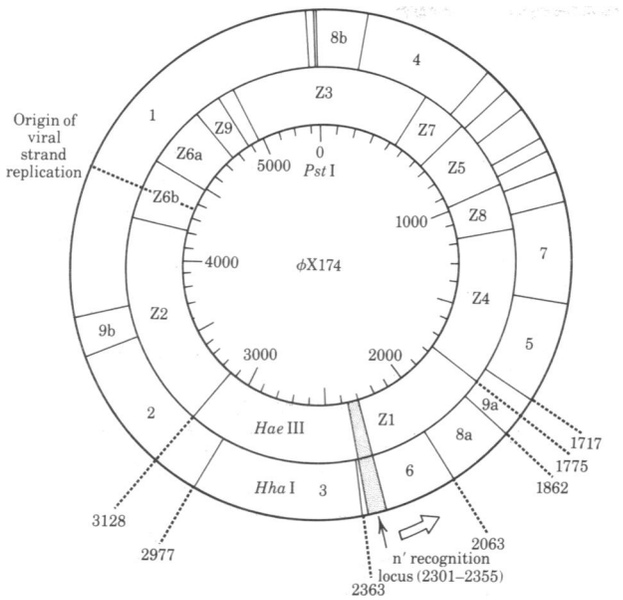

FIG. 4. Physical map of ϕX DNA. The *Hae* III and *Hha* I restriction endonuclease cleavage sites and the origin of viral DNA strand replication at nucleotide 4306 (*Pst* I site = 0) on the Sanger map are taken from refs. 25 and 27. The protein n′ recognition site is located at nucleotides 2301–2354 (14). The open arrow indicates the DNA elongation direction.

ϕX Complementary Strand Replication Origin and the Protein n′ Recognition Locus. *Hha* I nuclease cleaves ϕX DNA into 17 fragments (25), of which the 300-nucleotide fragment 6 contains the 55-nucleotide recognition locus of protein n′ (Fig. 4). *Hha* I digests of ϕX DNA supported DNA synthesis in the presence of prepriming proteins at 5% the level with untreated DNA (Table 1). Fragment 6 accounted for 80% of the DNA incorporation as judged by agarose gel electrophoresis (Fig. 1) and by hybridization to various regions of ϕX DNA (see below); about 20% was related to a fragment from incomplete digestion of ϕX DNA that migrated between *Hha* I fragments 1 and 2 and contained sequences of fragments 3 and 6 (see below). Omission of the prepriming proteins almost abolished DNA synthesis on the complete *Hha* I digest (Table 1), on fragment 6, and on fragments 3 plus 6 (data not shown). These results indicate that SSB-coated ϕX DNA contains a unique origin for complementary strand synthesis within a 300-nucleotide fragment at or near the protein n′ recognition site.

Mobility of priming system in a direction opposite chain synthesis

Models for Polarity of Primosome Migration. Multiple primers are synthesized on almost every region of the chromosome of SSB-coated ϕX DNA by prepriming proteins (n, n′, n″, i, *dnaC*, and *dnaB*) and primase when uncoupled from DNA synthesis (5, 7). Because SSB-coated ϕX DNA has a single origin for a complementary strand start at or near the protein n′ recognition site, the primosome, known to contain at least proteins *dnaB*, n′, and primase (refs. 5 and 28; unpublished results), must migrate progressively along the viral DNA strand to achieve primer synthesis at multiple sites. Two possible models for the polarity of primosome migration are considered (Fig. 5) One model assumes that the primosome migrates in a direction opposite to DNA and primer synthesis (antielongation), namely the 5′→3′ polarity of the ssDNA template; the other assumes migration in the same direction as priming and DNA elongation. Our results fit best with the antielongation direction model.

Sizes of DNAs Synthesized on *Hae* III Fragment Z1 and *Hha* I Fragment 6 as an Indicator of Polarity of Primosome Movement. The 5′ end of the protein n′ recognition locus at position 2301 on the Sanger map (14, 25) is separated from the 5′ end of the *Hae* III fragment Z1 by 526 residues and from the *Hha* I fragment 6 by 248 residues. Were primosome movement in the elongation direction (3′→5′ polarity of the template), DNA products shorter than 600 nucleotides on fragment Z1 and shorter than 250 on fragment 6 would be expected. Instead, the heterogeneous products synthesized on fragment Z1 were longer than 600 nucleotides, the most abundant length being 1200–1300 (data not shown), suggesting *de novo* chain initiations predominate on the 3′ side of the protein n′ recognition site. In fact, *Hha I* digestion of [^{32}P]DNA products synthesized on the *Hae* III fragment Z1 yielded three main fragments that comigrated with *Hha* I fragments 3, 6, and 8a on agarose gel electrophoresis (data not shown), the 614-nucleotide *Hha* I fragment 3 being located at the 3′ side of the protein n′ recognition site (Fig. 4). A similar result was obtained with the DNA synthesized on *Hha* I fragment 6. The products were homogeneous in size and essentially the full length of the template (data not shown).

Map Positions of DNA Synthesized on *Hae* III Fragment Z1 and *Hha* I Fragments 3 plus 6 as an Indicator of Polarity of Primosome Movement. Molecular hybridization can be used to map DNA synthesized on DNA fragments. Products of ϕX RF I digestion by endonuclease *Hae* III or *Hha* I were separated on

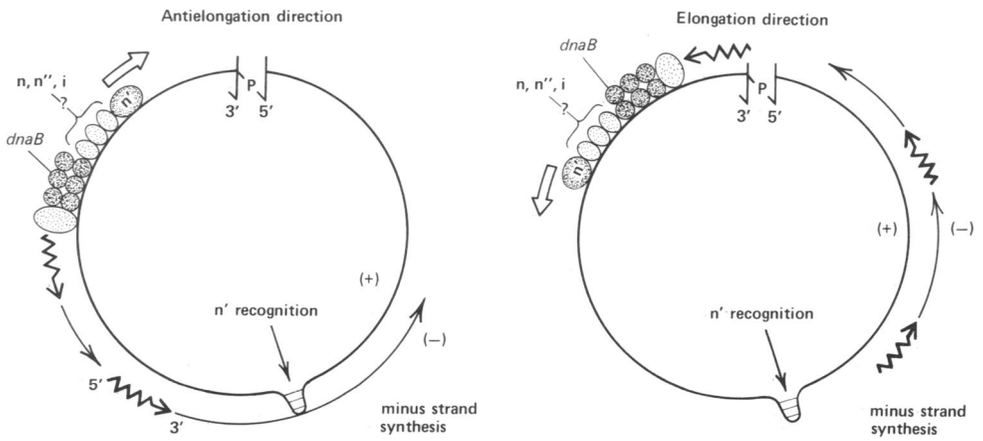

FIG. 5. Possible models for the polarity of primosome migration on ϕX DNA.

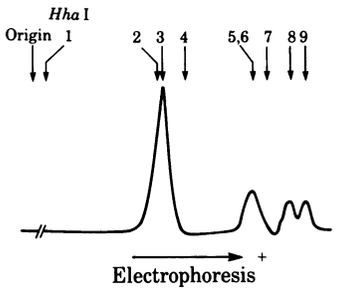

FIG. 6. Hybridization to *Hha* I fragments of DNA synthesized on the *Hae* III fragment Z1 of ϕX ssDNA. ϕX RF I DNA (2.5 μg) was incubated 2 hr at 37°C with *Hha* I (15 units) in 50 mM Tris·HCl (pH 7.5)/5 mM $MgCl_2$/1 mM dithiothreitol. DNA fragments (arrows) were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and transferred to DBM paper (21). ^{32}P-Labeled DNA synthesized on *Hae* III fragment Z1 was extracted from the agarose gel by electrophoresis and hybridized in 10% dextran sulfate 500 (Pharmacia) (22) at 42°C for 24 hr to the *Hha* I DNA fragments bound to DBM paper. The paper was washed and autoradiographed at −80°C, using an intensifying screen (Du Pont Cronex). The densitometric tracing of the ^{32}P autoradiogram is shown.

1.5% agarose gels, denatured, and transferred to DBM paper (22). With the *Hha* I digest (10 bands), [^{32}P]DNA synthesized on *Hae* III fragment Z1 hybridized mainly to *Hha* I fragments 3, 6, 8a, and 9a (Fig. 6). With the *Hae* III digest (nine bands), [^{32}P]DNA synthesized on *Hha* I fragments 6 (Fig. 7*A*) and 3 plus 6 (Fig. 7*B*) hybridized only to fragment Z1 as expected. The DNA synthesized on the latter as template hybridizes not only to *Hha* I fragment 6 but also to fragment 3. Inasmuch as the *Hha* I fragment 3 region is inert for initiating replication (Fig. 1), primosome movement on fragment Z1 and *Hha* I fragments 3 plus 6 in the 5′→3′ direction of the template may be inferred (Fig. 4).

Synchronized Initiation on Circular DNA as an Indicator of Polarity of Primosome Movement. Formation of the prepriming replication intermediate is rate limiting in the ϕX ssDNA → RF reaction (4). DNA replication is accelerated by preincubation of SSB-coated ϕX DNA with prepriming proteins in ATP

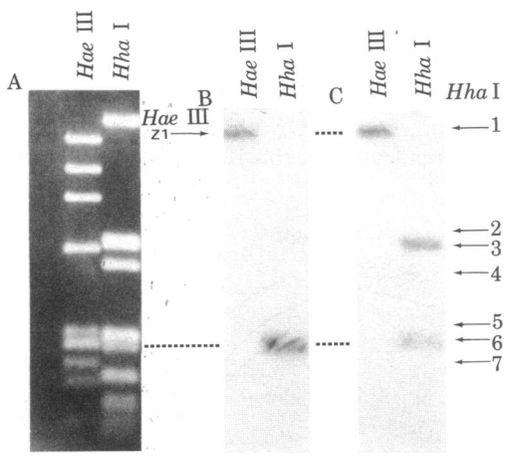

FIG. 7. DNA synthesized on ϕX ssDNA *Hha* I fragments 6 and 3 plus 6 hybridization to *Hae* III or *Hha* I fragments. ϕX RF I DNA (3 μg) was incubated 2 hr at 37°C with *Hae* III (15 units) or *Hha* I (15 units). After electrophoresis in 1.5% agarose gel, DNA fragments were visualized by staining with ethidium bromide (*A*) and transferred to DBM paper. The ^{32}P-labeled DNAs synthesized on a *Hha* I digest of ϕX ssDNA located in fragment 6 (*B*) or fragments 3 plus 6 (*C*) were extracted separately from the agarose gel by electrophoresis and hybridized at 42°C for 40 hr to the DNA *Hae* III and *Hha* I fragments bound to DBM paper. Other procedures were as in Fig. 6.

Table 2. Distribution of ^{32}P-labeled ribonucleotide primers on restriction fragments of ϕX RF II

Hae III fragment	Size		Preincubated with			Ratio	
	Residue	Ratio*	ATP A	p[NH]ppA B	p[NH]ppA† C	B/A	C/A
Z1	1353	1.00	100‡	100‡	100‡	1.00	1.00
Z2	1078	0.80	96	68	56	0.71	0.58
Z3	872	0.64	74	49	26	0.66	0.35
Z4	603	0.45	60	35	19	0.58	0.32

The reaction was in two stages. In the first stage, components were added in order at 0°C and incubated 20 min at 30°C: 10 μl of buffer A, 0.4 μmol of $MgCl_2$, 60 nmol of ATP or 50 nmol of p[NH]ppA, 1.5 nmol (as nucleotide) of ϕX DNA, 4.5 μg of SSB, 0.6 μg of protein n′, 0.6 μg of protein mixture n + n″, 0.7 μg of protein i, 0.5 μg of *dnaC* protein, 1.6 μg of *dnaB* protein, and water to 50 μl. Components were added to a second-stage mixture in order at 0°C: 10 μl of buffer A, 0.4 μmol of $MgCl_2$, 60 nmol of [α-^{32}P]ATP (2000 dpm/pmol), 50 nmol each of [α-^{32}P]GTP, UTP, and CTP (each at 10,000 dpm/pmol), 6 nmol each of [^{3}H]dATP, dGTP, dTTP, and dCTP (each at 200 dpm/pmol), 1.5 μg of primase, 10 μg of DNA polymerase III holoenzyme, and water to 50 μl. After incubation of the second-stage mixture at 30°C for 4 min, the first-stage mixture was added and the incubation was continued for 30 min. After addition of 10 μl each of 0.4 M EDTA and 10% sodium dodecyl sulfate and ϕX RF I (5 μg as carrier), the RF products, filtered through Bio-Gel A-5m (Bio-Rad), were incubated 2 hr at 30°C with *Hae* III endonuclease (100 units) in 250 μl of 50 mM Tris·HCl (pH 7.5)/5 mM $MgCl_2$/0.5 mM dithiothreitol. The ^{32}P-labeled digestion products were electrophoresed in 1.5% agarose gel and autoradiographed.

* Size relative to Z1.

† After 3 min, the second-stage reaction was supplemented with 1 mM each of the four unlabeled rNTPs.

‡ The ^{32}P radioactivity in each band was quantitated by densitometric tracing of autoradiograms and values relative to Z1 (set at 100) were calculated.

(4) or 5′-adenylyl imidodiphosphate (p[NH]ppA) (data not shown). As will be described elsewhere, migration of the primosome on SSB-coated DNA requires energy furnished by ATP hydrolysis. Initiation of priming of ϕX DNA replication was partially synchronized by forming a prepriming replication intermediate by preincubation with nonhydrolyzable p[NH]ppA. It is assumed that the primosome in the presence of ATP moves rapidly from its assembly point near the protein n′ recognition site to multiple locations around the circle before primer synthesis begins; substitution of p[NH]ppA should minimize this movement. After formation of the prepriming intermediate, components for priming and DNA elongation could be added, and the direction of primosome migration could be determined by the [^{32}P]RNA primer distribution around the circular map. Upon preincubation with ATP, [^{32}P]RNA primers were located relatively randomly (Table 2). However, upon preincubation with p[NH]ppA, primers were located preferentially on the *Hae* III fragment Z1 and the ratio of ^{32}P radioactivity (B/A and C/A, Table 2) on each fragment decreased in the order: Z2 > Z3 > Z4. These results indicate that the primosome migrates in the direction Z1 → Z2 → Z3 → Z4, consistent with the prediction of the antielongation direction model.

DISCUSSION

The prepriming system reconstituted *in vitro* from six *E. coli* proteins is highly specific for ϕX DNA coated with SSB (13). This specificity apparently derives from the capacity of protein n′ to recognize and bind a specific intergenic sequence. This initiating event in complementary strand synthesis is obscured by rapid movement of the priming system (primosome) around the circular template, leading to the apparently random priming and DNA syntheses observed *in vivo* and *in vitro*. In the

present studies, primosome movement was restrained by using DNA fragments generated by endonucleases *Hae* III and *Hha* I (17, 18). Only fragments containing the protein n′ recognition site were effective in sustaining the prepriming, priming, and DNA synthetic actions. Elevated levels of SSB were needed to suppress nonspecific priming and replication.

In view of the complexity of prepriming, it is remarkable that the 300-nucleotide *Hha* I fragment 6 suffices for the specificity for chain initiation on ϕX DNA. The DNA product size, 250 residues, suggests that primers are made at or near the hairpin within the 55-nucleotide protein n′ recognition locus where, presumably, primosome assembly takes place. The effect of protein n′ binding and primosome assembly at this site needs to be clarified. Because p[NH]ppA suffices for the formation of a prepriming replication intermediate (unpublished result), hydrolysis of ATP and movement of protein n′ or *dnaB* protein are not prerequisites for initial primosome assembly. The remarkable feature of the unique origin for ϕX complementary strand synthesis is that this site is not necessarily defined as the site where a unique primer is made. Rather it may be defined as the site for assembly of the primosome, which then may migrate processively along the viral circle. As will be described elsewhere, the energy of ATP and dATP hydrolysis utilized by protein n′ is essential to promote priming at many regions. Presumably protein n′ not only recognizes the origin of complementary strand replication but also drives the primosome along the DNA strand.

In the present study, polarity of primosome migration was shown to be uniquely in the *antielongation*, the 5′→3′, direction of the template (Fig. 5). This is consistent with the proposed mobility of the primosome on the lagging strand at the replication fork (3, 5), movement of the replication fork and primosome taking place in the same direction. Thus far, the polarity of primosome migration has been determined only when coupled to priming and DNA elongation. Approximately one primer is made per circle despite a capacity to synthesize multiple primers when uncoupled from DNA elongation (ref. 5; unpublished results). This indicates that the primosome can migrate in the 5′→3′ direction *before* priming. The random distribution of primers on ϕX DNA when preincubated with ATP to form the prepriming replication intermediate suggests that the primosome moves along the DNA template even without primase action. Whether this migration of a primosome lacking primase is *unidirectional* with a 5′→3′ polarity remains to be clarified.

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