Unique primed start of phage \( \phi X 174 \) 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)

KEN-ICHI ARAI* AND ARTHUR KORNBERG

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

Contributed by Arthur Kornberg, September 4, 1990

ABSTRACT A specific fragment of the \( \phi X 174 \) 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 \( \phi X 174 \) 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 [Sholomai, 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 \( \phi X 174 \) (\( \phi X \)) 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, \( \phi X \) 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 \( n' \), \( n'' \), \( i \), \( dnaC \), and \( dnaB \) interact to form a prepriming replication intermediate (4, 5, 7). Unlike phases M15 (8, 9) and G4 (10–12), which possess unique origins for priming complementary strand replication, \( \phi X \) permits multiple starts in DNA replication, as indicated by both in vitro (12) and in vitro studies (5, 7). However, the strict specificity of the prepriming system for \( \phi X \) DNA (7, 13) and the specific recognition by protein \( n' \) of a 55-nucleotide sequence in \( \phi X \) DNA, located in the same intergenic region as the G4 origin (14), strongly suggest a unique origin for \( \phi X \) 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 promotor 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 \( \phi X \) 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 \( \phi X \) or G4 DNA (30 \( \mu \)g) at 37°C for 15 hr with Hae III (400 units) or Hha I endonuclease (320 units) in 400 \( \mu \)l 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 \( \mu \)g 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 \( \mu \)l of buffer A, 1.2 nmol each of [\( ^{3} \)H]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 \( \mu \)mol of MgCl\(_2\), 450 pmol (as nucleotide) of \( \phi X \) ssDNA or its digestion products, 0.25 \( \mu \)g of rifampicin, SSB as indicated, 0.2 \( \mu \)g of DNA polymerase III holoenzyme, 0.4 \( \mu \)g of dnaB protein, 0.1 \( \mu \)g each of dnaC, i, and \( n' \) proteins, 0.14 \( \mu \)g of protein mixture \( n' + n'' \), 0.1 \( \mu \)g of primase, and water to 25 \( \mu \)l. 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 diazo benzoyloxymethyl (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.

Abbreviations: \( \phi X \), phage \( \phi X 174 \); ssDNA, single-stranded DNA; RF, double-stranded DNA in the circular replicative form; SSB, ssDNA-binding protein; DBM, diazo benzoyloxymethyl p[NH\_ppA, 5'-adenyl] imidodiphosphate.

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

† In some experiments, DNA fragments were transferred to aminoethylphenol paper, a procedure suggested by Brian Seed of the California Institute of Technology.
Table 1. Template activity of \textit{Hae} III and \textit{Hha} I endonuclease digests of phage DNA

<table>
<thead>
<tr>
<th>Nuclease treatment of template</th>
<th>Priming component(s)</th>
<th>DNA synthesis, pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>290 360</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>8 11</td>
</tr>
<tr>
<td>\textit{Hae} III</td>
<td>+</td>
<td>4 39</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>2 18</td>
</tr>
<tr>
<td>\textit{Hha} I</td>
<td>+</td>
<td>51 18</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>12 5</td>
</tr>
</tbody>
</table>

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.

No DNA synthesis was detected with a \textit{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 G4 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 unfractonated φX DNA \textit{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 \textit{dnaB} protein and primase (13), were examined with intact φX circular DNA and its \textit{Hae} III fragments. Nonspecific initiation on the intact circle was inhibited almost completely by SSB (Fig. 2A), 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). 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).

Of the DNA produced with only \textit{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 \textit{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. 1.** Autoradiogram of DNA products synthesized on phage DNA \textit{Hae} III or \textit{Hha} I endonuclease fragments. DNA synthesis was with ssDNA or its \textit{Hae} III or \textit{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 \textit{Hae} III endonuclease fragments (B) as templates. Reaction mixtures included only \textit{dnaB} protein, primase, and DNA polymerase III holoenzyme (○), or also prepriming proteins n, n', n", i, dnaC, and \textit{dnaB} (●).

**Fig. 3.** [%]DNA products synthesized on \textit{Hae} III endonuclease fragments of φX DNA. DNA synthesis was with 450 pmol (as nucleotide) of \textit{Hae} III digests of φX ssDNA, including in trace a only \textit{dnaB} protein, primase, DNA polymerase III holoenzyme, and 1.5 µg of SSB; trace b, prepriming proteins n, n', n", i, and dnaC, dnaB, 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 [%] autoradiograms. Arrows indicate the \textit{Hae} III fragments.
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 [32P]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. 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 4206 (Par 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.

- 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 dX ssDNA. dX 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 MgCl₂/1 mM dithiothreitol. DNA fragments (arrows) were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and transferred to DBM paper (21). [32P]-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 [32P]autoradiogram is shown.

1.5% agarose gels, denatured, and transferred to DBM paper (22). With the Hha I digest (10 bands), [32P]-labeled 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), [32P]-labeled DNA synthesized on Hae I fragments (Fig. 7A) and 3 plus 6 (Fig. 7B) 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 assumed 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 dX ssDNA → RF reaction (4). DNA replication is accelerated by preincubation of SSB-coated dX DNA with preincubating proteins in ATP

![Diagram](image)

FIG. 7. DNA synthesized on dX ssDNA Hha I fragments 6 and 3 plus 6 hybridization to Hae III or Hha I fragments. dX 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 [32P]-labeled DNAs synthesized on a Hha I digest of dX 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 60 hr to the DNA Hae III and Hha I fragments bound to DBM paper. Other procedures were as in Fig. 6.
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.

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