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Dynamics of DNA Polymerase III Holoenzyme of *Escherichia coli* in Replication of a Multiprimed Template*

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Movements of DNA polymerase III holoenzyme (holoenzyme) in replicating a template multiprimed with synthetic pentadecadeoxynucleotides (15-mers) annealed at known positions on a single-stranded circular or linear DNA have been analyzed. After extension of one 15-mer on a multiprimed template, holoenzyme moves downstream in the direction of chain elongation to the next primer. Holoenzyme readily traverses a duplex, even 400 base pairs long, to exploit its 3′-hydroxyl end as the next available primer. This downstream polarity likely results from an inability to diffuse upstream along single-stranded DNA. These holoenzyme movements, unlike formation of the initial complex with a primer, do not require ATP. Time elapsed between completion of a chain and initiation on the next downstream primer is rapid (1 s or less); dissociation of holoenzyme to form a complex with another primed template is slow (1–2 min). Thus, holoenzyme diffuses rapidly only on duplex DNA, probably in both directions, and forms an initiation complex with the first primer encountered. Based on these findings, schemes can be considered for holoenzyme action at the replication fork of a duplex chromosome.

DNA polymerase III holoenzyme is the multisubunit enzyme principally responsible for replication of *Escherichia coli* DNA (1–4). Upon activation by ATP, holoenzyme[1] within a few seconds forms a stable initiation complex with a primer-template, such as an RNA-primed, single-stranded, phage DNA circle (2, 3). With deoxynucleoside triphosphates present, replication proceeds processively at a rate of about 500 nucleotides/second at 30 °C (2, 3). Dissociation from the completed template, freeing holoenzyme to initiate another primed circle, requires ATP and is slow, consuming 1–2 min (3, 4). However, on a circle with several RNA primers, holoenzyme moves rapidly from the completed extension of one primer to another on the same template, bypassing the slow dissociation step (4). Thus the holoenzyme transfer to new primers on the same template is rapid in keeping with the physiological need for scarce holoenzyme molecules to extend primers on the discontinuous strand of the replication fork of a growing chromosome (1).

Questions about the polarity, dynamics, and energetics of holoenzyme movements are further explored by the present studies made possible by the use of multiple synthetic DNA primers at unique positions on circular and linear templates. The accompanying paper (5) examines how completely a template is replicated before the holoenzyme dissociates.

EXPERIMENTAL PROCEDURES

Materials—Sources were as follows: unlabeled and labeled nucleotides, Sigma and Amersham Corp., respectively; dAMP-PNP, gift of Dr. B. Alberts (San Francisco); φX, R199/G4*ori* and M13G*ori*1 viral DNAs, prepared as described (6); R199/G4*ori* phage, gift from G. N. Godson (New York); protected nucleotide dimers and polystyrene coupled protected nucleosides, Bachemgentec; Bio-Gel A-1.5m, Bio-Rad; restriction enzymes, New England Biolabs; T4 polynucleotide kinase, New England Nuclear; bovine serum albumin (Pentex), Miles Laboratories; SSB (4×10^4 units/mg) (7), gift from Dr. D. Soltis (this department); holoenzyme fraction V (7×10^6 units/mg), prepared as described (3); primase (3×10^6 units/mg, ≅ 70% pure) was purified (8) using an overproducing plasmid (9). Buffer A is 20 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 4% glycerol, 0.1 mM EDTA, 40 mM NaCl, 40 μg/ml of bovine serum albumin, and 5 mM dithiothreitol.

Synthesis of Primers—Oligodeoxyribonucleotide primers (15 mers) complementary to φX ssDNA were synthesized by the solid phase triester method using protected nucleotide dimers for coupling (10); synthesis of 15-mers complementary to M13G*ori*1 ssDNA used phosphoramidate nucleoside derivatives for coupling (11). Primer sequences were selected (Table I), with computer assistance, to minimize nonspecific annealing to other regions of the viral template.

Replication of Singly Primed DNA—To form singly primed DNA, a synthetic DNA 15-mer (1.6 μM as a 15-mer) was annealed at a molar ratio of 1000:1 to SSB-coated ssDNA (1.7 μg of SSB and 440 pmol, as nucleotide, of DNA) in buffer A (25 μl) for 5 min at 30 °C; hybridization was >95%.

Replication by holoenzyme of φX ssDNA primed with DNA primer 1 produced RF II DNA (>96%). In the absence of dNTPs, the initiation complex was unstable; incubation of holoenzyme with primed φX DNA and ATP (0.5 mM) led to a loss of replication activity due to the 3′→5′ exonuclease activity of the enzyme. Addition of 60 μM each of dCTP and dGTP (the 3′ terminal nucleotides of the primer and the first four nucleotides needed for synthesis) stabilized the initiation complex for at least 2 h. One dNTP and other combinations of two dNTP's failed to stabilize the system.

Replication of Multiply Primed DNA—Examining holoenzyme action on a multiprimed template (Fig. 1) depends on: (i) rapid and complete hybridization of several (*e.g.* four) unique synthetic DNA 15-mers to unique positions on φX ssDNA in the presence of SSB at 30 °C, (ii) formation of an initiation complex (on a uniquely positioned DNA primer) that will not dissociate and reassociate at other primer sites during the time of hybridization of other primers, and (iii) complete utilization of holoenzyme in initiation complexes, so that a significant amount of holoenzyme is not in free solution for association with other primers on the same template upon initiating elongation.

To examine the extent to which holoenzyme dissociates from the initiation complex and reassociates at other primer sites, holoenzyme

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[1] The abbreviations used are: holoenzyme, DNA polymerase III holoenzyme; ss, single-stranded; RF II, circular duplex with a small gap(s) or nick in one strand; φX, bacteriophage φX174; SSB, *E. coli* single-stranded DNA binding protein; dAMP-PNP, 2′-deoxy-5′-adenyl imidodiphosphate; rNTPs and dNTPs, ribo- and deoxynucleoside triphosphates, respectively; SDS, sodium dodecyl sulfate; kb, kilobase pair; bp, base pair.

TABLE I

Map positions and sequences of the synthetic oligodeoxyribonucleotide primers (15-mers)

The sum of the extinction coefficients $\epsilon_{260\,nm} \times 10^{-3}$ ($M^{-1}cm^{-1}$) of the individual bases was used to determine primer concentrations (as oligonucleotide); respective values for primers 1–7 were 166, 176, 178, 166, 184, 166, and 160.

Primer No.	Genome	Map position (5′ end)	Sequence (5′→3′)
1	φX174	2808	ATTGCCCGGCGTACG
2	φX174	887	GACCAGGGCGAGCGC
3	φX174	4792	CGCATAAAGTGCACC
4	φX174	4046	AGCCTCGATACGCTC
5	M13G*ori*1	2524	GAAACCATCGATAGC
6	M13G*ori*1	1376	TGCGGGATCGTCACC
7	M13G*ori*1	6802	GCTTTCGCCGTCCAT

was incubated for 1.5 min with a 5-fold molar excess of φX ssDNA primed with primer 1 and ATP, dGTP, and dCTP. Three other primers (primers 2, 3, and 4 in Table I) were then annealed at 50 μM each for either 1, 2, or 5 min. Synthesis was initiated upon addition of a mixture of dATP and [α-^{32}P]dTTP (1000–5000 cpm/pmol; final concentrations: 60 and 20 μM, respectively), followed in 2 s by a 1-min chase with 2 mM unlabeled dTTP to allow completion of the fragment which contained the initiation complex; 2 s is less than the time required to synthesize fragment 1 (1.92 kb). Hence, the percentage of ^{32}P incorporated in fragment 1 relative to the total ^{32}P incorporated is directly proportional to the percentage of holoenzyme remaining on primer 1 at the time of dATP and dTTP addition. Densitometric analysis of an alkaline agarose gel showed the ^{32}P in fragment 1 to be 95% of the total incorporation after an incubation time of 1 min, 92% after 2 min, and 84% after 5 min.

Complete hybridization of the other three primers within 1 min can be assumed, inasmuch as 5% or less of the holoenzyme dissociates from an initiation complex and transfers to another primer within 1 min. Replication of a multiprimed template from an initiation complex at primer 1 and lacking either primer 2, 3, or 4 will produce a fragment longer than 1.9 kb (3.4 kb if primer 2 is not annealed, 2.25 kb if primer 3 is missing, and 2.0 kb if primer 4 is missing). Upon repeating the above experiment and using various concentrations of primers 2, 3, and 4 in a 1-min incubation, a level of 27 μM of each of the primers yielded complete hybridization (>98%) to SSB-coated φX ssDNA, as determined from the nucleotide incorporation into the 1.9-kb fragment and into fragments greater than 1.9 kb.

The amount of holoenzyme in free solution after initiation complex formation was also determined by a challenge with another primed template circle. The initiation complex of holoenzyme with primer 1 annealed to φX DNA, formed in a 1.5-min reaction, was hybridized with primers 2, 3, and 4 (27 μM each for 1 min) and then challenged by addition of an equal amount (440 pmol) of RNA-primed R199/G4*ori* ssDNA circles (6.7 kb) in the presence of all four dNTPs. After 10 s of replication, the reaction was chased for 3 min with 2 mM unlabeled dTTP and then quenched with SDS/EDTA. Autoradiography of the DNAs separated by electrophoresis in a native agarose gel showed that less than 0.5% of the incorporated deoxynucleotides was associated with R199/G4*ori* DNA. Thus the initiation complex in the presence of an excess of primed φX ssDNA retains all the holoenzyme bound in a stable form.

Linearization of Circular φX ssDNA—The unique *Nci*I restriction nuclease site, formed by hybridization of primer 1 (3.2 nmol as 15-mer) with φX ssDNA (22 nmol as nucleotide) and SSB (85 μg) in 750 μl of buffer A for 20 min at 30 °C, was digested with 120 units of *Nci*I at 30 °C for 90 min. Linearization was confirmed by electrophoresis in a native 0.8% agarose gel. The digestion yields linear φX ssDNA with unique ends; upon cleavage of primer 1, the 6- and 9-base fragments dissociate from the template. The 6-base fragment at the 5′ end of primer 1, labeled with ^{32}P by T4 polynucleotide kinase, was not detected in the band of *Nci*I linearized DNA upon electrophoresis in a neutral 0.8% agarose gel; absence of the 9-base fragment at the 3′ end was indicated by the loss of replication activity upon linearization of the DNA.

Linearization of Circular M13Gori1 ssDNA—Hybridization of primer 5 (Table I) to viral M13G*ori*1 ssDNA forms a *Cla*I restriction nuclease site. Circular M13G*ori*1 ssDNA (160 nM as circles, 90 nmol as nucleotide) was hybridized with primer 5 (2.1 μM as 15-mer) in 65 μl of 10 mM Tris-HCl, 0.3 M NaCl, and 0.03 M sodium citrate (final pH 8.5) at 30 °C for 1 h, then diluted and linearized with 15 units of *Cla*I for 1 h at 37 °C in 315 μl containing 10 mM Tris-Cl (pH 7.5), 10 mM $MgSO_4$, and 1 mM dithiothreitol. The linearized ssDNA was extracted with phenol, precipitated with ethanol, and redissolved in a minimal volume of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA.

Two Kilobase Extension of Primer 7 on M13Gori1 DNA—M13G*ori*1 ssDNA was linearized using primer 5 and *Cla*I cleavage (see above). The linear DNA (2.0 pmol as linear molecules, 17.6 nmol as nucleotide) in 815 μl of buffer A containing 0.5 mM ATP, 60 μM each of dGTP and dATP, 20 μM [α-^{32}P]dTTP (600 cpm/pmol), and 53 μg of SSB was brought to 30 °C and primer 7 (a 15-mer complementary to the origin of G4 phage) was added to a final concentration of 1.6 μM (8.3 μl of a 160 μM solution); incubation was for 5 min at 30 °C to ensure full hybridization. A stoichiometric amount of holoenzyme was added (approximately 3 pmol) and incubated for 2 min to allow initiation complex formation. Then 18.5 μl of 3 mM dCTP was added (60 μM final) followed in 7 s by 74 μl of 0.5 M EDTA to rapidly halt polymerization. The DNA was precipitated with ethanol, redissolved in 150 μl of 0.5% SDS, 20 mM EDTA, and separated from remaining dNTPs by electrophoresis for 15 h at 40 V in a 0.8% neutral low melting agarose gel. The DNA product having an extended primer 7 (detected by long wavelength UV induced ethidium bromide fluorescence) was sliced out of the gel, phenol extracted twice, chloroform extracted, ethanol precipitated, and redissolved in 40 μl of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA (TE buffer). The size, 2 ± 0.2 kb, of extended primer 7 was determined by its position of migration in an alkaline gel relative to DNAs of known length.

Partial Extension of Primer 4 on Linear φX ssDNA—Primer 4 (1.6 μM) was hybridized to φX DNA (19 nM as circles, 33 nmol as nucleotide) and linearized by annealing of primer 1 and *Nci*I cleavage (see above) in the presence of 85 μg of SSB in 325 μl of buffer A. Then 0.5 mM ATP, 60 μM dCTP, and 60 μM dGTP were added with a 3-fold molar excess of holoenzyme (approximately 18 pmol) over linear DNA and incubated for 2 min at 30 °C to allow complete formation of the initiation complex. Primer 4 was extended approximately 400 nucleotides (determined by position of migration in an alkaline agarose gel relative to DNAs of known size) by a short pulse (<3 s) of replication by adding 13 μl of 1.5 mM dATP and 0.5 mM [α-^{32}P]dTTP (500 cpm/pmol) and quenching with 200 μl of 0.5 M EDTA. Incorporation of label indicated primer 4 was lengthened 500 bases. The agreement between incorporation of label and alkaline agarose gel electrophoresis in the length of extended primer 4 indicates all template molecules were utilized. The DNA was extracted with phenol, ethanol precipitated, resuspended in 200 μl of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and filtered over a Sephadex G-50 column in the same buffer to remove remaining dNTPs.

Complete Extension of Primer 4 on Linear φX ssDNA—A 250-μl reaction in buffer A contained 25 nmol (as nucleotide) of φX ssDNA (linearized as above), 61 μg of SSB, 60 μM each of dCTP, dGTP, and dATP, 20 μM [α-^{32}P]dTTP, and 1.6 μM primer 4; incubation at 30 °C was for 1 h. Extension of primer 4, initiated with 1.2 pmol of holoenzyme and monitored by acid-precipitable radioactivity, was complete in 30 min. After 45 min the reaction was quenched with 28 μl of 10% SDS and 11 μl of 0.5 M EDTA and filtered on a Bio-Gel A-1.5m column. The DNA in the excluded volume was precipitated with ethanol and redissolved in 50 μl of 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA.

RNA-primed R199/G4ori DNA—The 100-μl reaction containing 20 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 100 μg/ml of bovine serum albumin, 8 mM dithiothreitol, 4% glycerol, 0.5 mM ATP, 0.1 mM each of GTP, UTP, and CTP, 880 pmol (as nucleotide) of R199/G4*ori* ssDNA, 2.2 μg of SSB, and 440 ng of primase was incubated at 30 °C for 45 min to allow synthesis of the 28-nucleotide long RNA primer (12).

Product Analysis—Total incorporation of radiolabel was measured by mixing a 10-μl portion of the quenched sample with 0.1 ml of 0.1 M sodium pyrophosphate containing 20 μg/ml of salmon sperm DNA as carrier and 1.5 ml of 10% trichloroacetic acid. After 1 h at 0 °C, the precipitate was collected, washed, and dried on a GF/C filter and counted in a liquid scintillation counter.

Electrophoresis was in neutral 0.8% agarose gels in 90 mM Tris borate (pH 8.3), 2.5 mM EDTA and in alkaline 1.2% agarose gels in 30 mM sodium hydroxide, 1.0 mM EDTA. For autoradiography, dried gels were exposed to Kodak XAR-5 x-ray film for a series of times to ensure that the radioactive content of a gel band could be determined

by densitometry (Helena Laboratories Quick Scan, Jr.) in the linear range of film response; scan peaks were quantitated by weighing the paper.

RESULTS

Replication by Holoenzyme Is Processive on a Multiprimed Template—As is true for a circular template with multiple RNA primers (4), DNA synthesis was also processive on a ϕX DNA circle with multiple (*e.g.* four) synthetic DNA 15-mers as primers (Fig. 1). Autoradiography of a native agarose gel of products synthesized during replication of the template with four primers showed full length products within 14 s (Fig. 2*B*). Full length circles from singly primed DNA were formed within 11 s (Fig. 2*A*), indicating a turnover number of 500 nucleotides/s. The additional 3 s needed to replicate the 4-primer circle may be ascribed to the time consumed in transfers from a completed fragment to another primer on the circle. During the time in which full length products (RF II) were formed, the excess primed ssDNA remained unused (detected by UV-induced ethidium bromide fluorescence), showing that holoenzyme was processive in replication even on multiprimed DNA. Bands that migrated faster than RF II DNA were observed on the multiprimed (Fig. 2*B*) but not the singly primed DNA (Fig. 2*A*); presumably these products were partially replicated circles from which holoenzyme, after extending a primer, failed to transfer to another primer on the circle. Overall efficiency of replicating a 4-primer template was 75%, indicative of an efficiency of 90% or greater at each primer transfer.

Alkaline agarose gels of experiments, similar except that the multiprimed reactions were chased with unlabeled dTTP (to allow completion of fragments that were partially synthesized), showed four fragments of the sizes predicted from the location of each primer (Fig. 3). Thus the holoenzyme did not displace primers during replication. Fragment 1 (1.92 kb) was synthesized first, consistent with the location of the initiation complex; fragment 2 (1.50 kb) appeared next. As fragment 2 gained in abundance, fragment 3 (0.75 kb) and fragment 4 (1.24 kb) appeared with similar kinetics. The appearance of fragment 2 before fragment 3 and fragment 4 suggests that transfer of holoenzyme to another primer is preferentially in the elongation direction. However, the overall pattern of fragment synthesis in this particular experiment does not exclude holoenzyme movement in the opposite direction or some skipping over primers.

When the initiation complex was formed on primer 2 rather

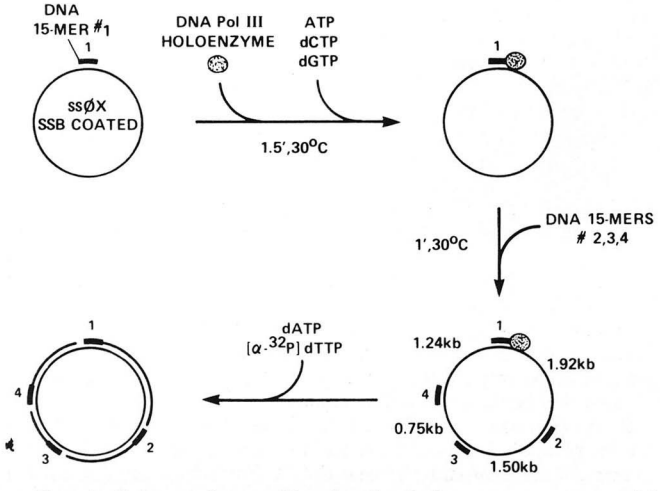

FIG. 1. **Scheme for replication by holoenzyme on a multiprimed template.**

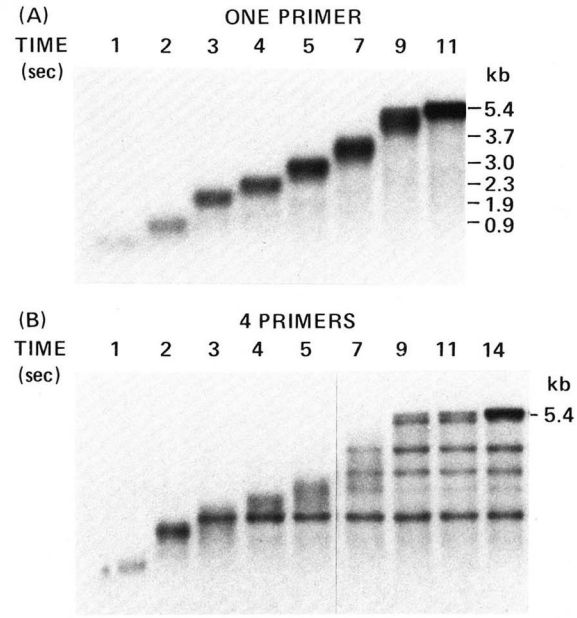

FIG. 2. **Time course of replication on singly primed and multiprimed ϕX DNA analyzed by native agarose gel electrophoresis.** Replication reactions (25 μl) contained 550 pmol (as nucleotide) of ϕX ssDNA, 2.1 μg of SSB, 0.5 mM ATP, 60 μM each of dCTP, dGTP, and dATP, 20 μM [α-^{32}P]dTTP, and approximately 0.020 pmol of holoenzyme (5-fold molar excess of DNA circles over holoenzyme) in buffer A. After quenching with 24 μl of 1% SDS and 40 mM EDTA, the products were electrophoresed on a native 0.8% agarose gel and autoradiographed. *A*, singly primed with primer 1. Holoenzyme (0.5 μl) was incubated with ϕX ssDNA primed with primer 1, SSB, ATP, dCTP, and dGTP in 24 μl of buffer A for 1.5 min at 30 °C to form an initiation complex. Replication was initiated with 1 μl of a solution containing dATP and [α-^{32}P]dTTP and quenched at the times indicated. The amount of duplex DNA on the circle (*kb*) was obtained by comparing size standards to the partially replicated products in an alkaline gel. *B*, multiprimed with four unique DNA primers. The initiation complex between holoenzyme and ϕX DNA primed with primer 1 was formed as described in *A* except the reaction volume was 18 μl. The DNA was multiply primed at 30 °C for 1 min with 6 μl of a mixture of 27 μM each of primers 2, 3, and 4. Replication was initiated and quenched as described. RF II DNA migrates as 5.4-kb DNA.

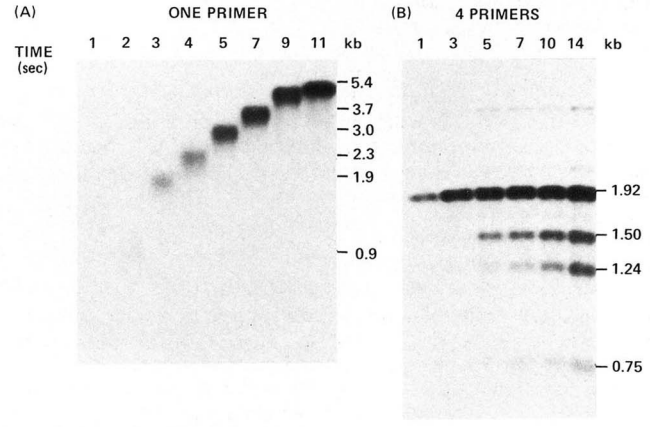

FIG. 3. **Time course of replication on singly primed and multiprimed ϕX DNA analyzed by alkaline agarose gel electrophoresis.** Autoradiograms of 1.2% alkaline agarose gels: *A*, replication reactions of Fig. 2*A*; *B*, replication reactions of Fig. 2*B* except that each reaction was chased for 30 s by adding 6 μl of 9.3 mM unlabeled dTTP before quenching with SDS/EDTA. Numbers to the right of *panel B* are sizes of DNA fragments in the gel that are predicted from the locations of the DNA primers on the ϕX genome. Band positions are consistent with the predicted sizes based on the migration of numerous size markers of 0.3–2.7 kb.

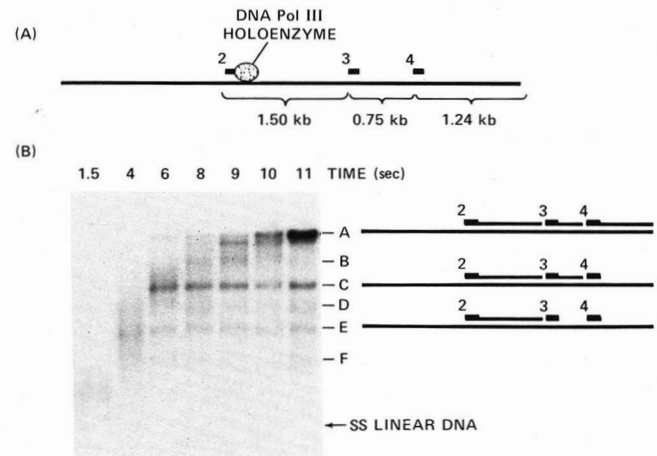

FIG. 4. **Time course of replication on a linear multiprimed template.** *A*, the initiation complex from which replication was initiated was formed as described in the legend to Fig. 2*B* except ϕX ssDNA linearized by *Nci*I and primed with primer 2 in 20 μl was used; 4 μl of 40 μM each of primer 3 and primer 4 was added to form the multiprimed template. *B*, an autoradiogram of a native 0.8% agarose gel of a series of replication reactions of the initiation complex in *A*. Replication was initiated with dATP and [α-^{32}P]dTTP and quenched with 25 μl of 1% SDS, 40 mM EDTA. Bands *A*, *C*, and *E* have structures shown to the right (see text) and *B*, *D*, and *F* are discussed in the text. The *arrow* marks the unreplicated multiprimed linear ϕX ssDNA detected by ethidium bromide UV fluorescence.

than primer 1, the patterns of replication were essentially identical. Thus the manner of holoenzyme transfer between intramolecular primers was not significantly affected by sequence or location of the initial primer or by the lengths of the product fragments.

Transfer to New Primers Occurs in the Downstream (Elongation) Direction—The polarity of holoenzyme transfers to primers on a circle (Fig. 1) was confirmed on a linear ϕX ssDNA template with three primers. Holoenzyme was incubated with ATP, dCTP, and dGTP and linear ϕX ssDNA primed with primer 2 for 1.5 min at 30 °C; then, primers 3 and 4 (27 μM each) were hybridized for 1 min (Fig. 4*A*). Replication was initiated with dATP and [α-^{32}P]dTTP and continued for various times before quenching with SDS/EDTA; products were analyzed on a native agarose gel (Fig. 4*B*). Full length products were formed within 11 s showing that holoenzyme transferred to the new primers in the elongation direction. Primer transfer was confirmed by autoradiography of the replication reactions on an alkaline agarose gel; three fragments of the sizes predicted by the locations of the primers were observed (data not shown). Densitometric analysis of this autoradiogram showed that following the appearance of fragment 2, both fragments 3 and 4 were produced. Fragment 3 was twice as abundant as fragment 4 after 8 s of replication. Linear DNA, singly primed with primer 2, required only 10 s for completion of replication (data not shown). Thus, transfer of holoenzyme to primers on the triply primed linear template required only about one-half s/transfer.

Holoenzyme Does Not Skip over Primers—During replication of the triply primed template with the initiation complex on primer 2 (Fig. 4*A*), holoenzyme did not skip over primer 3 and initiate synthesis on primer 4. The three major product bands (*A*, *C*, and *E* in Fig. 4*B*) correspond in size to the extensions of primer 2, of primers 2 and 3, and of all three primers. Two minor bands (*D* and *F*) migrate in the positions found after replication of a linear template with only primer 3 (at the position of band *D*) or with only primer 4 (at the position of band *F*). Inasmuch as neither product contains fragment 2, these minor products are not intermediates in replication of the template initiated on primer 2. Such low levels of these products are likely due to a small amount of dissociation and intermolecular (intertemplate) transfer of holoenzyme during the 1-min hybridization with primers 3 and 4.

The DNA of bands *C* and *E* (Fig. 4*B*) were further characterized by restriction enzyme digestion. *Xho*I and *Stu*I each cut duplex ϕX DNA once, *Xho*I in fragment 2 and *Stu*I in fragment 3. Band *E* was removed only by *Xho*I, whereas band *C* was removed by both *Xho*I and *Stu*I, consistent with their proposed structures (Fig. 4*B*). Accumulation of intermediates at these band positions may result from pausing of holoenzyme before transfer to a new primer. Were there a product in which primer 3 was skipped, its size would place it between bands *C* and *A*. Close inspection of Fig. 4*B*, does show a faint band in this region (band *B*). If this minor product were the gapped template containing fragments 2 and 4, but not fragment 3, it should not be cleaved by *Stu*I which cuts duplex ϕX DNA once at a position 307 bases from the 5′ end of fragment 3 and does not cleave ssDNA under these conditions. To examine this possibility, an 8-s replication reaction (as in Fig. 4*B*) was quenched with EDTA, precipitated with ethanol and digested with *Stu*I. Before *Stu*I digestion, products are seen at bands *C* and *E* (Fig. 5, *lane 1*); intermediates of the putative gapped template should be present in the smear above and below band *C*. However, *Stu*I removed more than 96% of the radioactivity above band *E* (Fig. 5, *lane 2*). (Band *G* was removed by digestion with *Xho*I, consistent with the structure shown (data not shown).) The absence of significant radioactivity in the region of *lane 2* corresponding to the smear in *lane 1* shows that the proposed gapped template is not present in a significant amount. Thus, holoenzyme proceeded in the elongation direction and did not skip a primer during replication of a template with multiple DNA primers.

Holoenzyme Can Traverse a Long Stretch of Duplex DNA—Transfer of holoenzyme to a downstream primer (15-mer) implies that the enzyme can diffuse over at least a 15-bp length of double-stranded DNA. To determine if the enzyme can traverse a longer stretch of duplex DNA, primer 4 was annealed to the linear ϕX DNA template and extended ap-

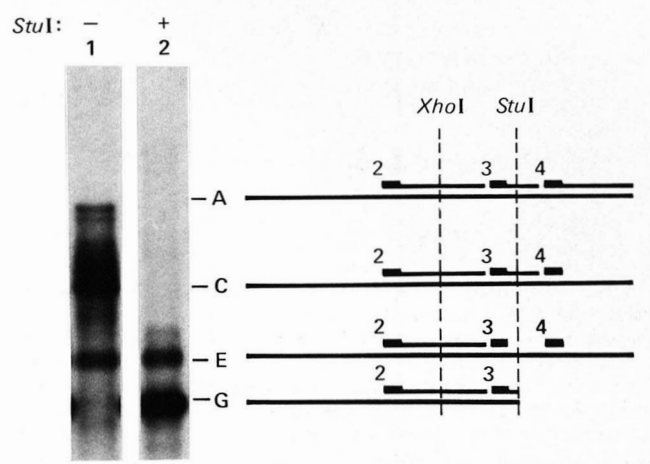

FIG. 5. **Analysis of replication intermediates of a multiprimed linear template by restriction nuclease cleavage.** The replication reaction was as described in the legend to Fig. 3 except it was scaled up 3-fold and quenched with 75 μl of 40 mM EDTA after 8 s of replication. The DNA was ethanol precipitated and redissolved in 200 μl of buffer A. Samples (36 μl) were incubated at 37 °C for 1 h with either no additions (*lane 1*) or with 2 units of *Stu*I (*lane 2*). Bands *A*, *C*, and *E* correspond to those of Fig. 4.

proximately 400 bases ("Experimental Procedures"). Hybridization of primer 3 and incubation with holoenzyme, ATP, dCTP, and dGTP for 1.5 min yielded stable initiation complexes with holoenzyme either at primer 3 or at the extended terminus of primer 4; densitometric analysis of autoradiograms of an alkaline gel of the replication reactions showed that these initiation complexes formed in approximately equal amounts. A molar ratio of 1 holoenzyme to 30 DNA templates was used to minimize template molecules with two initiation complexes. Primer 2 (27 μM) was then hybridized in a 1-min reaction. Elongation reactions were initiated on the resulting complexes (Fig. 6*A*) by adding dATP and [α-^{32}P]dTTP and quenched by SDS/EDTA after various times at 30 °C. An autoradiogram of a native gel analyzing the time course of replication is in Fig. 6*B*. Since [α-^{32}P]dTTP of low specific activity was used in extending primer 4, the starting triply primed template appears at position D (note the zero time point). Two elongation products were formed within 7 s (*B* and *C*, Fig. 6*B*) and most likely arose from the two initiation complexes (Fig. 6*A*). Analysis of the alkaline agarose gel patterns showed only fragments of the size predicted for fully triple primed templates (>98%). Elongation of the initiation complex on primer 3 gave fragment 3 and fully extended fragment 4 (band B) within 7 s; therefore, holoenzyme was capable of translocating across a stretch of 400 bp of duplex DNA. The low amount of product (band) *A*, containing fragments 2, 3, and 4, relative to products *B* and *C* indicates that holoenzyme did not move to a significant extent to a primer in the upstream (anti-elongation) direction. Measurement of the efficiency of holoenzyme transfer over the 400-bp duplex DNA was not possible in this experiment since extension of primer 3 to the 5′ end of primer 4 without reinitiation on primer 4 would yield template migrating at the position of band *C*.

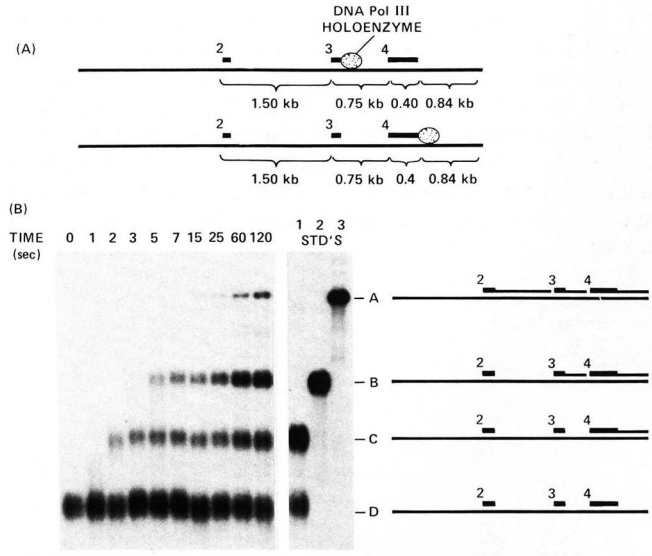

FIG. 6. **Products of replication on a multiprimed template including a 400-bp primer.** *A*, two possible initiation complexes formed with holoenzyme and a linear ϕX DNA containing primer 3 and an extended primer 4 ("Experimental Procedures"). The conditions of initiation complex formation were essentially as in the legend to Fig. 2*B* except holoenzyme was added to a linear template that contained both primer 3 and the extended primer 4 in 22 μl. Addition of 2 μl of 80 μM primer 2 and incubation for 1 min at 30 °C completed the construction of the two enzyme-DNA complexes shown. *B*, autoradiogram of a native 0.8% agarose gel of replication reactions initiated by 1 μl of dATP and [α-^{32}P]dTTP (9000 cpm/pmol) and quenched at the indicated times with 25 μl of 1% SDS, 40 mM EDTA. Standards *1*, *2*, and *3* were made by replicating linear templates containing only extended primer 4, only primer 3, or only primer 2, respectively. Replication products *A*, *B*, and *C* are likely the structures shown at the right.

The speed and efficiency of transfer over a longer stretch of duplex DNA was measured using a substrate (Fig. 7*A*) prepared from the larger M13G*ori*1 ssDNA genome (8.6 kb). The M13G*ori*1 ssDNA circle was linearized by a method analogous to that used to linearize ϕX DNA. Primer 7 was annealed to the linear template and extended approximately 2 kb ("Experimental Procedures"). Primer 6 (1.6 μM) was annealed in a 2-min reaction and incubation with holoenzyme, ATP, dCTP, and dGTP for an additional 2 min gave stable initiation complexes with holoenzyme either at primer 6 or at the extended terminus of primer 7 (Fig. 7*A*). A stoichiometry of 1 holoenzyme to 12 DNA templates was used to avoid forming two initiation complexes on the same template molecule. Synchronous elongation reactions of these complexes was initiated by adding dATP and [α-^{32}P]dTTP and quenched by SDS/EDTA after various times at 30 °C. An autoradiogram of a native agarose gel analysis of the replication time course is shown in Fig. 7*A*. The starting template is the smear at position *D*. Three replication products are observed (bands *A*, *B*, and *C*; the band between *A* and *B* will be discussed in Fig. 7*B*). Product *C* appeared within 6 s and co-migrates with the replication product of a control linear template having only primer 7 (data not shown). Hence product *C* resulted from elongation of the initiation complex formed on the 2-kb primer 7. Product *A* appeared within 13–22 s and co-migrates with the replication product of a control linear template with primer 6 fully extended to the end (data not shown). Therefore, product *A* was formed upon elongation by an initiation complex at primer 6 to the end of the 2-kb primer 7, rapid transfer across the 2-kb long primer 7, reinitiation, and replication to the end of the template. Product *B* appears complete within 13 s. Its kinetics, position, and diffuse appearance indicate the structure shown at the right wherein holoenzyme initiated synthesis at primer 6 and completely extended it but did not traverse the 2-kb duplex DNA of extended primer 7. Densitometric analysis of products *A* and *B* at 22 s indicates that approximately 65% of the initiation complex at primer 6 successfully transferred across the 2-kb primer 7 to reinitiate and complete the template. Densitometric analysis of product *C* relative to products *A* and *B* at 22 s indicates that the two initiation complexes were formed in approximately equal amounts (±5%).

The time course of replication from primer 6 on linear M13G*ori*1 DNA without a downstream primer is shown in Fig. 7*B*. The initiation complex is diagrammed at the top of Fig. 7*B* and below is the autoradiogram of a neutral agarose gel analysis of the replication time course. The full length product (band *A*) appears within 18–22 s. The less than full length band is due to pausing (or premature termination) since addition of excess holoenzyme results in the single full length product (data not shown). The replication time of the template either with or without the 2-kb stretch of duplex DNA (Fig. 7, *A* and *B*, respectively) was 18–22 s. Hence, the time for transfer across the 2-kb duplex did not exceed 4 s (*i.e.* the time required to replicate 2 kb of ssDNA).

Transfer of Holoenzyme from a Linear DNA Template to Another RNA-primed Template Is Slow—In the previous experiment (Fig. 6), holoenzyme may have failed to transfer intramolecularly to primer 2 due to a rapid dissociation from the end of linear duplex DNA upon completing fragment 4. In the intermolecular transfer of holoenzyme from RF II to another primed circle, dissociation requires ATP and takes 1–2 min; holoenzyme reassociates with another primed tem-

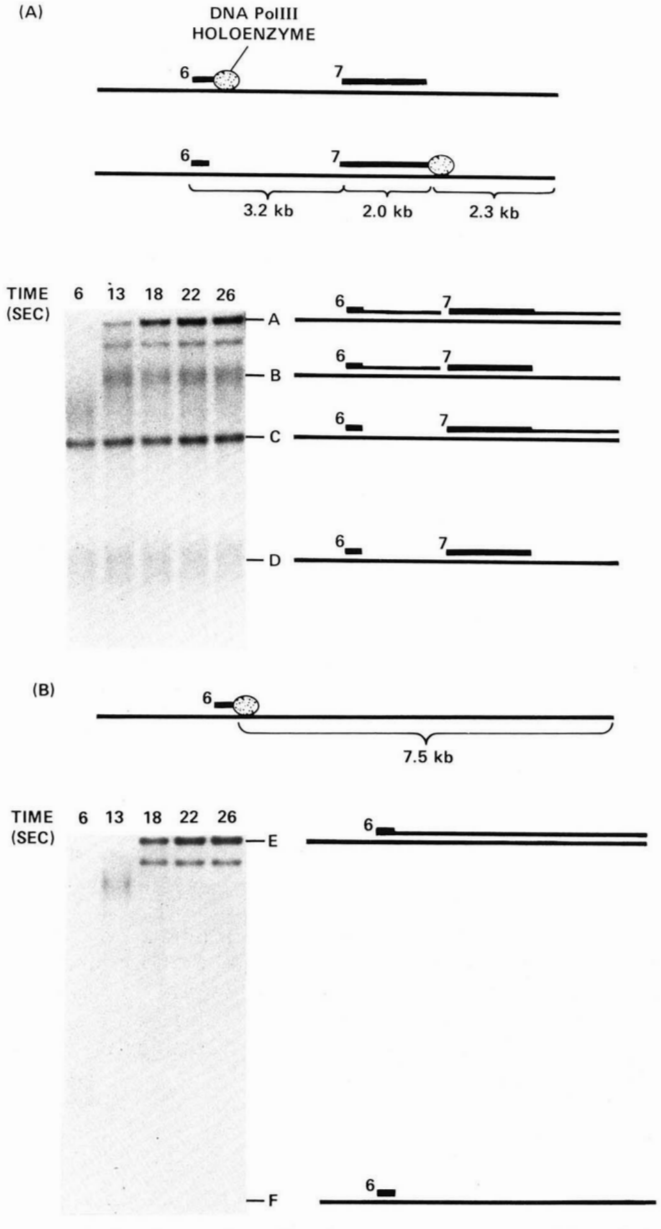

FIG. 7. **Products of replication on a multiprimed template including a 2-kb primer.** *A*, the top two linear DNA templates show the two possible locations of holoenzyme on a linear M13G*ori*1 DNA primed with primer 6 and extended primer 7 ("Experimental Procedures"). The conditions of initiation complex formation were essentially as described in the legend to Fig. 2*B* except holoenzyme was added to the doubly primed linear template in 24 μl. The autoradiogram is of a native 0.8% agarose gel of replication reactions initiated by adding 1 μl of dATP and [α-^{32}P]dTTP (5000 cpm/pmol) and quenched at the indicated times with 25 μl of 1% SDS, 40 mM EDTA. Standards prepared by replicating linear templates having only primer 6 or primer 7 migrated at the positions marked *A* and *C*, respectively. The smear at position *D* is the starting doubly primed template. Replication products *A*, *B*, and *C* are likely the structures shown at the right. *B*, the DNA structure at the top shows the initiation complex between holoenzyme and a linearized M13G*ori*1 DNA molecule primed with primer 6 and formed under essentially the same conditions as above. The autoradiogram is of a native 0.8% agarose gel of replication reactions initiated by adding 1 μl of dATP and [α-^{32}P]dTTP (5000 cpm/pmol) and quenched at the indicated times with 25 μl of 1% SDS, 40 mM EDTA. The position of starting material visualized by UV-induced ethidium bromide fluorescence is marked *F*. Band *E* is the full length product.

plate within 3 s (2, 3). To determine the relative rates of dissociation of holoenzyme from linear and circular duplexes, elongation by an initiation complex on a ϕX DNA circle was initiated in the presence of a 2-fold excess of RNA-primed R199/G4*ori* circles. (R199/G4*ori* ssDNA, a chimera of f1 DNA derivative R199 (13) with 276 bases of G4 DNA containing the origin for complementary strand synthesis (14), had been primed with RNA using primase.) Replication of the R199/G4*ori* DNA, by requiring dissociation of holoenzyme from replicated ϕX DNA, measures the dissociation rate. Whether the initiation complex was formed on primer 4 annealed to either circular or linear ϕX DNA, formation of R199/G4*ori* RF II DNA occurred at the same slow rate (Fig. 8), indicating that holoenzyme dissociates from a linear duplex as slowly as from a circular one. Holoenzyme either remained bound to the end of the replicated linear template or diffused along the duplex product. The low levels and slow appearance of template containing fragment 2 in the experiment of Fig. 6 (product *A*) are within the range expected for intermolecular cycling of holoenzyme from the replicated products *B* and *C*. This result may be taken as evidence that, while bound tightly to DNA, holoenzyme does not diffuse along SSB-coated ssDNA.

Processive Synthesis on Multiprimed DNA Does Not Occur in the Upstream (Anti-elongation) Direction—To test further the conclusion that primer transfer does not occur in the anti-elongation direction (Fig. 6), the products of replication on another linear template were determined (Fig. 9). The holoenzyme initiation complex was formed on primer 3 on a template which also included hybridized primer 2 and completed fragment 4. Only 10% of the products at 15 s (Fig. 9*B*) contain both fragments 2 and 3, indicating that holoenzyme rarely transferred to primer 2. Transfer to primer 2 was probably an intermolecular event that occurred during the hybridization of primer 2. There was no significant accumulation of product containing both fragments 2 and 3, even after 1 min when a 5-fold excess of RNA-primed R199/G4*ori* DNA was present during the hybridization of primer 2 (Fig. 9*C*). Thus holoenzyme transfer to an upstream intramolecular

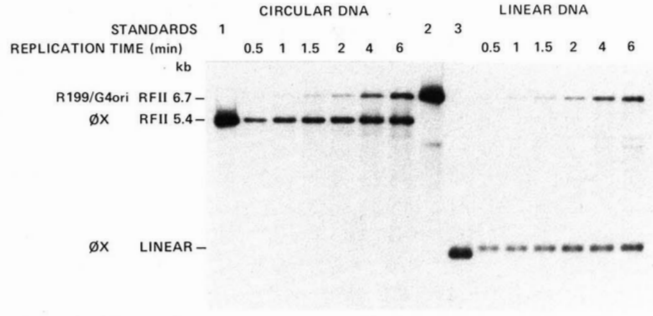

FIG. 8. **Transfer of holoenzyme from replicated circular or linear DNA to another primed circular DNA.** The autoradiogram of a native 0.8% agarose gel of a replication time course of synthesis initiated on circular (*left*) or linear (*right*) ϕX DNA. An initiation complex was formed in a 2-min incubation of holoenzyme (~20 fmol) with either circular or linear ϕX ssDNA (900 pmol as nucleotide, an 8-fold molar excess as circles) primed with primer 4 in 72 μl of buffer A containing 0.5 mM ATP, 60 μM dCTP, 60 μM dGTP, and 3.9 μg of SSB. Replication was initiated at 30 °C upon adding 78 μl of buffer A containing RNA primed R199/G4*ori* DNA (1800 pmol as nucleotide), 0.5 mM ATP, 60 μM dCTP, 60 μM dGTP, 120 μM dATP, 40 μM [α-^{32}P]dTTP, and 7.8 μg of SSB. At the times indicated 19-μl samples of the reaction were incubated 45 s at 30 °C with 1 μl of 87 mM unlabeled dTTP before quenching with 20 μl of 1% SDS, 40 mM EDTA. Standards 1, 2, and 3 are replication products of circular ϕX ssDNA primed with primer 4, RNA primed R199/G4*ori* DNA, and linear ϕX ssDNA primed with primer 4, respectively.

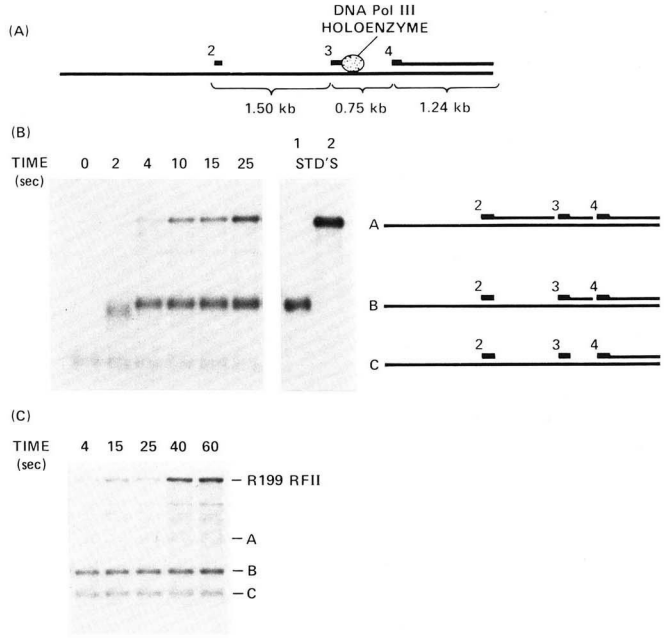

FIG. 9. **Replication of a multiprimed linear template with a completed downstream duplex structure.** *A*, initiation complex on primed ssDNA. Linear ϕX DNA having a completed fragment 4 was prepared as described under "Experimental Procedures." The initiation complex was formed on primer 3 and then primer 2 was annealed as in Fig. 6. *B*, autoradiogram of a native 0.8% agarose gel. Replication reactions were initiated with 1 μl of dATP and [α-^{32}P]dTTP and quenched at the indicated times with 25 μl of 1% SDS, 40 mM EDTA. Standards 1 and 2 were prepared by replicating linear ϕX DNA containing either primer 3 or primer 2, respectively. Bands *A* and *B* are likely the structures shown to the right and band *C*, the starting template, since [α-^{32}P]dTTP of low specific activity was used in the synthesis of fragment 4. *C*, autoradiogram of a native 0.8% agarose gel of replication reactions which differ from those of *B* in containing a 5-fold excess of RNA primed R199/G4*ori* DNA during the hybridization of primer 2 and being chased for 1 min by 2 mM unlabeled dTTP before quenching. *Lettered bands* correspond to those in *B*.

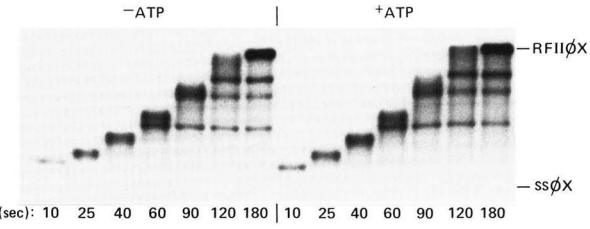

FIG. 10. **Effect of ATP on replication of a circle with multiple DNA primers.** An initiation complex of holoenzyme (600 fmol) and DNA primer 1 on ϕX ssDNA (16.5 nmol as nucleotide) was formed in 250 μl of buffer A containing 64 μg of SSB, 0.5 mM ATP, 60 μM dCTP, and 60 μM dGTP. After 1 min at 30 °C, the reaction was chilled and filtered over a 6-ml Bio-Gel A-1.5m column at 5 °C in buffer A containing 60 μM each of dCTP and dGTP to stabilize the initiation complex. The void fractions were pooled (300 μl total volume). The template (115 μl) was multiprimed with 77 μl of 17 μM each of primers 2, 3, and 4 in buffer A containing dCTP and dGTP. Replication was initiated with 8 μl of 1.5 mM dAMP-PNP and 0.5 mM [α-^{32}P]dTTP in the absence (*left*) or presence of 2 mM ATP (*right*). At the times indicated, 25-μl samples were quenched with 25 μl of 1% SDS, 40 mM EDTA, subjected to electrophoresis in a native 0.8% agarose gel, and autoradiographed.

primer requires a dissociation event. In addition, the rate of appearance of R199/G4*ori* RF II DNA (Fig. 9) was similar to that for cycling from a replicated singly primed circular ϕX DNA (data not shown).

The structure of the duplex end of the linear template (Fig. 8) did not influence the dissociation and upstream transfer of holoenzyme (from fragment 3 to primer 2). The linear template containing fragment 4 was treated with *Sac*II which removes 59 bases from the duplex end and leaves a two-base overhang on the 3′-hydroxyl strand. Replication of the *Sac*II-treated template gave the same pattern of products as those shown in Fig. 9 for the untreated template (data not shown).

Coating of ssDNA with SSB is not responsible for impeding holoenzyme transfer to an upstream primer. In experiments carried out in the absence of SSB, holoenzyme formed an initiation complex, was processive in synthesis on ssDNA, and transferred to downstream primers but failed to transfer to the upstream primer 2 under conditions given in Fig. 9 (data not shown).

ATP Is Not Required for Processivity in Replication of a Multi-DNA Primed Template—Although ATP hydrolysis is required to initiate processive DNA synthesis by holoenzyme on a singly primed template (2, 3), it is not needed for subsequent initiations on a multi-DNA primed template (Fig. 10). An initiation complex with primer 1 and circular ϕX DNA (as in Fig. 1) was filtered on Bio-Gel A-1.5m (in buffer A containing dCTP and dGTP) to remove ATP. After hybridization of primers 2, 3, and 4, elongation was initiated with the ATP analog, dAMP-PNP, and [α-^{32}P]dTTP. (dAMP-PNP is incorporated in place of dATP during replication but does not replace ATP in supporting initiation complex formation which requires hydrolysis of the β, γ-phosphodiester bond of ATP or dATP.) Autoradiography of a native gel of the time course of replication showed that full length circles were formed at the same rate in the absence as in the presence of ATP (Fig. 10); the rate of replication with dAMP-PNP is, however, only one-tenth that with dATP. Products of the replication reactions examined by alkaline agarose gel analysis showed four fragments of the predicted size, confirming that hybridization of the primers was complete and that all primers were used in elongation (data not shown). The absence of ATP or dATP contamination in the dAMP-PNP or the dCTP, dGTP, and dTTP preparations was demonstrated by the inability of these nucleotide samples to support initiation of processive replication by holoenzyme on primed ϕX DNA in the absence of ATP and dATP (data not shown).

Holoenzyme Can Traverse RNA Primers in the Elongation Direction—To determine whether holoenzyme processivity on a template multiply primed with RNA primers (4) is in the elongation direction as observed with DNA primers, a novel hybrid primer template was prepared (Fig. 11). M13G*ori*1 DNA is a chimera, 8623 nucleotides long, of M13 DNA and 2216 nucleotides of G4 DNA that includes the G4 origin for priming complementary strand synthesis (14). Hybridization of primer 6 to linearized M13G*ori*1 DNA ("Experimental Procedures") and addition of dCTP and dGTP enables holoenzyme to form a stable initiation complex with the primed DNA. The initiation complex was formed with sufficient primase to synthesize the 28-nucleotide RNA primer at the G4 origin in 1 min (Fig. 11) (15). Replication was initiated with dATP and [α-^{32}P]dTTP and quenched with SDS/EDTA at various times after initiation. The appearance of full length products (Fig. 11*B*) showed that holoenzyme traversed the RNA primer in the direction of elongation. An alkaline gel showed two major products (95% of total radioactivity) corresponding to the 3.2- and 4.3-kb fragments, thus confirming that RNA priming had occurred (data not shown). Priming required that the four rNTPs be present during the 1-min incubation with primase; ATP alone was not sufficient. Com-

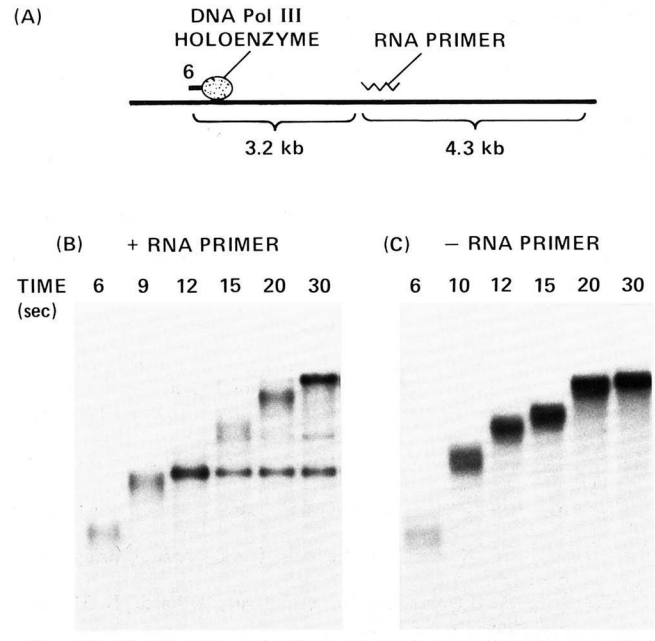

FIG. 11. **Replication of a linear template containing an RNA primer.** *A*, the initiation complex on DNA- and RNA-primed linear M13G*ori*1 DNA. Linear M13G*ori*1 template was prepared as described under "Experimental Procedures." The initiation complex was formed in a 25-μl reaction containing 550 pmol of linear ss-M13G*ori*1 DNA (as nucleotide), 1.9 μg of SSB, 60 μM each of dCTP, dGTP, and dATP, 20 μM [α-^{32}P]dTTP, 1 mM ATP, 100 μM each of CTP, GTP, and UTP in buffer A. Primer 6 was added to 1.6 μM and incubated for 10 min at 30 °C to allow complete hybridization. Holoenzyme (20 fmol) was added and incubated for 2 min to allow complete initiation complex formation. Primase (42 ng) was added and incubated for 1 min prior to initiating DNA synthesis to allow RNA primer formation at the G4 origin. *Panel B* shows an autoradiogram of a native 0.8% agarose gel of replication reactions that were initiated by adding 26 μl of a solution of buffer A containing the same concentration of nucleotides as above including 120 μM dATP, 40 μM [α-^{32}P]dTTP and, as a challenge template, 550 pmol of circular ϕX ssDNA (as nucleotide) primed with primer 1 and 1.9 μg of SSB. Reactions were quenched at the indicated times by adding an equal volume of SDS/EDTA. *Panel C* shows replication reactions which were performed as in *panel B* except primase was not added and the reactions were initiated by adding 1 μl of 1.5 mM dATP, 0.5 mM [α-^{32}P]dTTP without the challenge template.

parison of the time course of replication of linear M13G*ori*1 DNA primed only with DNA primer 6 (Fig. 11*C*) with that of the doubly primed template (Fig. 11*B*) showed that holoenzyme paused for about 3 s in traversing the RNA primer. This 3-s pause is in agreement with the previous estimate of 2–5 s required for intramolecular primer transfers by holoenzyme (4). That primase was not required for transfer across the RNA primer was shown by an experiment analogous to that of Fig. 6. The M13G*ori*1 DNA was primed by primase, treated with 0.5% SDS, and extracted with phenol; primer 6 was annealed and then holoenzyme (one-tenth the molar level of template) was added. Products of replication contained both 3.2- and 4.3-kb fragments on one template strand within 30 s showing that holoenzyme which had associated with the DNA primer could traverse the RNA primer in the absence of primase (data not shown).

DISCUSSION

A multiprimed template, primed with RNA (4) or with synthetic DNA 15-mers (this study), is fully replicated without dissociation of DNA polymerase III holoenzyme. The enzyme transfers in a second or less from a completed fragment to another primer on the same template. With DNA 15-mers as primers, the transfer was exclusively to the next primer downstream (direction of elongation) and synthesis was reinitiated without ATP or dATP. The enzyme rapidly traversed even a 2-kb stretch of duplex DNA. These rapid transfers suggest that holoenzyme moves on DNA by linear diffusion. Thus the ATP-activated initiation complex proceeds processively to complete replication of available template. Upon encountering a 5′ terminus in its path, the enzyme diffuses over it and any downstream duplex DNA (or RNA-DNA hybrid) to locate the next available primer terminus. Efficiency of transfer is indifferent to whether the 5′ terminus encounter is with a 5′-triphosphate of an RNA primer made by primase or a 5′-hydroxyl of a synthetic DNA primer.

Holoenzyme failed to transfer to a primer in the upstream (anti-elongation) direction, even when no downstream primer was available (Fig. 9). This polarity of holoenzyme movement in the absence of an energy (*e.g.* ATP) requirement suggests that holoenzyme is unable to diffuse rapidly over ssDNA. This conclusion is also supported by the observation that holoenzyme did not traverse ssDNA to skip an available primer when moving downstream (Fig. 5). In keeping with an inability to diffuse on ssDNA, holoenzyme, once bound to a primer, does not move to newly hybridized primers (with only dCTP and dGTP). This inability of holoenzyme to diffuse over ssDNA did not depend on the ssDNA being coated with SSB.

It is remarkable that the initiation complex is so stable. The half-time of holoenzyme dissociation from a primer terminus is greater than 15 min, but upon completing the replication of available template and encountering a duplex structure in its path, the holoenzyme releases from the terminus in a second or less in search for another primer terminus on the same template. The role of ATP in these apparently related but fundamentally different processes is significant. Hydrolysis of the ATP-activated holoenzyme is required to form the initiation complex at a primer 3′ terminus (2, 3). ATP is also required to dissociate the initiation complex

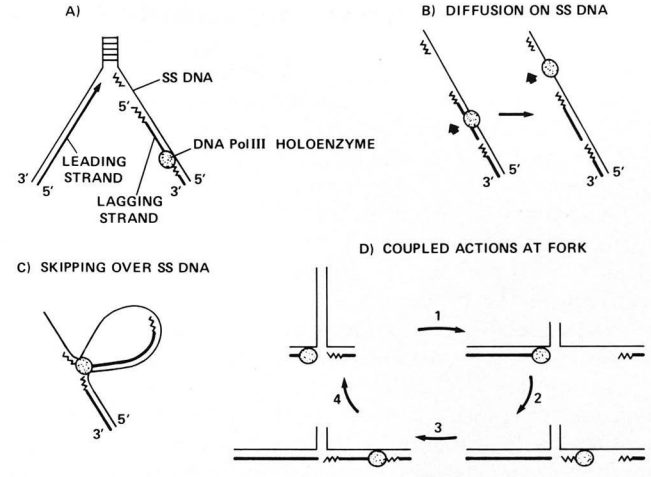

FIG. 12. **Schemes for holoenzyme transfer to an upstream primer without dissociation from the template.** *A*, model of a replication fork. *B–D*, schemes by which the holoenzyme may locate an upstream RNA primer on the lagging strand. *B*, sliding over duplex DNA and ssDNA. *C*, skipping over duplex DNA and ssDNA. *D*, movement of the lagging strand polymerase is coupled to that of the leading strand polymerase; elongation of the leading strand, priming of the lagging strand and diffusion of the holoenzyme over the primer to initiate a new Okazaki fragment (step 1) is followed by extension of the primer (step 2), and finally diffusion of the holoenzyme back upstream to the parental duplex to allow leading strand synthesis to resume.

during the slow release of holoenzyme from the template. However, the rapid release of holoenzyme from the 3′ terminus of a completed fragment to locate and initiate a new primer terminus on the same template molecule does not require ATP; holoenzyme remains bound to the template presumably through associating with duplex DNA and is still "activated." It would appear that ATP enables holoenzyme to grip the template rather than the 3′ terminus. Dissociation of holoenzyme from the initiation complex requires the breakage of this firm bond to DNA and is a slow and ATP-dependent process (4). In contrast, transfer from the 3′ terminus of a completed stretch of DNA and diffusion on duplex DNA retains the activated attachment of the holoenzyme.

In assessing the behavior of holoenzyme upon termination of its processive synthesis, it became important to define precisely how completely a template is replicated. The subsequent report (5) shows that synthesis proceeds right up to the 5′ terminus of a duplex, whether it be RNA or DNA, without leaving a gap.

Despite the information gained in these studies about the dynamics of holoenzyme, major questions remain about its structure and behavior at the replication fork of a duplex chromosome (Fig. 12). Does a holoenzyme molecule, in synthesizing the lagging strand, dissociate after completing the nascent (Okazaki) fragment and reassociate with the newly synthesized RNA primer upstream, all within a second? Does the holoenzyme diffuse on the template DNA to find this primer despite the intervening stretch of single-stranded DNA? These movements might require other factors to facilitate transfer of the holoenzyme over single-stranded DNA by either diffusion (Fig. 12*A*) or skipping (Fig. 12*B*). Despite the failure of holoenzyme in these studies (Fig. 6) to bind two primers on the same template, a dimeric structure might still exist as part of a larger replisome (16) and thus enable the holoenzyme to remain associated with more than one 3′-hydroxyl primer terminus. Movement on the parental duplex of the polymerase replicating the lagging strand template may be coupled to synthesis of the leading strand (Fig. 12*C*). A pause in the leading strand synthesis during initiation of an Okazaki fragment would prevent an upstream ssDNA gap and thus allow holoenzyme to remain activated and to diffuse back over the nascent fragment to the parental duplex. In view of our lack of knowledge about structure and organization of holoenzyme, these and other schemes can be considered that would enable the relatively few holoenzyme molecules in the cell to achieve the synthesis of leading and lagging strands, as well as participate in chromosomal repair and recombination processes, and in extrachromosomal DNA synthesis.

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