

Complete Replication of Templates by *Escherichia coli* DNA Polymerase III Holoenzyme*

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DNA polymerase III holoenzyme (holoenzyme) processively and rapidly replicates a primed single-stranded DNA circle to produce a duplex with an interruption in the synthetic strand. The precise nature of this discontinuity in the replicative form (RF II) and the influence of the 5' termini of the DNA and RNA primers were analyzed in this study. Virtually all (90%) of the RF II products primed by DNA were nicked structures sealable by *Escherichia coli* DNA ligase; in 10% of the products, replication proceeded one nucleotide beyond the 5' DNA terminus displacing (but not removing) the 5' terminal nucleotide. With RNA primers, replication generally went beyond the available single-stranded template. The 5' RNA terminus was displaced by 1–5 nucleotides in 85% of the products; a minority of products was nicked (9%) or had short gaps (6%). Termination of synthesis on a linear DNA template was usually (85%) one base shy of completion. Thus, replication by holoenzyme utilizes all, or nearly all, of the available template and shows no significant 5'→3' exonuclease action as observed in primer removal by the "nick-translation" activity of DNA polymerase I.

EXPERIMENTAL PROCEDURES

Materials—Sources were as follows: NAD⁺, Sigma; calf intestinal alkaline phosphatase (further purified by chromatography on Seapacryl-200), Boehringer Mannheim; exonuclease III and restriction enzymes, New England Biolabs; T4 polynucleotide kinase, New England Nuclear; *E. coli* DNA ligase, gift from I. R. Lehman (this department); NACS-52 resin, Bethesda Research Laboratories; NA45 DEAE membrane, Schleicher & Schuell; and low melting agarose (Seaplaque), Marine Colloids Div., FMC Corp. Buffer A is 20 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 4% glycerol, 0.1 mM EDTA, 40 µg/ml of bovine serum albumin, and 5 mM dithiothreitol. All other chemicals, nucleic acids, and enzymes not described here were from sources or prepared as in the previous paper (5).

Oligodeoxyribonucleotide-primed M13Gori1 DNA—An oligodeoxyribonucleotide (15-mer), 5'-GCTTTGCCGTCCAT-3', was synthesized by the solid phase triester method using protected nucleotide dimers for successive coupling reactions (6), separated from incomplete products by electrophoresis in a 20% polyacrylamide sequencing gel, and extracted from the gel by the crush and soak method (7). The 15-mer was further purified by binding to NACS-52 in 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and 0.25 M ammonium acetate followed by washing with the same buffer and elution with 4.0 M ammonium acetate, 10 mM Tris-HCl (pH 7.2), 1 mM EDTA, and freed of salt by repeated lyophilizations; its sequence was confirmed by analysis (8). Hybridization of the synthetic DNA 15-mer to 4500 pmol (as nucleotide) of M13Gori1 ssDNA was in 10 mM Tris-HCl, 0.3 M NaCl, and 0.03 M sodium citrate (final pH 8.5) using a 10-fold molar excess of the DNA primer (extinction coefficient at 260 nm of 160,000 M⁻¹ cm⁻¹). Hybridization was complete within 1 h at 30 °C as shown by replication (90%) of the input ssDNA to RF II DNA by holoenzyme.

Replication of DNA- and RNA-primed M13Gori1 DNA—The 200-µl reactions contained 3500 pmol (as nucleotide) of M13Gori1 DNA, 10.4 µg of SSB, 1.0 mM ATP, 10 µM each of CTP, GTP, and UTP, 50 µM each of dCTP, dGTP, and dATP, and 20 µM [α-³²P]dTTP (5000 cpm/pmol) in buffer A. For M13Gori1 DNA primed with the synthetic DNA 15-mer, replication was initiated with 4.8 µg of holoenzyme (approximately one-half the molar concentration of circles) and quenched after 5 min at 30 °C with 10 µl of 10% SDS. In the case of RNA priming coupled to DNA replication, 2 µg of primase (50-fold molar excess over circles) and 4.8 µg of holoenzyme were incubated with M13Gori1 DNA for 5 min at 30 °C before quenching with 10 µl of 10% SDS. Replication of the samples was complete as measured by trichloroacetic acid-precipitable counts.

Preparation of PvuII/AvaII Fragments—DNA products from replication reactions of DNA or RNA primed circular M13Gori1 DNA were extracted with phenol/chloroform (1:1), precipitated with ethanol, and resuspended in 30 µl of 10 mM Tris-HCl (pH 7.4), 10 mM MgSO₄, 1 mM dithiothreitol, and 50 mM NaCl. Each sample was digested with AvaII (10 units) and PvuII (5 units) for 30 min at 37 °C, quenched with 1.5 µl of 10% SDS, and analyzed by electrophoresis in a 2.0% agarose gel in 90 mM Tris borate (pH 8.1), 2.5 mM EDTA, and 1 µg/ml of ethidium bromide. After resolution from the 384-bp fragment, the 155-bp fragment was electroeluted into a NA45 DEAE membrane and removed from the membrane by three successive extractions with 100 µl of 20 mM Tris-HCl (pH 8.0), 1 M NaCl, and 0.1 mM EDTA at 70 °C for 20 min.

A PvuII/AvaII fragment uniquely labeled with ³²P at the PvuII 5' terminus was prepared from M13Gori1 RF I DNA. The M13Gori1 RF I DNA (15 µg) was separated from small nucleic acid contaminants by electrophoresis as described above except in a 0.8% low melting agarose gel. The RF I DNA was eluted from the gel, purified by

DNA polymerase III holoenzyme, the multisubunit replicative enzyme of *Escherichia coli*, rapidly and processively converts a primed, single-stranded DNA circle to the duplex form (1–4). The nature of the discontinuity in the synthetic strand of the replicated product (RF II¹) has remained uncertain. How closely the huge holoenzyme can approach the 5' terminus of an RNA or DNA primer, the influence of substituents at the 5' terminus, and the extent of nucleolytic or strand displacement activity of the enzyme on 5' termini have been important and unresolved questions. With more data available about the dynamics of holoenzyme movements on a template (5), the need for information about termination of replication by holoenzyme has become more pressing. The present studies establish that the holoenzyme almost invariably completes the replication of all available template residues, leaving the 5' end of the initiating primer strand intact.

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¹ The abbreviations used are: RF II, circular duplex with a small gap, nick, or a few extra nucleotides in one strand; holoenzyme, DNA polymerase III holoenzyme; ssDNA, single-stranded DNA; φX, bacteriophage φX174; bp, base pair; SSB, *E. coli* single-strand DNA binding protein; SDS, sodium dodecyl sulfate.

phenol/chloroform extractions, precipitated with ethanol, resuspended in 20 μ l of 10 mM Tris-HCl (pH 7.4), 10 mM MgSO₄, 50 mM NaCl, and 1 mM dithiothreitol, and linearized by incubating 1 h at 37 °C with *Pvu*II (12 units). The DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 50 μ l of 50 mM Tris-HCl (pH 9.0), 1.0 mM MgCl₂, 0.1 mM ZnCl₂, and 1 mM spermidine; the 5' phosphate was removed by treatment with calf intestinal alkaline phosphatase (0.5 units) for 5 min at 37 °C. The dephosphorylated DNA was extracted with phenol/chloroform, precipitated with ethanol, redissolved in 20 μ l of 50 mM Tris-HCl (pH 7.6), 2 μ M [γ -³²P]ATP (10⁷ cpm/pmol), 10 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM spermidine, and 0.1 mM EDTA, and incubated with T4 polynucleotide kinase (15 units) for 30 min at 37 °C to phosphorylate the 5' termini. The labeled DNA was extracted with phenol/chloroform, precipitated with ethanol, redissolved in 30 μ l of 10 mM Tris-HCl (pH 7.4), 10 mM MgSO₄, 50 mM NaCl, and 1 mM dithiothreitol, and digested with *Ava*II (24 units) for 1 h at 37 °C. The reaction was quenched with 1.5 μ l of 10% SDS and subjected to electrophoresis in a native 2.0% agarose gel; the 155-bp *Ava*II/*Pvu*II fragment was purified using a NA45 DEAE membrane as described above.

Replication of DNA-primed Linear ϕ X DNA—The ϕ X ssDNA (19 pmol, as circles; 105 nmol as nucleotide) was primed with 320 pmol (as 15-mer) of a primer (primer 1) (5) that hybridizes over the unique *Nci*I site at position 2802 in 30 μ l of 10 mM Tris-HCl, 0.3 M NaCl, 0.03 M sodium citrate (final pH 8.5). The primed circular ϕ X DNA (9000 pmol as nucleotide) was linearized by nuclease *Nci*I as described in Ref. 5 and then extracted with phenol/chloroform (1:1). The linear DNA (4300 pmol as nucleotide), from which the fragments of the cleaved 15-mer have dissociated, was primed again by incubating at 30 °C for 20 min with a 200-fold molar excess of another 15-mer (5) (primer 2, which anneals at position 4047) in 115 μ l of buffer A containing 12.8 μ g of SSB, 0.5 mM ATP, 50 mM each of dCTP, dGTP, and dATP, and 20 μ M [α -³²P]dTTP (5000 cpm/pmol). Replication was initiated with 5 μ g of holoenzyme and quenched after 5 min at 30 °C with 6 μ l of 10% SDS and 5 μ l of 0.5 M EDTA. A size standard for position of termination on linear ϕ X DNA was prepared from circular ϕ X ssDNA (8600 pmol as nucleotide) primed with a 200-fold molar excess of primer 2 (see above) at 30 °C for 20 min in 230 μ l of buffer A containing 25.6 μ g of SSB, 0.5 mM ATP, 50 μ M each of dCTP, dGTP, and dATP, and 20 μ M [α -³²P]dTTP (5000 cpm/pmol). Replication was initiated upon adding 10 μ g of holoenzyme and quenched after 5 min at 30 °C with 12 μ l of 10% SDS and 10 μ l of 0.5 M EDTA. Replication of the linear and circular DNA was essentially complete as measured by acid-insoluble radioactivity.

Preparation of *Sac*II/*Nci*I Fragments—DNA products from replication reactions of DNA-primed linear and circular ϕ X DNA were extracted with phenol/chloroform (1:1), precipitated with ethanol, and resuspended in 100 μ l of 10 mM Tris-HCl (pH 7.4), 10 mM MgSO₄, and 1 mM dithiothreitol. The circular, replicated DNA was digested with *Sac*II (30 units) and *Nci*I (24 units) for 1 h at 37 °C. Fragments shorter than 58 bases were prepared by incubating 75 μ l of the *Sac*II/*Nci*I digest of the replicated circular DNA with 0.5 unit of exonuclease III and quenched after 30 s at 30 °C with 25 μ l of 1% SDS, 40 mM EDTA. The linear replicated DNA (linearized with *Nci*I before replication) was digested only with *Sac*II (30 units) for 1 h at 37 °C. The samples were extracted with phenol/chloroform (1:1), precipitated with ethanol, washed, rinsed twice with 70% ethanol, and resuspended in 10 μ l of loading buffer for polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis—Electrophoresis was at 2000 V in an 8% polyacrylamide, 1% *N,N'*-methylenebisacrylamide gel containing 7 M urea, 36 mM Tris borate (pH 8.1), and 1 mM EDTA poured between 20 \times 50-cm glass plates using 0.4-mm spacers. Samples, resuspended from ethanol precipitation (holoenzyme-replicated samples) or lyophilization (base-specific cleavage reactions) in 8 μ l of 80% formamide, 0.2% xylene cyanol, 0.7% bromphenol blue, 45 mM Tris borate (pH 8.1), and 1.25 mM EDTA, were heated to 90 °C for 2 min and chilled on ice just prior to loading on the gel. A sequence ladder was made using the end-labeled *Ava*II/*Pvu*II fragment prepared from M13Gori1 RF I DNA. The base-specific cleavage reactions were performed as described by Maxam and Gilbert (9); the G, C, and C + T reactions were at 0 °C for 20 min and the G + A reaction was at 37 °C for 20 min. Autoradiography of dried gels and quantitation of autoradiograms were as described (5).

RESULTS

Replicated Circular DNA Primed with a DNA 15-mer Can Be Covalently Closed by *E. coli* DNA Ligase—A synthetic DNA 15-mer complementary to the 3' terminal portion of the 28-residue RNA primer synthesized by primase on G4 DNA (10, 11; see "Experimental Procedures") was labeled at its 5' terminus (with T4 polynucleotide kinase and [γ -³²P]ATP) and hybridized to M13Gori1 viral ssDNA. (The latter is a chimera of M13 DNA, 6407 residues, and G4 DNA, 2216 residues, that contains the G4 origin (12).) The primed DNA was replicated by DNA polymerase III holoenzyme and the purified products (98% conversion to RF II; Fig. 1, lane 2) upon treatment with *E. coli* DNA ligase were covalently closed to RF I DNA; the latter becomes supercoiled upon intercalation of ethidium bromide. Over half of the RF II was sealed by ligase within 5 min, consistent with the amount of ligase present; the reaction neared completion within 20 min with a limit of 92% conversion to RF I by 60 min. Trials with other preparations of *E. coli* ligase gave similar results. Inasmuch as this ligase typically seals DNA at a nick in a strand of

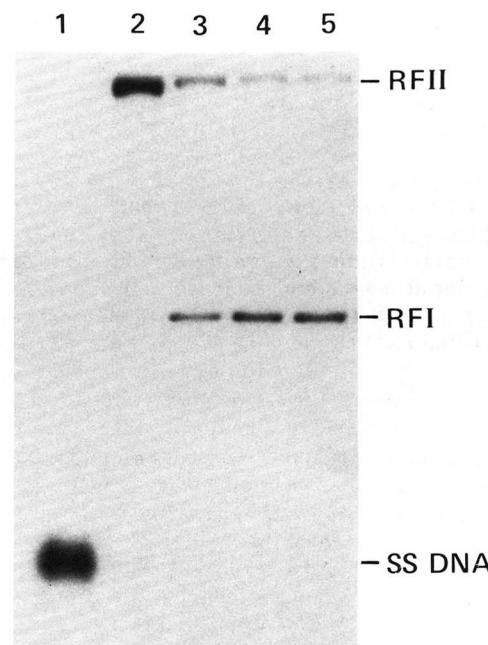


FIG. 1. Action of *E. coli* DNA ligase on replicated M13Gori1 primed with an oligodeoxyribonucleotide. Autoradiogram of a native agarose gel analysis of the products of *E. coli* DNA ligase action on M13Gori1 DNA primed with a DNA 15-mer and replicated by holoenzyme. The replication reaction contained 4532 pmol (as nucleotide) of M13Gori1 ssDNA primed with 5'-³²P-phosphorylated DNA 15-mer, 13 μ g of SSB, 0.5 mM ATP, 20 μ M dTTP, and 50 μ M each of dCTP, dGTP, and dATP in 100 μ l of buffer A. Replication was initiated with 6 μ g of holoenzyme and, after 5 min at 30 °C, quenched and extracted with 100 μ l of phenol/chloroform (1:1). After precipitation with ethanol, 168 pmol (as nucleotide) of RF II DNA was treated with 0.1 μ g of *E. coli* DNA ligase in 40 μ l of 30 mM Tris-HCl (pH 8.0), 4 mM MgCl₂, 50 mM NAD⁺, 1.2 mM EDTA, 1 mM dithiothreitol, and 50 μ g/ml of bovine serum albumin. Samples of 10 μ l were removed after 5, 20, and 60 min at 30 °C and added to 15 μ l of 1% SDS and 40 mM EDTA; 20- μ l portions were analyzed by electrophoresis in a 0.8% agarose gel in 90 mM Tris borate (pH 8.1), 2.5 mM EDTA, and 1 μ g/ml of ethidium bromide. Lane 1, primed M13Gori1 ssDNA (135 pmol as nucleotide); lane 2, replicated M13Gori1 DNA (90 pmol as nucleotide); lanes 3, 4, and 5, replicated M13Gori1 DNA (50 pmol as nucleotide) after phenol extraction and 5, 20, and 60 min of reaction with *E. coli* DNA ligase, respectively; positions of primed DNA (lane 1), replicated DNA (lane 2), and RF I DNA (visualized by UV-induced fluorescence of ethidium bromide) are marked.

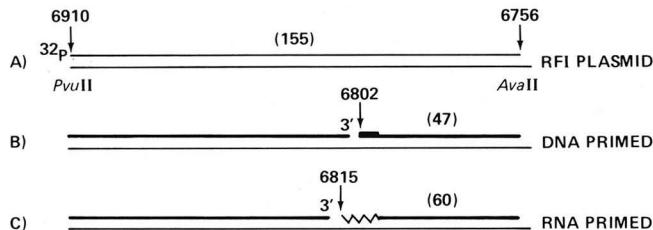


FIG. 2. Diagram of the 155-bp *Pvu*II/*Ava*II fragment of M13Gori1 DNA containing the G4 origin. The map positions are for M13Gori1 DNA. The 5' end of the DNA 15-mer primer (solid block) is position 6802. The 5' ATP of the RNA primer (sawtooth line) synthesized by primase is position 6815. The numbers in parentheses are the lengths of the small arms (including the primers) of the replicated DNA strands on the fragment. The map position of the *Ava*II end is the 3' terminal nucleotide of the complementary strand.

duplex DNA (13), it may be presumed that the predominant product of holoenzyme action in this instance has a nick in the synthetic strand.

The Terminus of Replicated DNA Circles Primed with DNA—The position at which holoenzyme terminates replication was determined by polyacrylamide gel analysis of the size of the product digested by restriction nucleases (Fig. 2). M13Gori1 DNA, primed with the synthetic DNA 15-mer, was replicated by holoenzyme. Digestion of the products with both *Pvu*II (single cut at position 6910) and *Ava*II (cuts at 6756 and 7296) yields fragments of 7928, 384, and 155 bp. The smallest fragment (separated from the larger ones by electrophoresis) contains the G4 origin and thus the primer and presumably the terminus of the synthetic strand (Fig. 2B).

The discontinuity in the synthetic strand of the 155-bp fragment creates a short arm of 47 nucleotides that includes the synthetic primer and a long arm that includes the terminus. The length of the long arm was determined in a polyacrylamide sequencing gel relative to fragments of known length (Fig. 3). The major species (about 90%) of the long arm of the synthetic DNA (Fig. 3, lane 3) coincides in its migration with a fragment of 108 residues ending at guanine 6802. Whereas the reference fragments produced by the base-specific cleavage reactions have a phosphate at both 3' and 5' termini, the long arm of the 155-bp fragment has phosphate only at the 5' end. Hence, this fragment migrates slower and really coincides with a standard that has one less nucleotide (e.g. 107 residues). Thus, holoenzyme terminates replication of the template by including the G residue at 6803 and abutting it to the G residue (6802) at the 5' end of the primer. With respect to the minor species (about 10%; Fig. 3, lane 3), its migration to a position one nucleotide longer than the major species indicates that the synthetic strand terminates at G 6802.

To account for the extra length of this small fraction of the product several possibilities may be considered: (i) displacement of the 5' terminal residue of the primer (G 6802), (ii) nucleolytic removal of G 6802, and (iii) contamination by a 14-mer in the 15-mer primer preparation. Explanation iii is unlikely because in the synthesis of the 15-mer, dimeric nucleotides are successively coupled to a single nucleotide 3'-linked to a resin and so should yield only odd numbered products. The virtual absence of 5'→3' exonuclease activity from holoenzyme preparations² leaves displacement of the 5' terminus as the most probable basis for extension of the synthetic strand by an extra nucleotide.

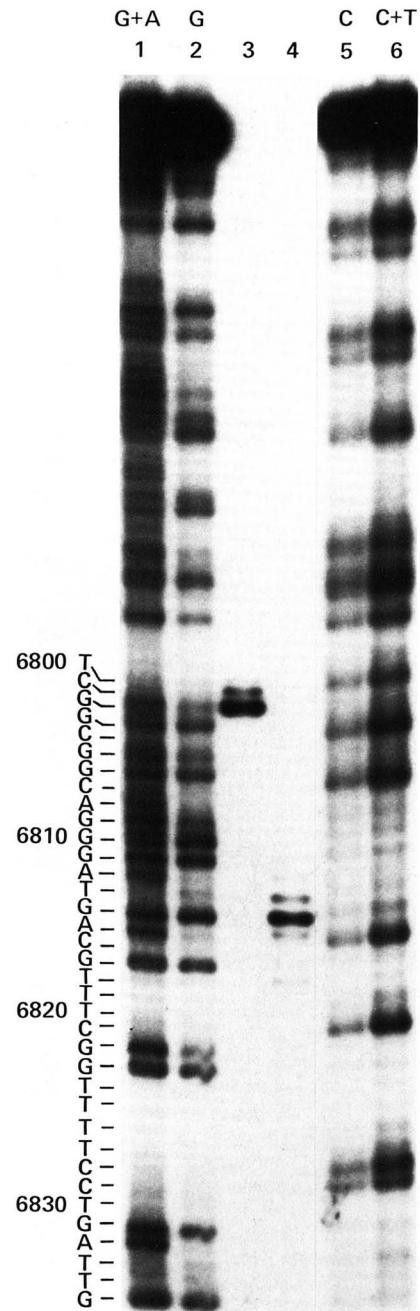


FIG. 3. Polyacrylamide gel electrophoresis of the long arm of the *Pvu*II/*Ava*II fragment of M13Gori1 DNA replicated by holoenzyme. Autoradiogram of an 8% polyacrylamide sequencing gel containing the *Pvu*II/*Ava*II fragments isolated from M13Gori1 DNA replicated by holoenzyme and primed *in vitro* with either the synthetic DNA 15-mer (lane 3) or the RNA primer synthesized by primase (lane 4). A sequencing ladder identical to the long arm of the *Pvu*II/*Ava*II fragment was prepared by performing the Maxam-Gilbert base-specific cleavage reactions (lane 1, G + A; lane 2, G; lane 5, C; lane 6, C + T) on the 155-bp *Pvu*II/*Ava*II fragment prepared from M13Gori1 RF I plasmid DNA and uniquely labeled at the *Pvu*II 5' terminus ("Experimental Procedures"). The sequence and numbers correspond to positions on the M13Gori1 sequence map. Although the cleavage at T's in the C + T cleavage reaction was suppressed, the sequence ladder was still unambiguous owing to the known sequence of the ϕ X genome (14). Faint bands are visible at every nucleotide in the pyrimidine ladders allowing placement of the missing T's at positions known to have T's from the published sequence and supported by the absence of A, G, and C cleavage fragments.

² H. Maki and A. Kornberg, personal communication.

G+A G C C+T

1 2 3 4 5 6

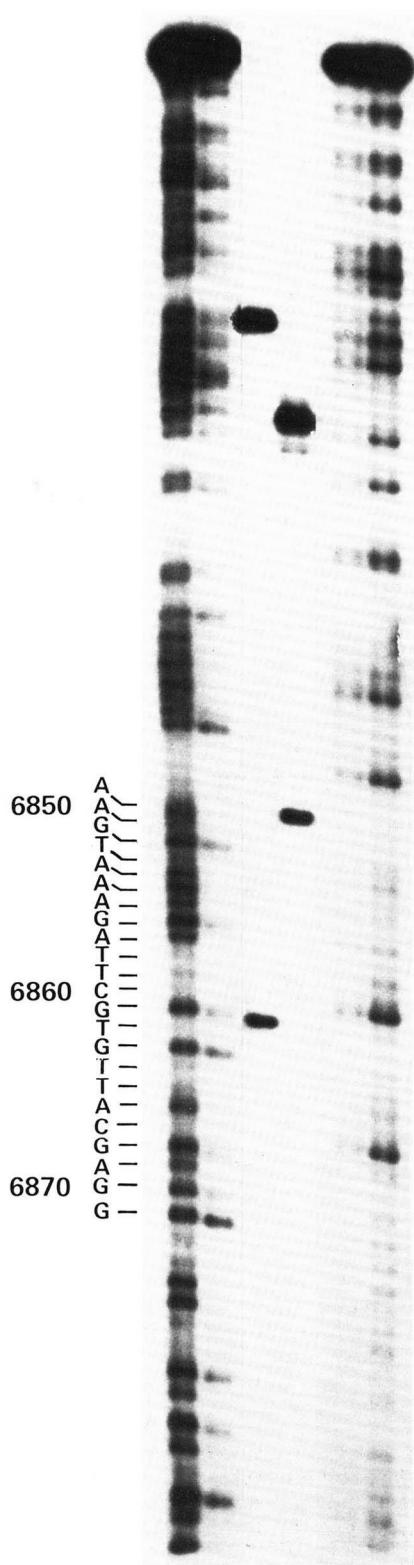


FIG. 4. Polyacrylamide gel electrophoresis of the short arm of the *Pvu*II/*Ava*II fragment of M13Gori1 DNA replicated by holoenzyme. Autoradiogram of an 8% polyacrylamide sequencing gel containing the base-specific cleavage fragments of the 155-bp *Pvu*II/*Ava*II fragment prepared from RF I DNA and the *Pvu*II/*Ava*II fragments from M13Gori1 DNA primed with a synthetic DNA primer (lane 3) or an RNA primer (lane 4) and replicated by holoenzyme. The base specific cleavage reactions are in lane 1 (G + A), lane 2 (G),

TABLE I
Termini of replication by DNA polymerase III holoenzyme on a circular DNA template primed with DNA or RNA

Primer	3' terminal nucleotide	Final DNA structure	Product
DNA	G 6803	Nick	90
	G 6802	Displaced G	10
RNA	T 6819	Gap of 3 residues	4
	T 6818	Gap of 2 residues	2
	G 6817	Gap of 1 residue	<1
	C 6816	Nick	9
	A 6815	Displaced A	67
	G 6814	Displaced AG	12
	T 6813	Displaced AGT	3
	A 6812	Displaced AGTA	2
	G 6811	Displaced AGTAG	<1

Direct measurement of the length of the short arm of the strand in the 155-bp fragment should disclose a minor species with 46 residues (in addition to a major one of 47 residues) were the 5' end of the primer absent at the outset or removed later. As determined by electrophoresis (Fig. 4, lane 3), only a single major band was observed even after a prolonged film exposure that would have detected a minor species (e.g. 46 nucleotides). The unique band migrated to the position of T 6803 (48 nucleotides) consistent with a strand of 47 nucleotides; the lack of 5' phosphate residues on the short arm strand partially accounts for this somewhat slower migration. Thus, holoenzyme in approaching the 5' terminus of a DNA primer in its path leaves no gap in most instances, but occasionally extends the strand by displacing the 5' terminal residue (Table I).

The Terminus of Replicated DNA Circles Primed with RNA—*E. coli* primase synthesizes an RNA primer on G4 DNA at the complementary strand origin from a unique point (10, 11). When coupled to replication, the length of RNA synthesized by primase depends on the concentrations of rNTPs and dNTPs (15, 16) and varies from 2 to 9 nucleotides under the conditions used here. The RF II product of the reaction was treated with *Pvu*II and *Ava*II to generate the 155-bp fragment as for the DNA-primed reaction (above). The lengths of the long arm of the synthetic strand determined by electrophoresis on a sequencing gel (Fig. 3, lane 4) are shorter than those from the DNA-primed product, consistent with the start of the 5' RNA primer terminus (Fig. 2C). A major species (67%) of 96 nucleotides terminates at position A 6815 (corrected for the lower charge to length ratio relative to the standard fragments), presumably displacing the 5' RNA terminal nucleotide. Slower migrating products indicate that holoenzyme can replicate and displace even further beyond the 5' RNA terminus. Fragments migrating faster than the major species indicate terminations of replication that generate a nick or leave a small gap (Table I).

As was true for the DNA-primed reaction, the short arm appears as a single band indicative of a unique start (Fig. 4, lane 4); no other bands were detected on longer exposure (data not shown). The short arm, with a 5'-ribonucleoside triphosphate and a 3'-hydroxyl moiety has the same net charge of its termini (minus four) as the reference base-specific cleavage fragments. The fragment migrates to a position between A 6849 and A 6850, 60 and 61 nucleotides in

lane 5 (C), and lane 6 (C + T). The sequence and numbers correspond to positions on the M13Gori1 sequence map. The slow migrating bands in the gel (lanes 3 and 4) are the long arms of the *Pvu*II/*Ava*II fragments.

length, respectively; a short arm initiated at A 6815, the primase origin, and ending at the *Pvu*II site should be 60 nucleotides long. Thus, holoenzyme generally continues replication until one or a few of the 5' terminal nucleotides of an RNA primer are displaced or, less frequently, a nick or small gap is left (Table I).

The Terminus of Replicated Linear Templates—The position at which holoenzyme terminates replication on a linear template was determined by polyacrylamide gel analysis of the size of product at the terminus of a linear template. A ϕ X174 ssDNA circle was linearized at position 2803 by annealing a synthetic DNA 15-mer at the unique *Nci*I site followed by restriction nuclease digestion (5); fragments of the DNA 15-mer produced by nuclease cleavage dissociate from the linearized ϕ X DNA. The linear DNA, primed by annealing a DNA 15-mer at position 4047, was replicated by holoenzyme and the product was cleaved with *Sac*II which removes a small fragment from the terminus (Fig. 5). The size of the *Sac*II terminal fragment was determined by comparing its migration in a sequencing gel (Fig. 5, lane 3) to that of a 58-base fragment prepared by *Sac*II and *Nci*I digestion of replicated circular ϕ X DNA primed at position 4047 (lane 1); a size ladder was made by slight digestion of the reference fragment with exonuclease III (lane 2). The major product (85%) of replication of the linear DNA (85%) was 58 nucleotides long showing that the holoenzyme generally stops one base short of full use of the template. The other products were full length (59 bases) and 57-base fragments.

DISCUSSION

These studies demonstrate that DNA polymerase III holoenzyme can replicate an available template flush to the 5' terminus of a pre-existing duplex DNA or an RNA primer-template hybrid. This property of the enzyme to create a complete duplex structure fits nicely with recent information

about the dynamics of holoenzyme movements on DNA (5). Holoenzyme, in an activated complex with a primer-template, carries on rapid, processive replication. Retaining a firm grip on the template strand, the holoenzyme diffuses readily over duplex DNA (or an RNA-DNA hybrid duplex) until it finds the next available primer terminus and thus replicates all available templates downstream. However, diffusion over single-stranded DNA does not occur at a significant rate, and thus holoenzyme fails to exploit an upstream primer separated by a single-stranded region. Another indication that interaction with single-stranded template downstream is not crucial to holoenzyme action is the capacity of the enzyme to replicate a linear template to one nucleotide short of its end.

The mode of termination on a primed circular DNA has proved to be slightly different depending on whether the primer is DNA or RNA (Table I). With a synthetic DNA 15-mer containing a 5'-hydroxyl terminus, the replication product was predominantly (90%) a nicked structure; in a minority of products, the chain was lengthened an extra nucleotide by displacing the 5' terminal residue. When the DNA 15-mer contained a 5'-phosphoryl terminus, the duplex circular replication product was almost completely (92%) convertible to a covalently closed structure by *E. coli* DNA ligase. Thus, termination of replication by holoenzyme completes the circular template, whether it meets a hydroxyl or a phosphate at the 5' end of the primer DNA strand. Two other prokaryotic DNA polymerases, DNA polymerase I (16) and bacteriophage T7 DNA polymerase (17) completely replicate primed viral ssDNA circles to form a structure sealable by ligase.

An RNA primer synthesized by primase, in the presence of holoenzyme and at the concentrations of nucleotides used here, is 2–9 residues long (18) and was generally (85%) extended beyond the open template; one or a few additional nucleotides were incorporated into the synthetic strand of the circular template by displacing residues at the 5' end of the initiating primer. In a minority of instances, the replication product was completed exactly to generate a nicked product (RF II) or was slightly shy of completion to leave a gap of 2 or 3 residues. A similar profile of products was observed with an RNA primer 28 residues long (produced by primase action not coupled to replication) as found with the short RNA primers (Table I).³

The tendency to displace the 5' end of the RNA primer and extend the synthetic strand may be due to one or more features of the primer: (i) the triphosphate substituent on the 5' end; (ii) the lower stability of the 5' terminal rA·dT pair end compared to the dG·dC base pair of the synthetic DNA primer; and (iii) pausing of holoenzyme upon encountering an RNA primer *versus* a DNA primer, as suggested by the 3-s transfer time for holoenzyme across an RNA primer compared to a 1-s transfer time across a DNA primer (5). Another possible explanation for the multiple forms of the terminal residue of the replication product, that include incomplete as well as overextended strands (Table I), may be the heterogeneity of subassemblies present in the multisubunit holoenzyme preparations.

In these holoenzyme studies, none of the 5'→3' exonuclease activity that enables DNA polymerase I to remove RNA primers and cleave nucleotides from the 5' terminus of a DNA duplex was detected (19). This is consistent with direct measurements that show no significant level of this exonuclease activity in preparations of DNA polymerase III core or holoenzyme.² For example, the ratio of 5'→3' exonuclease to

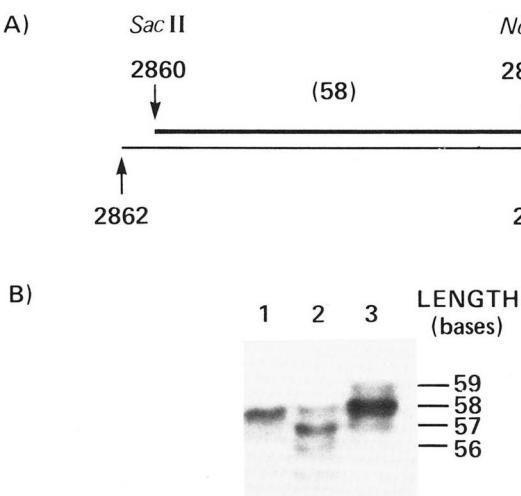


FIG. 5. Polyacrylamide gel electrophoresis of the product of replication of a linear template. *A*, unique cleavage sites (arrows) of *Sac*II and *Nci*I restriction nucleases on ϕ X duplex DNA. The strand synthesized by holoenzyme on the circular template (thick upper line) is 58 bases long. *B*, autoradiogram of a 12% polyacrylamide sequencing gel. Circular ϕ X ssDNA, primed at position 4047, was replicated by holoenzyme (using [α -³²P]dTTP) and digested with *Sac*II and *Nci*I to produce a labeled, 58-base fragment (lane 1). This *Sac*II/*Nci*I fragment was subjected to a short pulse of exonuclease III digestion to produce the fragments in lane 2. Linear ϕ X DNA primed 1245 nucleotides upstream (position 4047) was replicated by holoenzyme (using [α -³²P]dTTP) and digested with *Sac*II to generate a small terminal fragment containing the 3' terminus of the product strand (lane 3).

³ M. E. O'Donnell and A. Kornberg, unpublished results.

polymerase activity in DNA polymerase I is about 1×10^{-3} , but the ratio for DNA polymerase III core or holoenzyme is less than 2×10^{-6} (19).² These findings are in keeping with the physiological requirement for DNA polymerase I in removing RNA primers in replication (20) and mismatched residues in repair of DNA (21) and the implied failure of DNA polymerase III holoenzyme to perform these functions.

These studies together with those of the previous paper demonstrate that once holoenzyme is bound to an available ssDNA template the enzyme replicates the template to the very last nucleotide without dissociating, and will also use any available stretches of duplex DNA downstream as primers.

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REFERENCES

1. McHenry, C., and Kornberg, A. (1981) in *The Enzymes* (Boyer, P. D., ed) Vol. 14, pp. 39–50, Academic Press, New York
2. Burgers, P. M. J., and Kornberg, A. (1982) *J. Biol. Chem.* **257**, 11468–11473
3. Burgers, P. M. J., and Kornberg, A. (1982) *J. Biol. Chem.* **257**, 11474–11478
4. Burgers, P. M. J., and Kornberg, A. (1983) *J. Biol. Chem.* **258**, 7669–7675
5. O'Donnell, M. E., and Kornberg, A. (1985) *J. Biol. Chem.* **260**, 12875–12883
6. Ito, H., Ike, Y., Ikuta, S., and Itakura, K. (1982) *Nucleic Acids Res.* **10**, 1755–1769
7. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Banasuk, A. M., Deugau, K. V., Sherwood, J., Michalak, M., and Glick, B. R. (1983) *Anal. Biochem.* **128**, 281–286
9. Maxam, A. M., and Gilbert, W. (1980) *Methods Enzymol.* **65**, 499–560
10. Bouché, J.-P., Rowen, L., and Kornberg, A. (1978) *J. Biol. Chem.* **253**, 765–769
11. Sims, J., and Dressler, D. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3094–3098
12. Kaguni, J., and Ray, D. S. (1979) *J. Mol. Biol.* **135**, 863–878
13. Lehman, I. R. (1974) *Science* **186**, 790–797
14. Sanger, F., Coulson, A. R., Friedmann, T., Air, G. N., Barrell, B. G., Brawn, N. L., Fiddes, J. C., Hutchison, C. A., III, Slocombe, P. M., and Smith, M. (1978) *J. Mol. Biol.* **125**, 225–246
15. Rowen, L., and Kornberg, A. (1978) *J. Biol. Chem.* **253**, 770–774
16. Goulian, M., and Kornberg, A. (1967) *Proc. Natl. Acad. Sci. U. S. A.* **58**, 1723–1730
17. Engler, M. J., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11197–11205
18. Ogawa, T., Arai, K., and Okazaki, T. (1983) *J. Biol. Chem.* **258**, 13353–13358
19. Deutscher, M. P., and Kornberg, A. (1969) *J. Biol. Chem.* **244**, 3029–3037
20. Cozzarelli, N. R., Kelly, R. B., and Kornberg, A. (1969) *J. Mol. Biol.* **45**, 513–531
21. Kelly, R. B., Atkinson, M. R., Huberman, J. A., and Kornberg, A. (1969) *Nature* **224**, 495–501