

The Highly Processive DNA Polymerase of Bacteriophage T5

ROLE OF THE UNIQUE N AND C TERMINI*[§]Received for publication, July 26, 2004, and in revised form, September 9, 2004
Published, JBC Papers in Press, September 17, 2004, DOI 10.1074/jbc.M408428200Nathalie Andraos^{‡§}, Stanley Tabor[‡], and Charles C. Richardson^{‡¶}From the [‡]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and [§]Laboratoire de Minéralogie-Cristallographie Paris, CNRS UMR 7590, Université Paris 6, Case 115, 4 Place Jussieu, 75252 Paris, France

The DNA polymerase encoded by bacteriophage T5 has been reported previously to be processive and to catalyze extensive strand displacement synthesis. The enzyme, purified from phage-infected cells, did not require accessory proteins for these activities. Although T5 DNA polymerase shares extensive sequence homology with *Escherichia coli* DNA polymerase I and T7 DNA polymerase, it contains unique regions of 130 and 71 residues at its N and C termini, respectively. We cloned the gene encoding wild-type T5 DNA polymerase and characterized the overproduced protein. We also examined the effect of N- and C-terminal deletions on processivity and strand displacement synthesis. T5 DNA polymerase lacking its N-terminal 30 residues resembled the wild-type enzyme albeit with a 2-fold reduction in polymerase activity. Deletion of 24 residues at the C terminus resulted in a 30-fold reduction in polymerase activity on primed circular DNA, had dramatically reduced processivity, and was unable to carry out strand displacement synthesis. Deletion of 63 residues at the C terminus resulted in a 20,000-fold reduction in polymerase activity. The 3' to 5' double-stranded DNA exonuclease activity associated with T5 DNA polymerase was reduced by a factor of 5 in the polymerase truncated at the N terminus but was stimulated by a factor of 7 in the polymerase truncated at the C terminus. We propose a model in which the C terminus increases the affinity of the DNA for the polymerase active site, thus increasing processivity and decreasing the accessibility of the DNA to the exonuclease active site.

Upon infection of *Escherichia coli* by bacteriophage T5, a new DNA polymerase activity is induced (1). This activity is essential for phage DNA replication and phage growth (2, 3). The purified enzyme, like many prokaryotic DNA polymerases, has an associated 3'–5'-exonuclease activity that is active on single- and double-stranded DNA (4, 5). Early studies with the purified enzyme showed that it had two properties not found in most DNA polymerases: a processivity of polymerization of nucleotides on single-stranded DNA that was greater than that observed with other DNA polymerases tested (6), and the ability to catalyze extensive strand displacement synthesis on du-

plex DNA (7). One goal of the present study was to characterize these two interesting properties of T5 DNA polymerase purified from cells expressing the cloned T5 DNA polymerase gene and thus free of any contaminating phage-encoded proteins.

The gene for T5 DNA polymerase has been sequenced (8). Based on alignment of homologous regions, it is a member of the pol^I family of DNA polymerases (8, 9). The alignment between the large fragment of *E. coli* DNA polymerase I, T5 DNA polymerase, and T7 DNA polymerase, another phage replicative DNA polymerase, is shown in Fig. 1. T5 DNA polymerase has an extension on each end that is not found in other polymerases from the pol I family. There are an additional 159 residues at the N terminus not present in T7 DNA polymerase (Fig. 1), 130 of which are not present in the N terminus of the large fragment of *E. coli* DNA polymerase I. At the C terminus, there are 71 residues not found in T7 DNA polymerase or *E. coli* DNA polymerase I.

The role of *E. coli* DNA polymerase I is primarily in the repair of DNA; as such it has low processivity and does not interact strongly with other proteins. T5 and T7 DNA polymerases, being replicative DNA polymerases, must interact with other proteins. Most replicative DNA polymerases, such as *E. coli* DNA polymerase III and T7 DNA polymerase, interact with a processivity factor in order to increase their binding to the primer-template, which in turn allows them to incorporate thousands of nucleotides in a single binding event (10). In *E. coli*, the processivity factor is the β clamp, which assembles as a dimer to encircle duplex DNA, and then binds to the DNA polymerase multisubunit complex. T7 DNA polymerase, on the other hand, acquires the host *E. coli* thioredoxin as its processivity factor (11). Thioredoxin binds with high affinity to a unique 70-residue domain within the T7 DNA polymerase (Fig. 1); other members of the pol I family do not contain this insert (12).

The ability of T5 DNA polymerase to catalyze extensive strand displacement synthesis in the absence of an accessory protein is also intriguing (7). Other members of the pol I family of DNA polymerases catalyze strand displacement synthesis but only after modification of the enzyme or the primer-template, or in the presence of accessory proteins. For example, *E. coli* DNA polymerase I will not initiate strand displacement synthesis unless the 5' end of the non-template strand is already displaced, and the T7 DNA polymerase-thioredoxin complex will not catalyze such synthesis unless its exonuclease activity is reduced drastically (13). Phage T4 DNA polymerase likewise does not catalyze strand displacement synthesis (14). Instead, replicative DNA polymerases such as T7 and T4 DNA

* This work was supported in part by United States Public Health Service Grant GM-54397 and by United States Department of Energy Grant DE-FG02-96ER62251. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at <http://www.jbc.org>) contains sequences.

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¹ The abbreviations used are: pol, polymerase; Ni-NTA, nickel-nitrilotriacetic acid; DTT, dithiothreitol; SSB, single-stranded DNA-binding protein.

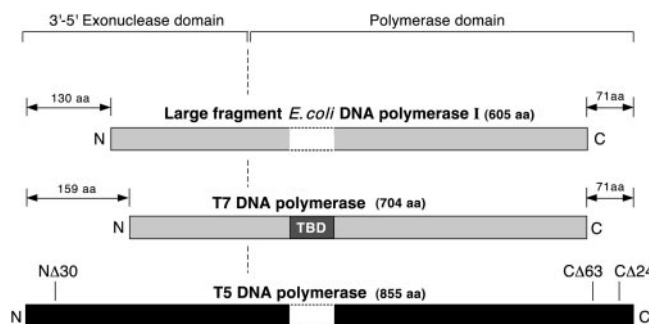


FIG. 1. Alignment of the large fragment of *E. coli* DNA polymerase I, T7 DNA polymerase, and T5 DNA polymerase. Alignment is based on regions of homology (8). Lengths are proportional to protein sizes. The location of the 3'-5'-exonuclease and polymerase domains are shown at the top. N and C represent N and C termini, respectively. N Δ 30 shows the start of the truncated T5 DNA polymerase missing the first 30 residues. C Δ 24 and C Δ 63 show the end of the truncated T5 DNA polymerases missing the C-terminal 24 and 63 residues, respectively. 130 aa and 159 aa correspond to the residues at the N terminus of T5 DNA polymerase not present in the large fragment of *E. coli* DNA polymerase I and T7 DNA polymerase, respectively. 71 aa corresponds to the residues at the C terminus of T5 DNA polymerase not present in the large fragment of *E. coli* DNA polymerase I or T7 DNA polymerase. TBD corresponds to the 70-residue thioredoxin binding domain present in T7 DNA polymerase that is not found in other DNA polymerases in the pol I family (12). A detailed alignment of the primary structures of three DNA polymerases based on superimposing the conserved exonuclease and polymerase domains present in all pol I DNA polymerases, and the conserved exonuclease and polymerase regions identified from known crystal structures, can be found in the supplemental material of the on-line version of this article.

polymerases are dependent on their cognate DNA helicases to unwind the duplex DNA to expose single-stranded template ahead of the advancing replication fork. Under conditions where the T7 DNA polymerase-thioredoxin complex and *E. coli* DNA polymerase I catalyze strand displacement synthesis in the absence of a helicase, the polymerase frequently displays a phenomenon known as strand-switching, in which it transfers to the displaced strand without interrupting synthesis. The products of such synthesis are abnormal in that they are branched and contain hairpin structures (15, 16).

Although the gene for T5 DNA polymerase has been cloned (17), the overproduced enzyme was reported to be insoluble, and thus its purification has not been described. In this paper, we report that the actual start site of the gene is 90 bp upstream of that reported previously, resulting in an enzyme of 97,600 daltons. We describe the purification and characterization of the overproduced wild-type T5 DNA polymerase. We also investigate the role of the unique regions at the ends of T5 DNA polymerase in the polymerase and exonuclease activities of the enzyme, and its ability to carry out processive DNA synthesis and extensive strand displacement synthesis.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains, Plasmids, and Bacteriophage—*E. coli* HMS174-(DE3) and BL21(DE3)/pLysS and the plasmids pET19b and pET21d were from Novagen. *E. coli* C43(DE3) is a derivative of *E. coli* BL21(DE3) that has been selected for increased expression of some toxic genes (18). The plasmid pRSET-C was from Invitrogen. Phage T5 was from the laboratory collection. Growth and manipulation of phage T5 and *E. coli* were as described (1).

Proteins—T5 DNA polymerase produced by phage T5-infected *E. coli* B cells was purified as described previously (1). The purification of T7 DNA polymerase (11), T7 DNA polymerase-thioredoxin complex (11), an exonuclease-deficient form of T7 DNA polymerase (T7 Δ 28 DNA polymerase)-thioredoxin complex (19), and *E. coli* single-stranded DNA-binding protein (SSB protein) (11) have been described. Restriction enzymes were from New England Biolabs.

DNA, Oligonucleotides, and Nucleotides—M13mp8 is a 9950-bp

derivative of M13mp8 that has been described previously (11). Salmon sperm DNA (type II) was from Sigma. The 17-nucleotide M13 primer ("–40") was from New England Biolabs. Other oligonucleotides were from Integrated DNA Technologies. Radioactive nucleotides were from Amersham Biosciences.

Protein Sequencing—To determine the N-terminal amino acid sequence of T5 DNA polymerase, purified proteins were separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After the gel was stained, the bands containing protein were cut out of the membrane and subjected to Edman degradation (20, 21) by the Biopolymers Facility at Harvard Medical School.

Cloning Strategy

Plasmids containing the desired sequences were constructed using standard PCR and cloning techniques (22). We initially cloned the gene for T5 DNA polymerase into the vector pRSET-C based on the sequence of the T5 DNA polymerase gene, reported previously (17), to generate the plasmid pRSET-T5. However, our terminal amino acid sequence analysis of the T5 DNA polymerase purified from phage T5-infected cells showed that the start of the gene was 90 bp upstream from that published previously. The correct start site is shown in Fig. 2A. Thus this original clone produced a protein with a deletion of 30 residues at the N terminus, which we will refer to here as T5 N Δ 30 DNA polymerase.

We subsequently cloned the full-length gene using a combination of synthetic oligonucleotides and PCR products. We amplified a PCR fragment that contained the entire gene except for the first six codons; these codons were generated using a synthetic duplex fragment that was used to ligate the PCR fragment to the 5' end of the expression vector pRSET-C (Fig. 2). The oligonucleotide used for PCR at the 3' end of the gene contained a BamHI restriction site at its 5' end. The oligonucleotide used for PCR at the 5' end of the gene was complementary to the region around codons six and seven of the polymerase gene; it introduced silent mutations in codons six (CAT to CAG) and seven (CTA to CTG) to create a SalI restriction site (CAGCTG) at this position. The resulting PCR product (2600 bp) was digested with BamHI and SalI and was then gel-purified. This fragment was then ligated to the vector pRSET-C that had been digested with NdeI and BamHI, along with a duplex synthetic linker encoding the first six codons of the T5 DNA polymerase gene that contained ends complementary to SalI and NdeI restriction sites. The full-length cloned T5 DNA polymerase

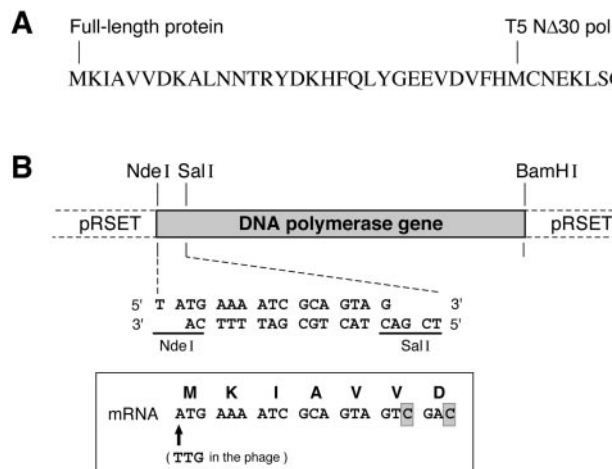


FIG. 2. Sequence at the N terminus of T5 DNA polymerase. A, protein sequence at the start of the wild-type T5 DNA polymerase. Full-length protein refers to the position of the initiating methionine determined by sequencing the first 10 residues of the DNA polymerase purified from the phage. T5 N Δ 30 pol refers to the position of the initiating methionine residue proposed previously (17). B, cloning of the gene for wild-type T5 DNA polymerase. The top panel shows the gene for the full-length T5 DNA polymerase in the vector pRSET. The middle panel shows the codons for the first six residues that were generated using synthetic oligonucleotides to generate NdeI and SalI restriction sites. The remainder of the gene was generated by PCR amplification, with a SalI site at the 5' end and a BamHI site at the 3' end. Two silent mutations were introduced in order to create a unique SalI site at the junction of the oligonucleotides and the PCR product; these are shown in the bottom panel. Also shown at the bottom is the initiation codon, which is TTG in the phage gene but ATG in the cloned gene.

gene from this plasmid was then transferred into the vector pET19b by digesting both the pRSET-C plasmid containing the T5 DNA polymerase gene and pET19b with XbaI and BamHI, and then ligating the appropriate fragments. The resulting construct we refer to as pET19b-T5.

Clones expressing the gene for T5 DNA polymerase with C-terminal deletions of 24 (CΔ24) and 63 (CΔ63) residues were constructed by standard PCR techniques using the appropriate primers and pET19b-T5 as a template. The resulting fragments were inserted into the vector pET21d, which resulted in the fusion of an eight-residue histidine tag to the C terminus of the induced protein. As a control, the wild-type T5 DNA polymerase gene was also inserted into this vector, such that the histidine tag was attached to the C terminus of the full-length T5 DNA polymerase. The DNA sequences of the T5 DNA polymerase gene in all constructs were confirmed by DNA sequence analysis.

Purification of Wild-type T5 DNA Polymerase

The scheme used for the purification of the wild-type T5 DNA polymerase was modified from that described by Stuart *et al.* (23). The purification is summarized in Table I. During purification, the protein was monitored by polymerase assays using salmon sperm single-stranded DNA and by SDS-PAGE. Protein concentrations were measured by the method of Bradford (24) using bovine serum albumin as a standard.

Growth of Cells—The plasmid containing the gene for wild-type T5 DNA polymerase, pET19b-T5, was toxic in *E. coli* cells that allowed elevated levels of uninduced expression. For this reason we used the cells C43(DE3), a strain that was selected to give increased stability and expression to genes that are toxic (18). 11 liters of *E. coli* C43(DE3) cells containing the plasmid pET19b-T5 were grown with aeration at 37 °C in LB broth containing 100 µg/ml ampicillin in a fermenter. When the A_{590} of the cultures reached 1.2, the cells were induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM, and the temperature was reduced to 25 °C. Six hours after induction, the A_{590} of the culture had reached 2.8, and the cells were harvested by centrifugation at 5,000 × *g* for 20 min at 4 °C. The cell pellet was resuspended in 200 ml of 20 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, and 10% glycerol, frozen in liquid N₂, and stored at -80 °C.

Preparation of Cell Extract—Frozen cells (200 ml) were thawed at 0 °C, and lysozyme was added to a final concentration of 500 µg/ml. After incubating for 30 min at 4 °C, NaCl was added to a final concentration of 0.5 M. The cells were then sonicated, and the lysate was centrifuged for 1 h at 50,000 × *g*. The supernatant (180 ml) was fraction I (Table I).

Precipitation of Nucleic Acids by Polyethyleneimine—To 180 ml of fraction I, 10% (w/v) polyethyleneimine (pH 7.5) was added to a final concentration of 0.1% with stirring for 20 min at 4 °C. The supernatant was decanted after centrifugation at 23,000 × *g* for 30 min at 4 °C. The supernatant (150 ml) was fraction II.

Ammonium Sulfate Precipitation—To 150 ml of fraction II, 54 g of ammonium sulfate (60% saturation) was added over a 25-min period at 4 °C and was stirred gently for an additional 15 min. The pellet was collected by centrifugation at 23,000 × *g* for 30 min at 4 °C. The pellet was redissolved in 420 ml of 20 mM KPO₄, pH 7.4, 0.1 mM EDTA, 0.1% mercaptoethanol, and 10% (v/v) glycerol (Buffer A). The resuspended pellet (450 ml) was fraction III.

Phosphocellulose Chromatography—200 ml of phosphocellulose P-11 (Whatman) was prepared according to the manufacturer and equi-

brated with Buffer A. Fraction III was diluted to 800 ml with Buffer A to bring the conductivity to that equivalent to Buffer A containing 100 mM KCl, and was then mixed with the phosphocellulose in batch for 30 min at 4 °C. The resin was collected by centrifugation at 6,000 × *g* for 15 min at 4 °C and then resuspended in 600 ml of Buffer A containing 100 mM KCl. After a second centrifugation, the resin was again washed with 300 ml of Buffer A containing 100 mM KCl. After the second wash, the resin was loaded onto a column (20 cm² × 20 cm) by gravity. The column was washed with 2 liters of Buffer A containing 100 mM KCl. Proteins were eluted using a 2-liter linear gradient from 100 to 500 mM KCl in Buffer A. T5 DNA polymerase eluted at 300 mM KCl. Fraction IV (250 ml) was dialyzed overnight at 4 °C against 2 liters of 20 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 0.1% β mercaptoethanol, and 10% glycerol (Buffer B) containing 50 mM NaCl.

Anion Exchange Chromatography—Fraction IV was further purified by anion exchange chromatography using a 6-ml POROS HQ column on a BioCAD Sprint perfusion chromatography system (Applied Biosystems). Dialyzed fraction IV was applied to the column at 10 ml/min. The column was washed with 60 ml of Buffer B containing 100 mM NaCl. Proteins were eluted using a linear gradient from 100 to 400 mM NaCl in Buffer B. The proteins were monitored by A_{280} , and fractions containing the single protein peak were pooled (fraction V, 90 ml). Fraction V was dialyzed overnight at 4 °C against 20 mM KPO₄, pH 7.4, 0.1 mM EDTA, 0.1 mM DTT, and 50% glycerol and then stored at -20 °C.

Purification of Genetically Altered T5 DNA Polymerase, Missing the First 30 Residues (T5 NΔ30)

The purification scheme was similar to that described for the wild-type T5 DNA polymerase above. During purification, the protein was monitored by polymerase assays using salmon sperm single-stranded DNA and by SDS-PAGE.

Growth of Cells—11 liters of *E. coli* HMS174(DE3) cells containing the plasmid pRSET-T5 were grown with aeration at 36 °C in LB broth containing 100 µg/ml ampicillin in a fermenter. When the A_{590} of the cultures reached 1.3, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM, and the temperature was reduced to 31 °C. Six hours after induction, the A_{590} of the culture had reached 2.5; the cells were harvested by centrifugation at 6,000 × *g* for 15 min at 4 °C. The cell pellet was frozen in a dry ice/ethanol bath and stored at -80 °C.

Preparation of Cell Extract—The cell pellet from the 11 liters of culture was suspended in 250 ml of Buffer A containing 300 mM NaCl and the protease inhibitors 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, and 1 µg/ml leupeptin (Sigma). The cells were lysed by sonication and then centrifuged for 1 h at 50,000 × *g*. The supernatant (250 ml) was fraction I.

Anion Exchange Chromatography (DEAE)—Fraction I was diluted 3-fold with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1% mercaptoethanol (Buffer C) to produce a final NaCl concentration of 100 mM. The diluted fraction I was loaded onto a column (20 cm² × 16 cm) containing the anion exchange resin DEAE-DE52 (Whatman) that had been equilibrated with Buffer C. The column was washed with 200 ml of Buffer C containing 100 mM NaCl, and then the bound proteins were eluted with a 900-ml gradient from 300 mM to 1 M NaCl in Buffer C. Fractions containing T5 DNA polymerase were pooled (fraction II; 150 ml).

Phosphocellulose Chromatography—A column (20 cm² × 18 cm) of phosphocellulose P-11 was prepared according to the manufacturer (Whatman) and equilibrated with Buffer A. Fraction II was dialyzed overnight at 4 °C against Buffer A containing 50 mM KCl and was then applied to the column. The column was washed with 300 ml of Buffer A containing 100 mM KCl. Proteins were eluted using a 750-ml linear gradient from 100 to 800 mM KCl in Buffer A. Fractions containing T5 DNA polymerase, which eluted at 600 mM KCl, were pooled (fraction III; 200 ml).

Anion Exchange Chromatography (MonoQ)—The high resolution anion exchange resin Mono Q (Amersham Biosciences) was used as the final purification step. MonoQ HR 10/10 (8 ml) was equilibrated with Buffer B. Fraction III was dialyzed overnight at 4 °C against Buffer B containing 50 mM NaCl. Dialyzed fraction III was applied to the column, and then the column was washed with 25 ml of Buffer B containing 50 mM NaCl. Proteins were eluted using a 120-ml linear gradient from 50 mM to 1 M NaCl in Buffer B. The proteins were monitored by A_{280} , and fractions containing the single protein peak, which eluted at 600 mM NaCl, were pooled (fraction IVA, 20 ml). Fraction IVA was dialyzed overnight at 4 °C against 20 mM KPO₄, pH 7.4, 0.1 mM EDTA, 0.1 mM DTT, and 50% glycerol. After dialysis, the sample (fraction IVB, 7 ml) was stored at -20 °C.

TABLE I

Purification of wild-type T5 DNA polymerase from 11 liters of *E. coli* C43(DE3) cells containing the plasmid pET19b-T5

DNA polymerase activity was assayed using salmon sperm single-stranded DNA as described under "Experimental Procedures." One unit of DNA polymerase activity is defined as the amount required to incorporate 10 nmol of all four dNMPs into DNA in 30 min at 37 °C under standard assay conditions.

Fraction	Step	Protein mg	Total units (×10 ⁶)	Specific Activity units/mg	Yield %
I	Extract	10,000	7.2	720	100
II	Polyethyleneimine	6,800	8.3	1,220	115
III	Ammonium sulfate	4,500	8.5	1,900	118
IV	Phosphocellulose	450	4.8	10,700	66
V	HQ	210	2.9	14,000	40

*Purification of Genetically Altered T5 DNA Polymerases
Containing a Histidine Tag at their C Termini*

Genetically altered T5 DNA polymerases with truncations of either 24 or 63 residues at their C termini were overproduced in the vector pET21d that resulted in the fusion of an 8-histidine tag onto their C termini. After overproduction of each protein in *E. coli* BL21(DE3)/pLysS, each was purified by using a Ni-NTA-agarose column (Qiagen) following the manufacturer's procedures. For each protein, 1 liter of culture was grown in LB broth containing 100 μ g/ml ampicillin at 37 °C with shaking. At an $A_{590} = 1.0$, the cells were induced by the addition of isopropyl-1-thio- β -D-galactopyranoside to a final concentration of 1 mM. Three hours after induction, the cells were pelleted by centrifugation at $5,000 \times g$ for 20 min and then resuspended in 50 ml of 50 mM KPO_4 , pH 8.0, and 500 mM NaCl (Buffer D) containing 10 mM imidazole at 4 °C. The resuspended cells were sonicated, and the extracts were prepared by centrifugation at $40,000 \times g$ for 30 min at 4 °C. The clear lysates were loaded onto a 5-ml column containing Ni-NTA-agarose at 4 °C. The column was washed with 50 ml of Buffer D containing 20 mM imidazole, and the proteins were eluted with 4 ml of Buffer D containing 500 mM imidazole. Fractions containing protein were identified by the method of Bradford (24); they were pooled and dialyzed overnight at 4 °C against 20 mM KPO_4 , pH 7.4, 0.1 mM EDTA, 0.1 mM DTT, and 50% glycerol. After dialysis, the proteins were stored at -20 °C.

Polymerase Assays

Activity on Salmon Sperm Single-stranded DNA—DNA polymerase activity was assayed by measuring the amount of 3H -nucleotide incorporated into alkali-denatured salmon sperm single-stranded DNA (23). Reaction mixtures (50 μ l) contained 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 mM NaCl, 0.15 mM dCTP, dATP, dGTP, and [3H]dTTP (2.9 cpm/pmol), 0.3 mM salmon sperm single-stranded DNA (expressed as nucleotide equivalents), and variable amounts of enzyme diluted in 20 mM Tris-HCl, pH 7.5, 10 mM mercaptoethanol, and 500 μ g/ml bovine serum albumin (SED). Reaction mixtures were incubated at 37 °C for 15 min, and then 45 μ l were spotted on DE81 filter disks (Whatman). The filters were washed to remove unincorporated radioactivity as described (25).

Activity on Primed M13 DNA—DNA polymerase activity on a 9,950-nucleotide, single-stranded M13 DNA with a 25-nucleotide primer annealed to it was determined by measuring the amount of 3H -nucleotide incorporated essentially as described (11). Reaction mixtures (45 μ l) contained 80 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 0.15 mM dCTP, dATP, dGTP, and [3H]dTTP (2.9 cpm/pmol) and 80 μ M of the primed M13mGP1-2 DNA (expressed as nucleotide equivalents). Reactions were initiated by the addition of 5 μ l of the DNA polymerase diluted in SED. Reactions were carried out by incubation at 37 °C for 10 min and then terminated by the addition of 10 μ l of 50 mM EDTA, pH 8.0. 55 μ l were spotted on DE81 filter disks. The filters were washed to remove unincorporated radioactivity as described (25).

Processivity Assay—The processivity of DNA synthesis was measured on a 9,950-nucleotide, single-stranded circular M13 DNA template containing a 5'- ^{32}P -labeled 17-mer primer, as described previously (11). The reaction mixture (17 μ l) contained 15 pmol of ^{32}P -labeled primer-template molecules (110,000 cpm), 20 mM Tris-HCl, pH 7.5, 7.5 mM $MgCl_2$, 5 mM DTT, and 250 μ M of all four dNTPs. The reaction mixture was preincubated at 37 °C for 2 min, and then 3 μ l of varying amounts of DNA polymerase diluted in SED were added. The reactions were allowed to proceed for varying times, as indicated. The samples were then analyzed by either native agarose gel electrophoresis or denaturing PAGE.

For native agarose gel electrophoresis, the reactions were terminated by the addition of 10 μ l of 30% glycerol, 100 mM EDTA, 0.25% bromphenol blue, and 0.25% xylene cyanol FF. The DNA products were separated by electrophoresis in a 0.6% agarose gel for 10 h at 40 V in 40 mM Tris acetate, pH 8.3, 1 mM EDTA and a 1–10,000 dilution of GelStar (Cambrex, Inc.). After electrophoresis, gels were dried under vacuum.

For denaturing PAGE, the reactions were terminated by the addition of 10 μ l of 90% formamide, 25 mM EDTA, 0.25% bromphenol blue, and 0.25% xylene cyanol. The samples were heated for 2 min at 95 °C and then loaded on a 8% polyacrylamide gel containing 7 M urea in 100 mM Tris borate, pH 8.3, and 2 mM EDTA. After electrophoresis, the gel was fixed in 10% acetic acid, 15% methanol, transferred to Whatman No. 1 paper, and dried under vacuum. After drying, gels were analyzed by autoradiography to determine the location and amount of the radioactively labeled products.

Strand Displacement Assay—The assay for strand displacement synthesis measured the length of radioactive products synthesized on

primed M13 DNA. The 5'- ^{32}P -labeled primer annealed to the M13 DNA template was extended by DNA polymerase under the same conditions described in the processivity assay. Upon complete synthesis of the complementary strand of the M13 single-stranded DNA to form a nicked circular duplex molecule, strand displacement synthesis will increase the length of the ^{32}P -labeled product beyond that of the M13 DNA. The lengths of the radioactive products were measured by electrophoresis on alkaline agarose gels. After incubation for varying times, the reactions were stopped by the addition of 4 μ l of 300 mM NaOH, 6 mM EDTA, 18% Ficoll 400, 0.15% bromcresol green, and 0.25% xylene cyanol. The fragments were separated by electrophoresis in a 0.6% alkaline agarose gel for 15 h at 30 V in 50 mM NaOH and 1 mM EDTA. After electrophoresis, the gels were dried under vacuum and then analyzed by autoradiography to determine the location and amount of the radioactively labeled products.

Exonuclease Assay—Exonuclease activity was measured by the rate of release of soluble radioactivity from a uniformly 3H -labeled M13 DNA substrate as a function of the concentration of DNA polymerase, as described by Kumar *et al.* (26). A 25-nucleotide primer was annealed to M13mGP1-2 DNA and then extended using all four dNTPs (including [3H]dTTP at 30 cpm/pmol). Unincorporated radioactivity was removed by gel filtration. Single-stranded DNA substrate was obtained by denaturation of the product of synthesis with alkali immediately prior to use. Reaction mixtures for the exonuclease assay (100 μ l) contained 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 mM NaCl, 1 nmol of 3H -labeled single- or double-stranded M13 DNA (expressed as nucleotide equivalents), and enzyme dilutions in SED. Reaction mixtures were incubated at 37 °C for 15 min and then terminated by the addition of 30 μ l of 10 mg/ml bovine serum albumin and 30 μ l of 100% (w/v) trichloroacetic acid. After incubating on ice for 10 min, the precipitated DNA was collected by centrifugation at $12,000 \times g$ for 30 min. The acid-soluble radioactivity was measured by mixing 100 μ l of the supernatant with 400 μ l of H_2O and 5 ml of Ultima scintillation fluor (PerkinElmer Life Sciences and Analytical Sciences, Inc.).

RESULTS

N Terminus of Phage T5 DNA Polymerase

The gene for T5 DNA polymerase was first sequenced in 1989 (8). In 1991, Chatterjee *et al.* (17) corrected the sequence, showing that the original report had a frameshift at the beginning of the gene. The start site in the revised sequence was reported to be the first ATG in the open reading frame, which would result in the production of a 94,000-dalton protein. We initially cloned the gene for T5 DNA polymerase based on this prediction, fusing the initiating ATG into a vector that contained a strong ribosome-binding site (pRSET). We purified the gene product that was overproduced from this construct.

To confirm the integrity of our protein, we determined the sequence at the N terminus and compared it to that of a preparation of T5 DNA polymerase we purified from phage T5-infected *E. coli*. As expected, the first 10 residues of the cloned gene product (MCNEKLSGRL) were the same as those predicted by Chatterjee *et al.* (17) (Fig. 2A). However, the first 10 residues of the DNA polymerase purified from phage T5-infected cells were MKIAVVDKAL. This latter sequence corresponds to a site 90 nucleotides upstream from the start site predicted by Chatterjee *et al.* (17). The initiation codon used by the phage gene is TTG (Fig. 2B), resulting in a protein of predicted molecular weight 97,600. A recently deposited sequence of the T5 genome to NCBI (accession number 005859) is in agreement with our finding.

Based on these results, we constructed a new clone that contained the codons for these additional 30 residues (Fig. 2B). In this paper, we refer to the full-length protein purified from this clone as T5 DNA polymerase, and the protein purified from the initial construct as T5 N Δ 30 DNA polymerase.

*Overproduction and Purification of Wild-type and
Truncated T5 DNA Polymerases*

A summary of the purification of the wild-type T5 DNA polymerase is shown in Table I. From 11 liters of induced cells,

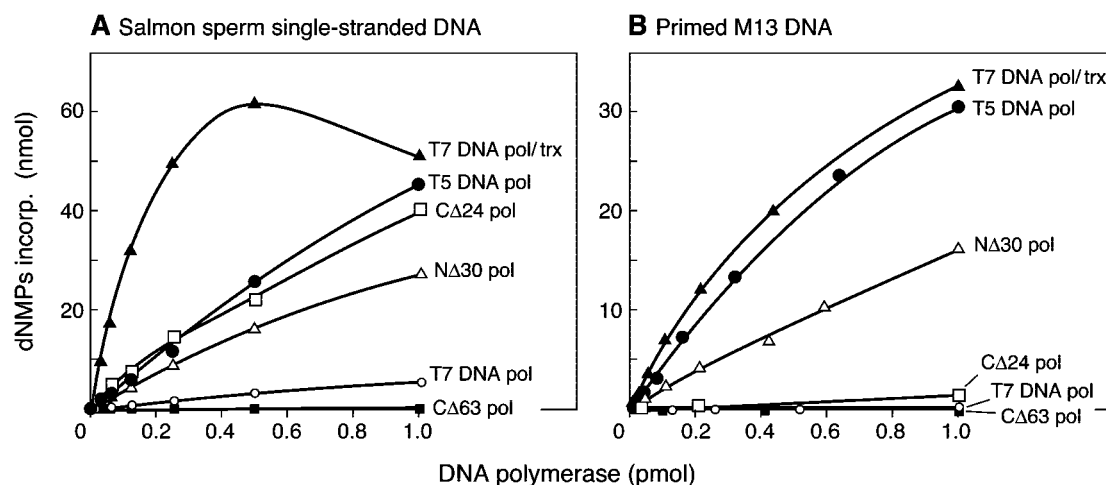


FIG. 3. Comparison of DNA synthesis rates on salmon sperm single-stranded DNA versus primed M13 DNA substrates. The amount of DNA synthesized by the indicated enzymes is plotted as a function of the concentration of each enzyme. Reaction conditions are as described under "Experimental Procedures." A, DNA synthesis on salmon sperm single-stranded DNA. B, DNA synthesis on primed M13 DNA. ●, wild-type T5 DNA polymerase; △, T5 NΔ30 DNA polymerase; □, T5 CΔ24 DNA polymerase; ■, T5 CΔ63 DNA polymerase; ▲, T7 DNA polymerase with thioredoxin (*trx*); ○, T7 DNA polymerase without thioredoxin.

TABLE II

Comparison of polymerase and exonuclease activities of T5 DNA polymerases and T7 DNA polymerase-thioredoxin complex

DNA polymerase and exonuclease assays were determined as described under "Experimental Procedures." The amount of DNA incorporated or degraded per min per mg of protein is expressed as nmol/min/mg. WT, wild type; *trx*, thioredoxin.

Enzyme	Polymerase activities		Exonuclease activities	
	Salmon sperm single-stranded DNA	Primed M13 DNA	Double-stranded	Single-stranded
	nmol/min/mg		nmol/min/mg	
WT T5 DNA pol	8,400	4,700	1,300	1,900
T5 NΔ30 DNA pol	6,200	2,600	280	1,000
T5 CΔ24 DNA pol	8,600	150	4,000	5,300
T5 CΔ63 DNA pol	150	0.24	9,000	4,700
T7 DNA pol/ <i>trx</i>	46,000	5,300	100,000	25,000
T7 DNA pol	1,700	1.3	450	19,000

210 mg of T5 DNA polymerase were obtained, representing an overall recovery of 40% relative to the activity found in the extract. The purity was greater than 95% as measured by SDS-PAGE. For comparison, Steuart *et al.* (23) reported the purification of 0.6 mg of T5 DNA polymerase from 100 liters of T5 phage-infected *E. coli* B. In our preparation of T5 DNA polymerase from 6 liters of T5 phage-infected *E. coli* B, we obtained 1.5 mg of purified T5 DNA polymerase.

The T5 NΔ30 DNA polymerase was purified from cells containing a plasmid that overexpressed its gene. From 10 liters of cells, we obtained 12 mg of T5 NΔ30 DNA polymerase that was greater than 95% pure as measured by SDS-PAGE.

Genetically altered T5 DNA polymerase with deletions in the C termini of 24 (CΔ24) and 63 (CΔ63) residues were purified by using His-tagged affinity purification. The expression vector pET-21d used in these experiments results in the fusion of an 8-histidine tag onto the C terminus of the truncated proteins. The proteins were then purified by binding to a Ni-NTA column and eluting with imidazole as described under "Experimental Procedures". As a control, we also purified the full-length T5 DNA polymerase containing the histidine tag at its C terminus. From 1 liter of cells overproducing His-tagged full-length T5 CΔ24 and CΔ63 DNA polymerases, we obtained 6, 20, and 12 mg of protein, respectively. In each case the protein was greater than 95% pure as measured by SDS-PAGE.

Polymerase Activity of Wild-type and Truncated T5 DNA Polymerases

Salmon Sperm DNA Primer-Template—Salmon sperm single-stranded DNA provides a useful primer-template for as-

sessing the ability of a DNA polymerase to polymerize nucleotides over relatively short stretches (11). Because the length of the available single-stranded DNA template in this template is relatively short, it is useful for measuring the ability of a polymerase to add a relatively few nucleotides to a primer without concerns over processivity. We compared the ability of varying concentrations of wild-type and mutant T5 DNA polymerases to incorporate [³H]dTMP by using this template (Fig. 3A and Table II). T5 NΔ30 and CΔ24 DNA polymerases had comparable activity to that of wild-type T5 DNA polymerase, whereas the activity of T5 CΔ63 DNA polymerase was reduced 56-fold. For comparison, the activity of wild-type T5 DNA polymerase was 15% that of the T7 DNA polymerase-thioredoxin complex.

Primed M13 DNA Primer-Template—DNA polymerases with low processivity often display a rate of synthesis considerably less than that observed with processive DNA polymerases on primed M13 DNA single-stranded DNA (11). By using a 9,950-nucleotide single-stranded M13 template with a single primer per molecule, the activity of T5 DNA polymerase was 90% that observed for the T7 DNA polymerase complex (Fig. 3B and Table II). Deletion of 30 residues at the N terminus of T5 DNA polymerase (NΔ30) resulted in 55% of the activity of the wild-type T5 DNA polymerase, compared with 70% of the activity of the wild-type enzyme on salmon sperm DNA.

Deletion of 24 residues at the C terminus had a major effect on its activity on primed M13 DNA. Although the activity of T5 CΔ24 DNA polymerase was equal to that of wild-type T5 DNA polymerase on salmon sperm DNA, it was 31-fold less than wild-type T5 DNA polymerase on primed M13 DNA. The ratio

of activity of T5 C Δ 63 DNA polymerase to wild-type T5 DNA polymerase on salmon sperm DNA and primed M13 DNA was 60- and 20,000-fold, respectively. These results suggest strongly that the deletions at the C terminus are reducing the processivity of polymerization to a much greater extent than the deletion at the N terminus. The fact that deletion of 63 residues from the C terminus reduces the polymerase activity on salmon sperm DNA to a much greater extent than deletion of 24 residues suggests that this larger deletion is affecting more than just the processivity of polymerization.

The ability of T5 DNA polymerase, with no accessory protein, to catalyze DNA synthesis on primed M13 DNA at a rate similar to that observed with T7 DNA polymerase-thioredoxin complex at the same protein concentration is quite remarkable. In the absence of the processivity factor thioredoxin, DNA synthesis by T7 DNA polymerase on primed M13 DNA was greatly reduced (Fig. 3B). Elimination of the unique C-terminal extension of T5 DNA polymerase reduced the rate of synthesis of T5 DNA polymerase to a comparable level.

Processivity of Wild-type and Truncated T5 DNA Polymerases

T5 DNA polymerase has been shown to have a processivity higher than that of several other polymerases (6). However, the relatively short length of the available template in those earlier studies (poly(dA)₃₀₀-oligo(dT)₁₀) limited the measurement of processivity to ~150 nucleotides per cycle of binding, well below the values now known to pertain to replicative DNA polymerases with their processivity factors (27). In order to determine the upper limit of processivity for T5 DNA polymerase, we used an enzyme dilution procedure, in which a 5'-³³P-labeled primer was annealed to a circular 9,950-nucleotide M13 DNA template and was incubated with a range of concentrations of DNA polymerase (Figs. 4 and 5). In some of the reactions only a fraction of the primers are extended; under these conditions, the reactions exhibit "single-hit" kinetics, in which each primer is extended by a single binding event of the DNA polymerase (28).

We compared reaction products by using a range of concentrations of wild-type T5 DNA polymerase to those carried out using the T7 DNA polymerase-thioredoxin complex by denaturing PAGE (Fig. 4). With each enzyme, at low polymerase concentrations (an excess of primer-template), only a small percentage of primers were extended, but those that had been extended have an average of greater than several hundred nucleotides polymerized. Although these results show that T5 DNA polymerase is highly processive, they suggest that the T7 DNA polymerase-thioredoxin complex may have slightly higher processivity, because high molecular products are observed at lower concentrations of the T7 DNA-polymerase complex.

In view of the dramatic reduction in the macroscopic rate of DNA synthesis catalyzed by T5 C Δ 24 and C Δ 63 DNA polymerases on long M13 DNA templates, it was of interest to analyze their processivity directly (Fig. 5). In this experiment, the products of the reactions were separated by native agarose gel electrophoresis. With wild-type T5 DNA polymerase, some of the primers had been extended the entire distance around the M13 molecule, ~10,000 nucleotides, under conditions where most of the primers have not been extended (Fig. 5, lane 2). The processivity of the T5 N Δ 30 DNA polymerase was reduced, but the enzyme was still highly processive, because some of the primers were fully extended at polymerase concentrations where there was no synthesis on most of the primers (Fig. 5, lane 10). On the other hand, T5 C Δ 24 DNA polymerase had dramatically reduced processivity (Fig. 5, lanes 13–16),

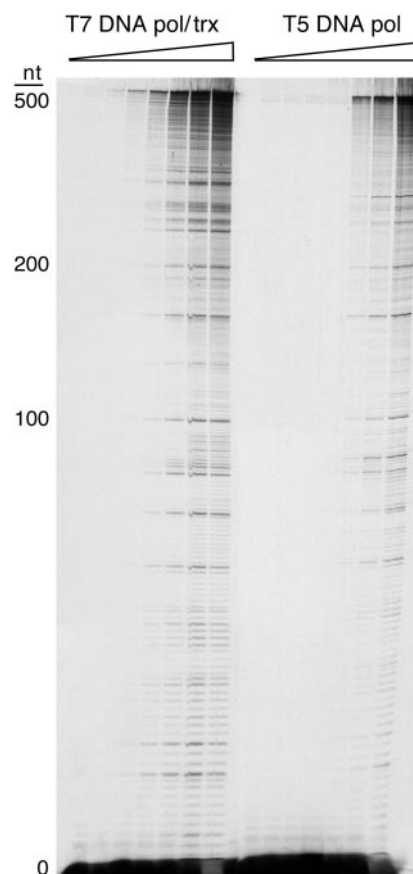


FIG. 4. Processivity of T5 DNA polymerase and T7 DNA polymerase. The processivity of T5 and T7 DNA polymerases was measured as described under "Experimental Procedures." Reactions were carried out using varying amounts of DNA polymerase and 20 fmol of a 5'-³³P-labeled 17-nucleotide primer annealed to a circular M13 DNA. The amount of T7 DNA polymerase in the seven reaction mixtures (from left to right) was 0, 0.35, 1.7, 8.7, 43, 210, and 1000 fmol. The amount of T5 DNA polymerase in the seven reaction mixtures (from left to right) was 0.23, 1.1, 5.7, 29, 140, 720, and 3600 fmol. After 90 s at 37 °C, the DNA was denatured, and the fragments were separated by electrophoresis in an 8% polyacrylamide gel containing 7 M urea. After electrophoresis, the gel was dried and visualized by autoradiography. The markers on the left indicate 0 as the position of unsynthesized primer, and the position of primers that had been extended 100, 200, and 500 nucleotides. *trx*, thioredoxin.

whereas there was no DNA synthesis detected with the T5 C Δ 63 DNA polymerase (Fig. 5, lanes 17–20). In fact, in the presence of high concentrations of T5 C Δ 63 DNA polymerase the primer was degraded (Fig. 5, lane 20). The histidine tag on the C termini of T5 C Δ 24 and C Δ 63 DNA polymerases was unlikely to be affecting their polymerase activity because fusion of this tag on the C terminus of the full-length T5 DNA polymerase did not significantly affect its polymerase activity or processivity (Fig. 5, lanes 5–8).

Strand Displacement Activity of Wild-type and Truncated T5 DNA Polymerases

In order to examine the ability of T5 DNA polymerase to carry out strand displacement synthesis, we determined the extent of DNA synthesis on a ³³P-labeled primer annealed to M13 single-stranded DNA by electrophoretic analysis of the products on alkaline-agarose gels. Using 20 pmol of wild-type T5 DNA polymerase, a 1,000-fold excess over the amount of M13 molecules, extensive strand displacement synthesis was observed by 4 min, with very little pausing observed at the position corresponding to full-length M13 (Fig. 6, lane 6). Using 100 times less T5 DNA polymerase, 0.2 pmol, strand displace-

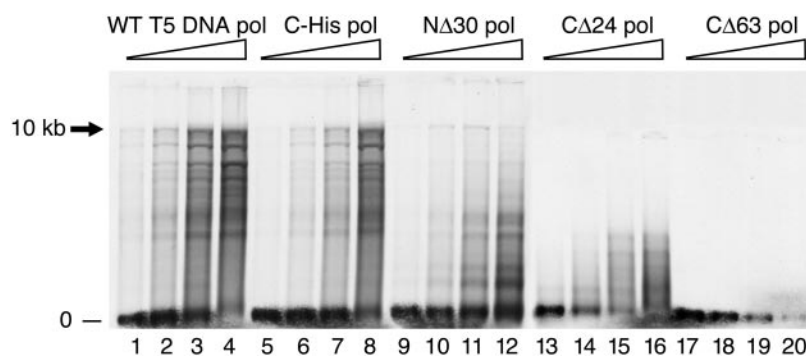


FIG. 5. **Processivity of truncated T5 DNA polymerases.** The processivity of the truncated T5 DNA polymerases was measured as described under "Experimental Procedures." Reactions were carried out using varying amounts of DNA polymerase and 250 fmol of a 5'-³²P-labeled 17-mer annealed to circular M13 DNA. The amount of wild-type T5 DNA polymerase in the reaction mixtures shown in lanes 1–4 was 30, 140, 720, and 3,600 fmol. The amount of C-His T5 DNA polymerase in the reaction mixtures shown in lanes 5–8 was 8, 40, 200, and 1,000 fmol. The amount of T5 NΔ30 DNA polymerase in the reaction mixtures shown in lanes 9–12 was 8, 40, 200, and 1,000 fmol. The amount of T5 CΔ24 DNA polymerase in the reaction mixtures shown in lanes 13–16 was 40, 200, 1,000, and 5,000 fmol. The amount of T5 CΔ63 DNA polymerase in the reaction mixtures shown in lanes 17–20 was 20, 100, 500, and 2,500 fmol. After 90 s at 37 °C, the products of the reaction were separated by electrophoresis in a 0.6% native agarose gel. After electrophoresis, the gel was dried and visualized by autoradiography. The markers on the left indicate 0 as the position of unsynthesized primer, and 10 kb as the position of primer that had been synthesized completely around the circular M13 template.

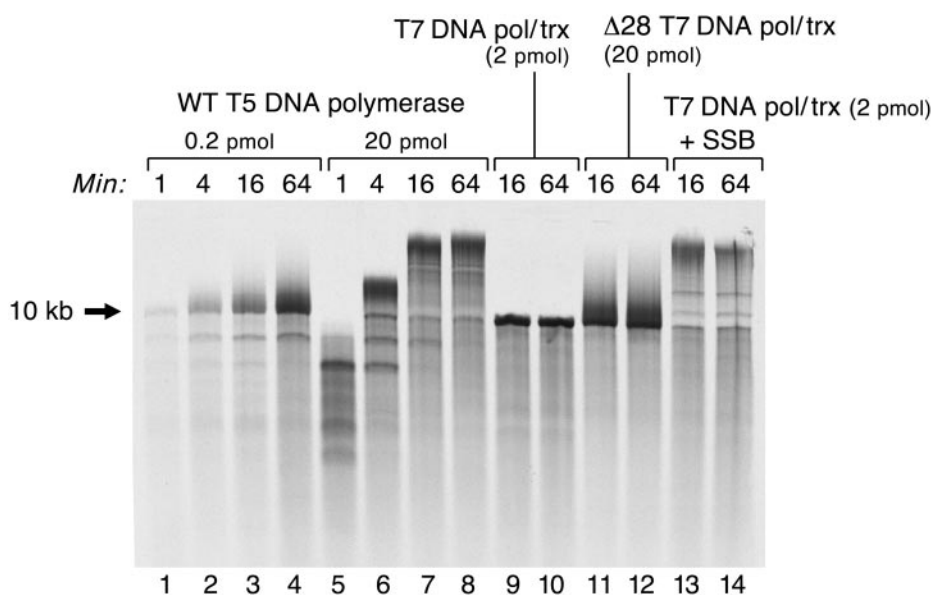


FIG. 6. **Strand displacement activity of T5 and T7 DNA polymerases.** Strand displacement synthesis was measured as described under "Experimental Procedures." Reactions were carried out using 20 fmol of a 5'-³²P-labeled 17-mer annealed to circular M13 DNA. Reaction mixtures contained the indicated amounts of wild-type T5 DNA polymerase (lanes 1–8), T7 DNA polymerase-thioredoxin complex (lanes 9, 10, 13, and 14), and an exonuclease-deficient form of T7 DNA polymerase (Δ28)/thioredoxin (*trx*) complex (lanes 11 and 12). Reaction mixtures in lanes 13 and 14 contain 5 μg of *E. coli* SSB. After incubation for the indicated times, the products were separated by electrophoresis in a 0.6% agarose gel under alkaline conditions. After electrophoresis, the gel was dried and visualized by autoradiography. The marker on the left corresponds to the length (10,000 nucleotides) of the M13 DNA.

ment synthesis was still observed by 4 min, but the extent of synthesis on each primer was much less (Fig. 6, lane 2). The finding that the length of the products did not increase from 4 to 64 min by using 0.2 pmol of DNA polymerase, taken together with the reduced total amount of DNA synthesis, suggests that strand displacement synthesis has low processivity. Once the polymerase dissociates from the primer during strand displacement synthesis, the displaced strand can re-anneal to the template, displacing the 3' end of the primer and rendering it unavailable for further extension.

With 0.2 pmol of T5 DNA polymerase, the predominant product synthesized after 1 min corresponded to that of full-length M13 DNA (Fig. 6, lane 1). Using 100 times more T5 DNA polymerase, the average length of the products synthesized in the same time period was significantly shorter (Fig. 6, lane 5). Thus, the rate of primer extension is reduced by increased amounts of T5 DNA polymerase. Perhaps the excess polymer-

ase binds to the DNA template ahead of the replication fork, interfering with its movement. Alternatively, the polymerase may form higher order complexes at the high concentrations, interfering with its activity.

For controls, the T7 DNA polymerase-thioredoxin complex synthesized DNA around the M13 molecule but did not displace the duplex region, resulting in a homogeneous band at a position corresponding to 10,000 nucleotides; there was no change in the amount of synthesis from 16 to 64 min (Fig. 6, lanes 9 and 10). In the presence of *E. coli* single-stranded DNA-binding protein (SSB protein), the primer was extended much greater than 10,000 nucleotides, indicative of extensive strand displacement synthesis (Fig. 6, lanes 13 and 14). The ability of SSB protein to stimulate T7 DNA polymerase/thioredoxin to carry out strand displacement synthesis has been shown previously (29). When an exonuclease-deficient form of T7 DNA polymerase (Δ28 T7 DNA polymerase) complexed with thiore-

doxin was used, strand displacement synthesis was also observed, but the length of the products was considerably less than that observed with wild-type T7 DNA polymerase in the presence of SSB protein; the absence of further strand displacement synthesis after 16 min was likely because of strand switching (16).

We examined the ability of T5 Δ 24 DNA polymerase to catalyze strand displacement synthesis (Fig. 7). After 10 min, 0.1 pmol of T5 Δ 24 DNA polymerase synthesized the primers on average approximately half the distance around the 10,000-nucleotide M13 DNA template (Fig. 7, lane 5). With 1 pmol of enzyme the predominant product was full-length M13 DNA

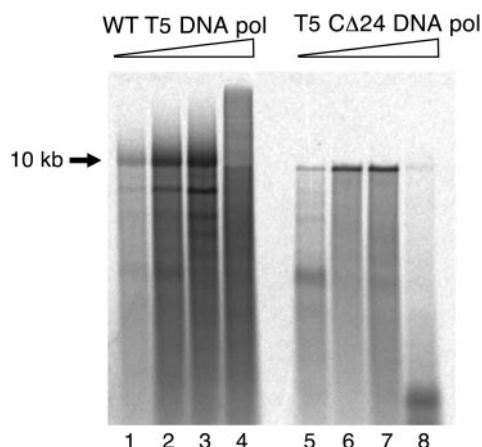


FIG. 7. Strand displacement activity of the truncated T5 Δ 24 DNA polymerase. Strand displacement synthesis catalyzed by T5 Δ 24 DNA polymerase was compared with strand displacement synthesis catalyzed by T5 DNA polymerase, as described in the legend of Fig. 6. Reactions were carried out using 20 fmol of a 5'-³³P-labeled 17-mer annealed to circular M13 DNA. The amount of wild-type T5 DNA polymerase in the reaction mixtures shown in lanes 1–4 was 18, 180, 1,800, and 18,000 fmol. The amount of T5 Δ 24 DNA polymerase in the reaction mixtures shown in lanes 5–8 was 100, 1,000, 10,000, and 100,000 fmol. After incubation for 30 min at 37 °C, the reactions products were separated by electrophoresis in a 0.6% agarose gel under alkaline conditions. After electrophoresis, the gel was dried and visualized by autoradiography. The marker on the left corresponds to the length (10,000 nucleotides) of the M13 DNA.

(Fig. 7, lane 6). No evidence of strand displacement synthesis was observed, as measured by the absence of any product greater than 10,000 nucleotides in length, even in the presence of 10 pmol of T5 Δ 24 DNA polymerase (Fig. 7, lane 7). Attempts to increase the concentration of T5 Δ 24 DNA polymerase beyond this amount (Fig. 7, lane 8) resulted in the disappearance of product. These results support our conclusion that T5 Δ 24 DNA polymerase has low processivity and show that it cannot catalyze strand displacement synthesis. For comparison, the strand displacement synthesis catalyzed by wild-type T5 DNA polymerase is shown in Fig. 7, lanes 1–4. Even at the lowest amount of enzyme, some of the primers had been synthesized greater than the 10,000-nucleotide M13 molecule, indicative of strand displacement synthesis (Fig. 7, lane 1); with increasing amounts of enzyme, there was an increasing amount of molecules that have carried out strand displacement synthesis, and the maximum length of the products increased (Fig. 7, lanes 2–4). T5 Δ 63 DNA polymerase has such reduced DNA polymerase activity (20,000-fold on primed M13 DNA) that it was not possible to obtain sufficient DNA synthesis to examine strand displacement synthesis in this assay. T5 Δ 30 DNA polymerase, on the other hand, carried out the same extent of strand displacement synthesis as that observed with wild-type T5 DNA polymerase (data not shown).

Exonuclease Activity of Wild-type and Truncated T5 DNA Polymerases

We determined the exonuclease activities of wild-type and mutant T5 DNA polymerases (Fig. 8 and Table II). The level of exonuclease activity of wild-type T5 DNA polymerase was similar on single- and double-stranded DNA. Deletion of the 30 residues at the N terminus reduced the activity on single- and double-stranded DNA by 2- and 5-fold, respectively. Most surprisingly, deletions at the C terminus of the T5 DNA polymerase increased dramatically the exonuclease activity. For example, the exonuclease activity of the T5 Δ 63 DNA polymerase was higher than that of the wild-type T5 DNA polymerase by 3- and 7-fold on single- and double-stranded DNA, respectively. The higher levels of exonuclease found in the C-truncated T5 DNA polymerases provide an explanation for the disappearance of DNA products when high amounts of these enzymes

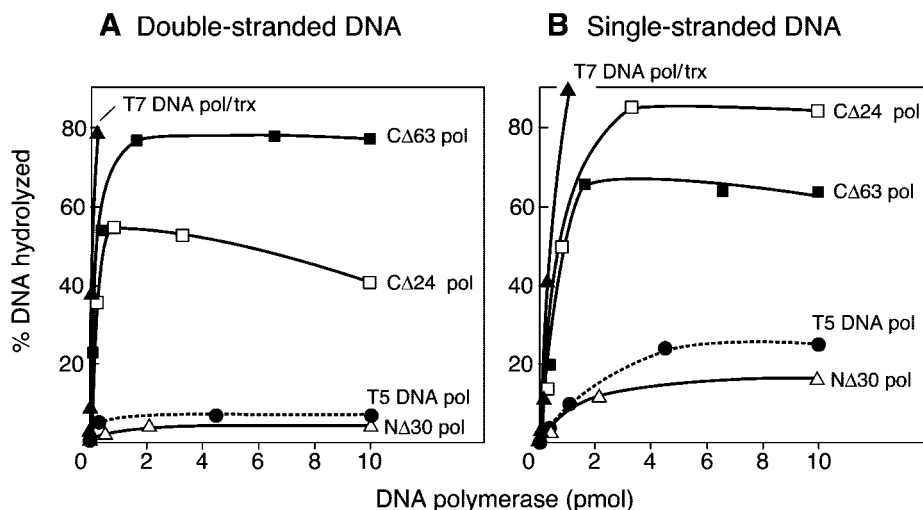


FIG. 8. Exonuclease activities of T5 and T7 DNA polymerases. The exonuclease activities of T5 wild-type, Δ 30, Δ 24, and Δ 63 DNA polymerases and the T7 DNA polymerase-thioredoxin (trx) complex, were measured on single- and double-stranded DNA substrates as described under "Experimental Procedures." The single- and double-stranded DNA was M13 DNA that was uniformly labeled with [³H]thymidine. The reaction mixtures contained the indicated amounts of each polymerase. The amount of DNA hydrolyzed during the 30-min incubation at 37 °C was determined by measuring the amount of acid-soluble radioactivity formed. The percentage of DNA hydrolyzed is plotted as a function of the polymerase concentration. 100% corresponds to 300 pmol of DNA (expressed as total nucleotides). A, double-stranded DNA substrate. B, single-stranded DNA substrate. ●, wild-type T5 DNA polymerase; △, T5 Δ 30 DNA polymerase; □, T5 Δ 24 DNA polymerase; ■, T5 Δ 63 DNA polymerase; ▲, T7 DNA polymerase/thioredoxin.

were employed for processivity (Fig. 5, lanes 19 and 20) and strand displacement (Fig. 7, lane 8) experiments.

By comparison, the exonuclease activity of the T7 DNA polymerase-thioredoxin complex was much more active than that of T5 DNA polymerase as follows: 75-fold more active on double-stranded DNA and 15-fold more active on single-stranded DNA (Fig. 8 and Table II). The double-stranded DNA exonuclease activity of T7 DNA polymerase was 200-fold higher in the presence of thioredoxin than in its absence. Thus the increase in exonuclease activity on double-stranded DNA observed with T5 DNA polymerase mutants that had reduced processivity was opposite to that expected based on these activities observed with T7 DNA polymerase. Although the double-stranded DNA exonuclease activity of the T7 DNA polymerase-thioredoxin complex was 77-fold higher than that of T5 DNA polymerase, the level of this activity with T7 DNA polymerase in the absence of thioredoxin was 20-fold lower than that of T5 $\Delta 63$ DNA polymerase.

DISCUSSION

Replication of the genomes of essentially all known organisms is carried out by DNA polymerases in a highly processive manner, a *modus operandi* that is critical for the rapid, coordinated synthesis of the two strands at the replication fork. In most replication systems, the DNA polymerase is one subunit of a multiprotein complex, the replisome. Other basic components of most replisomes include a helicase, a primase, processivity factors, and DNA-binding proteins. In most cases the replicative DNA polymerases, in the absence of accessory proteins, have low processivity.

Bacteriophage and viruses frequently encode many of their own replication proteins, thus bypassing the more complex replication machinery of their host. However, occasionally they do acquire one or more of the host proteins for their own replisome. In general, the relatively smaller size of the viral genome negates the requirement for an intricate regulatory system, and hence the number of components in the viral replisome is relatively small compared with that of the host. As such, these relatively small systems provide ideal models to study the fundamental reactions that occur during DNA replication. Studies on several bacteriophage replication systems have revealed the diversity in the mechanisms that have evolved to carry out processive DNA synthesis. The DNA polymerase from phage $\Phi 29$ is highly processive in the absence of any factors (30). The DNA polymerase from phage T7 has very low processivity by itself, but it binds tightly in a one-to-one complex with *E. coli* thioredoxin, and this complex is able to synthesize thousands of nucleotides without dissociating from a primer-template (11). The DNA polymerase from phage T4 also has low processivity by itself, but it requires a complex of three additional proteins, one of which is a trimer that encircles the duplex DNA, to carry out processive DNA synthesis (10). Of these three processivity mechanisms, that of phage T4 most closely resembles that of the host.

Early studies showed that the DNA polymerase from phage T5 had relatively high processivity in the absence of any factors (6). T5 DNA polymerase was compared with the non-replicative DNA polymerase *E. coli* DNA polymerase I, and the replicative DNA polymerases T4 DNA polymerase and calf thymus DNA polymerase α and β , each in the absence of their processivity factors. T5 DNA polymerase was found to have a processivity of at least 150 nucleotides per cycle of polymerization at 37 °C, compared with 3–12 nucleotides for the other DNA polymerases tested; the length of the poly(dA)₃₀₀ template used precluded the determination of higher rates of processivity. However, the processivity of replicative DNA polymerases and their processivity factors far exceed these values, usually

reaching into the thousands of nucleotides (31).

The first goal of the present study was to extend the earlier studies on processivity in order to determine whether the processivity of T5 DNA polymerase, in the absence of accessory proteins, approaches that of other replicative polymerases in the presence of their processivity factors. In order to ensure the absence of any phage encoded accessory proteins we purified the protein from cells harboring only the T5 DNA polymerase gene. Once we found that T5 DNA polymerase did indeed have a processivity typical to that of replicative DNA polymerases with their processivity factors, a second goal was to determine whether the unique extensions at the N and C termini of T5 DNA polymerase played a role in its inherent processivity. Deletion of 30 residues from the N terminus had a minimal effect, whereas deletion of 24 residues from the C terminus had a dramatic effect on reducing its processivity.

A second property of T5 DNA polymerase that is unique among pol I-type DNA polymerases is its ability to carry out extensive strand displacement synthesis on duplex DNA templates in the absence of accessory factors (7). In order to polymerize nucleotides through duplex DNA, most DNA polymerases require either the catalytic, energy-dependent unwinding activity of a DNA helicase (16), or the stoichiometry destabilization of duplex DNA by a single-stranded DNA-binding protein (32). Deletion of 30 residues at the N terminus of T5 DNA polymerase had no effect on its ability to carry out strand displacement, whereas deletion of 24 residues from the C terminus eliminated this activity. Thus deletion of even a small part of the unique 71-residue C terminus of T5 DNA polymerase has a dramatic effect at reducing both the processivity of the enzyme and its ability to carry out strand displacement synthesis. Although these two effects are likely related, it is also evident from our results that the strand displacement synthesis observed by the T5 DNA polymerase occurs with relatively low processivity compared with synthesis on single-stranded DNA.

The physiological significance of the ability of T5 DNA polymerase to catalyze strand displacement synthesis in the absence of other proteins is not clear. Sequence analysis of the T5 genome (NCBI accession number 005859) shows that it encodes an NTP-dependent DNA helicase homologous to that used in other phage replication systems such as T7 and T4. The low processivity observed during strand displacement synthesis by T5 DNA polymerase suggests the requirement for such a helicase at the T5 replication fork. In addition, it is in fact detrimental for a DNA polymerase to initiate strand displacement synthesis from a nick during replication of the lagging strand, because it will prevent the ligation of adjacent Okazaki fragments. For example, exonuclease-deficient mutants of T7 DNA polymerase are able to initiate strand displacement synthesis at nicks (33), and it is likely that this property is the basis for their inability to support the growth of T7 (19). It is likely that in T5 phage-infected cells a mechanism exists to prevent strand displacement synthesis on the lagging strand.

T5 DNA polymerase has a low level of 3'-5'-exonuclease activity, similar to that associated with *E. coli* DNA polymerase I (11) and much weaker than that of T7 DNA polymerase. It is possible that the high level of exonuclease in T7 DNA polymerase is an *in vitro* artifact and that *in vivo* this activity is held in check by being part of a large replication complex. Deletion of 30 residues at the N terminus of T5 DNA polymerase reduces its double-stranded DNA exonuclease activity by 5-fold. This reduction is not surprising, because the exonuclease domain is located toward the amino end of the enzyme. It is surprising, however, that the double-stranded DNA exonuclease activity of T5 DNA polymerase increases by 3- and 7-fold

when 24 or 63 residues are removed from the C terminus, respectively. These results suggest that the C-terminal domain normally prevents the entry of the DNA into the proofreading exonuclease site, perhaps serving as a regulator of this activity during DNA synthesis.

What do our results suggest that are the roles of the unique regions at the ends of T5 DNA polymerase? Because deletion of 30 residues from the N terminus of T5 DNA polymerase does not significantly effect its polymerase activity, processivity, or ability to strand displace, one possibility is that it is involved in interactions with other T5 proteins at the replication fork. Termini of other DNA polymerases have been shown to be critical for protein-protein interactions. For example, a domain near the N terminus of Φ 29 DNA polymerase interacts with the protein responsible for priming DNA replication (34), and DNA polymerases that use sliding clamp processivity factors, such as T4 DNA polymerase and *E. coli* DNA polymerase III, tether to the clamps via their C termini (10).

Our data show that the C terminus of T5 DNA polymerase is important in processivity, strand displacement synthesis, and exonuclease activity. A model consistent with these data is that this region functions as an intramolecular processivity factor, increasing the affinity of the polymerase for the template during DNA synthesis. How might this occur? In the absence of a three-dimensional structure of the T5 DNA polymerase, one can only infer possibilities from other examples of processivity factors. In the sliding clamp mechanism of processivity, a dimer or trimer, in the case of *E. coli* DNA polymerase III and T4 DNA polymerase, respectively, is loaded onto the duplex primer-template via a clamp loader (10). The proteins assemble on the duplex to form a ring around the DNA. The clamp, in turn, is tethered to the C-terminal domain of the DNA polymerase, thus preventing the dissociation of the DNA from the enzyme. In the case of phage T7, the processivity factor thioredoxin binds to a unique segment in the thumb subdomain of the polymerase, and this complex resides over the DNA binding crevice of the polymerase through which the duplex DNA follows to the active site (35).

The structure of a number of other polymerases in the pol I family have been solved in a complex with the primer-template. In the structure of T7 DNA polymerase, the C-terminal residue, histidine, forms a bond with the penultimate phosphate diester of the primer strand (35). Changing this histidine to an alanine results in a drastic reduction in processivity (26). This residue is located in the palm region, on the opposite side of the polymerase from the thioredoxin binding domain. Thus it is difficult to imagine that an extension at the C terminus could serve a role of clamping the DNA to the enzyme in a role analogous to that of thioredoxin. In the only known structure of a polymerase in the pol II family bound to a primer-template, the replicative DNA polymerase from the phage T4 relative RB69, a region near the C terminus makes electrostatic interactions with three phosphate groups of the bound DNA template (36). Deletion of the C-terminal 13 residues of Φ 29 DNA polymerase severely reduces its processivity and its ability to bind duplex DNA (37). Thus, for the C termini of DNA poly-

merases from both pol I and pol II families, the C terminus interacts with the primer-template and can influence the processivity of the enzyme. We propose that the unique C-terminal domain of T5 DNA polymerase plays a critical role keeping the polymerase bound tightly to DNA, giving it high processivity in the absence of accessory proteins. The crystal structure of this DNA polymerase will be of considerable interest to understand the structural basis for its novel mechanism for carrying out processive DNA synthesis.

Acknowledgment—We thank Samir Hamdan for critical reading of the manuscript.

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