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A Unique Region in Bacteriophage T7 DNA Polymerase Important for Exonucleolytic Hydrolysis of DNA*

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Jaya K. Kumar, Erica T. Chiu‡, Stanley Tabor, and Charles C. Richardson§

From the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

A crystal structure of the bacteriophage T7 gene 5 protein/*Escherichia coli* thioredoxin complex reveals a region in the exonuclease domain (residues 144–157) that is not present in other members of the *E. coli* DNA polymerase I family. To examine the role of this region, a genetically altered enzyme that lacked residues 144–157 (T7 polymerase (pol) Δ144–157) was purified and characterized biochemically. The polymerase activity and processivity of T7 pol Δ144–157 on primed M13 DNA are similar to that of wild-type T7 DNA polymerase implying that these residues are not important for DNA synthesis. The ability of T7 pol Δ144–157 to catalyze the hydrolysis of a phosphodiester bond, as judged from the rate of hydrolysis of a *p*-nitrophenyl ester of thymidine monophosphate, also remains unaffected. However, the 3′-5′ exonuclease activity on polynucleotide substrates is drastically reduced; exonuclease activity on single-stranded DNA is 10-fold lower and that on double-stranded DNA is 20-fold lower as compared with wild-type T7 DNA polymerase. Taken together, our results suggest that residues 144–157 of gene 5 protein, although not crucial for polymerase activity, are important for DNA binding during hydrolysis of polynucleotides.

Bacteriophage T7-encoded gene 5 protein is a replicative DNA polymerase that also has an associated 3′-5′ exonuclease activity that is active on both single-stranded (ss)[1] and double-stranded (ds) DNA (1). By itself, the 80-kDa gene 5 protein (gp5) is distributive for DNA synthesis and catalyzes the addition of less than 15 nucleotides before dissociating from a primer-template terminus (2). Association in a 1:1 complex with the 12-kDa *Escherichia coli* host protein thioredoxin increases the processivity of polymerization by gp5; the gp5·thioredoxin complex catalyzes the addition of thousands of nucleotides per cycle of polymerization at a rate of 300 nucleotides per second (3, 4). The association with thioredoxin also increases the exonuclease activity of gp5 on dsDNA several hundred-fold but has little effect on the hydrolysis of ssDNA (2). The complex of gp5 and thioredoxin will be referred to as T7 DNA polymerase in this report.

T7 DNA polymerase, like other members of the *E. coli* DNA polymerase I (Pol I) family, has a bipartite architecture with distinct C-terminal polymerase and N-terminal exonuclease domains (5). A 2.2-Å resolution crystal structure of T7 DNA polymerase in complex with a primer-template and an incoming dideoxynucleoside triphosphate was solved in the polymerization mode (see Fig. 1). This structure captured the polymerase in the act of adding a nucleotide to a growing chain of DNA. The polymerase domain is likened to a right hand composed of thumb, fingers, and palm subdomains that converge to form a DNA binding groove that leads to the polymerase active site. The processivity factor, *E. coli* thioredoxin, is bound to the thumb subdomain. In this structure, there are no visible contacts between the exonuclease domain and DNA. The exonuclease and polymerase active sites are separated by ~35 Å.

Although T7 DNA polymerase and the Klenow fragment of *E. coli* Pol I are structurally similar (5), they differ strikingly in a number of ways. T7 DNA polymerase is a replicative DNA polymerase and hence must physically and functionally interact with the other proteins of the T7 replication system, whereas *E. coli* Pol I is mainly devoted to DNA repair and thus does not have strong interactions with other *E. coli* proteins. T7 DNA polymerase, by virtue of its bound processivity factor, thioredoxin, has a processivity of polymerization in the range of thousands of nucleotides, whereas Klenow fragment polymerizes less than 10 nucleotides per polymerization cycle (2, 6). Remarkably, T7 DNA polymerase incorporates a dideoxynucleotide almost as well as a deoxynucleotide while Klenow fragment discriminates against these analogues several hundred-fold (7). Finally, the 3′-5′ exonuclease activity of T7 DNA polymerase on dsDNA is several hundred-fold higher than that of Klenow fragment (2).

One approach to understanding the molecular basis for these differences in activities is to compare T7 DNA polymerase and Klenow fragment for unique differences. Such an approach has been used successfully to identify a loop in the DNA binding crevice that interacts with the DNA primase domain of T7 gene 4 protein (8), a unique segment in the thumb subdomain of T7 gene 5 protein that binds thioredoxin (6), and a single amino acid residue in the active site that accounts for the discrimination against dideoxynucleotides (7).

Following this approach, we identified an elongated helix spanning residues 120–150 that is unique to T7 DNA polymerase (*helix E**, see Fig. 1). There is limited sequence homology in this region between T7 DNA polymerase and the Klenow fragment; two glutamic acid residues are present in both T7 DNA polymerase (Glu-148 and Glu-149) and Klenow fragment (Glu-474 and Glu-475). However, in T7 DNA polymerase, res-

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‡ Present address: Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111.

§ To whom correspondence should be addressed: Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-1864; Fax: 617-432-3362; E-mail: ccr@hms.harvard.edu.

[1] The abbreviations used are: ss, single stranded; ds, double stranded; nt, nucleotide(s); BSA, bovine serum albumin; DTT, dithiothreitol; pol, polymerase; TBD, thioredoxin-binding domain; *p*NP-TMP, 5′-*p*-nitrophenyl ester of thymidine monophosphate.

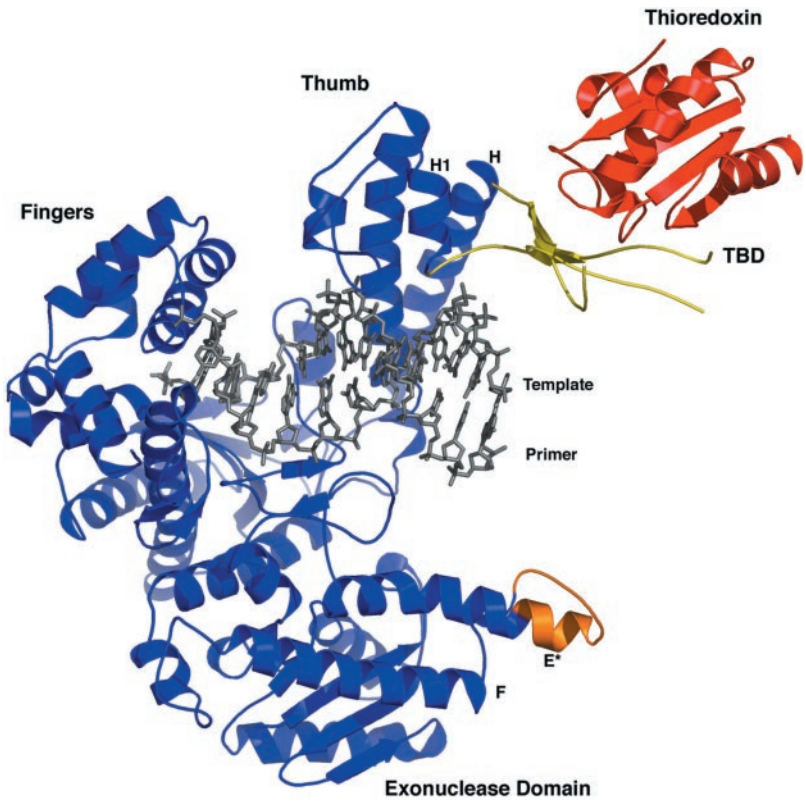

FIG. 1. **Crystal structure of T7 DNA polymerase.** A ternary complex of T7 gene *5* protein (*blue*) with its processivity factor, thioredoxin (*red*), a primed DNA template (*gray*), and an incoming 2′,3′-dideoxynucleotide (ddGTP) determined at 2.2-Å resolution (7). Residues 144–157 (KRMLEEQGEEYVDG) are in *orange*. The thioredoxin binding domain (*TBD*) comprising residues 262–338 is in *yellow*.

idues 120–157 form an elongated helix and residues 144–157 protrude out from the rest of the exonuclease domain. This region includes helix E* and the loop between helices E* and F (see Fig. 1).

The location and composition of this segment makes it particularly attractive for study. Most replicative DNA polymerases achieve high processivity by functioning as sliding clamps. Processivity factors such as proliferating cell nuclear antigen, the β subunit of *E. coli* DNA polymerase III, and the gene 45 proteins of bacteriophage T4 and RB69 all form closed sliding clamps that can accommodate duplex DNA in their central cavities, effectively anchoring the catalytic subunits to DNA (9). Such a closed clamp structure was not observed in the published ternary complex of T7 DNA polymerase (5). Instead, *E. coli* thioredoxin was bound to a 76-amino acid extended loop between helices H and H1 of the thumb subdomain positioning the loop over the duplex region of the primer-template, but was rotated away from the primer-template (Fig. 1). It is possible that, in this particular structure, T7 DNA polymerase was frozen in an "open" conformation and the polymerase does in fact flicker between open and closed conformations (10). During such a conformational switch, the thioredoxin-binding domain (TBD) could dock over duplex DNA, contacting another site on the polymerase, and thereby encircling the DNA.

The TBD is comprised of 71 amino acid residues enriched in glycines, prolines, and lysines. The average temperature factors of the atoms in this loop are nearly twice as high as the rest of the molecule indicating a region of high flexibility (5). Twenty-five residues (219–317) at the distal end of the loop are disordered. Of these, five are basic (Lys-268, Lys-285, Lys-300, Lys-302, and Lys-304), and Lys-300, Lys-302, and Lys-304 have been shown to be important in DNA binding and processivity of polymerization (11). The loop between helices E* and F is poised on the opposite of the DNA binding crevice in the exonuclease domain. Five residues at the tip of this loop have acidic side chains (Glu-148, Glu-149, Glu-153, Glu-154, and Asp-157) providing the possibility that the TBD swings over the primer-template and docks at this site in the exonuclease domain. The resulting clamp could increase the stability of the duplex primer-template/polymerase complex and thus bestow high processivity on either the polymerase or exonuclease reaction.

In the current study, 14 amino acids (residues 144–157) within the unique loop were deleted and the biochemical properties of the genetically altered T7 DNA polymerase (T7 polΔ144–157) examined. The deletion of this charged region of the loop has no effect on polymerase activity but, instead, plays a critical role in the exonucleolytic activity on polynucleotides.

EXPERIMENTAL PROCEDURES

Materials

DNA—M13mp18 was from New England Biolabs. M13 mGP1–2 is a 9950-nt derivative of vector M13 mp8 containing an insert of phage T7 DNA (*2*). Oligo-68 (5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCACGAATTCGCGGATCCAGCTGCAGGTAT-3′), Oligo-70 (5′-TCCGCGAATTCGTGCGACCCAGTGATAGGTATTGTTGGTGATTGTTGGTGGATTAGTAAGGACCCATACCTGCAGCTGGA-3′), the 24-nt (−47) M13 sequencing primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′), the 17-nt (−40) M13 sequencing primer (5′-GTTTTCCCAGTCACGAC-3′), and the 100-nt oligonucleotide (5′-GTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCTGGAAAGACGACAAAACTTTAGATCGTTACGCTAACTATGAGGGCTGTCTGTGGAATGC-3′) were from Integrated DNA Technologies. The 24-nt M13 sequencing primer and M13 mGP1–2 DNA were mixed in a 1:1 molar ratio (100 nM) in 40 mM Tris-Cl, pH 7.5, and 50 mM NaCl and annealed by heating to 95 °C for 5 min, followed by slowly cooling to room temperature. Oligonucleotide concentrations were determined spectrophotometrically. Oligonucleotide concentrations are expressed in terms of primer 3′-ends. M13 DNA concentrations are expressed in terms of number of molecules of M13.

E. coli Strains, Bacteriophage, and Plasmids—*E. coli* C600 was from the laboratory collection. *E. coli* HMS174 (DE3) and DH5α were from Invitrogen. T7Δ5 (gene *5* deletion) phage was from the laboratory collection. Plasmid pGP5-3 contains T7 gene *5* under the control of the T7 RNA polymerase promoter Φ 10 (*2*). Growth and manipulation of bacteriophage T7 and *E. coli* were carried out as described previously (12).

Mutagenesis of T7 Gene 5—Plasmid pGP5 Δ144–157 was constructed using standard PCR and cloning techniques. The identity of the clone was confirmed by DNA sequencing.

Enzymes and Proteins—Native T7 gene 5 protein and the genetically altered gene 5 protein (T7 pol Δ144–157) were overproduced in *E. coli* HMS174 (DE3) cells containing plasmid pGP5-3, and pGP5 Δ144–157, respectively. The 1:1 complex of polymerase and thioredoxin was purified to apparent homogeneity as described (2). Protein concentrations were determined by the method of Bradford (13). Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were from New England Biolabs.

Nucleotides—Sequencing grade nucleotides (dATP, dCTP, dGTP, and dTTP), [^{3}H]dTTP (3000 Ci/mmol), [*methyl*-^{3}H]thymidine (25 Ci/mmol), [γ-^{33}P]ATP (2500 Ci/mmol), and [α-^{33}P]dCTP (2500 Ci/mmol) were from Amersham Biosciences. The 5′-*p*-nitrophenyl ester of thymidine monophosphate (*p*NP-TMP) was from Sigma.

Methods

Plating Efficiencies—Plating efficiencies of T7Δ5 phage were measured on *E. coli* C600 cells harboring either pGP5-3 or pGP5Δ144–157. The cells were grown to a density of 2×10^8 cells/ml in LB media (1% Tryptone, 0.5% yeast, 0.5% NaCl, pH 7.0, at 50 °C). Dilutions of phage solutions in TB media were mixed with 0.5 ml of cells, 3 ml of top agar (1% Tryptone, 0.5% yeast, 0.5% NaCl, 0.7% agar, pH 7.0, at 50 °C) and 200 μg of ampicillin, and plated on TB (1% Tryptone, 0.5% yeast, 0.5% NaCl, 1.5% agar, pH 7.0) plates. The plates were incubated at 37 °C for 2–5 h before they were analyzed for the appearance of plaques.

Measurement of T7 Burst Size—The burst size of T7Δ5 phage was measured by infecting logarithmically growing *E. coli* C600 cells harboring pGP5-3 or pGP5Δ144–157 at a multiplicity of infection of 0.1 at 37 °C. After 15 min, the infected cells were centrifuged to remove unadsorbed phage, and then were diluted 10,000-fold. The infected cells were titered on *E. coli* C600 cells harboring plasmid pGP5-3 or pGP5Δ144–157 every 5 min from 15 to 40 min after infection.

DNA Polymerase Assays—Reaction mixtures (50 μl) contained 40 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 mM NaCl, 500 μM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (2 cpm/pmol), 50 μg/ml bovine serum albumin, 20 nM M13 mGP1–2 DNA annealed to a 24-nt M13 DNA sequencing primer, and varying amounts of T7 DNA polymerase. The reactions were incubated at 37 °C for 10 min and terminated by the addition of 10 μl of 0.5 M EDTA, pH 7.5. The incorporation of [^{3}H]dTMP into DNA was measured on DE81 filter discs as described previously (14).

Polymerase Processivity Assays—The DNA used for processivity assays was a 5′-^{33}P-labeled, 24-nt primer annealed to M13 mGP1–2 ssDNA as described before (2). Reactions were carried out at 37 °C as previously described (15). Reaction mixtures contained 40 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 mM NaCl, 500 μM each of dATP, dCTP, dGTP, and dTTP, the indicated amounts of primer-template, and 0–325 nM T7 DNA polymerase.

Strand Displacement DNA Synthesis—A pre-formed replication fork was constructed by ligating the 70-nt Oligo-70 into a circle using Oligo-68 as a splint. To anneal the two oligonucleotides, Oligo-68 (50 pmol) was mixed with Oligo-70 (50 pmol) in a reaction mixture (120 μl) containing 125 mM Tris-Cl, pH 7.5, and 0.15 M NaCl, incubated at 70 °C for 5 min and then slowly cooled to room temperature. This mixture was then incubated with T4 DNA ligase (40 units) and T4 polynucleotide kinase (10 units) at 37 °C for 30 min in a reaction mixture (145 μl) containing 345 μM ATP, 15 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM β-mercaptoethanol, 20 μg/μl bovine serum albumin, and 35 μM DNA template.

Strand displacement reactions (50 μl) contained 20 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 3 mM DTT, 37.5 mM NaCl, 500 μM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (2 cpm/pmol), and 20 nM pre-formed replication fork. Reactions were initiated by the addition of 63 kDa of T7 helicase/primase (gene 4 protein) and T7 DNA polymerase to final concentrations of 24 nM and 0–2 nM, respectively. The reaction mixtures were incubated at 37 °C for 10 min, and the reactions were terminated by addition of 10 μl of 0.5 M EDTA, pH 7.5. The incorporation of [^{3}H]TMP was measured as described (14).

T7 Gene 4 Primase-dependent DNA Synthesis by T7 DNA Polymerase—Reaction mixtures (50 μl) contained 40 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 mM NaCl, 300 μM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (60 cpm/pmol), 500 μM ATP and CTP, 50 μg/ml bovine serum albumin, 4 nM M13 mGP1–2 DNA, from 0 to 80 nM 63-kDa gene 4 protein, and 15 nM T7 DNA polymerase. The mixtures were incubated at 37 °C for 15 min and terminated by the addition of 10 μl of 0.5 M EDTA, pH 7.5. The incorporation of [^{3}H]dTMP into DNA was measured on DE81 filter discs as described (14).

Hydrolysis of pNP-TMP—Reaction mixtures (250 μl) contained 50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 5 mM DTT, 1 mM $MnCl_2$, 3 mM *p*NP-TMP (dissolved in 50 mM Tris-Cl, pH 8.0, and 50 mM NaCl), and 500 nM T7 DNA polymerase. The rate of hydrolysis of *p*NP-TMP was determined spectrophotometrically by monitoring *p*-nitrophenol production at 420 nm in a Hewlett-Packard 8452A diode array spectrophotometer. The molar extinction coefficient of *p*-nitrophenol at pH 8.0 and 420 nm is 12,950 $M^{-1}cm^{-1}$ (16).

TABLE I

Ability of gene 5 plasmids to complement T7 phage growth

Efficiency of plating of wild-type and Δ5 T7 phage on *E. coli* C600 was measured as described under "Experimental Procedures." *E. coli* C600 cells harboring the indicated plasmids were infected with wild-type or Δ5 T7 phage. The efficiency of plating is calculated by dividing the number of plaque-forming units observed when plated on cells containing the indicated plasmid by the number of plaque-forming units on pGP5-3 and represents an average of three experiments.

Plasmid[a]	Mutation	Efficiency of plating		Burst size[b]
		T7Δ5	T7 wt	
No plasmid		$<10^{-6}$	1	
pT7-7	No gene *5*	$<10^{-6}$	1	
pGP5-3	WT	1	1	45
pGP5Δ144–157	Δ144–157	1	1	32

[a] Plasmid pGP5-3 contains T7 gene *5* under the control of the T7 RNA polymerase promotor φ10, pGP5Δ144–157 contains T7 gene *5* lacking the codons encoding residues 144–157, and pT7-7 is the parent vector of pGP5-3 missing the gene *5* insert.

[b] Burst sizes were determined using T7Δ5 phage and are the average of three experiments.

Exonuclease Activity—The 3′-5′ exonuclease activity of T7 DNA polymerase was measured using uniformly ^{3}H-labeled M13 ssDNA or dsDNA. The substrates were prepared, and reactions were carried out as previously described (15). Reaction mixtures (100 μl) contained 40 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 mM NaCl, 20 nM of ^{3}H-labeled M13mp18 ssDNA or dsDNA, and varying amounts of T7 DNA polymerase.

Processivity of Exonuclease Activity—The DNA used for dsDNA processivity assays was a 5′-^{33}P-labeled, 100-nt oligonucleotide annealed to M13 mp18 ssDNA. The oligonucleotide (1 pmol) was first labeled at its 5′ terminus with ^{33}P by incubation in a reaction mixture (20 μl) containing 70 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 5 μl of [γ-^{33}P]ATP (2500 Ci/mmol), and 1 μl of T4 polynucleotide kinase at 37 °C for 30 min, and then annealed to M13 DNA. The 5′-^{33}P-labeled, 100-nt ssDNA substrate used for processivity measurements on ssDNA was prepared by alkali denaturation of the dsDNA substrate by treatment with 50 mM NaOH at 20 °C for 15 min, followed by neutralization with HCl.

The reaction mixtures (12 μl) contained 40 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 mM NaCl, and 2 nM M13 DNA. Reactions were initiated by the addition of 4 μl of varying concentrations of T7 DNA polymerase (0–16 nM). After 1 min at 30 °C, the reactions were stopped by the addition of 12 μl of 95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% Bromphenol Blue. The products were separated by electrophoresis in an 8% polyacrylamide denaturing gel containing 50% urea (w/v) in 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA. After electrophoresis, the gels were dried and autoradiographed.

RESULTS

T7 pol Δ144–157 Supports T7 Phage Growth—To examine the role of residues 144–157 in T7 DNA polymerase, an altered gene *5* lacking the codons encoding these residues was constructed. The effect of the genetically altered gene *5* on the growth of wild-type T7 phage and T7 phage lacking gene *5* (T7Δ5 phage) was examined. T7Δ5 phage is dependent on the expression of plasmid encoded gene *5* for viability. Plasmid-encoded T7 pol Δ144–157 supports the growth of T7Δ5 phage (Table I). While the efficiencies of plating of T7Δ5 phage on *E. coli* C600 cells expressing wild-type T7 DNA polymerase or T7 pol Δ144–157 are similar, plaques formed by T7 pol Δ144–157 are smaller. T7 pol Δ144–157 also has a slightly smaller burst size (Table I) than wild-type T7 DNA polymerase implying an overall defect in phage growth.

DNA Synthesis in Vitro—The polymerase activity of T7 pol Δ144–157 was compared with wild-type T7 DNA polymerase

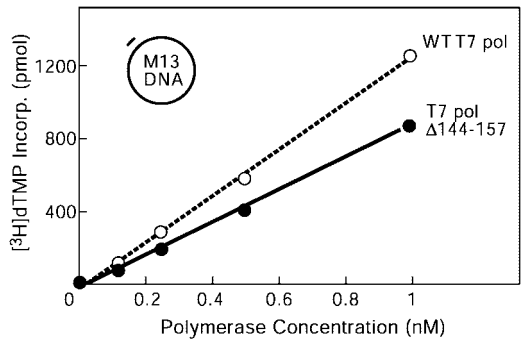

FIG. 2. **DNA synthesis catalyzed by wild-type T7 DNA polymerase and T7 pol Δ144–157.** A 24-nt oligonucleotide annealed to circular M13 ssDNA was used as the primer-template in a standard polymerase assay. The incorporation of [^{3}H]dTMP in 10 min at 37 °C was measured as described under "Experimental Procedures." The length of the template is 9950 bases. Reaction mixtures contained 20 nM DNA.

by measuring the incorporation of [^{3}H]dTMP into primed M13 ssDNA (Fig. 2). The concentration of the DNA primer-template (20 nM) was at least ~20-fold in excess over the polymerase. As shown in Fig. 2, the polymerase activity of T7 pol Δ144–157, measured as the initial rate of DNA synthesis, is within a factor of two of the activity of the wild-type polymerase.

Polymerase Processivity Assays—The similar rates of DNA synthesis by wild-type T7 polymerase and T7 pol Δ144–157 suggest that they have a similar processivity of polymerization. We measured directly the lengths of the products synthesized during a single round of DNA synthesis. M13 ssDNA annealed to a 5′-^{33}P-labeled, 24-nt oligonucleotide was used as the primer-template. In one experiment, the primer-template was maintained in 8-fold molar excess over the polymerase. Aliquots of the reaction mixture were removed at various times and subjected to electrophoresis in an agarose gel (Fig. 3*A*). In a second experiment, the polymerase concentration was varied and the lengths of products formed in a 5-min reaction were observed (Fig. 3*B*). T7 pol Δ144–157 is as processive as wild-type T7 DNA polymerase as observed from the accumulation of the full-length 9950-nt product in <5 min. This experiment illustrates the similar processivity of the two enzymes in that both enzymes catalyze the formation of the full-length DNA product under conditions where DNA synthesis has not yet been initiated on most of the primers (Fig. 3*A*, 2-min time points).

Helicase-dependent Strand Displacement DNA Synthesis by T7 DNA Polymerase—The ability of T7 pol Δ144–157 to interact functionally with the bacteriophage T7 helicase encoded by gene *4* (gp4) was evaluated on a minicircle template using purified proteins (8). On a small minicircle template, once the polymerase encounters duplex regions of the template, it cannot catalyze further DNA synthesis; gp4 is required to unwind duplex DNA for T7 DNA polymerase to further synthesize DNA via a rolling circle mechanism (17, 18). In the absence of gp4, there is no observable DNA synthesis (data not shown). Thus, the resulting strand displacement synthesis requires a functional coupling of the polymerase and helicase via a direct physical interaction between the two proteins (19). As shown in Fig. 4, T7 pol Δ144–157 catalyzes strand displacement DNA synthesis in complex with gp4, implying that it interacts in a functional mode with gp4 in a manner analogous to that of wild-type T7 DNA polymerase.

Primase-dependent DNA Synthesis by T7 DNA Polymerase—The primase domain of gp4 catalyzes the synthesis of tetraribonucleotides at primase recognition sites in the presence of ATP and CTP (20). These tetraribonucleotides are stabilized on

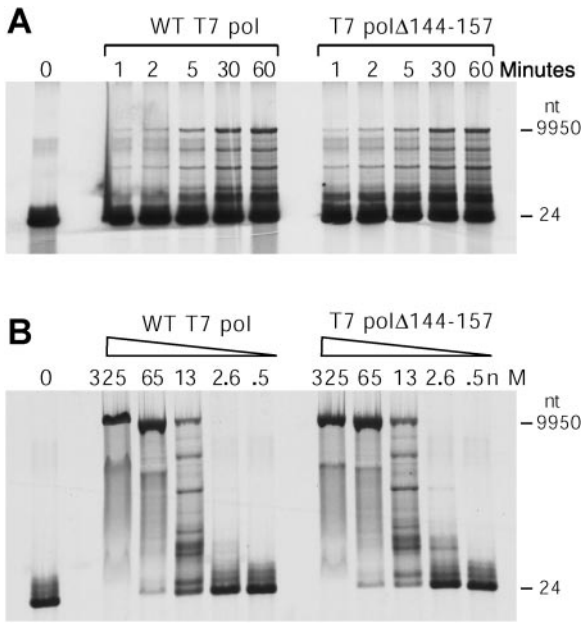

FIG. 3. **Processivity of polymerization catalyzed by wild-type T7 DNA polymerase and T7 pol Δ144–157.** A 5′-^{33}P-labeled 24-nt primer was annealed to a M13 ssDNA template. The primer was extended by the incorporation of 4 dNMPs in a standard polymerase assay at 37 °C with either wild-type T7 DNA polymerase or T7 pol Δ144–157. *A*, the molar ratio of polymerase (0.25 nM) to primer-template (4 nM) was 1:16. For each reaction, aliquots were removed at the indicated times and mixed with an equal volume of 0.2% SDS and 25 mM EDTA. The reaction products were separated by electrophoresis in a 0.6% agarose gel containing 0.06 μg/ml ethidium bromide and visualized by autoradiography. *B*, effect of increasing polymerase concentration on the length of products. The reaction time was 5 min. The markers on the *right* correspond to the length of the labeled primer annealed to M13 DNA.

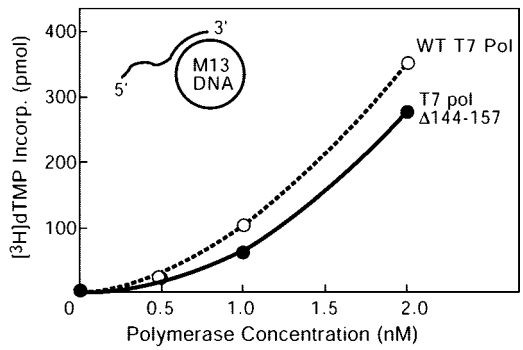

FIG. 4. **Gene *4* helicase-dependent DNA synthesis.** DNA synthesis catalyzed by T7 DNA polymerase and T7 gene 4 protein was measured using a 70-nt circular dsDNA template with a 40-nt 5′-oligo(dT) tail (see *inset*). The 5′-tail allows the helicase to load onto the DNA template. The helicase activity of gene 4 protein unwinds duplex region of the template, allowing the T7 DNA polymerase to catalyze DNA synthesis on the circular ssDNA template. The incorporation of [^{3}H]dTMP in 10 min at 37 °C was measured as described under "Experimental Procedures." Reaction mixtures contained 20 nM DNA, 24 nM gp4, and from 0 to 2 nM T7 DNA polymerase.

the DNA template by gp4 until utilized by T7 DNA polymerase for priming DNA synthesis (21, 22). T7 DNA polymerase cannot extend the primers in the absence of gp4. The gene 4 primase-dependent DNA synthesis catalyzed by T7 pol Δ144–157 on M13 ssDNA was compared with that catalyzed by wild-type T7 DNA polymerase (Fig. 5). The rates of DNA synthesis are comparable indicating that T7 pol Δ144–157 interacts functionally with the T7 gene 4 primase.

Hydrolysis of the 5′-p-Nitrophenyl Ester of Thymidine 5′-Monophosphate—The *p*-nitrophenyl ester of thymidine 5′-

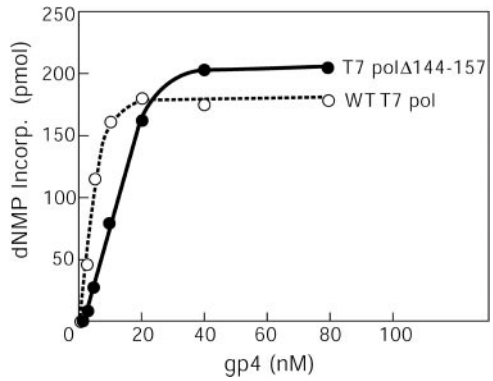

FIG. 5. **T7 gene 4 primase-dependent DNA synthesis.** The ability of wild-type T7 DNA polymerase and T7 pol Δ144–157 to use primers synthesized by T7 63-kDa gp4 on M13 ssDNA in the presence of ATP and CTP (500 μM) was measured in a reaction containing 4 nM DNA, 15 nM T7 DNA polymerase, and from 0 to 80 nM gp4. The incorporation of [^{3}H]dTMP in 15 min at 37 °C was measured as described under "Experimental Procedures." The data have been presented as the incorporation of deoxyribonucleoside monophosphate (4× dTMP).

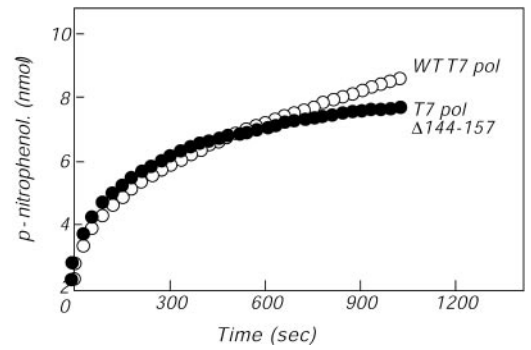

FIG. 6. **Hydrolysis of the 5′-*p*-nitrophenyl ester of thymidine monophosphate (*p*NP-TMP).** The rate of hydrolysis of *p*NP-TMP catalyzed by T7 DNA polymerase and T7 pol Δ144–157 at 25 °C was determined spectrophotometrically by monitoring *p*-nitrophenol production at 420 nm. Reaction mixtures contained 3 mM *p*NP-TMP and 50 nM T7 DNA polymerase.

monophosphate (*p*NP-TMP) was used to examine the integrity of the 3′-5′ exonuclease active site of T7 DNA polymerase. Examining the hydrolysis of a mononucleotide substrate allows the isolation of specific interactions with the residues in the active site from the more extensive interactions of longer polynucleotide substrates (16, 23). The rates of phosphodiester bond hydrolysis catalyzed by wild-type T7 DNA polymerase and T7 pol Δ144–157 were compared by monitoring the formation of *p*-nitrophenol, the product of *p*NP-TMP hydrolysis (Fig. 6). The substrate, *p*NP-TMP, was maintained in 6000-fold molar excess over the polymerase for pseudo first-order kinetic measurements. The catalytic rate constant (k_{cat}) was determined from the initial velocity of the reaction. From initial rate measurements, the pseudo first-order rate constants for wild-type T7 DNA polymerase (k_{cat} = 0.026 s^{-1}) and T7 pol Δ144–157 (k_{cat} = 0.023 s^{-1}) are comparable. Thus, deletion of residues 144–157 of the polymerase has little effect on its exonuclease active site.

3′-5′ Exonuclease Activity on Polynucleotide Substrates—Gp5 possesses 3′-5′ exonuclease activity on both ssDNA and dsDNA. Thioredoxin greatly stimulates the activity on dsDNA (24). We measured the 3′-5′ exonuclease activities of T7 pol Δ144–157 on both ssDNA and dsDNA at 37 °C (Fig. 7). The fraction of DNA hydrolyzed was measured as a function of the enzyme concentration. The 3′-5′ exonuclease activities of T7 pol Δ144–157 on both ssDNA and dsDNA are reduced drastically compared with wild-type T7 DNA polymerase. The activity of T7 pol Δ144–157 on ssDNA is reduced by ~10-fold compared with wild-type T7 DNA polymerase (Fig. 7*A*), and on dsDNA is reduced by ~20-fold (Fig. 7*B*). Because the exonuclease active site of T7 pol Δ144–157 is intact, as evidenced by rate measurements with *p*NP-TMP, this decrease in exonuclease activity on polynucleotide substrates suggests a defect in DNA binding.

Processivity of Hydrolysis of DNA—Because the processivity of the 3′-5′ exonuclease of T7 DNA polymerase has not been reported, we first analyzed the processivity of hydrolysis catalyzed by T7 DNA polymerase by visualizing the length of the products of degradation of a DNA substrate by the method of Bambara *et al.* (25). A 5′-^{33}P-labeled, 100-nt oligonucleotide was used to examine exonuclease activity and processivity on ssDNA. This oligonucleotide, which is complementary to M13 DNA, was annealed to M13 ssDNA to form the dsDNA substrate used for exonuclease activity measurements on dsDNA. In all experiments, the DNA concentration was maintained at

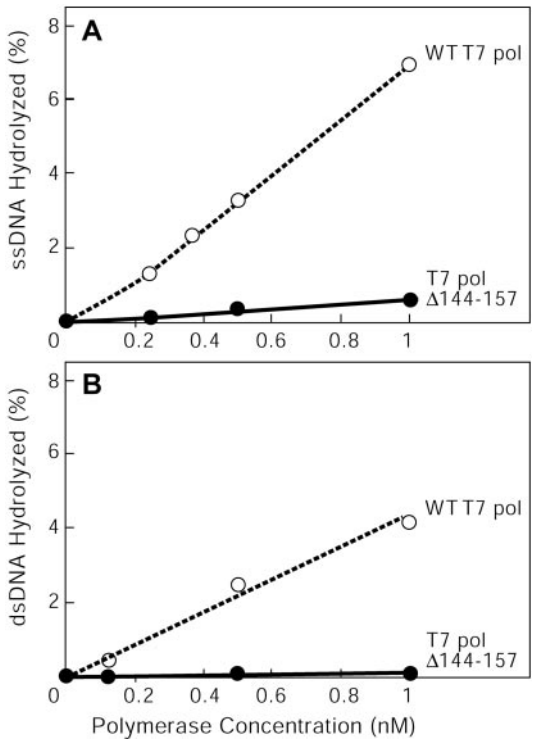

FIG. 7. **3′-5′ exonuclease activity on ^{3}H-labeled M13 DNA.** *A*, single-stranded DNA exonuclease activity. M13[^{3}H] ssDNA was prepared by alkali denaturation of M13[^{3}H] dsDNA. *B*, double-stranded DNA exonuclease activity. Uniformly labeled ^{3}H-labeled M13 dsDNA was prepared by annealing a 24-nt oligonucleotide to M13 DNA and then extending the primer using T7 DNA polymerase in the presence of [^{3}H]dTTP. Acid-soluble radioactivity was measured as described under "Experimental Procedures." The percentage of DNA hydrolyzed has been presented as a function of polymerase concentration. 100% ssDNA (*A*) or dsDNA (*B*) corresponds to 1 nmol of M13 DNA (in terms of total nucleotides).

2 nM, whereas the polymerase concentration was varied from 0 to 16 nM. Reactions were initiated by the addition of polymerase at 30 °C and stopped after 1 min. The products of the reaction were analyzed by electrophoresis in an 8% denaturing polyacrylamide gel (Figs. 8 and 9).

The processivity of the 3′-5′ exonuclease activity on ssDNA is shown in Fig. 8. The undigested 100-nt substrate is shown in *lane 1*. The end-products of hydrolysis that include nucleoside monophosphates and short oligonucleotides (up to 5-mers) can be observed at the *bottom* of the gel in *lanes 2–4*. Under the conditions used here, short oligonucleotides less than 5 nucleotides in length are not resolved. In *lanes 6–9*, where there is a

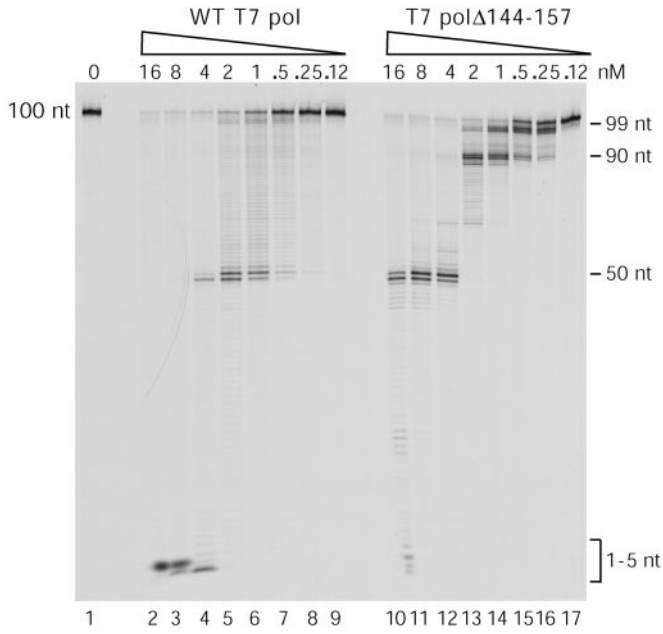

FIG. 8. **Processivity of the 3′-5′ ssDNA exonuclease activity of T7 DNA polymerase and T7 pol Δ144–157.** A 5′-^{33}P-labeled, 100-nt primer (2 nM) was used as the substrate. Reaction mixtures contained 2 nM DNA and from 0 to 16 nM T7 DNA polymerase. The reaction mixtures were incubated for 1 min at 30 °C and then terminated by the addition of 95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% Bromphenol Blue. Reaction products were separated by electrophoresis in an 8% denaturing polyacrylamide gel containing 50% urea and visualized by autoradiography. Markers are shown on the *left* (starting material) and *right* (degradation products).

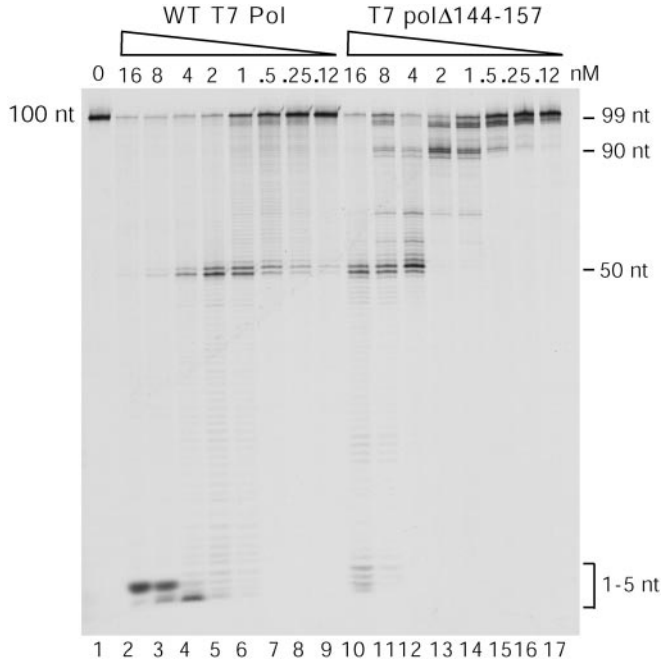

FIG. 9. **Processivity of the 3′-5′ dsDNA exonuclease activity of T7 DNA polymerase and T7 pol Δ144–157.** A 5′-^{33}P-labeled, 100-nt primer (2 nM) was annealed to M13 DNA and used as the substrate for reactions. Reaction mixtures contained 2 nM DNA and from 0 to 16 nM T7 DNA polymerase. Reaction mixtures were incubated for 1 min at 30 °C and terminated by the addition of 95% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% Bromphenol Blue. Reaction products were separated by electrophoresis in an 8% denaturing polyacrylamide gel containing 50% urea and visualized by autoradiography. Markers are shown on the *left* (starting materials) and *right* (degradation products).

4- to 17-fold excess of template over polymerase, most of the DNA substrate is not hydrolyzed, suggesting that the products observed are due to a single cycle of polymerase binding and exonuclease degradation. Under these conditions, the processivity of wild-type T7 DNA polymerase appears to be less than 50 nucleotides. With higher amounts of T7 DNA polymerase (*lanes 4* and *5*), there are a pair of strong bands corresponding to fragments ~50 nucleotides in length. This represents a site that is a strong kinetic pause for T7 DNA polymerase, presumably due to secondary structure in the single-stranded DNA. It is only at higher enzyme concentrations (*lanes 2–4*) where there is an excess of wild-type T7 DNA polymerase over ssDNA that the end-products of exonucleolytic hydrolysis are observed.

The processivity of the 3′-5′ exonuclease of T7 pol Δ144–157 on ssDNA is much lower than that of wild-type T7 DNA polymerase (Fig. 8). Under conditions where the DNA substrate is in 2- to 16-fold excess over the polymerase (*lanes 14–17*), the polymerase dissociates from the substrate after the degradation of less than 5 nucleotides. T7 pol Δ144–157 dissociates from DNA at two additional pause sites as judged by the accumulation of premature degradation products of ~95 and 90 nt. This result suggests that T7 pol Δ144–157 is more prone to dissociation from ssDNA than the wild-type polymerase. The lower processivity of T7 pol Δ144–157 is further demonstrated by the fact that, at concentrations where T7 pol Δ144–157 is in 8-fold molar excess over DNA (*lane 10*), the DNA is not yet fully degraded, with the predominant fragments still ~50 nucleotides in length, whereas at a comparable concentration of wild-type T7 DNA polymerase, the DNA is essentially completely degraded (<5 nucleotides, *lane 2*).

The processivity of 3′-5′ exonucleolytic hydrolysis on a dsDNA template was measured using the 5′-^{33}P-100-nt primer annealed to M13 DNA (Fig. 9). As the binding of thioredoxin to gp5 bestows a high processivity for polymerization, as well as dramatically increases the exonuclease activity on dsDNA, it is likely that it also increases the processivity of the exonuclease (2). Fig. 9 shows that wild-type T7 DNA polymerase does appear to processively catalyze the hydrolysis of dsDNA. Under conditions where most of the primer is not degraded (*lanes 8* and *9*), where there is an 8–16 fold excess of DNA over polymerase, the primers that have been degraded are reduced in size by about 50 nucleotides.

T7 pol Δ144–157 has much lower processivity than wild-type T7 DNA polymerase on dsDNA (Fig. 9). T7 pol Δ144–157 hydrolyzes only a few nucleotides before dissociating from DNA as judged by the distinct band at ~98 nt (Fig. 9, *lanes 15* and *16*). Even under conditions where the enzyme is in molar excess over DNA, T7 pol Δ144–157 dissociates at additional pause sites (*lanes 10–13*), resulting in the accumulation of products that are ~90 nt in length. These pause sites are not observed with wild-type T7 DNA polymerase, indicating that T7 pol Δ144–157 stalls or dissociates from the dsDNA substrate upon encountering these sequence-specific sites, whereas wild-type T7 DNA polymerase can navigate through these regions in a more processive manner.

DISCUSSION

T7 DNA polymerase has a higher processivity of polymerization and an exonuclease activity that is several hundred-fold higher than that of *E. coli* Pol I (6). Both proteins belong to the pol I family of polymerases and share extensive sequence and structural homology. One approach toward understanding differences in activity is to identify regions that are unique to either protein. The crystal structure of T7 DNA polymerase shows that residues 144–157 in the exonuclease domain form an extended helix that is absent in *E. coli* Pol I.

This region was examined for a possible role in processive DNA polymerization and exonucleolytic hydrolysis of DNA, because it is located in a position to serve as a docking site for the thioredoxin-binding domain. The structure of T7 gene 5 protein in complex with thioredoxin and a primer-template showed the processivity factor and its binding domain poised on the thumb subdomain over the DNA binding crevice. This particular structure provides a snapshot of the complex that is stabilized by the inability of the 3′-dideoxy-terminated primer chain to carry out a nucleophilic attack on the incoming nucleoside 5′-triphosphate. However, processive polymerization requires the stabilization of the polymerase·DNA complex during the translocation of the primer-template with each cycle of polymerization. We postulate that the binding of thioredoxin confers this stability by one of two mechanisms. In the first mechanism, thioredoxin and its TBD would dock at an unspecified site located in the exonuclease domain of the DNA binding crevice. The resulting clamp, one half composed of the DNA binding crevice itself, and the other half provided by the TBD, would then function essentially as a sliding clamp. In the second possible mechanism, the TBD, which is highly basic, would make electrostatic contacts with DNA to lock it into place.

From the crystal structure, residues 144–157 seem uniquely poised at the tip of the exonuclease domain to make contact with the TBD. In addition, this region is rich in acidic residues (5 out of 14 residues), making the model of electrostatic interactions with the highly basic TBD tractable. However, we find that this relatively large deletion of 14 amino acids has only a slight effect on DNA polymerase activity, less than 2-fold. Likewise the processivity of polymerization of nucleotides by the altered enzyme on a primed M13 DNA template was comparable to that observed with the wild-type enzyme. We conclude that residues 144–157 do not play a critical role in processivity of polymerization. Because there are no other apparent sites on T7 DNA polymerase where the TBD might dock, our results suggest that the TBD does not form a closed clamp structure around DNA during polymerization, and may in fact confer processivity by direct electrostatic interactions with duplex DNA.

Residues 144–157 of T7 DNA polymerase are also not involved in interactions with the T7 helicase/primase encoded by gene *4*. Bacteriophage T7 DNA polymerase interacts with a number of accessory proteins to reconstitute a replisome *in vitro* (26, 27). One of the most important protein-protein interactions is with the T7 helicase/primase. T7 DNA polymerase requires the helicase to unwind sections of duplex DNA to carry out strand displacement DNA synthesis on the leading strand, and a primase to initiate the synthesis of Okazaki fragments on the lagging strand. These interactions are also crucial for coordinating leading and lagging strand DNA synthesis (26). To examine the ability of T7 pol Δ144–157 to function with gp4 on a replication fork *in vitro*, we examined strand displacement DNA synthesis via a rolling circle mechanism catalyzed by the two proteins. T7 pol Δ144–157 catalyzes strand displacement DNA synthesis in complex with gp4 at approximately the same rate as the wild-type T7 DNA polymerase. T7 pol Δ144–157 can also extend DNA from tetraribonucleotide primers synthesized by the primase domain of gp4 as well as the wild-type protein. These results establish that T7 pol Δ144–157 interacts functionally with both the helicase and primase domains of gp4.

We find that residues 144–157 are important in the hydrolysis of polynucleotides, a reaction catalyzed by the exonuclease domain of T7 DNA polymerase. Residues 144–157 are not a part of any of the three conserved 3′-5′ exonuclease motifs (Exo I, Exo II, and Exo III) (28). Nevertheless, to examine whether the deletion of residues 144–157 perturbs the exonuclease active site, the rate of hydrolysis of a single phosphodiester bond was examined using the *p*-nitrophenyl ester of thymidine 5′-monophosphate (*p*NP-TMP). This substrate allows one to analyze the interactions of the key residues of the exonuclease active site separately from the more extensive interactions with a polynucleotide substrate. The similar pseudo first-order rate constants (k_{cat}) of 0.026 and 0.023 s^{-1} determined at 25 °C and pH 8.0 for wild-type and T7 pol Δ144–157, respectively, establishes that the phosphodiester bond hydrolysis reaction catalyzed by the polymerase is not affected by the deletion. The rate of hydrolysis of *p*NP-TMP catalyzed by T7 DNA polymerase is much lower compared with the rate of hydrolysis of polynucleotide substrates. The rate constant attending hydrolysis of ssDNA is 700 s^{-1} (29). Previous studies that have used *p*NP-TMP to measure the 3′-5′ exonuclease activity of proofreading enzymes also report lower rates of hydrolysis of *p*NP-TMP in comparison to rates of hydrolysis of polynucleotide substrates (16, 23). Lehman *et al.* (23) measured the rate of hydrolysis of *p*NP-TMP catalyzed by Klenow fragment (k_{cat} = 0.065 s^{-1} at 37 °C and pH 9.0) and found the corresponding k_{cat} for a polynucleotide substrate to be 4- to 40-fold higher. The ϵ subunit of *E. coli* pol III catalyzes the hydrolysis of *p*NP-TMP (16) with a k_{cat} = 0.32 s^{-1} at 25 °C and pH 8.0 and of ssDNA (30) with a k_{cat} = 200 s^{-1} at pH 7.5. The higher catalytic efficiency of hydrolysis of polynucleotide substrates has been ascribed to the DNA substrates making more extensive interactions within the exonuclease domain, thereby positioning the substrate in an orientation that favors phosphodiester bond hydrolysis (16).

The major consequence of deleting residues 144–157 is the reduction in the rates of hydrolysis of single- and double-stranded M13 DNA, which are only 9 and 4%, respectively, of the activity observed with the wild-type polymerase. This pronounced effect on the 3′-5′ exonuclease activity on DNA, but not on *p*NP-TMP, suggests that T7 pol Δ144–157 has a reduced affinity for polynucleotides. We show that T7 pol Δ144–157 has a dramatically lower processivity for hydrolysis of ssDNA and dsDNA compared with wild-type T7 DNA polymerase, consistent with a reduced affinity for DNA.

The exonuclease activity on dsDNA is reduced more than on ssDNA. This result may also be suggestive of a defect in shuttling the primer terminus of dsDNA to the exonuclease active site. A kinetic partitioning mechanism has been proposed for T7 DNA polymerase whereby duplex DNA binds preferentially to the polymerase site and only the 3′-primer terminus shuttles intramolecularly between the polymerase and exonuclease active sites (29). Crystal structures of polymerases in the Pol I family bound to a primer-template either in the polymerization or editing mode also show that the duplex region of the primer-template resides in the same region of the polymerase (31, 32). Yet, the primer terminus needs to traverse the ~35 Å that separate the polymerase and exonuclease active sites. This switch is effected by conformational changes in the polymerase as well as in the orientation of the helical axis of DNA, such as those observed in the crystal structure of RB69 DNA polymerase in the editing mode (33). The fact that the exonuclease activity on dsDNA is lowered even more than on ssDNA raises the possibility that T7 DNA polymerase undergoes a conformational change during the proofreading reaction and that residues 144–157 may play a role during the postulated conformational change.

In summary, we find that the deletion of residues 144–157 has no effect on the processive polymerization of nucleotides or on the integrity of the exonuclease active site. However, exonuclease activity on polynucleotide substrates is drasti-

cally reduced. We conclude that residues 144–157 of T7 DNA polymerase are important for DNA binding during exonucleolytic hydrolysis.

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REFERENCES

1. Grippo, P., and Richardson, C. C. (1971) *J. Biol. Chem.* **246,** 6867–6873
2. Tabor, S., Huber, H. E., and Richardson, C. C. (1987) *J. Biol. Chem.* **262,** 16212–16223
3. Huber, H. E., Russel, M., Model, P., and Richardson, C. C. (1986) *J. Biol. Chem.* **261,** 5006–5012
4. Huber, H. E., Tabor, S., and Richardson, C. C. (1987) *J. Biol. Chem.* **262,** 16224–16232
5. Doublie, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998) *Nature* **391,** 251–258
6. Bedford, E., Tabor, S., and Richardson, C. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 479–484
7. Tabor, S., and Richardson, C. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6339–6343
8. Chowdhury, K., Tabor, S., and Richardson, C. C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12469–12474
9. Breyer, W. A., and Matthews, B. W. (2001) *Prot. Sci.* **10,** 1699–1711
10. Doublie, S., Sawaya, M. R., and Ellenberger, T. (1999) *Structure Fold. Des.* **7,** R31–R35
11. Yang, X. M., and Richardson, C. C. (1997) *J. Biol. Chem.* **272,** 6599–6606
12. Kumar, J. K., Kremsdorf, R., Tabor, S., and Richardson, C. C. (2001) *J. Biol. Chem.* **276,** 46151–46159
13. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
14. Bryant, F. R., Johnson, K. A., and Benkovic, S. J. (1983) *Biochemistry* **22,** 3537–3546
15. Kumar, J. K., Tabor, S., and Richardson, C. C. (2001) *J. Biol. Chem.* **276,** 34905–34912
16. Hamdan, S., Bulloch, E. M., Thompson, P. R., Beck, J. L., Yang, J. Y., Crowther, J. A., Lilley, P. E., Carr, P. D., Ollis, D. L., Brown, S. E., and Dixon, N. E. (2002) *Biochemistry* **41,** 5266–5275
17. Lechner, R. L., Engler, M. J., and Richardson, C. C. (1983) *J. Biol. Chem.* **258,** 11174–11184
18. Lechner, R. L., and Richardson, C. C. (1983) *J. Biol. Chem.* **258,** 11185–11196
19. Notarnicola, S. M., Mulcahy, H. L., Lee, J., and Richardson, C. C. (1997) *J. Biol. Chem.* **272,** 18425–18433
20. Tabor, S., and Richardson, C. C. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 205–209
21. Nakai, H., and Richardson, C. C. (1986) *J. Biol. Chem.* **261,** 15217–15224
22. Kusakabe, T., and Richardson, C. C. (1997) *J. Biol. Chem.* **272,** 12446–12453
23. Lehman, I. R., and Richardson, C. C. (1964) *J. Biol. Chem.* **239,** 233–241
24. Hori, K., Mark, D. F., and Richardson, C. C. (1979) *J. Biol. Chem.* **254,** 1598–1604
25. Bambara, R. A., Fay, P. J., and Mallaber, L. M. (1995) *Methods Enzymol.* **262,** 270–280
26. Lee, J., Chastain, P. D., Kusakabe, T., Griffith, J. D., and Richardson, C. C. (1998) *Mol. Cell* **1,** 1001–1010
27. Debyser, Z., Tabor, S., and Richardson, C. C. (1994) *Cell* **77,** 157–166
28. Shevelev, I. V., and Hubscher, U. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 364–375
29. Donlin, M. J., Patel, S. S., and Johnson, K. A. (1991) *Biochemistry* **30,** 538–546
30. Miller, H., and Perrino, F. W. (1996) *Biochemistry* **35,** 12919–12925
31. Beese, L. S., Derbyshire, V., and Steitz, T. A. (1993) *Science* **260,** 352–355
32. Eom, S. H., Wang, J. M., and Steitz, T. A. (1996) *Nature* **382,** 278–281
33. Shamoo, Y., and Steitz, T. A. (1999) *Cell* **99,** 155–166