Role of the C-terminal Residue of the DNA Polymerase of Bacteriophage T7*

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The crystal structure of the DNA polymerase encoded by gene 5 of bacteriophage T7, in a complex with its processivity factor, Escherichia coli thioredoxin, a primer-template, and an incoming deoxynucleoside triphosphate reveals a putative hydrogen bond between the C-terminal residue, histidine 704 of gene 5 protein, and an oxygen atom on the penultimate phosphate diester of the primer strand. Elimination of this electrostatic interaction by replacing His704 with alanine renders the phage nonviable, and no DNA synthesis is observed in vivo. Polymerase activity of the genetically altered enzyme on primed M13 DNA is only 12% of the wild-type enzyme, and its processivity is drastically reduced. Kinetic parameters for binding a primer-template (Km), nucleotide binding (Km), and kcat for dissociation of the altered polymerase from a primer-template are not significantly different from that of wild-type T7 DNA polymerase. However, the decrease in polymerase activity is concomitant with increased hydrolytic activity, judging from the turnover of nucleoside triphosphate into the corresponding nucleoside monophosphate (percentage of turnover, 65%) during DNA synthesis. Biochemical data along with structural observations imply that the terminal amino acid residue of T7 DNA polymerase plays a critical role in partitioning DNA between the polymerase and exonuclease sites.

Gene 5 protein encoded by bacteriophage T7 is a replicative DNA polymerase with an associated 3′-5′ exonuclease activity (1). This 80-kDa enzyme, by itself, is distributive for DNA synthesis and can only incorporate less than 15 nucleotides before dissociating from a primer terminus (2). However, in a 1:1 complex with the host Escherichia coli protein, thioredoxin (KD = 5 nM), gene 5 protein processively catalyzes the addition of thousands of nucleotides at rates approaching 300 nucleotides/s (2–6). The gene 5 protein-thioredoxin complex will be referred to as T7 DNA polymerase in this manuscript.

A recent crystal structure of T7 DNA polymerase in complex with a primer-template and an incoming deoxynucleoside triphosphate solved at 2.2 Å resolution (7) reveals a bipartite structure with distinct C-terminal polymerase and N-terminal exonuclease domains (Fig. 1). Most polymerases of the polymerase family have a similar bipartite architecture (8–12). A “right hand” with distinct fingers, palm, and thumb subdomains forms a distinct DNA-binding groove that leads to the polymerase active site (13). The fingers, palm, and thumb grip the primer-template by a number of direct and water-mediated contacts mainly to the phosphodiester backbone of DNA such that the 3′-end of the primer strand is positioned next to the nucleotide-binding site. The 3′-5′ exonuclease activity on ssDNA and dsDNA serves to excise mismatches during progressive DNA synthesis (14, 15). The polymerase and the exonuclease active sites are ~35 Å apart.

Although the error frequency during base selection by the polymerase activity occurs at the low rate of one misincorporation per 106 turnovers, the 3′-5′ proofreading activity further reduces the error frequency by 10–200-fold depending on the method of measurement (16, 17). It is believed that the 3′-5′ exonuclease activity maximizes its contribution to replication fidelity by minimal hydrolysis of correctly base paired DNA (18). The pre-steady-state kinetic pathways for the polymerization and the exonuclease reactions have been described in detail for T7 DNA polymerase (17–19) and the Klenow fragment of E. coli DNA polymerase I (20–25). An equilibrium exists between DNA binding to the polymerase or exonuclease active site, with binding to the polymerase site being thermodynamically more favored (18). Any process that slows the rate of the elongation reaction, for example a mismatch, allows time for the transfer of DNA to the exonuclease active site (17, 25). The data also suggest that DNA is transferred intramolecularly in both directions between the polymerase and exonuclease sites without dissociating from the enzyme. However, the mechanisms that underlie the occupancy of DNA in the polymerase versus the exonuclease site are not well understood.

Residues that contact DNA may be expected to govern the partitioning of DNA between the two active sites. The crystal structure of T7 DNA polymerase reveals a putative hydrogen bond between the C-terminal residue His704 of gene 5 protein and an oxygen atom on the penultimate phosphate diester of the primer strand (Fig. 1). This C-terminal residue is conserved in T7 DNA polymerase (His704) and in E. coli DNA polymerase I (His228). In the DNA polymerases from Bacillus stearothermophilus (26, 27) and Thermus aquaticus (28–30), the homologous residues are Lys876 and Lys832, respectively. To probe the role of this interaction with the 3′-OH primer terminus, His704 of T7 DNA polymerase was substituted with alanine. The loss of this interaction could result either in reduced affinity of the polymerase for the primer-template or in reduced affinity for just the primer strand. In this report, we show that the C-terminal histidine of T7 DNA polymerase plays a critical role in

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1 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; nt, nucleotide; BSA, bovine serum albumin; DTT, dithiothreitol.
orrienting the primer terminus in position for catalysis and possibly in the controlled shutting of DNA between the polymerase and exonuclease sites.

**EXPERIMENTAL PROCEDURES**

**Materials**

DNA—M13 mGP1-2 is a 9950-nt derivative of vector M13 mp8 containing an insert of phage T7 DNA (31). The 24-nt M13 sequencing primer (5'-GGCCAGGTTTTTCCCCAGACGAC-3') and oligonucleotides for *in vitro* mutagenesis were from Oligo Etc. Activated calf thymus DNA (Type XV) was obtained from Sigma. Poly(dA)280 was obtained from Amersham Pharmacia Biotech, and oligo(dT)22 was obtained from Integrated DNA Technologies. Poly(dA)280, poly(dT)22, and oligo(dT)22 were dissolved in a 1:1 molar ratio (20 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 slow cooling to room temperature. The 24-nt M13 sequencing primer and M13 mGP1-2 DNA were mixed in a 1:1 molar ratio (100 nM) and annealed using the same protocol. Oligonucleotide concentrations were determined spectrophotometrically. DNA concentrations are expressed in terms of primer 3'-ends.

**E. coli Strains, Bacteriophage, and Plasmids**—*E. coli* strains C600 and HMS174 are from the laboratory collection. *E. coli* HMS174 (DE3)/pLysS cells are from Novagen. Wild-type bacteriophage T7 and mutant T7Δ5 (gene 5 deletion) phage are from the laboratory collection. Plasmid pGP5-H704A was digested with restriction enzymes StyI and Hin107I and then cloned into the *E. coli* strain HMS174. Mutagenesis of T7 Gene 5—*E. coli* tutoring 340 base pairs of the T7 DNA sequence containing gene 5 that encodes gp5-H704A under control of the T7 phage promoter 40T10. The codon in bold type corresponds to the amino acid alteration. Restriction enzymes were from New England Biolabs.

**Enzymes**—Gene 5 proteins were overproduced from *E. coli* HMS174 (DE3)/pLysS cells carrying plasmids. 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 (34) and were confirmed by amino acid analysis. Restriction enzymes were from Epicentre Technologies.

**Methods**

**Plating Efficiencies**—Plating efficiencies of wild-type and Δ5 T7 phage were measured on *E. coli* C600 cells harboring either the plasmid pTP7T, pGP5-3, or pGP5-H704A. The cells were grown to a density of 2 × 10^8 cells/ml. Dilutions of phage solutions 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) and ampicillin (200 μg/ml) 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 5 h before being analyzed for plaques.

**[3H]Thymidine Incorporation Assays**—Thymidine incorporation assays were carried out at 30 °C (35). *E. coli* C600 cells harboring plasmid pGP5-3 or pGP5-H704A were grown to a density of 3 × 10^8 cells/ml in Davis medium (0.7% potassium diphosphate, 2% potassium monophosphate, 0.05% sodium citrate, 0.1% magnesium sulfate, 0.1% ammonium sulfate) supplemented with glucose, thiamine, casamino acids, and ampicillin (80 μg/ml). The cells were infected with either wild-type T7 phage or T7 phage containing a deletion of gene 5 (Δ5 phage) at a multiplicity of infection of ~5 (35). At indicated time intervals, 200 μl of the samples were removed, and [3H]thymidine was added to a final concentration of 50 μCi/ml. Radioactive labeling was terminated after 90 s by the addition of 3 ml of ice-cold 0.3 n trichloroacetic acid. Acid-insoluble radioactivity was collected via filtration on glass microfiber filters and washed three times with 1 M HCl (3 ml) and twice with ethanol (3 ml). The acid insoluble radioactivity was measured using a scintillation counter.

**DNA Polymerase Assays**—The reaction mixtures (50 μl) with primed M13 mGP1-2 DNA contained 40 mM Tris-Cl, pH 7.5, 10 mM MgCl2, 5 mM DTT, 50 mM NaCl, 20 mM M13 DNA annealed to a 24-nt oligonucleotide, 500 μM each of dATP, dCTP, dGTP, and [3H]dTTP (2 cpmm/μl), 50 μg/ml BSA, and 0.3 nM DNA polymerase. The reaction mixtures were incubated at 37 °C for the indicated periods of time. The reactions were stopped by the addition of 10 μl of 0.5 n EDTA, pH 7.5. The incorporation of [3H]dTTP was measured on DE81 filter discs as described (20).

To measure a single round of DNA synthesis, (dA)400(dT)22 was used as a primer-template, and the reactions were carried out at 22 °C as described (6). The DNA polymerase was preincubated with the primer-template in the absence of Mg2+ and dTTP for 5 min. The reactions were initiated by the addition of Mg2+, dTTP, and challenger DNA to trap any free polymerase. The aliquots were withdrawn at the indicated times and quenched with a final concentration of 100 nM EDTA. The control reactions to measure background incorporation by polymerase not trapped by challenger DNA were carried out by adding challenger DNA to the preincubation mix. This background reaction was subtracted wherever applicable. The preincubation mixture (30 μl) contained 270 nM poly(dA)400 oligo(dT)22 and 40 nM DNA polymerase. The reaction was initiated by the addition of 25 mM MgCl2, 0.75 mM [3H]dTTP (2 cpmm/μl), and 10 μg calf thymus DNA in 20 μl. Final concentrations were 160 nM poly(dA)400 oligo(dT)22, 0.3 μM [3H]dTTP, and 0.5 μM MgCl2.
25 nm DNA polymerase, 10 mM MgCl₂, and 200 μg/ml calf thymus DNA. All reaction mixtures also contained 40 nm Tris-Cl, pH 7.5, 5 mM DTT, 50 mM NaCl, and 50 μg/ml BSA.

3'-5' Exonuclease Assays—The 3'-5' exonuclease activity was measured using uniformly [³²P]-labeled M13 ssDNA or dsDNA. This substrate was purified by dialyzing the 24-nt oligonucleotide to M13 mGP1-2 DNA and then extending the primer in a reaction mixture (300 μl) that contained 30 nm Tris-Cl, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 50 mM NaCl, 50 μM each of dATP, dCTP, dGTP, and [³²P]dTPP (300 Ci/mmol), and 200 nm T7 DNA polymerase. After incubation at 37 °C for 8 min, the DNA was extracted with an equal volume of buffer-saturated phenol, pH 9.8, 1% sodium dodecyl sulfate (SDS), and the labeled DNA was purified by a Sepharose CL-6B (Amersham Pharmacia Biotech) column. [³²P]-Labeled M13 mGP1-2 ssDNA was prepared by alkali denaturation of [³²P]-labeled dsDNA by treatment with 50 mM NaOH at 20 °C for 15 min, followed by neutralization with HCl. M13 [³²P]-labeled ssDNA was used immediately. [³²P]-Labeled M13 ssDNA was stored at 4 °C.

The reaction mixtures for exonuclease assays (100 μl) contained 40 nm Tris-Cl, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 50 mM NaCl, 1 nmol (in terms of total nucleotides) [³²P]-labeled M13 mGP1-2 ssDNA or dsDNA, and (0.06–6 nm) DNA polymerase. After incubation at 37 °C for 10 min, the reaction was quenched by the addition of 30 μl of BSA (10 mg/ml) and 30 μl of trichloroacetic acid (100% w/v). After incubation at 0 °C for 15 min, precipitated DNA was collected by centrifugation at 12,000 × g for 30 min, and the acid-soluble radioactivity was measured by scintillation counting in Ultra fluor (Packard). One unit of exonuclease activity catalyzes the release of 1 pmol of total nucleotides into an acid-soluble form in 1 min.

Processivity Assays—The DNA used for processivity assays was a 5'-³²P-labeled primer annealed to M13 mGP1-2 ssDNA or dsDNA (described below). The reaction mixture (22.5 μl) contained 40 nm Tris-Cl, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 50 mM NaCl, 500 μM each of dATP, dCTP, dGTP, and dTTP, and 8 μM primer-template. This mixture was preincubated at 37 °C for 3 min, and the reaction was started by the addition of 2.5 μl of T7 DNA polymerase. Final concentration of the polymerase was 0.3 nm. After the indicated times, the reaction was stopped by the addition of 5 μl of stop solution that contained 40% sucrose, 20 mM EDTA, and 0.05% bromphenol blue. The reaction mixtures were subjected to electrophoresis on a 0.6% agarose gel in a buffer containing 100 mM Tris–HCl, pH 8.0:chloroform:isoamyl alcohol (24:24:1), and the labeled DNA was visualized by autoradiography. The efficiency of cleolytic hydrolysis by the enzyme was quantified by TLC. An aliquot of the reaction mixture was quenched by the addition of 30 μl of trichloroacetic acid (100% w/v). After incubation at 0 °C for 15 min, precipitated DNA was collected by centrifugation at 12,000 × g for 30 min, and the acid-soluble radioactivity was measured by scintillation counting in Ultra fluor (Packard). One unit of exonuclease activity catalyzes the release of 1 pmol of total nucleotides into an acid-soluble form in 1 min.

Nucleotide Turnover Assays—The reaction mixture for turnover assays was essentially the same as for polymerase assays with the following exceptions. After varying periods of time (5–60 s), the polymerase reaction was started by the addition of MgCl₂ and dTTP and stopped after 30 s by the addition of EDTA to a final concentration of 100 mM. Final concentrations of all the reagents were identical to those used in the polymerase assay with poly(dA)₂₈₀ oligo(dT)₉₂ and adding challenger DNA at time 0. After

| Plasmid | Mutation | Efficiency of plating |
|---------|----------|-----------------------|
| No Plasmid |          | 10⁻⁶                  |
| pT7-7 | No gene 5 | 10⁻⁶                  |
| pGP5-3 | Wild type | 1                    |
| pGP5-H704A | H704A | 10⁻⁶                   |

- Plasmid pGP5-3 contains T7 gene 5 under the control of the T7 RNA polymerase promoter ã610, pGP5-H704A contains T7 gene 5 with the H704A codon substitution, and pT7-7 is the parent vector of pGP5-3 missing the gene 5 insert.

Gp5-H704A Cannot Support the Growth of T7 Phage—To ascertain the role of the C-terminal histidine in T7 DNA polymerase, a gene 5 mutant was constructed in which histidine 704 was replaced with alanine (pGP5-H704A). The effect of the genetically altered gene 5 protein on the growth of T7 phage was tested (Table I). When gp5-H704A is produced from a plasmid, it is unable to support the growth of T7 phage in which the wild-type gene 5 has been deleted (T7Δ5). T7Δ5 phage are dependent on the expression of plasmid encoded gene 5 protein for viability. Furthermore, gp5-H704A inhibits the growth of wild-type T7 phage that is expressing the wild-type gene 5. In contrast, production of wild-type gene 5 protein from a plasmid supports the growth of T7Δ5 phage and has no effect on the growth of wild-type T7 phage. Thus, the histidine to alanine substitution is dominant lethal for T7 phage growth.

DNA Synthesis in Vivo—The rates of in vivo DNA synthesis were measured by monitoring the incorporation of [³²P]thymidine into DNA in phage-infected cells (Fig. 2). E. coli C600 cells harboring a plasmid (pGP5-3 or pGP5-H704A) were infected with either T7Δ5 phage (Fig. 2A) or wild-type T7 phage (Fig. 2B). T7 DNA synthesis in infected cells starts ~10 min after infection, presumably after the shut down of host DNA synthesis (33). Fig. 2A shows a plot of the incorporation of [³²P]thymidine as a function of time upon infection with T7Δ5 phage. When wild-type gene 5 protein is produced from the plasmid, DNA synthesis starts to increase 10 min after infection and continues to increase up to 30 min after infection. In contrast, when gp5-H704A is produced from the plasmid, DNA synthesis is strongly inhibited 10 min after infection with T7Δ5 phage.

Even more striking is the kinetics of DNA synthesis upon infection with wild-type T7 phage (Fig. 2B). With wild-type gene 5 protein, T7 DNA synthesis starts 10 min after infection, reaching a maximum at 40 min after infection. In contrast, with gp5-H704A, DNA synthesis starts to decrease immediately upon infection. These data indicate that the altered protein, gp5-H704A, cannot restore DNA synthesis in T7Δ5 phage-infected cells. Furthermore, gp5-H704A inhibits DNA synthesis in wild-type T7 phage-infected cells.

DNA Synthesis in Vitro—To determine how the mutation in gene 5 leads to decreased DNA synthesis in vivo, gp5-H704A was overproduced, purified, and characterized bio-

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Fig. 2. Time course of in vivo DNA synthesis after infection with T7 phage. E. coli C600 cells (3 × 10³/ml) containing plasmid pGP5-3 (○) or pGP5-H704A (○) grown in Davis minimal medium at 30 °C were infected at a multiplicity of 5. At the indicated times after infection, the cells were pulse-labeled with [³H]thymidine for 90 s. The labeling was terminated by the addition of trichloroacetic acid. The acid-insoluble radioactivity was counted. The plots represent [³H]thymidine incorporated (cpm) as a function of time after infection. A, infecting phage contains a deletion of gene 5 (T7Δ5). B, infecting phage is wild-type T7 phage.

Fig. 3. Rates of DNA synthesis catalyzed by wild-type T7 DNA polymerase and gp5-H704A. A 24-nt oligonucleotide annealed to circular M13 ssDNA was used as the primer-template in a standard polymerase assay with gp5 or gp5-H704A. The rate of incorporation of [³H]dTTP was measured at 37 °C on DE81 filter discs as described under "Experimental Procedures." The data have been presented as the rates of incorporation of deoxyribonucleoside monophosphate (4 × dTMP). The length of the template is 9950 bases. The reaction mixtures contained 20 nM DNA and 0.30 nM polymerase.

Chemically. Initially, gp5-H704A was compared with wild-type T7 DNA polymerase by measuring the rate of polymerization of nucleotides. Initial rates of DNA synthesis with purified enzymes were measured by following the incorporation of [³H]dTTP into DNA using primed M13 ssDNA (Fig. 3) or poly(dA)280·oligo(dT)22 (Fig. 4) as primer-templates. The reactions were carried out under conditions of excess nucleotides and DNA. The steady-state rate constant for polymerization (kpol) was determined by dividing the rate of incorporation of nucleotides by the polymerase concentration. The polymerase activity has been expressed in terms of kpol. Gp5-H704A has 8-fold lower polymerase activity (kpol = 30 s⁻¹) than the wild-type polymerase (kpol = 240 s⁻¹) on M13 ssDNA (Fig. 3). With poly(dA)280·oligo(dT)22, the polymerase activity of gp5-H704A (kpol = 60 s⁻¹) is 3-fold lower than of the wild-type polymerase (kpol = −200 s⁻¹) (Fig. 4A). One possible explanation of the lower rate of DNA synthesis on the long M13 DNA template relative to that observed on the shorter poly(dA)280·oligo(dT)22 template is that gp5-H704A is less processive.

DNA synthesis for a single processive cycle was measured by preincubating the polymerase with poly(dA)280·oligo(dT)22 and initiating the polymerase reaction by the addition of Mg³dTTP along with activated calf thymus DNA added to trap any polymerase dissociating from the primer-template (6). The back-ground incorporation from polymerase not trapped by challenger DNA was subtracted (Fig. 4C). Under these conditions, where polymerase activity is measured for a single primer-template binding, polymerization, and dissociation reaction, gp5-H704A retains 50% of the activity observed with the wild-type polymerase (Fig. 4B). It has been suggested that with short templates, the rate of dissociation and reassociation of T7 DNA polymerase with DNA becomes rate-limiting, and processivity is unimportant (2). Taken together, these results are indicative of a lower processivity of DNA synthesis for gp5-H704A. The processivity of DNA synthesis catalyzed by gp5-H704A has also been measured directly by measuring the length of products formed by gel electrophoresis.

Effect of Ionic Strength on Polymerase Activity—Rates of DNA synthesis were measured as a function of NaCl concentration by following the incorporation of [³H]dTMP into primed M13 ssDNA. 100 mM NaCl is known to reduce the processivity of T7 DNA polymerase (2) because the higher ionic strength may stabilize secondary structures in the template. The rate of DNA synthesis is reduced at the higher ionic strength (Table II) for both the wild-type and gp5-H704A polymerases. Although at 50 mM NaCl, gp5-H704A retains 12% of the activity of the wild-type polymerase, at 100 mM NaCl concentration, gp5-H704A retains only 3% of the activity of the wild-type...
polymerase. Since the alanine substitution removes a putative electrostatic interaction with DNA, it is plausible that higher salt concentrations preferentially destabilize the binding of gp5-H704A to DNA.

**Processivity Assays**—The processivity of DNA synthesis catalyzed by the polymerase was measured by the method of von Hippel and co-workers (36, 37). M13 ssDNA annealed to a 5′-32P-labeled 24-nt oligonucleotide was used as the primer-template. To measure the length of products formed from a single polymerase-DNA binding event, the concentration of the primer-template was maintained in ~25-fold molar excess over the polymerase. Aliquots of the reaction mixture were removed at different times and were subjected to electrophoresis in an agarose gel (Fig. 5). The processivity of T7 wild-type DNA polymerase is in the order of thousands of nucleotides as observed from the accumulation of the full-length 9950-nt product in less than 5 min (2). A rate of incorporation of 240 nts/s is observed in steady-state assays for short periods of incubation (Table II), this value may not be extrapolated for longer incubations on long templates that contain secondary structure. In direct contrast, the length of products synthesized by gp5-H704A is drastically shorter with no full-length product formed from the primer-template and, thus, reduced processivity, because the polymerase concentration is higher than the concentration of the primer-template. Nevertheless, the results establish that the limited extensions are a result of dissociation from the primer-template and, thus, reduced processivity, rather than a lower rate of elongation.

**3′-5′ Exonuclease Activity**—Gene 5 protein has a 3′-5′ exonuclease activity on both ssDNA and dsDNA. Thioredoxin greatly stimulates the 3′-5′ exonuclease activity on dsDNA (15). The 3′-5′ exonuclease activity of the two enzymes was measured on both ssDNA and dsDNA (Table III) in the absence of added nucleotides. The intrinsic exonuclease activity of the two enzymes is comparable on both ssDNA and dsDNA.

**Nucleotide Turnover**—Nucleotide turnover is defined as the DNA-dependent conversion of deoxynucleoside triphosphates into their corresponding monophosphates under conditions of DNA synthesis. The polymerization of nucleotides into a primer followed by exonucleolytic degradation by the 3′-5′ exonuclease activity associated with the polymerase results in nucleotide turnover. Thus, it reflects the levels of “editing” (38, 39) that take place in a reaction. To determine whether the substitution of His704 with alanine that removes an interaction with the primer 3′-terminus in the polymerase domain results in an increase in exonucleolytic editing, the nucleotide turnover associated with gp5-H704A and wild-type T7 DNA polymerase was measured. The rate of hydrolysis of deoxynucleoside triphosphates to the corresponding monophosphate (turnover) during DNA synthesis was measured using primed M13 ssDNA and [α-32P]dTTP. Enzyme concentrations were normalized to yield similar amounts of DNA synthesis, and the rates of DNA synthesis were measured by the incorporation of [α-32P]dTMP. To measure the exonucleolytic release of [α-32P]dTMP, an aliquot of the reaction mixture was removed at the indicated time and analyzed by TLC (Fig. 6). For similar amounts of DNA synthesis, the amount of dTMP formed with gp5-H704A was 11 times higher than that with wild-type polymerase (Table IV). Because the exonuclease activity on ssDNA of gp5-H704A is comparable with that of the wild-type polymerase, the increased nucleotide turnover observed with gp5-H704A must reflect an increased rate of switching of DNA from the polymerase to exonuclease active site rather than an intrinsically higher exonuclease activity.

**Dissociation Kinetics of Preformed Complexes**—DNA is held in position by the fingers, palm, and thumb subdomains of T7 DNA polymerase by a number of contacts, both direct and water-mediated, primarily to the phosphodiester backbone (7). To determine whether the removal of a single hydrogen bond to the primer terminus perturbs the overall binding of the polymerase to DNA, the primer was extended by the incorporation of four deoxyribonucleoside monophosphates in a standard polymerase assay at 37°C with either gp5 or gp5-H704A. DNA synthesis conditions are described under “Experimental Procedures.” A, the molar ratio of polymerase to primer-template was 1:25. For each reaction, the 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 0.1% ethidium bromide and visualized by autoradiography. B, effect of increasing polymerase concentration on the length of products. The reaction time was 1 h. bp, base pairs.

**TABLE II**

| NaCl (mM) | Incorporation rates a |
|-----------|----------------------|
|           | Wild-type | gp5-H704A | |
| 50        | 240       | 30        |
| 100       | 130       | 4         |
| 150       | 35        | ND b      |
| 200       | 8         | ND        |

* a Rate of incorporation expressed in terms of moles of deoxyribonucleoside monophosphates incorporated per mole of enzyme per second. The reaction mixtures contained 0.60 nM polymerase and 20 nM DNA.
* b ND, not determined.

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**Fig. 5. Processivity of wild-type T7 DNA polymerase and gp5-H704A.** A 5′-32P-labeled 24-nt primer was annealed to a M13 ssDNA template. The primer was extended by the incorporation of four deoxyribonucleoside monophosphates in a standard polymerase assay at 37°C with either gp5 or gp5-H704A. DNA synthesis conditions are described under “Experimental Procedures.” A, the molar ratio of polymerase to primer-template was 1:25. For each reaction, the 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 1 h. bp, base pairs.
C-terminal Residue of T7 DNA Polymerase

**TABLE III**

Exonuclease activity on single- and double-stranded DNA

| Polymerase | ssDNA | dsDNA |
|------------|-------|-------|
| Wild-type  | 100   | 100   |
| gp5-H704A | 60    | 130   |

*Specific activity has been expressed as the percentage of the activity observed for wild-type T7 DNA polymerase. One unit of exonuclease activity catalyzes the release of 1 pmol of total nucleotides into an acid soluble form in 1 min. 100% double-stranded activity corresponds to 9100 units/mg, and 100% single-stranded activity corresponds to 6800 units/mg.

**FIG. 6.** Turnover of nucleotides during DNA synthesis. The DNA-dependent conversion of deoxyribonucleoside triphosphates into their corresponding monophosphates during DNA synthesis was measured with wild-type T7 DNA polymerase and gp5-H704A. A 24-nt oligonucleotide annealed to circular M13 ssDNA was used as the primer-template in a standard polymerase assay at 37 °C. DNA synthesis was measured by following the incorporation of [3H]dTMP as described under "Experimental Procedures." The amount of the two polymerases was adjusted to give the same amount of dTMP incorporation. The hydrolysis of [α-32P]dTMP to [32P]dTMP was monitored by TLC analysis of the reaction mixture (Fig. 6).

**TABLE IV**

Incorporation and turnover of dTMP during DNA synthesis on primed M13 DNA

| Polymerase | dTMP incorporated (a) | dTMP formed (b) | Turnover % |
|------------|-----------------------|----------------|------------|
| Wild-type  | 12.0                  | 2.0            | 13         |
| gp5-H704A | 12.0                  | 22             | 65         |

Consequent to the addition of a base to a growing DNA chain in the polymerase active site, there are three competitive sinks for the polymerase: (i) further polymerization with catalysis of phosphodiester bond formation, (ii) dissociation from the primer terminus, or (iii) exonucleolytic cleavage of the phosphodiester bond. An equilibrium exists between DNA binding to the polymerase or exonuclease active site with binding to the polymerase site being thermodynamically more favored (18). It is the balance between these two opposing reactions that governs high fidelity of replication with minimal hydrolysis of nucleotides. Although the polymerization and exonuclease re-
actions have been the subject of detailed kinetic study, the mechanisms that underlie the occupancy of DNA in the polymerase versus exonuclease active site are not well understood. In this report, we describe a mutant of T7 DNA polymerase, T7 gp5-H704A, that has an increased turnover of nucleotides during DNA polymerization. We hypothesize that this increase represents an increase in shutting of the primer strand to the exonuclease active site.

T7 DNA polymerase makes a number of contacts with base pairs near the 3′ terminus of the primer (7). Substitution of a number of analogous residues in E. coli polymerase I results in altered affinity of the enzyme for DNA (40, 41). These residues have been implicated in scanning for errors during polymerization. It has been postulated that such residues could be involved in the transfer of mismatched nucleotides to the 3′-5′ exonucleolytic active site in T7 DNA polymerase (11).

In a ternary crystal structure of T7 DNA polymerase in complex with a primer-template and the incoming deoxyribonucleotide triphosphate (7), the C-terminal amino acid residue of T7 DNA polymerase, His704, makes a putative hydrogen bond with the penultimate phosphate diester of the 3′-OH terminus of the primer strand. From the structure, this residue may orient the 3′-OH terminus of the primer strand in position for the next phospholyl transfer reaction. Removal of this contact could either promote the local denaturation of the 3′-end that would result in the movement of the primer terminus to the exonuclease site or perturb the overall binding to DNA. To probe the contribution of this single electrostatic interaction in DNA binding and catalysis, histidine was substituted with alanine.

The single amino acid substitution in T7 DNA polymerase results in a polymerase that not only cannot support growth of T7 phage but that is also inhibitory to the T7 wild-type allele. DNA synthesis cannot be detected in vivo upon infection by T7 phage of cells harboring a plasmid encoding for the genetically altered protein. To understand the biochemical basis for this phenotype, the genetically altered protein, gp5-H704A, was overproduced from E. coli overexpressing the gene and purified to apparent homogeneity. Biochemical experiments with purified protein show that gp5-H704A retains only 12% of the polymerase activity of the wild-type enzyme using primed M13 ssDNA as a substrate. Furthermore, although wild-type T7 DNA polymerase is extremely processive for DNA synthesis and can catalyze the addition of hundreds of nucleotides in a single encounter with DNA, the processivity for nucleotide polymerization of gp5-H704A is severely reduced. Thus, removal of this canonical electrostatic interaction with the phosphate backbone of DNA lowers the efficiency of translocation of T7 DNA polymerase on DNA. Processivity measurements are dependent on a number of parameters that affect both the polymerase and the template. Consequently, we do not know the precise molecular mechanism for the decrease in processivity.

Certainly one parameter that decreases processivity may be a change in the exonuclease/polymerase activity ratio. The higher the ratio, with stalling of the polymerase at secondary structures, the shorter the products.

To examine the contribution of His704 in binding DNA, the rate of dissociation of preformed polymerase-DNA complexes and the apparent binding affinity of the polymerase for a primer-template were determined. The off rate of gp5-H704A ($k_{off} = 0.04 \text{ s}^{-1}$) from poly(dA)$_{280}$-oligo(dT)$_{22}$ is not significantly different from that of the wild-type polymerase ($k_{off} = 0.02 \text{ s}^{-1}$). The $K_{Dp}$ of a gp5-H704A-poly(dA)$_{280}$-oligo(dT)$_{22}$ complex is also not significantly different relative to the wild-type polymerase. These results are not surprising given that the primer-template is held in position by the polymerase via a number of direct and water mediated interactions with the phosphodiester backbone. The substitution of histidine with alanine removes only a single electrostatic contact with DNA. Hence, a large perturbation in the overall binding constant of DNA to enzyme is not expected.

The position of His704 from the crystal structure does not suggest a functional role for this residue in catalysis via interaction with the incoming nucleotide or the metal ions in the polymerase active site. However, His704 contacts Glu655, one of the three highly conserved carboxylates in the polymerase I family of polymerases. Glu655 is located near the metal-ligating residues Asp775 and Asp654 but does not contact the metal ions itself. Substitution of the analogous residue in Klenow (Glu883) with alanine results in a modest increase in the $K_m$ for dNTP binding (40). To investigate the role of the interaction of Glu655 with His704 in nucleotide binding and catalysis, $K_m$ (dNTP) was measured using steady-state kinetics. The $K_m$ for dNTP binding is 2-fold higher than that of the wild-type enzyme. A more drastic change in $K_m$ (dNTP) is not expected from the information from the crystal structure. Because the measured apparent dissociation constant is the overall dissociation constant of the ternary polymerase-dNTP-DNA complex, the increase in $K_m$ for gp5-H704A may reflect a perturbation in maintaining the DNA primer in position for catalysis.

Thus, removal of a single hydrogen bond between His704 and the primer terminus does not significantly alter the binding affinity of the polymerase for DNA or for nucleotides. However, gp5-H704A has a much higher percentage of nucleotide turnover than the wild-type polymerase during DNA synthesis than what would be expected from the level of its exonucleolytic activity. When the hydrolytic function of the enzyme was monitored by the turnover rate of dTTP during DNA polymerization, the amount of free dTMP formation with gp5-H704A was 11 times higher than that with wild-type enzyme at 37 °C. While wild-type T7 DNA polymerase removes 13 of every 100 nucleotides it inserts, for every 100 nucleotides that are inserted by gp5-H704A, 65 are hydrolyzed to the monophosphate. Because the exonucleolytic rate of gp5-H704A on ssDNA is comparable with that of the wild-type polymerase, the higher nucleotide turnover is not a result of a higher intrinsic exonuclease activity. It is possible that the local denaturation of the primer-template upon removal of this interaction with the polymerase increases the single-stranded nature of the 3′-end, thus enhancing the propensity for exonucleolytic editing.

### Table V

| Polymerase | $K_{D}$ | $k_{cat}$ | $K_{Dp}$ | $k_{off}$ |
|------------|---------|----------|---------|----------|
| Wild-type  | 40      | 170      | 30      | 0.02     |
| gp5-H704A | 90      | 65       | 40      | 0.04     |

a Apparent dissociation constant of the enzyme from dTTP using poly(dA)$_{280}$-oligo(dT)$_{22}$ as a template.

b Maximum rate of incorporation of dTMP.

c Apparent dissociation constant of enzyme from poly(dA)$_{280}$-oligo(dT)$_{22}$ for a single cycle of DNA synthesis.

d Rate of dissociation of the enzyme from poly(dA)$_{280}$-oligo(dT)$_{22}$ assuming a first order decay process.

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C. While wild-type T7 DNA polymerase removes 13 of every 100 nucleotides it inserts, for every 100 nucleotides that are inserted by gp5-H704A, 65 are hydrolyzed to the monophosphate. Because the exonucleolytic rate of gp5-H704A on ssDNA is comparable with that of the wild-type polymerase, the higher nucleotide turnover is not a result of a higher intrinsic exonuclease activity. It is possible that the local denaturation of the primer-template upon removal of this interaction with the polymerase increases the single-stranded nature of the 3′-end, thus enhancing the propensity for exonucleolytic editing.

Interactions with the primase-helicase of bacteriophage T7 encoded by gene 4 (gp4) were also examined in assays with purified proteins. Gp5-H704A catalyzes strand displacement DNA synthesis in complex with gp4, implying that it interacts with gp4 in a functional mode comparable to wild-type DNA.

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2 J. K. Kumar, S. Tabor, and C. C. Richardson, unpublished observations.
polymerase (42, 43). The ability of gp5-H704A to interact with a primase-primer complex was also examined. The primase domain of gene 4 catalyzes the synthesis of tetraribonucleotides at primase recognition sites in the presence of ATP and CTP (44). These tetraribonucleotide primers are stabilized on the DNA template by gp4 until used by T7 DNA polymerase (45, 46). The gp4-dependent DNA synthesis rate of T7 DNA polymerase was measured on M13 ssDNA. In this experiment, gp5-H704A has ~4-fold lower activity than the wild-type polymerase.2 This difference in activity is not substantial given that gp5-H704A has ~8-fold lower rate of DNA synthesis on DNA primed M13 ssDNA by itself.

In experiments where bacteriophage T7 was used to infect cells that harbored the plasmid pGP5-H704A, gp5-H704A inhibited the growth of wild-type T7 phage. Biochemical experiments have not clarified the basis for this strong in vivo dominant lethal phenotype of the H704A substitution. DNA synthesis catalyzed by wild-type T7 DNA polymerase in vitro on primed M13 ssDNA is not inhibited by the addition of gp5-H704A. However, in the complementation assay, gp5-H704A, overexpressed from plasmid pGP5-H704A, is present at levels many times greater than wild-type gene 5 protein encoded by T7 phage. Thus, this gene dosage effect may account for the dominant lethal phenotype.

In summary, His704 is an essential residue of T7 DNA polymerase. A systematic dissection of the reaction pathway supports a critical role of this residue in positioning the 3'-OH terminus of the primer strand in the polymerase domain in place for phosphodiester bond catalysis. Removal of this residue modifies the partitioning of DNA between the polymerase and exonuclease active sites. The higher turnover of nucleotides most likely is a manifestation of the polymerase spending a higher proportion of time with DNA in its exonuclease site. Because high DNA replication fidelity with minimal turnover of nucleotides is incumbent upon a balance between the competing polymerase and exonuclease activities, disruption of this balance may become untenable for T7 phage growth.

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Role of the C-terminal Residue of the DNA Polymerase of Bacteriophage T7
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