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Citation
Yang, Xiao-Ming, and Charles C. Richardson. 1997. “Amino Acid Changes in a Unique Sequence of Bacteriophage T7 DNA Polymerase Alter the Processivity of Nucleotide Polymerization.” Journal of Biological Chemistry 272 (10): 6599–6606. https://doi.org/10.1074/jbc.272.10.6599.

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Amino Acid Changes in a Unique Sequence of Bacteriophage T7 DNA Polymerase Alter the Processivity of Nucleotide Polymerization*

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T7 gene 5 DNA polymerase forms a complex with Escherichia coli thioredoxin (its processivity factor), and a 76-amino acid sequence (residues 258–334), unique to gene 5 protein, has been implicated in this interaction. We have examined the effect of amino acid substitution(s) in this region on T7 phage growth and on the interaction of the polymerase with thioredoxin. Among the mutations in gene 5, we found that a substitution of either Glu or Ala for Lys-302 yielded a protein that could not complement T7 phage lacking gene 5 (T7Δ5) to grow on E. coli having reduced thioredoxin levels. One triple mutant (K300E,K302E,K304E) could not support the growth of T7Δ5 even in wild type cells. This altered polymerase is stimulated 4-fold less by thioredoxin than is the wild type enzyme and the polymerase-thioredoxin complex has reduced processivity. The exonuclease activity of the altered polymerase is not stimulated to the same extent as that of the wild type enzyme by thioredoxin. The observed dissociation constant of the gene 5 protein K(300,302,304)E-thioredoxin complex is 7-fold higher than that of the wild type complex. The altered polymerase also has a lower binding affinity for double-stranded DNA.

Protein-protein interactions are essential for the coordination of the multiple reactions that occur at a replication fork. Although the replication machinery of bacteriophage T7 is simple in comparison to that of its host, Escherichia coli, specific interactions among the relatively few proteins are important (1–3). In fact, the economy of proteins involved in T7 DNA replication has made it an attractive model for dissecting their interactions. An essential component of the T7 replisome is the T7 DNA polymerase, the 80-kDa product of gene 5 of the phage. Gene 5 protein physically interacts with the hexameric gene 4 protein of the phage, a protein that provides both helicase and primase activities at the replication fork to coordinate both leading and lagging strand synthesis (2, 4–6). Both the gene 5 and gene 4 proteins in turn interact with the T7 gene 2.5 protein, a single-stranded DNA (ssDNA)1-binding protein that stimulates both polymerase and primase activities (4, 7, 8). Thus, all three of these phage-encoded proteins physically interact with one another.

In addition to the interactions of the T7 DNA polymerase with the other two phage-encoded proteins, the gene 5 protein also physically interacts with one host protein, E. coli thioredoxin (9–12). Thioredoxin forms a stable one-to-one complex with T7 gene 5 protein with an apparent dissociation constant of 5 nM (13). The consequence of the interaction is to convert gene 5 protein from a polymerase with low processivity of polymerization of nucleotides to one of high processivity (9–12). The increased processivity arises as a result of the 80-fold greater affinity of the DNA polymerase-thioredoxin complex for a primer-template (13, 14). T7 gene 5 protein also has a 3′ to 5′ exonuclease activity that is active on both ssDNA and double-stranded DNA (dsDNA) (9, 15). Thioredoxin stimulates activity on dsDNA but the activity on ssDNA is not affected. The enhanced activity on dsDNA is most likely due to a higher affinity of the polymerase-thioredoxin complex to the 3′-termini, resulting in increased processivity of hydrolysis (14).

Studies on both E. coli thioredoxin and T7 gene 5 protein have provided information on the domains in each protein that are important for the interactions of the two proteins. Thioredoxin, a 12-kDa protein that contains an active center consisting of two cysteine residues (Cys-32 and Cys-35) that can form a disulfide bridge, provides the reducing power for a number of reactions in E. coli (16–18). However, the active-center cysteines are not essential for the interaction of thioredoxin and gene 5 protein, since genetically altered thioredoxins in which either one or both Cys have been replaced with Ser, form a functional complex with gene 5 protein and support T7 phage growth (13). Other studies have implicated a group of residues (Gly-33, Pro-34, Ile-75, Pro-76, Gly-92, and Ala-93) that form a hydrophobic surface as being involved in interactions with a number of other proteins (17, 19). The three-dimensional structures of both oxidized (20) and reduced thioredoxin (21) are known; these residues are located in three loops formed between β-sheet 2 (β2)-α-helix 2 (α2), a3-β4, and β5-α4, and all face the same side of the thioredoxin molecule. A comparison of the structures of oxidized and reduced thioredoxin reveals that the transition of Cys-32 and Cys-35 between reduced and oxidized forms introduces a significant change in the position of Pro-34, resulting in a local conformational change around Cys-35. This conformational change could explain why only reduced thioredoxin binds to gene 5 protein (20). Genetic (22) and biochemical analyses (13, 23) of thioredoxin mutants that are defective in supporting T7 phage growth have shown that mutations at Cys-32, Cys-34, Gly-74, and Gly-92 affect the binding of thioredoxin to gene 5 protein, suggesting that the same...
hydrophobic surface of thioredoxin is also involved in its interaction with T7 gene 5 protein. Several other residues in thioredoxin have also postulated to be involved in the interaction with gene 5 protein: Pro-34, Gly-74, and Gly-92 (13, 22); recently, it has been proposed that the active-site cysteines, Gly-74, and Gly-92 define part of the thioredoxin surface that contacts gene 5 protein (23, 24).

Although the three-dimensional structure of T7 DNA polymerase is not known, it shares a high degree of homology to the large fragment of E. coli DNA polymerase I (25), whose crystal structure is known (26, 27). Furthermore, a number of studies indicate that the active sites of the enzyme are similar and that the two proteins share some structural similarities. For example, the polymerase activity site of T7 DNA polymerase, like that of E. coli DNA polymerase I, resides within the carboxy-terminal half of the molecule, while the 3' to 5' proofreading domain is located within the amino-terminal half of the molecule (24, 28). Furthermore, relatively large segments of the polypeptide chain that constitutes the polymerase active site of E. coli DNA polymerase I can be exchanged with the homologous region of T7 DNA polymerase with retention of polymerase activity (29).

Two lines of evidence suggest that at least a portion of the thioredoxin binding domain of gene 5 protein resides within the polymerase domain. T7 DNA polymerase purified in the absence of thioredoxin is subject to proteolytic attack at three clustered sites that lie within the COOH-terminal region of the protein, and this proteolysis is inhibited by thioredoxin (9). Mapping of these sites showed the proteolytic cleavage to reside at positions Ile-289, Lys-299, and Ala-323 of the T7 gene 5 protein (24). These results implicated the sequence Ile-289 to Lys-299 and Ala-323 as a region that physically interacts with thioredoxin. This sequence, which does have a homologous counterpart in E. coli DNA polymerase I (30, 31), has an unusually high content of hydrophilic residues. In the structure of the large fragment of E. coli DNA polymerase I, this 76-amino acid sequence is located in a region referred to as the “thumb” that partially covers the crevice through which the DNA passes, a region that has been proposed to be involved in the interaction of the polymerase with duplex DNA (27). Further evidence suggesting that this unique amino acid sequence interacts with thioredoxin comes from studies on suppressor mutations that allow T7 phage to use a genetically altered thioredoxin (22). One suppressor mutation (Glu-319 $\rightarrow$ Lys) resides within this region and restores the ability of T7 DNA polymerase to interact with this particular mutant thioredoxin (23).

In this report we have introduced specific amino acid changes into this unique hydrophilic region of T7 gene 5 protein. We show that alteration of amino acid within this sequence results in the failure to support the growth of T7 phage due to decreased binding of the polymerase to thioredoxin and reduced processivity of the polymerase-thioredoxin complex.

**Experimental Procedures**

### Materials

**E. coli strains, Bacteriophage, and Plasmids—** E. coli strains A307ompT (HfrC, $\Delta$trxA307, ompT $^-$), HMS249 (F $^-\text{opt}$, $\text{Al}$, dapD4), C600, C600trxA (trxA gene deletion), HMS157 (F $^-\text{recC22}$, sbcA5, endA $^-$, gal $^+$, thi $^+$, sup $^+$), HMS157trxA $^-$, and HB101 are from the laboratory collection. Wild type bacteriophage T7 is from the laboratory collection, and mutant phages T7Δ5 (gene 5 deletion), T7trxA (E. coli trxA gene inserted into T7 phage genome between genes I and J) were from S. Tabor (Harvard Medical School). Plasmid pGP5 $^-3$ containing wild type T7 gene 5 under control of T7 RNA polymerase promoter was obtained from S. Tabor (Harvard Medical School) (9). M13mpGP1 $^-2$ is a 9950-base pair derivative of phage M13 containing an insert of phage T7 DNA, which expresses T7 RNA polymerase upon induction by isopropyl-1-thio-$\beta$-d-galactopyranoside (9). Growth and manipulation of bacteriophage T7 and E. coli were performed as described (28, 32, 33).

**Nucleotides, Oligonucleotides, and DNA—** M13mp18 DNA and the 23-mer universal cycle sequencing primer were obtained from Amersham Life Sciences, Inc. Calf thymus DNA was from Sigma. Oligonucleotides of the xy52 series used for mutagenesis (each harbors one of the mutated amino acid codons), JH10 (5'-CCCTTAATCCTGCGGCGG-3'), JH11 (5'-CCCTTAATCCTGCGGCGG-3'), and JH12 (5'-TACGACTCATCTAGGGAAGC-3') complementary to T7 gene 5 and Liu12 (5'-TACGACTCATCTAGGGAAGC-3') complementary to T7 RNA polymerase primer of plasmid pGP5 $^-3$, are from the laboratory collection. Nucleoside 5'-triphosphates (dNTPs) were from Pharmacia Biotech Inc. [$\gamma$-$^32P$]dATP (800 Ci/mmol, 1 Ci = 37 Gbq), [$\gamma$-$^32P$]dATP (3000 Ci/mmol), and [$\gamma$-$^32P$]dATP (15 Ci/mmol) were products of DuPont NEN for Amersham Life Science, Inc.

**Proteins—** Purified bacteriophage T7 gene 5 protein and gene 2.5 protein were obtained from S. Tabor (Harvard Medical School). T7 gene 4 proteins were provided by S. Notarnicola and B. B. Beauchamp (Harvard Medical School). E. coli thioredoxin and endonuclease AvoI are products of Amersham Life Science, Inc. Bovine serum albumin was from Miles Laboratories. Ampli-Taq DNA polymerase was from Perkin Elmer. Polyonal antisera specific to gene 5 protein and to E. coli thioredoxin was from Hazelton Research Products, Inc.

**Other Materials—** dsDNA-cellulose was obtained from B. B. Beauchamp (Harvard Medical School). DEAE-Sephadex A-50, Sephase- rose CL-2B cellulose (P-11), and DE-51 filter discs were obtained from Whatman. Microspin Columns were products of Pharmacia Biotech Inc. Bio-Spin Chromatography columns, Mini-Protein ready gels, and protein silver stain kits were purchased from Bio-Rad. Protein electrophoresis standards were from Amersham Life Science, Inc. Premixed polyacrylamide solutions were from Boehringer Mannheim or National Diagnostics. 1,4-Dithiothreitol (DTT) is the product of ICN Biochemicals Inc.

### Methods

**DNA Manipulation—** If not indicated specifically, DNA manipulation was performed according to the protocols described (34). DNA sequence analysis was performed using the method of dideoxyribonucleotide chain-termination (35) with Sequenase 2.0 (Amersham Life Science, Inc.).

**Site-directed Mutagenesis—** In vitro mutagenesis of bacteriophage T7 gene 5 was carried out by using a modified “overlap extension” method as described elsewhere (36). Two partially overlapping oligonucleotide primers (xy52 series), each bearing appropriate codons corresponding to the desired amino acid residue substitution in the overlapping region, were used in the polymerase chain reaction (PCR) (37) to generate each mutant T7 gene 5. Each mutagenesis involved three separate PCR reactions. For example, two oligonucleotide primers, xy52-5 (5'-TCTC-GGTTGGCTTCTTTCGAGTTTTAAAAGATACC-3') and xy52-5c (5'-GGTACTTTTTAAAGGTGACACCTGACAGCCAGCAGG-3'), were used to replace the Lys-302 (AAG) to a Glu (GAG) in gene 5 protein. The underlined codon corresponds to the amino acid residue alteration. In the first PCR, the oligonucleotide primer (xy52-5) and one upstream primer (Liu12) were used to generate a 980-base pair (bp) fragment. Another oligonucleotide primer (xy52-5c) and a downstream primer (JH10) were used in the second PCR to generate a fragment of 900 bp. The 980- and 900-bp fragments from the first two PCR were mixed and used as a template in the third PCR in the presence of the upstream (Liu12) and downstream (JH10) primers. The product from the third PCR (1800 bp) was digested with restriction enzymes MunI and HpaI, and the resulting fragment was ligated into the MunI and HpaI site of pGP5 $^-3$ giving rise to pGP5 $^-$3K302E. All the mutants were prepared in a similar way as described above. All clones were confirmed by DNA sequencing.

### Preparation of DNA Substrates—** Circular M13 DNA to which a 25-nucleotide oligonucleotide primer was annealed was used in the polymerase and processivity assays. In the latter assay, the oligonucleotide primer was radioactively labeled at its 5'-terminus using polynucleotide kinase and [$\gamma$-$^32P$]ATP (34). After incubation at 37 °C for 30 min followed by 15 min at 70 °C, the labeled oligonucleotide was annealed to M13mp18 DNA in the presence of MgCl$_2$ (10 mM) and NaCl (100 mM). After purification using an S300 Spin-Column, the DNA was extracted with an equal volume of phenol/chloroform. The DNA was precipitated with ethanol and dissolved in buffer.

DNA for use in the exonuclease assay was uniformly labeled [$^32$H]dXDNA and ssDNA. dsDNA (1,800 base pairs) was amplified by PCR using primers JH10 and Liu12 in the presence of [$^32$H]dTPP and three other nucleotide triphosphates. The PCR products were isolated by $800$ Spin-Columns and digested with endonuclease AvoI, generating a fragment of 1600 base pairs with protruding cohesive 5' termini.
Mutations Affecting Processivity of T7 DNA Polymerase

After separation on an agarose gel, the desired DNA was purified by GeneClean (Bio 101, Inc.) and stored in TE buffer (specific activity 15–20 cpmp/μmol). ssDNA was prepared by incubating the [3H]dsDNA with 1 μl NaOH for 5 min at room temperature. The solution was neutralized by adding 1 μl of 1 M HCl and 1 μl EDTA.

Construction of Phage T7ΔSTRXα—In order to produce the mutation of thioredoxin in E. coli infected with phage T7ΔSTRXα, E. coli C600 and C600StrXα, each harboring the plasmid pGP5–3 (C600/pGP5–3, C600StrXα/pGP5–3), were grown in LB medium (33) at 37°C. At an A600 of 1.0, the cells were infected with wild type T7 and with T7ΔSTRXα phage, at a multiplicity of infection of 5 plaque-forming units per cell. Aliquots of the culture were transferred before and after infection, and the cells were grown to 2 × 108 cells/ml. The shorter fragment from T7ΔSTRXα and the longer fragment from T7ΔW5 were isolated and ligated, and the ligated DNA was transfected into E. coli HMS157 harboring plasmid pGP5–3 (HMS157/pGP5–3). The transformed cells were plated on E. coli C600 harboring plasmid pGP5–3 (C600/pGP5–3). Individual plasmid plaques were picked, and the phages were screened for their ability to grow on HMS157StrXα/pGP5–3.

Determination of Thioredoxin Production in Cells Infected with Phage T7ΔSTRXα—In order to measure the production of thioredoxin in E. coli infected with phage T7ΔSTRXα, E. coli C600 and C600StrXα, each harboring the plasmid pGP5–3 (C600/pGP5–3, C600StrXα/pGP5–3), were grown in LB medium (33) at 37°C. At an A600 of 1.0, the cells were infected with wild type T7 and with T7ΔSTRXα phage, at a multiplicity of infection of 5 plaque-forming units per cell. Aliquots of the culture were transferred before and after infection, and the cells were grown to 2 × 108 cells/ml. The shorter fragment from T7ΔSTRXα and the longer fragment from T7ΔW5 were isolated and ligated, and the ligated DNA was transfected into E. coli HMS157 harboring plasmid pGP5–3 (HMS157/pGP5–3). The transformed cells were plated on E. coli C600 harboring plasmid pGP5–3 (C600/pGP5–3). Individual protein plaques were picked, and the phages were screened for their ability to grow on HMS157StrXα/pGP5–3.

RESULTS

As described in the Introduction, earlier studies have implicated a 76-amino acid sequence (residues 258–334) in T7 gene 5 protein as the thioredoxin binding domain (23, 24). This sequence, unique to T7 DNA polymerase, is inserted into the thumb region of the polymerase domain based on the homology of T7 DNA polymerase to E. coli DNA polymerase I (25). To further examine the role of this domain in T7 DNA polymerase, we have made amino acid substitutions in the 76-amino acid sequence and examined the effect of these changes on T7 DNA polymerase function both in vivo and in vitro.

Construction of Gene 5 Protein Mutants—Within the 76-amino acid sequence composing the postulated thioredoxin binding domain, there are 9 lysine residues. We have made single amino acid substitutions for 5 of these lysine residues, three in the cluster of 5 lysine residues located at one terminus of the segment and one each in the center and at the other terminus. In addition we have also constructed gene 5 mutants that contain either double or triple substitutions in the cluster of five lysine residues. Basically, for the construction of each mutant, directed mutagenesis was carried out using PCR and the appropriate primers, two of which contained the desired mutation (see “Experimental Procedures”). Each construction was verified by DNA sequence analysis. The resulting gene 5 mutants are presented in Table I.

Complementation System for Gene 5 Protein-thioredoxin Interaction—The basis for the complementation assay used to examine the gene 5 mutants is the requirement for a functional T7 gene 5 protein and E. coli thioredoxin for T7 DNA replication and phage growth (1, 13). Since a functional interaction between the gene 5 protein and thioredoxin is dependent

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on the intracellular concentration of thioredoxin (22), we have designed the complementation system such that the thioredoxin concentration can be reduced below that normally present in E. coli. In this manner it is possible to identify altered gene 5 proteins that support T7 growth at high levels of thioredoxin but not at lower levels, a consequence of a decreased affinity of the two proteins.

As illustrated in Fig. 1A, we have inserted the structural gene for E. coli thioredoxin, trxA, into the phage T7 genome to create the recombinant phage T7ΔtrxA. The trxA gene is located just downstream from T7 gene 1; hence, upon infection of E. coli, it is transcribed by the host RNA polymerase (38). Once the product of gene 1, T7 RNA polymerase, is produced, it transcribes the majority of the phage genes, one of which, gene 2, encodes an inhibitor of E. coli RNA polymerase (38). Consequently, the amount of thioredoxin produced in a E. coli trxA− cells infected with T7ΔtrxA phage should be limited. T7ΔtrxA phage also contains a deletion of gene 5, the structural gene for T7 DNA polymerase. Hence, phage growth is dependent on expression of a plasmid-encoded gene 5, pGP5−3. Plasmid pGP5−3 harbors T7 gene 5 proteins that support T7 growth at high levels of thioredoxin, in contrast, is maintained at approximately constant level. T7ΔtrxA-infected E. coli C600trxA− /pGP5−3 begin to produce thioredoxin at 10 min after infection, but the level obtained is approximately 5-fold lower than that normally present in E. coli, as judged by densitometry of the gels shown in Fig. 1B. Gene 5 protein is present at levels similar to that found in wild type phage-infected cells at 30 min after infection.

**Analysis of Gene 5 Mutants**—The effect of each of the mutations in gene 5 on phage T7 growth was examined by measuring the ability of the altered gene 5 proteins expressed from the cloned genes to complement T7 lacking gene 5 (Table 1). The complementation assays were carried out using infection of E. coli C600 and C600trxA− by T7ΔtrxA phage, respectively. Expression of the trxA gene located on the phage leads to intracellular levels of thioredoxin lower than that obtained when the E. coli chromosomal gene is expressed.

As shown in Table I, phage T7 lacking gene 5 grow normally when gene 5 protein is produced from a plasmid encoding the wild type gene, but not in its absence. Furthermore, T7ΔtrxA capable of supplying its own thioredoxin can grow on E. coli lacking the trxA gene provided that gene 5 protein is also expressed from the cloned gene, E. coli C600trxA− /pGP5−3.

When plated on E. coli C600, all of the plasmids containing a single or double mutation in gene 5 can complement and support the growth of T7ΔtrxA (Table 1). However, the triple mutant, T7 gp5K(300,302,304)E, cannot. Under more stringent conditions in which the intracellular concentration of thioredoxin is reduced by supplying thioredoxin from the phage, gene 5 proteins containing the single amino acid changes K302A and K302E cannot complement T7ΔtrxA, nor can the double mutant, K300E,K302E. Under this condition another single mutation, K300E, also resulted in a 10-fold lower plating efficiency compared with that of wild type gene 5.

The polymerase and 3′ to 5′ exonuclease activities of all the altered gene 5 proteins were examined by overexpressing each mutant gene in E. coli BL21(DE3). Both activities of all the genetically altered gene 5 proteins were indistinguishable from that of wild type gene 5 protein when assayed in cell extracts containing thioredoxin (Table 1). This result strongly implies that this unique region of gene 5 protein is not involved with either the polymerase or exonuclease active site, a conclusion that is in agreement with our previous results that proteolytic fragments missing this region retain polymerase and exonuclease activity (24).

**Purification of Mutant Gene 5 Protein**, gp5K(300,302,304)E—Based on the results of the complementation analysis, implying that the triple mutant protein, gp5K(300,302,304)E, is more defective than any of the single mutant proteins, we purified gp5K(300,302,304)E from extract of cells over-producing this protein and characterized it biochemically. The five-step purification procedure includes NH₄₂SO₄ precipitation and DEAE- and phosphocellulose chromatography (9). SDSPAGE analysis of the isolated protein revealed that the predominant polypeptide migrates at a position corresponding to a

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**Table I**

| Plasmid          | Amino acid substitution | T7Δ5 (C600) | T7Δ5trxA (C600trxA−) | Pola | Exob |
|------------------|-------------------------|-------------|---------------------|------|------|
| No plasmid       |                         | <0.001      | <0.001              | <1   | <1   |
| pT7              | No gene 5               | <0.001      | <0.001              | <1   | <1   |
| pGP5–3           | Wild type               | 100         | 100                 | 100  | 100  |
| pT7–gp5–3        | Wild type               | 110         | 110                 | 100  | 100  |
| pGP5–3K265E      | K265E                   | 90          | 90                  | 95   | 96   |
| pGP5–3K285A      | K285A                   | 90          | 100                 | 94   | 96   |
| pGP5–3K285G      | K285G                   | 80          | 90                  | 93   | 93   |
| pGP5–3K285E      | K285E                   | 90          | 80                  | 94   | 96   |
| pGP5–3K300E      | K300E                   | 50          | 5                   | 93   | 90   |
| pGP5–3K302E      | K302E                   | 40          | <0.001              | 94   | 96   |
| pGP5–3K302A      | K302A                   | 50          | <0.001              | 95   | 96   |
| pGP5–3K304E      | K304E                   | 70          | 70                  | 93   | 95   |
| pGP5–3K          | K300E,K302E             | 3           | <0.001              | 85   | 73   |
| pGP5–3K         | K300A,K302A             | 5           | <0.001              | 91   | 75   |
| pGP5–3K         | K300E,K302E,K304E       | <0.001      | <0.001              | 90   | 70   |

a E. coli C600 or C600trxA− cells harboring the indicated plasmids were infected with phage T7Δ5 or phage T7Δ5trxA, respectively. The number of plaques on each plate were counted, and an average from three experiments are presented as phage formation units (pfu/ml) of phage solution.

b DNA polymerase and exonuclease activities in extracts prepared from E. coli BL21(DE3) cells transformed with each of the gene 5-containing plasmids were determined in the presence of E. coli thioredoxin (5 μg) using heat-denatured calf thymus DNA and [3H]dNTPs as described under “Experimental Procedures.” Based on the observation that all of the level of expression of all of the cloned genes was approximately the same, the activities are presented as a percentage of the wild type gene 5 protein activity. The specific activity of polymerase (~2000 pmol of dNTP incorporated/min/mg of protein) and 3′ to 5′ exonuclease (~2500 pmol of dNMP hydrolyzed/min/mg of protein) of the wild-type enzyme were presented as 100%.
molecular mass of approximately 80 kDa and reacts to antibody specific to gene 5 protein (data not shown). The protein is 85% pure, as estimated by densitometry analysis of the Coomassie Brilliant Blue-stained gel.

**DNA Polymerase and Exonuclease Activities of the gp5K(300,302,304)E Protein**—Both the polymerase and 3' to 5' exonuclease activities of the purified gp5K(300,302,304)E were determined and compared with those of the wild type enzyme, both in the presence and absence of thioredoxin (Table II). In the absence of thioredoxin, the polymerase specific activity of the mutant enzyme is essentially identical to that of wild type gene 5 protein. However, in the presence of thioredoxin, the wild type gene 5 protein is stimulated approximately 600-fold, whereas the mutant protein is stimulated only 150-fold. Like the wild type enzyme, the mutant protein also catalyzes the hydrolysis of ssDNA, a reaction that is not stimulated by thioredoxin (Table II). Although the exonuclease activity of both enzymes on dsDNA is stimulated by thioredoxin, the stimulation is 3-fold less with the mutant enzyme.

**Processivity of Mutant Gene 5 Protein**—Thioredoxin stimulates the activity of wild type gene 5 protein by increasing the processivity of polymerization of nucleotides (9). Therefore, the most likely explanation for the lack of stimulation of the polymerase activity of gp5K(300,302,304)E by thioredoxin is due to a failure to achieve processivity. We have compared the processivity of wild type gene 5 protein and the mutant enzyme in the presence of thioredoxin using a dilution experiment in which DNA synthesis was carried out using a 32P-end-labeled primer-M13 template with a 4-fold excess of primer-template (Fig. 2). The DNA synthesis observed from the extension of a given primer or the result of a single binding event by the polymerase (9). The processivity of wild type gene 5 protein in the presence of thioredoxin is in the order of hundreds of nucleotides resulting in the accumulation of full-length 7000-nucleotide M13 molecule within the short period of the incubation. In contrast, the products of synthesis by the mutant enzyme in the presence of thioredoxin are considerably shorter, and no full-length molecules are observed (Fig. 2). Furthermore, there is a decrease in the amount of unreplicated primer-template indicative of the rapid cycling of the mutant enzyme. Although not shown, in the absence of thioredoxin the products synthesized by both enzymes are less than 50 nucleotides in length at 1 min of incubation, as judged by polyacrylamide gel electrophoresis analysis.

**T7 gene 2.5 protein**, as well as *E. coli* ssDNA-binding protein, stimulate the processivity of nucleotide polymerization catalyzed by the gene 5 protein-thioredoxin complex, presumably by removing secondary structures within ssDNA (8, 9). However, T7 gene 2.5 protein also physically interacts with T7 DNA polymerase, an interaction that may affect processivity through a different mechanism (8). As shown in Fig. 3, although the gene 5 protein-thioredoxin complex alone is highly processive, the presence of gene 2.5 protein dramatically increases processivity. However, the increase in processivity by gene 2.5 protein is without effect on gene 5 protein alone. Again, the T7 gp5K(300,302,304)E-thioredoxin complex has lower processivity, but interestingly the addition of gene 2.5 protein increases processivity resulting in the formation of full-length product molecule (Fig. 3).

**Binding Affinity of gp5K(300,302,304)E and Thioredoxin—**T7 gene 5 protein and *E. coli* thioredoxin physically interact with a stoichiometry of 1:1 (10–12). The consequence
of the added total thioredoxin, we calculated the plex and that the free thioredoxin concentration is equal to that tional to the concentration that the gene 5 protein-thioredoxin complex and that the free thioredoxin concentration is equal to that of the added total thioredoxin, we calculated the $K_{\text{obs}}$ for the binding of gp5K(300,302,304)E with thioredoxin at 37 °C to be 56 nm and that of the wild type gene 5 protein with thioredoxin to be 8 nm. This latter value is in agreement with that of 5 nm determined earlier at 30 °C (13). We conclude that the mutations in gp5K(300,302,304)E lead to a decreased affinity for thioredoxin, resulting in a reduced processivity of polymerization.

**DNA Binding**—Amino acid changes in the mutant gene 5 protein represent changes of three positively charged residues to three negatively charged residues. We compared the affinity of the mutant protein for DNA to that of the wild type protein. Interestingly, the gp5K(300,302,304)E binds less tightly to a DNA-cellulose column than does the wild type gene 5 protein (Fig. 5). As in the case of the wild type gene 5 protein-thioredoxin complex, the mutant gene 5 protein-thioredoxin complex has a higher affinity for DNA-cellulose. The possible significance of this result is addressed under “Discussion.”

**DISCUSSION**

High processivity, the ability to polymerize thousands of nucleotides during each binding cycle, is an important property for DNA polymerases involved in the replication of a chromosome. The diversity with which the DNA polymerases of different organisms have acquired this essential function is illustrated by eukaryotes, *E. coli*, and bacteriophages φ29, T4, T5, and T7. The DNA polymerases of both phage φ29 (14) and T5 (40, 41) are highly processive by themselves, although the molecular mechanism by which they achieve this property is not at present known. By contrast, the T4 DNA polymerase and the replicative DNA polymerases of *E. coli* and eukaryotes associate with separate proteins that function as clamps to tether the polymerase to the primer-template (42). These homologous proteins (T4 gene 45 protein, *E. coli* β subunit, and eukaryotic proliferating cell nuclear antigen) form multimers that encircle the duplex region of the primer-template (43–45). Bacteriophage T7 DNA polymerase, the subject of this report, we believe to lie between these two extremes in that the polymerase and its processivity factor, *E. coli* thioredoxin, form the clamp that encircles the dsDNA. Thus, as discussed in more detail below, we propose that the DNA polymerase itself serves as one side of the clamp, and thioredoxin the other.

In the Introduction we discussed the high affinity of thioredoxin for T7 gene 5 protein, the increased affinity of the complex for a primer-template, and the resulting increase in processivity of polymerization of nucleotides. Here we show that amino acid changes in a 76-amino acid region of gene 5 protein (residues 258–333) decrease its affinity for thioredoxin and the processivity of the polymerization reaction. The rationale for selecting this particular sequence for site-directed mutagenesis
Thioredoxin partially protects the gene polymerase to the large fragment of amino acid sequence and does not bind thioredoxin (23, 47). Enhanced binding to the mutant thioredoxin, and the amino acid region with glutamate, on the assumption that the charged group would most likely have an affect on the binding of the protein to thioredoxin. However, replacement of individual lysine residues with glutamate did not alter the ability of the gene 5 protein to complement in wild type E. coli cells infected with T7 phage lacking gene 5. Only when the cellular concentration of thioredoxin was reduced did a gene 5 protein having a single amino acid change, K302A, fail to complement, although the K300E complemented relatively poorly. The requirement of high concentrations of thioredoxin to achieve complementation suggests strongly that the affinity of the altered gene 5 proteins and thioredoxin is reduced, a result observed previously (13). A more severe defect was observed when all three lysines were simultaneously replaced by glutamates. In this instance wild type levels of thioredoxin could not support growth of T7.

In order to determine the basis of the defect in the T7 gp5K300,302,304E, we purified the protein and characterized it biochemically. Although both the polymerase and 3’ to 5’ exonuclease activities of the enzyme were equivalent to that of the wild type gene 5 protein, it had a 7-fold lower affinity for E. coli thioredoxin and, as a consequence, the processivity of nucleotide polymerization catalyzed by the polymerase-thioredoxin complex was not nearly as high as that of the wild type complex. The severe affect of only a 7-fold reduction in binding of the mutant polymerase to thioredoxin on DNA replication and phage growth is not unanticipated, since the single amino acid change E319K in gene 5 protein increases its affinity for a mutant thioredoxin only 6-fold but restores phage growth on cells harboring this thioredoxin. We did not purify the T7 gp5K302E, gp5K302A, or gp5K300,302A, but we presume that their affinity for thioredoxin is reduced by an even lesser extent, since the defect is observed in vivo only when the intracellular concentration of thioredoxin is reduced.

The effect of thioredoxin on the dsDNA exonuclease activity of the mutant gene 5 protein also supports a decrease in processivity of the enzyme. The stimulation of the dsDNA exonuclease activity of wild type gene 5 protein by thioredoxin reflects the increased binding of the enzyme to the 3’-termini of a duplex DNA molecule in the presence of thioredoxin (14). Our finding that thioredoxin does not stimulate the dsDNA exonuclease activity of the mutant protein to the same extent as that observed with the wild type gene 5 protein suggests that the mutant gene 5 protein-thioredoxin complex binds less tightly to the DNA. We did not determine the apparent dissociation constant, $K_{d}$. From these data, but based on the similar values of $K_{d}$ for polymerase and dsDNA exonuclease reported from this laboratory (13), we assume it should be similar to that derived from the effect of thioredoxin on the polymerase activity of gene 5 protein.

By what mechanism do these mutations reduce the binding of the protein to thioredoxin? In the case of the previously described E319K mutation in gene 5 protein that was isolated as a suppressor mutation that allowed the phage to grow on a strain containing an altered thioredoxin (G74D), we believe that the residue at position 319 provides a contact point for residue 74 in thioredoxin. This conclusion is based on both in vivo and in vitro results, as well as the nature of the amino acid changes (20, 21). We suspect, however, that the affect of the changes in the three lysine residues described here on binding to thioredoxin are due to more complex interactions between the two protein. Although there is good evidence that the structure of T7 gene 5 protein resembles closely that of the large fragment of E. coli DNA polymerase I the 76-amino acid sequence in which these mutations reside is unique to T7 gene 5 protein. Not only is the structure of this segment unknown, but its effect on adjacent domains must await structure determination of T7 DNA polymerase. One approach to extending the

![Graph](http://www.jbc.org/)

**Fig. 5.** DNA binding activity of gene 5 proteins in the presence and absence of thioredoxin. Gene 5 proteins, wild type or gp5K300,302,304E, were reconstituted with thioredoxin as described under “Experimental Procedures.” The reconstituted gene 5 protein-thioredoxin complex (+Trx) or the gene 5 protein alone (−Trx) were loaded onto a dsDNA-cellulose column (0.5 ml), and the column was then washed with successive washes of 4 ml of Binding Buffer containing the indicated concentration of NaCl. Fractions were assayed for polymerase activity using heat-denatured calf thymus DNA as described under “Experimental Procedures.” B, before loading; F, flow-through; W, washing buffer.

is based on several considerations. First, T7 gene 5 protein is subject to a specific proteolytic cleavage within this region that enables the separation of the carboxyl-terminal polymerase domain from the amino-terminal 3’ to 5’ exonuclease domain (23). Thioredoxin partially protects the gene 5 protein from cleavages within this region. Second, proteolytic cleavage at residue Lys-299 gives rise to a carboxyl-terminal domain containing polymerase activity that is not stimulated by the addition of thioredoxin (23). Third, one mutation in gene 5 protein, Gln-319 → Lys, which lies within this 76-amino acid sequence, suppresses a mutation in thioredoxin that cannot support the growth of T7 phage (21). The suppressor gene 5 protein has enhanced binding to the mutant thioredoxin, and the amino acid changes suggest a contact point between the two proteins (22). Fourth, E. coli DNA polymerase I, an enzyme highly homologous to T7 gene 5 protein (23), does not have this 76-amino acid sequence and does not bind thioredoxin (23, 47). Finally, the T7 unique sequence, based on homology of T7 DNA polymerase to the large fragment of E. coli DNA polymerase I whose structure is known (25), is inserted into the thumb region of the polymerization domain. The thumb region has been proposed to play an important role in binding E. coli DNA polymerase I to duplex DNA, providing it with a processivity of approximately 20 nucleotides/binding cycle (26).

We initially replaced five lysine residues within the 76-amino acid region with glutamate, on the assumption that the replacement of a negatively charged group with a positively charged group would most likely have an affect on the binding of the protein to thioredoxin. However, replacement of individual lysine residues with glutamate did not alter the ability of the gene 5 protein to complement in wild type E. coli cells infected with T7 phage lacking gene 5. Only when the cellular concentration of thioredoxin was reduced did a gene 5 protein having a single amino acid change, K302A, fail to complement, although the K300E complemented relatively poorly. The requirement of high concentrations of thioredoxin to achieve complementation suggests strongly that the affinity of the altered gene 5 proteins and thioredoxin is reduced, a result observed previously (13). A more severe defect was observed when all three lysines were simultaneously replaced by glutamates. In this instance wild type levels of thioredoxin could not support growth of T7.

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Mechanism by which amino acid changes in this 76-amino acid segment decrease binding to thioredoxin would be to identify suppressor mutations in the trxA gene. The resulting amino acid changes in thioredoxin and the biochemical properties of the altered thioredoxin should provide additional information on the interaction of the two proteins.

It also appears that the triple amino acid changes in gp5K(300,302,304)E to thioredoxin, although the measurement not the major effect of the observed higher affinity of altered gene 5 protein-thioredoxin complex to DNA is activity by this mechanism. We believe that the decreased since thioredoxin binding to this domain may in part mask the higher than that of wild type gp5. The decreased binding of enzyme with DNA the amino terminus of the H1 helix fits into fragment of a suppressor mutations in the segment decrease binding to thioredoxin would be to identify mechanism by which amino acid changes in this 76-amino acid sequence is a major constituent of the thioredoxin binding domain.

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J. Biol. Chem. 1997, 272:6599-6606.
doi: 10.1074/jbc.272.10.6599

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