Structural and Functional Organization of the DNA Polymerase of Bacteriophage T7

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Accessibility
The 80-kDa gene 5 protein encoded by bacteriophage T7 shares significant amino acid homology with the large fragment of Escherichia coli DNA polymerase I (Klenow fragment). Like the Klenow fragment, T7 gene 5 protein has both DNA polymerase and 3' to 5' exonuclease activities. However, unlike the Klenow fragment, T7 gene 5 protein binds tightly to E. coli thioredoxin to form a complex that has a high processivity of nucleotide polymerization. In order to identify the domains of gene 5 protein responsible for polymerization, hydrolysis, and binding of thioredoxin, we have analyzed proteolytic fragments of gene 5 protein. Cleavage of the protein within one protease-sensitive region (residue 250–300) yields two molecular weight species of peptides of 32–37 and 43–51 kDa derived from the N-terminal and C-terminal region, respectively. DNA polymerase activity is found within the C-terminal fragments and exonuclease activity within the N-terminal fragments. Thioredoxin stimulates the DNA polymerase activity of the C-terminal fragments. All fragments bind to DNA. In addition to delineating the polymerase and exonuclease domains, the protease-sensitive region appears to interact with E. coli thioredoxin. Thioredoxin protects this region from proteolysis, and alteration of this region reduces the ability of thioredoxin to stimulate polymerase activity.

The DNA polymerase encoded by bacteriophage T7 offers several advantages for examining structure-function relationships and for studying protein-protein interactions at a replication fork. T7 DNA polymerase, the 80-kDa product of gene 5 of the phage, is responsible for the polymerization of nucleotides at the replication fork where it physically interacts with each of the other four components of the T7 replisome-processivity factor, helicase, primase, and single-stranded DNA binding protein (1, 2). With the exception of the processivity factor, the relatively few proteins of the T7 replisome are encoded by the virion. The processivity factor, Escherichia coli thioredoxin, binds tightly to T7 DNA polymerase in a 1:1 stoichiometry (3–5) and converts the low processivity gene 5 protein to a highly processive enzyme that can polymerize thousands of nucleotides without dissociation (6, 7).

Like many DNA polymerases (8), T7 DNA polymerase has a 3' to 5' exonuclease activity (9) that increases the fidelity of DNA synthesis by removing misincorporated nucleotides (10). The exonuclease activity of T7 gene 5 protein is active on both single-stranded (ss) and double-stranded (ds) DNA; thioredoxin increases the activity on dsDNA several hundred-fold while it has little effect on the activity on ssDNA (9, 11–13).

Although the three-dimensional structure of T7 DNA polymerase is not yet known, it does, on the basis of sequence homology (14), belong to the E. coli DNA polymerase I family of DNA polymerases (15, 16). The crystal structure of the large fragment of E. coli DNA polymerase I (Klenow fragment) has been solved both in the presence (17) and absence of DNA (18). The crystal structure reveals that the molecule is folded into two distinct domains, one composed of residues from the N-terminal region and the other of residues from the C-terminal region. Several lines of genetic and biochemical evidence show that the exonuclease and polymerase activities reside in the N- and C-terminal domains, respectively (19).

Based on the high degree of homology between T7 DNA polymerase and Klenow fragment, it seemed likely that the polymerase and exonuclease activities of T7 DNA polymerase would reside in similar domains of the gene 5 protein, a hypothesis supported by biochemical evidence. For example, in vitro mutagenesis of residues located in the N-terminal region of the polypeptide around residue 126 results in varying reductions of the exonuclease activity (20, 21) while mutation of a single residue (residue 526) in the C-terminal region affects nucleoside triphosphate utilization (22).

One approach to establish directly the existence of two distinct domains in the gene 5 protein is to disrupt the polypeptide chain and to show that exonuclease and polymerase activities can be physically separated. Such an approach was initially used to demonstrate that the 5' to 3' exonuclease activity of E. coli DNA polymerase I, an activity not found in T7 DNA polymerase, is located on a separate domain (23, 24). Limited proteolysis of the intact protein cleaves the molecule into two fragments, a C-terminal fragment (Klenow fragment) that has polymerase and 3' to 5' exonuclease activities and an N-terminal fragment containing the 5' to 3' exonuclease activity. Subsequently, in vitro mutagenesis has been used to physically separate the polymerase domain from the exonuclease domain (25). Limited proteolysis has also been used to show that the polymerase and exonuclease activities of bacteriophage T4 DNA polymerase and herpes simplex virus DNA polymerase reside in distinct domains (26, 27). These latter studies illustrate the overall similarities in the architecture of DNA polymerases even when they belong to different families (28).

In this report we have analyzed proteolytic fragments of gene 5 protein to generate direct experimental evidence for the func-

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1 The abbreviations used are: ss, single-stranded; ds, double-stranded; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis.

Structural and Functional Organization of the DNA Polymerase of Bacteriophage T7*

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tional and structural separation of the activities of gene 5 protein. Our results also support the presence of a unique domain involved in the binding of thioredoxin.

EXPERIMENTAL PROCEDURES

Materials

Proteins—Highly purified recombinant T7 gene 5 protein was provided by Dr. Stanley Tabor (Harvard Medical School). E. coli thioredoxin, Δ28 T7 DNA polymerase (Sequenase version 2.0), bovine serum albumin, E. coli DNA polymerase I Klenow fragment, E. coli exonuclease III, and endonuclease HindIII were purchased from Amersham Life Sciences, Inc. Trypsin was from Sigma. Polyclonal antiserum specific to gene 5 protein was from Hazelton Research Products, Inc.

DNA and Nucleotides—Calf thymus DNA was from Sigma. 32P-labeled nucleoside triphosphates were from Amersham Life Sciences, Inc. Ultrapure nucleoside triphosphates were from Pharmacia Biotech, Inc.

Other—Double-stranded and single-stranded DNA cellulose were provided by Drs. Q.-Y. Liu and B. B. Beauchamp (Harvard Medical School). Microspin columns were products of Pharmacia. Bio-Spin Chromatography columns, Mini-Protein ready gels, Trans-Blot transfer membranes (PVDF membrane), and protein silver stain kits were purchased from Bio-Rad. Rainbow protein electrophoresis standards were from Amersham Life Sciences, Inc. Premixed polyacrylamide solutions were from Boehringer Mannheim. Polyclonal gene 5 protein-thioredoxin-specific antiserum was from Hazelton Research Products, Inc.

Methods

Preparation of DNA Substrates—For exonuclease activity gel assay 10 μg of calf thymus DNA was first digested with restriction endonuclease HindIII and then incubated with 25 μCi of [α-32P]dATP (1 Ci = 37 Gbq) and 10 units of Δ28 T7 DNA polymerase at room temperature for 10 min. After heating the reaction mixture at 70 °C for 15 min, DNA was isolated using a Microspin Column S-400. The final product in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA was used as dsDNA. ssDNA was prepared by heating the dsDNA for 5 min at 95 °C followed by incubation with 1 mM NaOH for 5 min at room temperature. The solution was neutralized by the addition of 1 mM HCl and 1 mM EDTA. For polymerase activity gel assay, calf thymus DNA (1 mg/ml in 10 mM Tris-HCl, pH 7.5) was heated at 100 °C for 5 min before use.

Electrophoresis Analysis—Protein was analyzed on polyacrylamide gels under denaturing conditions (29) with mini-gels (8 × 10 mm) or standard gels (16 × 20 mm). Molecular weights of proteins and proteolytic fragments were estimated from standards (30). Gels were stained either by silver or Coomassie Brilliant Blue and analyzed by densitometry using a LKB Ultroscan XL Laser Densitometer.

SDS-DNA Activity Gel Analysis—Polymerase activity gel assay was carried out as described (22, 31). Heat-denatured calf thymus DNA (0.2 mg/ml final concentration) was mixed with the resolving gel solution and polymerized into 10 or 12% SDS-polyacrylamide gels (16 × 20 mm). Protein samples were mixed with the Low SDS Sample Buffer (36 mM Tris-HCl, pH 6.8, 0.14% SDS, 80 mM mercaptoethanol, 11% glycerol, and 0.014% bromphenol blue). All samples were heated at 37 °C for 10 min prior to loading on duplicate gels. Gel electrophoresis was carried out under denaturing condition according to Laemmli (29). To remove SDS and renature the proteins, the gels were washed several times with 500 ml of TMG Buffer containing 50 mM Tris-HCl, pH 7.5, 0.05 mM EDTA, 6 mM magnesium acetate, 40 mM KCl, 0.4 mM bovine serum albumin, 1 mM dithiothreitol, and 16% glycerol and were soaked in 1 liter of the same buffer for a minimum of 8 h at 4 °C followed by 2 h at room temperature. Reactions were carried out in 10 ml of TMG buffer at room temperature with 0.02 ml each of dGTP, dATP, dCTP, and dTTP, and 30 μCi of [α-32P]dATP (1 Ci = 37 Gbq) with or without E. coli thioredoxin (10 μM). After washing four times in 400 ml of 5% trichloroacetic acid and 13% sodium pyrophosphate solution to remove the unincorporated [α-32P]dAMP, the gels were dried, and the DNA product was identified by autoradiography or Phospholmager analysis.

The exonuclease activity gel assay was a modification of the method described (32). Protein samples were mixed with the Low SDS Buffer and heated at 37 °C for 5 min prior to loading on duplicate gels. The SDS-polyacrylamide gels contain 32P-labeled calf thymus DNA (4.25 × 105 cpm). After electrophoresis (2 mA/cm), SDS removal and phenolblue and were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained and analyzed by densitometry.

RESULTS

In wild type E. coli cells infected with phage T7 essentially all of the phage gene 5 protein is found in a 1:1 complex with E. coli thioredoxin (3–5). In order to obtain gene 5 protein free of thioredoxin, we have overproduced the protein from a cloned gene 5 harbored in an E. coli strain deleted for the chromosomal thioredoxin gene (trxA) (6). Under these conditions, in the absence of thioredoxin, the preparation of purified gene 5 protein contains a number of peptides that are smaller than the intact 80-kDa gene 5 protein as determined by SDS-PAGE analysis (6). Several lines of evidence suggest that these smaller peptides arise from the actions of protease(s) rather than contamination during the purification procedure. For example, very few if any of these small peptides are found in preparation of gene 5 protein from cells containing thioredoxin (6). This finding suggests that thioredoxin binds to and protects gene 5 protein from proteolysis.

Proteolysis of Gene 5 Protein; Effect on Enzymatic Activities—When the gene 5 protein purified from an E. coli trxA strain is analyzed by SDS-PAGE (Fig. 1A), six major small peptides (bands b–g) in addition to the full-length gene 5 protein (band a) are observed. These small peptides represent approximately 10% of the full-length gene 5 protein, and they fall into two major molecular species, three between 43 and 51 kDa (bands b–d) and three between 32 and 37 kDa (bands e–g). Interestingly, each band of the higher molecular weight peptides could be matched with one of the bands of lower molecular weight peptides based on their staining intensity by Coomassie Brilliant Blue (Fig. 1A). Each pair of fragments (b + g, e + f, and d + e) has a combined mass of approximately 80 kDa, the mass of the full-length gene 5 protein. These small fragments are only separated from the full-length gene 5 protein under denaturing conditions; no small peptides are seen during non-denaturing polyacrylamide gel electrophoresis or by gel filtration (data not shown).

In order to determine if any of the fragments found in the
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gene 5 protein preparation have polymerase or exonuclease activity, we used SDS-DNA activity gel assays. To measure the polymerase activity, the peptides in the gene 5 protein preparation were separated by electrophoresis in a polyacrylamide gel containing ssDNA. After electrophoresis, the SDS was removed, allowing the proteins to renature, and polymerase activity was determined directly in the gel by incubating with \([\alpha-\text{32P}]\text{dATP}\) and all four dNTPs. As shown in Fig. 1B, all three of the fragments (b, c, and d), as well as the intact gene 5 protein, had polymerase activity, whereas the lower molecular weight fragments were not active in this assay.

A similar in vitro assay was used to detect exonuclease activity. In this assay SDS gels contain 32P-labeled dsDNA. After electrophoresis, the SDS was removed, and exonuclease activity was determined directly in the gel by incubating in the presence of Mg2+ and E. coli thioredoxin (Fig. 1C). In this assay all three of the lower molecular weight fragments catalyzed the hydrolysis of the DNA, as did the intact gene 5 protein; no exonuclease activity was observed for the higher molecular weight fragments.

Since the most likely basis for the generation of the peptides found in the gene 5 protein preparation is proteolysis during expression and purification, we have examined the sensitivity of the gene 5 protein to the protease, trypsin. At a trypsin to gene 5 protein ratio of 1/750 the 80-kDa gene 5 protein is cleaved into two major molecular weight fragments, one of approximately 32 kDa and one 48 kDa (Fig. 2A). These fragments are relatively stable for up to 3 h of digestion under these conditions but are subsequently degraded further.

SDS-DNA activity gel assays for polymerase and exonuclease activity revealed that the 32-kDa species contains exonuclease activity (Fig. 2B), whereas the 48-kDa species contains polymerase activity (Fig. 2C). A small amount of DNA disappears at the 48-kDa position in the exonuclease assay. However, we believe that this activity arises from pyrophosphorylisis rather than hydrolysis since the amount of degradation is reduced by the addition of pyrophosphatase (data not shown). Thus, the in vitro proteolysis roughly mimics that of the presumed proteolysis that occurs during expression and purification.

Identification of Peptides by Amino Acid Sequencing—In order to establish definitively that the fragments obtained in the gel in Fig. 1 are derived from gene 5 protein and to locate the peptides within the intact gene 5 protein, we determined the N-terminal amino acid sequence of each peptide. The purified gene 5 protein and the trypsin-treated gene 5 protein were subjected to SDS-PAGE, and all fragments were transferred onto a PVDF membrane. The proteins were stained with Coomassie Brilliant Blue, and the peptides bands were cut out of the membrane for sequence analysis. In each case an N-terminal sequence could be obtained and matched with a corresponding sequence in the gene 5 protein (Fig. 3). Fragments b, c, and d, corresponding to the fragments shown in Fig. 1, were all derived from the C terminus of the intact gene 5 protein with Ile-289, Lys-299, and Ala-323 as the N-terminal amino acid, respectively. Fragments e, f, and g each contained the N terminus of the gene 5 protein, and based on the apparent molecular weight as judged by SDS-PAGE, they probably terminated at Glu-322, Lys-298, and Arg-288, respectively. The N-terminal Met residue in the fragments e, f, and g is the initiation codon. The N terminal of the two tryptic fragments were also sequenced, and the data show that the N terminus of the 48-kDa fragment begins at residue 300, and the N terminus of the 32-kDa fragment is identical with the N terminus of the intact gene 5 protein.

Interaction of Gene 5 Protein with E. coli Thioredoxin—T7 gene 5 protein forms one to one complex with E. coli thioredoxin to increase the processivity of the polymerization reaction and consequently the macroscopic rate of polymerization. The high affinity of the complex for a primer-template (7) also increases the 3’ to 5’ exonuclease activity on dsDNA but not on ssDNA (6, 9, 11). In order to determine if thioredoxin can physically interact with any of the proteolytic fragments, we have developed a gel activity assay that measures the effect of thioredoxin on the polymerase and exonuclease activities of gene 5 protein.

![Fig. 1. Electrophoretic analysis of proteolytic fragments of gene 5 protein and their activities.](image)

Purified gene 5 protein (6 µg) was incubated with trypsin at a 1/750 ratio at room temperature. Aliquots were transferred to an equal volume of 2 × Low SDS Sample Buffer at the indicated time points and analyzed by SDS-PAGE and SDS-DNA activity gel assays as described under “Experimental Procedures.” After electrophoresis, the gels were silver-stained (panel A), assayed for exonuclease activity on dsDNA (panel B), and for polymerase activity (panel C) in the presence of E. coli thioredoxin (10 µM). The positions of molecular mass standards are indicated at the left.
Fig. 3. Identification of the proteolytic fragments. Purified gene 5 protein from an E. coli trxA strain (Fig. 1) and trypsin-treated gene 5 protein (Fig. 2) were subjected to SDS-PAGE. After electrophoresis, the proteins were transferred onto a PVDF membrane and were stained with Coomassie Brilliant Blue. Each fragment was excised and subjected to N-terminal autosequencing. The proteolytic fragments a–g (Fig. 1), the tryptic fragments, and their apparent molecular mass as judged by SDS-PAGE are listed on the left. The N-terminal sequences are presented in the center, and the activity identified in each fragment (from Fig. 1) is shown on the right. The numbers above the sequences represent the N/C-terminal residue number relative to intact gene 5 protein. *, predicted carboxyl end points based on their apparent molecular weight and the determined N terminus of the corresponding peptides of the protein. **, activity is a summary of Fig. 1, and Pol is the polymerase activity (Fig. 1B), and Exo is the exonuclease activity (Fig. 1C).

<table>
<thead>
<tr>
<th>PROTEIN FRAGMENTS</th>
<th>ACTIVITY**</th>
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<tr>
<td>a (80 kDa)</td>
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<tr>
<td>b (51 kDa)</td>
<td>704</td>
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<td>c (47 kDa)</td>
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<td>e (37 kDa)</td>
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<td>f (33 kDa)</td>
<td>298*</td>
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<td>g (31 kDa)</td>
<td>288*</td>
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gene 5 protein (Fig. 4). In these assays polymerase and exonuclease activities are measured as in the experiments described above but in the presence or absence of purified E. coli thioredoxin. Both the polymerase activity (Fig. 4A, band a) and the exonuclease activity (Fig. 4B, band a) of gene 5 protein are stimulated by thioredoxin whereas the similar activities of Klenow fragment are not (Fig. 4C).

The polymerase activity of all three of the proteolytic fragments in the size range 43–51 kDa are stimulated by the addition of thioredoxin (Fig. 4A), whereas the 3′ to 5′ exonuclease activity found in the 31–37-kDa species is not (Fig. 4B). The stimulation of polymerase activity by thioredoxin is considerably greater for the larger species of the 40–51-kDa species since the amount of protein in the larger species (band b in Fig. 1A) is at least half that in band d. This result indicates that the amino acid residues Lys-299 through Gly-322 play an important role in the interaction of gene 5 protein with thioredoxin, in agreement with earlier studies that suggested Glu-319 of gene 5 protein provides a contact point for thioredoxin (33).

Earlier studies (6) showed that in contrast to proteolysis observed in gene 5 protein overproduced and purified in the absence of thioredoxin, the presence of thioredoxin during the purification prevented the proteolysis. Consequently, we have examined the ability of purified thioredoxin to protect gene 5 protein from proteolysis by trypsin. As shown in Fig. 5, thioredoxin provides significant protection of gene 5 protein from proteolysis by trypsin. In the presence of 5 μg of thioredoxin per 3 μg of gene 5 protein (10:1 molar ratio), the cleavage of gene 5 protein was reduced over 10-fold. Since the cleavage site in gene 5 protein for trypsin is residue Lys-300 (Fig. 3), the protection by thioredoxin supports our interpretation that residues in this region are involved in the binding of thioredoxin.

**DISCUSSION**

Previous observations revealed that several specific fragments of gene 5 protein arise during the purification of gene 5 protein, a phenomena that could be reduced by overexpressing gene 5 in E. coli trxA strain that contain thioredoxin (6). The possibility that these fragments arose from proteolysis is supported by the finding that the amount of fragments is reduced markedly by the use of E. coli OmpT (Outer Membrane Protease deletion) strains. In the present study we have shown that the fragments observed in these earlier studies are indeed derived from gene 5 protein and most likely arise by proteolysis at a sensitive region of gene 5 protein. Three pairs of peptides were identified in which one member of each pair is derived from the N terminus of the gene 5 protein and the other from the C terminus (Figs. 1 and 3); the sum of the molecular

2 S. Tabor, personal communication.

![Identification of the proteolytic fragments](image-url)
Weights of each pair approximate that of the intact gene 5 protein. The three sites of cleavage that give rise to the three pair of peptides occur within a relatively short region between residues 270 and 330. We have shown that thioredoxin protects this region from cleavage by trypsin suggesting that this region or a portion of it physically interacts with the processivity factor, thioredoxin.

The three-dimensional structure of the Klenow fragment of *E. coli* DNA polymerase is known, and from sequence analysis it shares a high degree of homology to T7 gene 5 protein (14). Indeed, biochemical (13, 20, 22) and genetic (33) studies on gene 5 protein support a similar overall structure for the two proteins including the localization of the polymerase domain to the C-terminal region of the protein and the exonuclease domain of the N terminus of the intact protein through residues around 280–290. In support of these data we have recently cloned the N-terminal region extending from the N terminus to Leu-257 and shown that the purified fragment has exonuclease activity. The polymerase and exonuclease domains are structurally distinct in several DNA polymerases. However, there is considerable evidence that they are associated both physically and functionally (8, 19). Furthermore, in the case of gene 5 protein the physical association appears to be such that the two domains remain associated even after disruption of the peptide chain; the proteolytic fragments remain associated with the intact enzyme through multiple purification steps (6). Similar findings have also been observed with the DNA polymerases of herpes simplex (27) and the thermoacidophilic archaean Sul-

![Fig. 5. Effect of thioredoxin on proteolysis of gene 5 protein.](#)

Purified gene 5 protein (3 μg) was incubated with varying amounts of thioredoxin at 0 °C followed by incubation with 0.06 μg of trypsin (50:1) for 60 min at room temperature. The amount of thioredoxin was 0 (lane 1), 0.1, 0.5, 2.0, and 5 μg. Proteolytic products were analyzed by 10–20% gradient SDS-PAGE, and proteins were visualized by silver staining. The two tryptic fragments are marked by arrowheads. *F*, full-length gene 5 protein, *Trx*, thioredoxin. The protein standards (kDa) are indicated at the left.

![Fig. 6. Alignment of the amino acid sequences of polymerase I type DNA polymerases.](#)

Sequences are shown in single-letter amino acid code. Numbers in brackets indicate the amino acid residues relative to the N terminus of each enzyme. Identical residues are bold. The homologue segments are indicated by filled bars and labeled on the top of each bar. The *Exo III motif* is derived from a published alignment (40), and the lettered bars refer to the homologue of *E. coli* DNA polymerase I (Pol I) (17), where β and α represent the regions in β-sheet and α-helix, respectively. The unique sequence in T7 DNA polymerase is indicated by dashed-line brackets, and the favored trypsin cleavage site is marked by an arrow.

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2 X.-M. Yang and C. C. Richardson, unpublished results.

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fonobus solfataricus (34). The functional association of the two
domains could explain, at least in part, the greatly reduced
polymerase and exonuclease activities found in the proteolytic
fragments. The C-terminal domain of Klenow fragment has
been isolated, and it too has at least a 10-fold reduction in
polymerase activity relative to the intact Klenow fragment
(25). It was proposed that the absence of the N-terminal do-
main introduced an unfavorable entropy contribution to sub-
strate binding. T4 DNA polymerase is also organized into a
polymerase and exonuclease domain (26, 35, 36), and the iso-
lated exonuclease domain has greatly reduced exonuclease ac-
tivity that arises from a higher $K_m$ for the DNA substrate (26).

Although T7 gene 5 protein and E. coli DNA polymerase I are
structurally similar they differ in several important features.
Perhaps the most obvious is the fact that T7 DNA polymerase
is responsible for the replication of a genome, whereas E. coli
dNA polymerase I is mainly responsible for repair and recomb-
ination (8). As a replication polymerase, the gene 5 protein
physically interacts with other T7 replication proteins such as
E. coli thioredoxin, its processivity factor (3, 4, 12), gene 4
protein, a helicase/primase (37), and gene 2.5 protein, as DNA
polymerase and exonuclease domain (26, 35, 36), and the iso-
lated exonuclease domain has greatly reduced exonuclease ac-
tivity that resembles the putative thioredoxin binding domain, and T3 also requires thioredoxin for growth. The DNA polymerase encoded by SP01, a Bacillus subtilis phage, contains a domain of approximately 45 residues that
resemble the putative thioredoxin binding domain of T7 DNA polymerase (42). However, it is not known if this SP01
DNA polymerase interacts with thioredoxin.

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