Deoxyribonucleic Acid Polymerase of Bacteriophage T7

PURIFICATION AND PROPERTIES OF THE PHAGE-ENCODED SUBUNIT, THE GENE 5 PROTEIN

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DNA polymerase of bacteriophage T7 is composed of two subunits, the gene 5 protein of the phage and the host-specific thioredoxin. The gene 5 protein has been purified 7400-fold to homogeneity from bacteriophage T7-infected Escherichia coli 7400 trxA cells that lack thioredoxin. The purification procedure has been monitored by using a complementation assay in which thioredoxin interacts with the gene 5 protein to form an active DNA polymerase. The purified gene 5 protein is a single polypeptide having a molecular weight of 87,000. The gene 5 protein itself has only 1 to 2% of the polymerase activity of T7 DNA polymerase. However, T7 DNA polymerase can be reconstituted by the addition of homogeneous thioredoxin to the gene 5 protein. Optimal reconstitution is obtained when the molar ratio of thioredoxin/gene 5 protein is 150. Under these conditions, the gene 5 protein attains approximately 80% of the activity of an equal amount of T7 DNA polymerase. However, T7 DNA polymerase is responsible for the polymerization of nucleotides while the gene 4 protein facilitates the unwinding of the two strands of the template (11–15). In the presence of rNTPs and DNA binding protein, the gene 5 protein catalyzes the synthesis of tetradeoxynucleotides on the displaced single strand that results from leading strand synthesis (14, 17, 18). The extension of the RNA primers by T7 DNA polymerase thus provides a mechanism for lagging strand synthesis.

Upon infection of Escherichia coli, bacteriophage T7 induces the synthesis of a T7 DNA polymerase (1, 2). The T7 DNA polymerase has been purified to near homogeneity (3–5) and shown to be composed of two subunits (3, 6, 7), the 84,000-dalton gene 5 protein encoded by the phage and the 12,000-dalton thioredoxin of the host. Phage mutants defective in gene 5 synthesize an altered DNA polymerase (1) and are unable to replicate T7 DNA in vivo (8). Similarly, when E. coli cells carrying a mutation in the structural gene for thioredoxin (trxA) are infected with phage T7, neither T7 DNA replication nor an active T7 DNA polymerase can be detected (6, 9, 10).

Obviously, T7 DNA polymerase plays an essential role in T7 DNA replication. Purified T7 DNA polymerase alone, however, cannot polymerize nucleotides on duplex DNA templates (1, 2, 11, 12), presumably its template in vivo. On the other hand, in vitro studies have shown that only T7 DNA polymerase can interact with the gene 4 protein, another T7 protein essential for T7 DNA replication (11–13). T7 DNA polymerase, together with the gene 4 protein of the phage, can account for most of the enzymatic events occurring at the replication fork during synthesis on duplex DNA templates. The T7 DNA polymerase is responsible for the polymerization of nucleotides while the gene 4 protein facilitates the unwinding of the two strands of the template (11–15), a reaction that requires the hydrolysis of NTPs (16). Furthermore, in the presence of NTPs and DNA binding protein, the gene 4 protein catalyzes the synthesis of tetradexynucleotides on the displaced single strand that results from leading strand synthesis (14, 17, 18). The extension of the RNA primers by T7 DNA polymerase thus provides a mechanism for lagging strand synthesis.

One approach to understanding the role of T7 DNA polymerase in the complex reactions occurring at the replication fork is to purify each of its two subunits and to determine the properties of each. Extracts of T7-infected E. coli trxA mutants, lacking thioredoxin, contain gene 5 protein but no T7 DNA polymerase activity (6). Such extracts provide the basis of assays for the purification of either thioredoxin or gene 5 protein. Complementation of these extracts with extracts of E. coli containing thioredoxin restores polymerase activity and initially enabled us to purify, characterize, and identify the thioredoxin subunit (3, 6, 7). In this paper we purify the gene 5 protein from extracts of T7-infected E. coli trxA mutants using a similar complementation assay in which gene 5 protein is measured by its ability to interact with purified thioredoxin to restore polymerase activity. We examine in detail the properties of the homogeneous gene 5 protein and its interaction with homogeneous thioredoxin. In the accompanying paper (19) we describe the exonuclease activities of the gene 5 protein and of the reconstituted polymerase.

**EXPERIMENTAL PROCEDURES**

**Materials**

_Bacterial Strains and Bacteriophages—_E. coli B/1, E. coli 7004 (a trxA mutant derived from E. coli B/1 (9)), and E. coli 0111 su* thy have been described previously (6). T7 wild type phage and T7 amber mutants were obtained from Dr. F. W. Studier (Brookhaven National Laboratory). T7 amber mutants are designated by subscript notation indicating the mutant gene only. The amber mutations used are: gene 3, amber; gene 4, amber; gene 5, amber; gene 6, amber. T7 su+ was used for the isolation of T7 gene 5 protein. T7 phages were grown on E. coli 0111 su* thy as described by Studier (20–22).

_DNA—_Undeleted T7 DNA and T7 [32P]DNA were prepared as previously described (23) and, when indicated, were denatured by heating in a boiling water bath for 5 min and then quickly chilling in an ice bath. Calf thymus DNA and salmon sperm DNA were purchased from Sigma Chemical Co. and denatured as previously de-
scribed (1). DNA concentrations are expressed as equivalents of nucleotide phosphorus.

Nucleotides—Unlabeled nucleotides were purchased from Schwartz Bio-Rsearch. [3H]dATP and [α-32P]dATP were obtained from New England Nuclear.

Enzymes and Proteins—T7 DNA polymerase was prepared from T7-infected E. coli as described by Adler and Modrich (5). The gene 4 protein was Fraction V of Kolodner et al. (12). E. coli DNA binding protein was prepared by the method of Weiner et al. (24). T7 DNA binding protein was purified by the method of Reuben and Gelfter (25). Homogeneous E. coli thioredoxin (26) was a gift from Dr. Arne Holmgren, Karolinska Institutet. E. coli RNA polymerase was a gift from Dr. M. Chamberlin, University of California, Berkeley. Crystalline bovine serum albumin and ovalbumin were purchased from Pentex. Lysozyme (code LYSF) and porcine pepsin (code PM) were obtained from Worthington. Bovine hemoglobin and bovine catalase were obtained from Sigma.

Buffer Solutions—The following buffer solutions were used: Buffer A was 20 mM Tris (pH 7.5), 10 mM 2-mercaptoethanol, 10% (w/v) glycerol; Buffer B was 20 mM potassium phosphate (pH 7.5), 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% (w/v) glycerol; Buffer C was 20 mM potassium phosphate (pH 7.5), 0.5 mM dithiothreitol, 20% (w/v) glycerol; and Buffer D was 20 mM potassium phosphate (pH 7.5), 0.5 mM dithiothreitol, 60% (w/v) glycerol.

Other Materials—Bio-Rex 70 was obtained from Bio-Rad. Quaternary aminoethyl (QAE)-Sephadex A-55 was purchased from Pharmacia. Hexyl-Sepharose was prepared as described by Shaltiel (27). DEAE-cellulose (Whatman DE52) and phosphocellulose (Whatman P-11) were obtained from Whatman. Native and denatured calf thymus DNA-cellulose were prepared by the method of Alberts and Herrick (28).

Methods

Enzyme Assays—T7 gene 5 protein, present in extracts or in affinity-purified fractions derived from T7-infected E. coli 7004 trxA, was determined by in vitro complementation with thioredoxin to restore T7 DNA polymerase activity (3). The assay measures the conversion of [3H]- or [α-32P]-labeled dNTPs into an acid-insoluble product. The incubation mixture (0.3 ml) contained: 88 mM potassium phosphate (pH 7.5), 0.7 mM MgCl2, 5 mM 2-mercaptoethanol; 0.5 mM heat-denatured salmon sperm DNA; 0.15 mM concentrations of dCTP, dATP, dGTP, and [3H]- or [α-32P]dATP; 0.4 μg of homogeneous thioredoxin; and 0.05 to 0.4 unit of gene 5 protein. Dilutions of gene 5 protein for assay were routinely made in a solution composed of 50 mM Tris (pH 7.5), 10 mM 2-mercaptoethanol, 10% (w/v) glycerol, 0.15 mM EDTA, 10% (w/v) glycerol, 10% (w/v) sucrose, and 1% (w/v) bovine serum albumin.

Enzyme assays were performed at 30°C for 30 min. The reaction was stopped by the addition of 3 ml of 1 M HCl, 0.1 M sodium pyrophosphate, and the acid-insoluble radioactivity was determined as previously described (13). One unit of gene 5 activity is defined as the amount catalyzing the incorporation of 10 nmol of total nucleotide into an acid-insoluble product in 30 min.

Bio-Rex 70 Chromatography—Bio-Rex 70 fractions assayed at the same conditions as the gene 5 protein except that thioredoxin was omitted from the incubation mixture and 0.08 to 0.4 unit of T7 DNA polymerase was added to the reaction mixture in place of gene 5 protein. One unit of T7 DNA polymerase is defined as the amount catalyzing the incorporation of 10 nmol of total nucleotide into an acid-insoluble product in 30 min.

Sedimentation Analysis—Glycerol density gradient sedimentation analyses were carried out in 5 to 20% glycerol gradients as described by Maitland and Anus (39). Gels were stained with Coomassie brilliant blue and destained as described by Fairbanks et al. (32).

Protein Determination—Protein was determined by the method of Lowry et al. (34) using bovine serum albumin as a standard.

RESULTS

Purification of the T7 Gene 5 Protein

The procedure described here is a modification and extension of that described by Modrich and Richardson (3) for the 70-fold purification of the T7 gene 5 protein from T7, ϕ-infected E. coli 7004 trxA. The assay used for monitoring the purification is based on the ability of thioredoxin to complement gene 5 protein to restore T7 DNA polymerase activity. A summary of the purification from 150 g of T7, ϕ-infected E. coli 7004 trxA is presented in Table I. Unless indicated otherwise, all steps were performed at 4°C, and centrifugation was at 15,000 × g for 15 min.

Growth of Phage-infected Cells—E. coli 7004 trxA was grown and infected with T7, ϕ phage as previously described (13). The cells were harvested, and the cell paste (150 g) was suspended in 600 ml of 50 mM Tris (pH 7.5), 10% sucrose. Aliquots (200 ml) in 250-ml polycarbonate bottles were frozen in liquid nitrogen.

Preparation of Cell Extract—The infected cells were thawed overnight in an ice bath and lysed as previously described (13). The lysates then were centrifuged for 90 min at 18,000 rpm in an International A54 rotor. The supernatant fluid was recovered and adjusted to A260 = 200 with 50 mM Tris (pH 7.5), 0.1 M NaCl, 10% sucrose (Fraction I).

Streptomycin and Ammonium Sulfate Fractionation—Fraction I (740 ml) was treated with 44 ml of 25% (w/v) streptomycin sulfate and, after stirring for 30 min, the precipitate was removed by centrifugation. Solid (NH4)2SO4 (0.39 g/ml of supernatant fluid) was slowly added to the supernatant fluid and the solution was stirred for 30 min. The precipitate was collected by centrifugation, dissolved in 150 ml of Buffer A, and dialyzed overnight against 2 liters of Buffer A to yield Fraction II (240 ml).

DEAE-Cellulose Chromatography—Fraction II was applied at a flow rate of 10 ml/min to a column of DEAE-cellulose (44 cm2 × 25 cm) previously equilibrated with Buffer A. The column was washed with 300 ml of Buffer A and then with 800 ml of Buffer A containing 0.1 M NaCl. The proteins were eluted with 6 liters of a linear gradient from 0.1 to 0.4 M NaCl in Buffer A; the flow rate was 4 ml/min and 20-ml fractions were collected. Gene 5 protein activity eluted at approximately 0.23 M NaCl. The fractions having greater than 2.0 units/mg of protein (A260) were pooled (825 ml) and the protein was precipitated with (NH4)2SO4 (0.47 g/ml). The precipitate was collected by centrifugation, dissolved in 60 ml of Buffer B, and then dialyzed 12 h against 2 liters of the same buffer to yield Fraction III (74 ml).

Bio-Rex 70 Chromatography—Fraction III was applied at a flow rate of 1.2 ml/min, to a column of Bio-Rex 70 (4.9 cm2 × 30 cm) previously equilibrated with Buffer B. The column was washed with 200 ml of Buffer B and then with 200 ml of Buffer B containing 0.1 M NaCl. The proteins were eluted

| Table I Purification of T7 gene 5 protein from T7, ϕ-infected Escherichia coli 7004 trxA |
|---------------------------------|-----------------|-----------------|
| Fraction | Activity | Protein | Specific Activity | Recovery |
|----------|----------|---------|------------------|---------|
|          | units    | mg      | units/mg protein | %       |
| I. Extract | 11,250 | 8040 | 1.4 (100) |
| II. Ammonium sulfate | 10,310 | 6450 | 1.6 95 |
| III. DEAE-cellulose | 5,110 | 980 | 5.2 45 |
| IV. Bio-Rex 70 | 3,220 | 6.1 | 524 29 |
| Va. QAE-Sephadex | 2,100 | 0.49 | 4300 18 |
| Vb. Phosphocellulose | 2,060 | 0.38 | 5390 17 |
| Concentrate | | | | |
| VI. Phosphocellulose | 1,650 | 0.19 | 8695 14 |
| VII. Hexyl-Sepharose | 1,050 | 0.10 | 10357 9* |

* Only 80% of Fraction VI was subjected to chromatography on hexyl-Sepharose. The recovery is corrected for this fact.
with a 1000-ml linear gradient from 0.1 to 0.7 M NaCl in Buffer B; the flow rate was 4 ml/min and 10-ml fractions were collected. Gene 5 protein activity eluted at approximately 0.35 M NaCl. The fractions containing greater than 100 units/mg of protein (A_{250}) were pooled (210 ml) and concentrated by precipitation with (NH_4)_2SO_4 as described in the previous step. The precipitate was dissolved in 10 ml of Buffer C and then dialyzed for 12 h against the same buffer to yield Fraction IV (10 ml).

**QAE-Sephadex Chromatography**—Fraction IV was applied, at a flow rate of 1 ml/min, to a column of QAE-Sephadex A-25 (1.8 cm x 15 cm) previously equilibrated with Buffer C. The column was washed with 100 ml of Buffer C and then with 100 ml of Buffer C containing 0.01 M NaCl. The proteins were eluted with a 1-liter linear gradient from 0.01 to 0.3 M NaCl in Buffer C; the flow rate was 0.6 ml/min and 6-ml fractions were collected. The gene 5 protein activity eluted at approximately 0.2 M NaCl and fractions containing greater than 250 units/mg of protein (A_{250}) were pooled to yield Fraction Va (48 ml).

In order to concentrate the pooled fractions, they were dialyzed against 2 liters of Buffer C for 4 h and applied, at a flow rate of 0.5 ml/min, to a column of phosphocellulose (0.8 cm x 6 cm) previously equilibrated with Buffer C. The column then was washed successively with 20 ml of Buffer C and 30 ml each of Buffer C containing 0.1, 0.2, and 1.0 M NaCl. More than 90% of the gene 5 protein activity applied to the phosphocellulose column was recovered in the 1 M NaCl eluate fractions and represented a concentration of 10-fold. The eluate then was dialyzed against 1 liter of Buffer C for 12 h to yield Fraction Vb (4.3 ml).

**Phosphocellulose**—Fraction Vb was diluted with an equal volume of Buffer C and applied, at a flow rate of 0.5 ml/min, to a column of phosphocellulose column (0.8 cm x 25 cm) previously equilibrated with Buffer C. Approximately 80% of the gene 5 protein activity passed through the column (see below). The pass-through fractions containing gene 5 activity were concentrated by dialyzing against Buffer D for 12 h to yield Fraction VI (6 ml).

**Hexyl-Sepharose Chromatography**—Fraction VI (5 ml) was applied, at a flow rate of 0.2 ml/min, to a column of hexyl-Sepharose (0.8 cm x 4 cm) previously equilibrated with Buffer C. The column was washed with 10 ml each of Buffer C and of Buffer C containing 0.2, 0.3, and 0.5 M NaCl. The flow rate was 0.5 ml/min and 2-ml fractions were collected; approximately 15% of gene 5 protein activity was found in the 0.2 M NaCl eluate and 65% in the 0.3 M NaCl eluate. Fractions of 0.3 M NaCl eluates containing gene 5 activity were pooled (8 ml) and dialyzed against Buffer D for 12 h to yield Fraction VII (3 ml).

**Modifications in the Purification Procedure**—The fractions of gene 5 protein activity obtained by elution from QAE-Sephadex are approximately 20% pure as measured by electrophoresis through polyacrylamide gels containing sodium dodecyl sulfate (Fig. 1A). If gene 5 protein of this purity is satisfactory, then these fractions can be concentrated by precipitation with (NH_4)_2SO_4 (0.43 g/ml) and the precipitate collected by centrifugation at 35,000 rpm in a Spinco 45 Ti rotor. However, if the purification is to be continued by passing through phosphocellulose, then it is essential to concentrate the QAE-Sephadex fractions by adsorption to, and elution from, the small phosphocellulose column. Otherwise, maximal purification is not obtained with the passage through phosphocellulose. The explanation for this result is unknown since concentration of the gene 5 protein activity by adsorption to phosphocellulose does not appear to affect its purity significantly (Fig. 1B).

During some purifications of the gene 5 protein, only 40 to 50% of the gene 5 protein activity passes through the phosphocellulose column (Step VI). The remainder of the gene 5 protein activity can be eluted from the column with Buffer C containing 1 M NaCl. After dialysis against Buffer C, the residual activity does not adsorb to phosphocellulose and can be recovered in the pass-through and pooled with the first fraction of gene 5 activity that was passed through phosphocellulose.

Fraction VI is approximately 60% pure (Fig. 1C) and has been useful in experiments where homogeneous gene 5 protein is not required.

**Properties of the Gene 5 Protein**

**Purity and Stability**—The most purified fraction, Fraction VII, represents a 7400-fold purification over the starting ex-
tract and contains 9% of the activity initially present (Table I). Fraction VII (30 μg of protein/ml) has been stored at -18°C for up to 6 months in Buffer D without any appreciable loss of activity (<10%).

Fraction VII of the gene 5 protein was denatured, reduced, and then analyzed by electrophoresis on a polyacrylamide gel containing sodium dodecyl sulfate. After staining with Coomassie blue, a single band was observed (Fig. 1D) whose molecular weight (see below) corresponds to that previously determined for the gene 5 protein. We estimate Fraction VII of gene 5 protein to be greater than 90% pure.

Molecular Weight—The molecular weight of the denatured gene 5 protein was determined by electrophoresis through 7.5% polyacrylamide gels containing sodium dodecyl sulfate. Comparison of its mobility with the mobilities of several proteins of known molecular weight (35) yields an apparent molecular weight for the denatured and reduced gene 5 protein of 87,000 ± 3,000 (Fig. 2A). This value is in good agreement with those previously determined for the denatured gene 5 protein (3, 4, 8).

The molecular weight of the native gene 5 protein has been determined by electrophoresis of the purified protein through nondenaturing polyacrylamide gels by the procedure of Hedrick and Smith (29). As shown in Fig. 2B, the homogeneous protein migrated to two positions corresponding to molecular weights of 87,000 (80% of the protein) and 170,000 (20% of the protein) as determined by comparison with proteins of known molecular weight. From the molecular weight of the two protein species, it appears that the majority of the gene 5 protein exists as a monomer under these conditions while approximately 20% is present as a dimer.

Sedimentation Properties and Isoelectric Point—The sedimentation coefficient (s_{20, w}) of gene 5 protein was determined by sedimenting the purified protein through a 5 to 20% glycerol gradient. The sedimentation coefficient of the gene 5 protein was calculated to be 5.3 S using catalase and hemoglobin as molecular weight standards (33). The isoelectric point (pI) of the gene 5 protein was pH 5.9 ± 0.1.

Reconstitution of T7 DNA Polymerase Activity

T7 DNA polymerase is composed of two subunits, the gene 5 protein, whose purification and properties are described in this paper, and the host specified thioredoxin (3). The purified gene 5 protein (Fraction VII) alone has only 1 to 2% of the DNA polymerase activity of homogeneous T7 DNA polymerase. However, as shown in Fig. 3, the inclusion of thioredoxin in the DNA polymerase reaction mixture results in a marked stimulation of DNA polymerase activity upon addition of the gene 5 protein. The sigmoidal curve obtained at relatively low concentration of gene 5 protein (Fig. 3) may reflect a cooperative reaction in the association of the two subunits. The most efficient reconstitution (nucleotides polymerized/μg of gene 5 protein added) is obtained when the molar ratio of thioredoxin/gene 5 protein is in the range of 50 to 200. Under these conditions the rate of DNA synthesis is 80% of that observed for an equal amount of homogeneous T7 DNA polymerase. If the gene 5 protein is first incubated with thioredoxin for 30 s at 37°C or for 60 min at 0°C in Buffer C, the reconstituted enzyme has 95% of the polymerase activity found in homogeneous T7 DNA polymerase.

The dependence of polymerase activity on the presence of both subunits is also shown in the experiment described in Fig. 4 in which thioredoxin is added to reaction mixtures containing a fixed amount of gene 5 protein. The apparent K_m for thioredoxin, calculated from the Lineweaver-Burk plot shown in the inset to Fig. 4, is 2.8 × 10^{-8} M.

Properties of the Reconstituted DNA Polymerase

Requirements for Activity—Maximal activity depends, in addition to gene 5 protein and thioredoxin, on the presence of all four deoxyribonucleoside 5′-triphosphates, Mg^{2+}, DNA, and 2-mercaptoethanol (Table II). A small but significant incorporation of [3H]dTMP into acid-insoluble material occurs in the absence of other dNTPs and probably represents limited addition to the ends of DNA chains (36). There is no detectable activity in the absence of DNA or Mg^{2+}. When 2-mercaptoethanol, present in all steps of the purification procedure, is omitted from the incubation mixture and the dilution buffer, the reaction proceeds at only half the optimal rate. N-Ethylmaleimide, in the absence of 2-mercaptoethanol results in 98% inhibition. Thus, the reconstituted DNA polymerase is indistinguishable from T7 DNA polymerase (Table II).

**Fig. 2. Determination of the molecular weight of denatured and native gene 5 protein.** A, gene 5 protein (Fraction VII) was denatured and reduced and subjected to electrophoretic analysis in the presence of sodium dodecyl sulfate (SDS) using gels of 7.5% polyacrylamide containing 1% 2-mercaptoethanol as described under “Experimental Procedures.” BSA, ovalbumin, bovine serum albumin (BSA), and E. coli RNA polymerase subunits (α, β, β′) were used as molecular weight standards. Mobilities are relative to the tracking dye, bromphenol blue. B, native gene 5 protein (Fraction VII) was subjected to electrophoretic analysis on 6, 7, 8, and 9% polyacrylamide disc gels according to the procedure of Hedrick and Smith (29). Bovine serum albumin (BSA) was electrophoresed through 5, 6, 7, 8, and 10% polyacrylamide gels. Staining of the gels was carried out as described under “Experimental Procedures.”
II) with regard to its requirements for polymerase activity.

**Effect of pH, Temperature, and Ionic Strength**—The optimal pH range for the reconstituted enzyme is 7.7 to 8.1 with potassium phosphate buffer and 8.0 to 8.4 with Tris/maleate buffer. The enzyme is 50% less active in Tris/maleate buffer than in potassium phosphate buffer at the optimal pH for each buffer. At pH 7.0 and 8.5 in potassium phosphate buffer, the activity was 60 and 85%, respectively, of optimal.

The optimal temperature of DNA synthesis was obtained at 34°C. The activity was reduced to 50% at 25 and 41°C. In the presence of 0.1 and 0.2 M KCl, the rate of DNA synthesis decreased to 60 and 10%, respectively, of the optimal rate in 0.05 M potassium phosphate buffer. Identical results were obtained for T7 DNA polymerase.

![Graph showing the requirement for gene 5 protein for DNA polymerase activity.](image1)

**Fig. 3.** Requirement for gene 5 protein for DNA polymerase activity. DNA polymerase activity was measured in the standard assay described under “Experimental Procedures.” The indicated amounts of gene 5 protein (Fraction VII) were added to reaction mixtures with (●) or without (○) 0.4 µg of homogeneous thioredoxin.

![Graph showing the requirement for thioredoxin for DNA polymerase activity.](image2)

**Fig. 4.** Requirement for thioredoxin for DNA polymerase activity. DNA polymerase activity was measured in the standard assay described under “Experimental Procedures.” The indicated amounts of thioredoxin were added to reaction mixtures containing 0.8 unit of gene 5 protein (Fraction VI). The inset is a Lineweaver-Burk plot of the data.

| Reaction mixture                  | Relative activity Gene 5 protein | Relative activity T7 DNA polymerase |
|----------------------------------|----------------------------------|------------------------------------|
| Complete                         | 100                              | 100                                |
| - Thioredoxin                    | 2                                | 1                                  |
| - Gene 5 protein                 | <1                               | <1                                 |
| - dATP, dCTP, dGTP               | 2                                | 5                                  |
| Mg²⁺                             | <1                               | <1                                 |
| - DNA                            | <1                               | <1                                 |
| - 2-Mercaptoethanol              | 54                               | 50                                 |
| - 2-Mercaptoethanol              | 2                                | 1                                  |
| + N-ethylmaleimide               |                                  |                                    |
| (10 mM)                          |                                  |                                    |

**Table II**

**Requirements for DNA synthesis**

DNA polymerase activity was measured in the standard assay described under “Experimental Procedures” using 0.8 unit of Fraction VI of gene 5 protein or 1.0 unit of T7 DNA polymerase. In the complete system 3.4 and 4.2 nmol of [3H]dTTP were incorporated into an acid-insoluble product with gene 5 protein and T7 DNA polymerase, respectively.

| Deoxyribonucleotides incorporated | DNA polymerase pmol | Gene 5 protein + thioredoxin pmol |
|-----------------------------------|---------------------|----------------------------------|
| -                                 | 12                  | 8                                |
| +                                 | 718                 | 400                              |

**Table III**

**Stimulation of reconstituted T7 DNA polymerase on a duplex DNA template by gene 4 protein**

The reaction mixture (0.1 ml) contained: 20 mM Tris (pH 7.5); 10 mM 2-mercaptoethanol; 20 mM MgCl₂; 0.3 mM concentrations of ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, and [3H]dTTP; and 6 nmol of T7 DNA. Gene 4 protein (0.8 unit), T7 DNA polymerase (0.8 unit), gene 5 protein (0.8 unit), and thioredoxin (0.8 µg) were present as indicated. After incubation for 20 min at 30°C, the amount of acid-insoluble radioactivity was measured as described under “Experimental Procedures.”

![Graph showing the stimulation of reconstituted T7 DNA polymerase on duplex DNA templates by gene 4 protein.](image3)

**Fig. 5.** Stimulation of reconstituted T7 DNA polymerase by gene 4 protein. The reaction mixture (0.1 ml) contained: 20 mM Tris (pH 7.5); 10 mM 2-mercaptoethanol; 20 mM MgCl₂; 0.3 mM concentrations of ATP, CTP, GTP, UTP, dATP, dCTP, dGTP, and [3H]dTTP; and 6 nmol of T7 DNA. Gene 4 protein (0.8 unit), gene 5 protein (0.8 unit), and thioredoxin (0.8 µg) were present as indicated. After incubation for 20 min at 30°C, the amount of acid-insoluble radioactivity was measured as described under “Experimental Procedures.”

**DISCUSSION**

The ability to complement gene 5 protein of phage T7 with thioredoxin to restore T7 DNA polymerase activity has made possible the purification of the gene 5 protein. Extracts of E. coli...
coli 7004 trx do not contain any detectable thioredoxin either by assay with ribonucleotide reductase or by radioimmune assay (37). Therefore, extracts of E. coli 7004 trx infected with phage T7 contain gene 5 protein but not an active T7 DNA polymerase since the functional T7 DNA polymerase consists of two subunits, gene 5 protein and E. coli thioredoxin (3, 7).

The native gene 5 protein we have purified is a single poly-peptide chain with a molecular weight of 87,000 ± 3,000, a value that is in agreement with that of 81,000 found by Studier (8) during analysis of proteins synthesized after T7 infection. A major difficulty in the purification of the gene 5 protein was its contamination through the QAE-Sephadex step with four proteins having molecular weights of 140,000, 120,000, 100,000, and 80,000. Attempts to separate the gene 5 protein from these proteins by chromatography on hydroxyapatite, DNA-cellulose, DEAE-cellulose, QAE-Sephadex, and Bio-Rex 70 were all unsuccessful. However, chromatography of the gene 5 protein on phosphocellulose alters its chromatographic properties such that removal of these contaminating proteins is possible. If fractions containing gene 5 protein are applied to phosphocellulose, the major portion of gene 5 activity is adsorbed to the resin and can then be eluted with higher salt concentrations. When the gene 5 protein is now applied to a second phosphocellulose fraction, it does not adsorb to the resin and can be recovered in the pass-through fraction. The contaminating proteins remain adsorbed to the column (Fig. 1). The explanation for this interesting behavior of the gene 5 protein is not known but probably reflects the removal of some protein or factor from the gene 5 protein during the first chromatography on phosphocellulose.

All preparations of the gene 5 protein catalyze the polymerization of nucleotides albeit at a rate of only 1 to 2% of T7 DNA polymerase. We have not been able to determine if this low activity is an inherent property of the gene 5 protein or reflects a minor contamination with thioredoxin or an altered thioredoxin. It is clear, however, that both thioredoxin and gene 5 protein are required to obtain a polymerase whose specific activity approaches that of T7 DNA polymerase normally synthesized in vivo. The addition of thioredoxin to preparations of gene 5 protein lead to a reconstituted enzyme that has 80% of the activity of T7 DNA polymerase. The most efficient reconstitution occurs at a molar ratio of thioredoxin/gene 5 protein in the range of 50 to 200. From our results we calculate that approximately 150 molecules of gene 5 protein are present in a phage-infected cell and it has been previously shown that an E. coli cell contains 3000 to 5000 molecules of thioredoxin (6). Since the apparent $K_m$ for thioredoxin in the in vitro reconstitution reaction is quite low, 2.8 × 10⁻⁸ M, the in vitro synthesis of T7 DNA polymerase should readily occur as long as both subunits are present.

The properties of the reconstituted T7 DNA polymerase are indistinguishable from those of the T7 DNA polymerase purified from wild type E. coli infected with wild type T7 phage. The two enzymes have the same requirements for optimal activity and the purified gene 5 protein migrates together with the large subunit of T7 DNA polymerase through a sodium dodecyl sulfate-polyacrylamide gel (data not shown). Two stringent criteria for identity are the ability of the reconstituted enzyme to function in DNA replication in an in vitro replication system and to interact with the gene 4 protein of phage T7 to copy duplex DNA templates. We have previously shown that thioredoxin can restore replication to an in vitro system in which only gene 5 protein is present (6), and we have shown that the reconstituted enzyme and gene 4 protein can replicate duplex T7 DNA.

T7 DNA polymerase is the second example of an enzyme comprised of both a phage-coded subunit and a host-coded subunit. QB replicase is comprised of one subunit specified by the phage and three subunits derived from the host (38–41) and which have been implicated in the catalytic activity (42, 43). The studies reported here have not determined the specific roles of thioredoxin or gene 5 protein in the polymerization reaction. However, now that both subunits are available in homogeneous form, we hope to determine which of the two subunits has the catalytic site for polymerization and the exonuclease activity specific for duplex DNA described in the accompanying paper (19) as well as the binding sites for deoxyribonucleoside 5'-triphosphates, primer, and template.

REFERENCES

1. Grippo, P., and Richardson, C. C. (1971) J. Biol. Chem. 246, 6867–6873
2. Oey, J. L., Stratling, W., and Knippers, R. (1971) Eur. J. Biochem. 23, 497–504
3. Modrich, P., and Richardson, C. C. (1975) J. Biol. Chem. 250, 5515–5522
4. Scherzinger, E., and Seifert, D. (1975) Mol. & Gen. Genet. 141, 213–232
5. Adler, S. R., and Modrich, P. (1979) J. Biol. Chem. 254, 11605–11614
6. Modrich, P., and Richardson, C. C. (1975) J. Biol. Chem. 250, 5508–5514
7. Mark, D. F., and Richardson, C. C. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 780–784
8. Studier, F. W. (1972) Science 176, 367–376
9. Chamberlin, M. (1974) J. Virol. 14, 509–516
10. Mark, D. F., Chase, J. W., and Richardson, C. C. (1977) Mol. & Gen. Genet. 155, 145–153
11. Scherzinger, E., and Kotz, G. (1975) Mol. & Gen. Genet. 141, 233–249
12. Kolodner, R., Massamune, Y., LeClerc, J. L., and Richardson, C. C. (1978) J. Biol. Chem. 253, 566–573
13. Hinkle, D. C., and Richardson, C. C. (1975) J. Biol. Chem. 250, 10529–10532
14. Scherzinger, E., Lanks, E., Morelli, G., Seifert, D., and Yuki, A. (1977) Eur. J. Biochem. 72, 543–558
15. Kolodner, R., and Richardson, C. C. (1978) J. Biol. Chem. 253, 574–584
16. Kolodner, R., and Richardson, C. C. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1529–1535
17. Romano, L. J., and Richardson, C. C. (1979) J. Biol. Chem. 254, 10476–10482
18. Romano, L. J., and Richardson, C. C. (1979) J. Biol. Chem. 254, 10483–10489
19. Horii, K., Mark, D. F., and Richardson, C. C. (1979) J. Biol. Chem. 254, 11589–11594
20. Studier, F. W. (1969) Virology 39, 562–574
21. Studier, F. W. (1973) J. Mol. Biol. 79, 227–236
22. Simon, M. N., and Studier, F. W. (1973) J. Mol. Biol. 79, 249–265
23. Richardson, C. C. (1966) J. Mol. Biol. 18, 49–61
24. Weiner, J. H., Bertsch, L. L., and Kornberg, A. (1976) J. Biol. Chem. 250, 1972–1980
25. Reuben, R. C., and Gefter, M. L. (1974) J. Biol. Chem. 249, 3843–3850
26. Laurent, T. C., Moore, E. C., and Reichard, P. (1964) J. Biol. Chem. 239, 3436–3444
27. Shaltiel, S. (1974) Methods Enzymol. 34, 126–140
28. Ausubel, F. M., and Herrick, G. (1971) Methods Enzymol. 22, 198–217
29. Hendrick, J. I., and Smith, A. J. (1968) Arch. Biochem. Biophys. 126, 155–164
30. Laemmli, U. K. (1970) Nature 227, 680–685
31. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021
32. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
33. Martin, R. G., and Amoe, B. N. (1961) J. Biol. Chem. 236, 1372–1379
34. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
35. Weber, K., and Osborn, M. (1967) J. Biol. Chem. 244, 4406–4412
36. Adler, J., Lehman, R. I., Beeman, M. J., Simms, E. S., and Kornberg, A. (1958) Proc. Natl. Acad. Sci. U. S. A. 44, 641–647
37. Holmgren, A., Ohlsson, I., and Grankvist, M.-L. (1978) J. Biol. Chem. 253, 37–43

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38. Kamen, R. I. (1970) *Nature* **228**, 527-533
39. Kondo, M., Gallerani, R., and Weissmann, C. (1970) *Nature* **228**, 525-527
40. Blumenthal, T., Landers, T. A., and Weber, K. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 1313-1317
41. Wahba, A. J., Miller, M. J., Nivel, A., Landers, T. A., Carmichael, G. G., Weber, K., Hawley, D. A., and Soblin, L. I. (1974) *J. Biol. Chem.* **249**, 3314-3316
42. Hori, K., Harada, K., and Kuwano, M. (1974) *J. Mol. Biol.* **86**, 699-708
43. Landers, T. A., Blumenthal, T., and Weber, K. (1974) *J. Biol. Chem.* **249**, 5801-5808
Deoxyribonucleic acid polymerase of bacteriophage T7. Purification and properties of the phage-encoded subunit, the gene 5 protein.

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