Gene 4 Protein of Bacteriophage T7

PURIFICATION, PHYSICAL PROPERTIES, AND STIMULATION OF T7 DNA POLYMERASE DURING THE ELONGATION OF POLYNUCLEOTIDE CHAINS*

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With the use of an in vitro complementation assay to measure activity, the gene 4 protein of bacteriophage T7 has been purified 1000-fold to yield a nearly homogeneous protein. The purified gene 4 protein is a single polypeptide having a molecular weight of 58,000. In addition to being essential for T7 DNA replication in vitro and in vivo, the gene 4 protein is required for DNA synthesis by the purified T7 DNA polymerase on duplex T7 DNA templates. In the absence of ribonucleoside 5'-triphosphates, DNA synthesis by the gene 4 protein and the T7 DNA polymerase is dependent on phosphodiester bond interruptions containing 3'-hydroxyl groups (nicks) in the duplex DNA. The reaction is specific for the T7 DNA polymerase, but any duplex DNA containing nicks can serve as template. The Km for nicks in the reaction is 3 x 10^-10 M.

Genetic analysis of bacteriophage T7 (1-3) has shown that the product of gene 4 is the phage for the replication of the phage DNA. In cells infected with T7 phage containing an amber mutation in gene 4, only a small amount of phage DNA is synthesized. Studico and cell free systems (4, 5) have demonstrated that the product of gene 4 is also required for T7 DNA replication in vitro. This in vitro requirement for the gene 4 protein has been the basis of assays for the gene 4 protein that have led to its partial purification (6-9).

The purified gene 4 protein effects a marked stimulation of DNA synthesis by the T7 DNA polymerase on duplex DNA templates (7, 9, 10); T7 DNA polymerase alone is unable to use such templates (11, 12). Our early studies (7) indicated that some of the product was not attached to template DNA,

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implicating the gene 4 protein in the initiation of polynucleotide strands. Recent studies indicate that the stimulation of DNA synthesis by the gene 4 protein on duplex DNAs may be accounted for by two novel activities of the protein: an oligoribonucleotide-synthesizing activity (9); and a DNA-dependent nucleoside 5'-triphosphatase activity (13). The former activity is involved in initiation of polynucleotide chains by the T7 DNA polymerase, while the latter may facilitate unwinding of duplex DNA during elongation of polynucleotide chains.

In vivo studies on mutants of phage T7 suggested a role of the gene 4 protein in the initiation of DNA synthesis. Wolfson and Dressler (14) found that, after shifting a gene 4 temperature-sensitive mutant to the nonpermissive temperature, large single-stranded gaps were formed on one side of each growing fork, suggesting that the gene 4 protein functions in the initiation of DNA synthesis on the lagging parental strand. Consistent with this interpretation is Strätling and Knippers' observation (6) that the DNA synthesized in the absence of a functioning gene 4 protein hybridized to the H strand exclusively. In vitro, significant amounts of DNA synthesized on T7 DNA templates by T7 DNA polymerase and gene 4 protein are not covalently attached to the template molecules (7, 9). Scherzinger and Litfin (15) showed that extracts of T7-infected Escherichia coli require the gene 4 protein for conversion of single-stranded circular phage DNAs to circular duplexes. Recently, Scherzinger et al. (9) have demonstrated that the DNA synthesized on single-stranded circular DNA molecules by T7 DNA polymerase and gene 4 protein in the presence of DNA binding protein contains an oligoribonucleotide at its 5'-terminus, a reaction requiring rNTPs. Similar evidence for a role of the gene 4 protein in the synthesis of oligoribonucleotide primers on displaced single strands arising during synthesis on duplex DNA has been obtained using these purified proteins to replicate duplex T7 DNA.

In addition to playing a role in the initiation of DNA synthesis, the gene 4 protein is also involved in the elongation of the polynucleotide chain during DNA synthesis on duplex DNA. We have shown that homogeneous preparations of the gene 4 protein catalyze the hydrolysis of NTPs to NDPs and P in the presence of single-stranded DNA, but not in the presence of double-stranded DNA (13). More important, however, hydrolysis of NTPs accompanies, and is necessary for, synthesis on duplex T7 DNA catalyzed by the gene 4 protein.

† L. J. Roman0 and C. C. Richardson, unpublished results.
and T7 DNA polymerase (13). Thus, the energy of hydrolysis of NTPs catalyzed by the gene 4 protein may facilitate the unwinding of the DNA during synthesis.

Although the multiple activities of the gene 4 protein in T7 DNA replication make it an extremely interesting and novel enzyme, they also present experimental difficulties. First, it is important to show that the multiple activities in preparations of gene 4 protein reside in the gene 4 protein. Consequently, in this paper we describe its purification to near homogeneity along with some of its physical properties. Second, it is advantageous to define conditions that make possible the characterization of each of the multiple activities of the gene 4 protein. Thus, we show here that, in the absence of rNTPs, DNA synthesis on duplex DNA requires the presence of nicks in the template DNA. In the accompanying paper (16), we present an extensive characterization of the product synthesized by T7 DNA polymerase and gene 4 protein and the mechanism by which it arises under conditions where the gene 4 protein does not synthesize oligoribonucleotide primers.

**EXPERIMENTAL PROCEDURES**

**Materials**

- **Bacterial Strains and Bacteriophages**
  - Escherichia coli D110 su' thy end polA1 has been described previously (17).
  - T7 wild type phage.
  - T7 amber mutants.
  - T7 deletion mutants were obtained from Dr. F. W. Studier (Brookhaven National Laboratory).

- **DNA**
  - Bacteriophage T7 DNA was prepared as previously described (20).
  - Bacteriophage PM2 DNA was prepared as described by Richardson (21)

- **Nucleotides**
  - Unlabeled nucleotides were purchased from P-L Biochemicals.

- **Enzymes**
  - DNA polymerase was the DNA-cellulose fraction of Modrich and Richardson (29).
  - Bacteriophage T4 DNA polymerase was the hydroxylapatite fraction purified by the method of Goulion et al. (23).
  - E. coli DNA polymerase I was purified by the method of Jovin et al. (24).
  - E. coli DNA polymerase III was Fraction VIII of Livingston et al. (25).
  - E. coli DNA polymerase II was a gift from Dr. D. S. Gefer (Massachusetts Institute of Technology).
  - Bacteriophage T4 polynucleotide ligase was Fraction VII of Weiss et al. (26).
  - Standard vials of pancreatic DNase were obtained from Worthington.
  - Crystalline bovine serum albumin and ovalbumin were from Pentex.
  - Lysozyme (code LYSF), β-galactosidase (code BGC), trypsin (code TRL), and alcohol dehydrogenase (code ADHII) were from Worthington.
  - Bovine hemoglobin, bovine catalase, and equine ferritin were from Sigma.

- **Other Materials**
  - DEAE-Sephadex A-25 and Sephadex G-150 and G-200 were from Pharmacia.
  - Whatman DEAE-cellulose (DE52) was equilibrated according to the manufacturer, and Whatman phosphocellulose (P-11) was preceluated as previously described (27).
  - Ultrapure ammonium sulfate and sucrose were from Schwarz/Mann.
  - Streptomycin sulfate from Merck, Sharp, and Dohme.
  - Ammonium persulfate and N,N,N',N'-tetramethyl ethylenediamine were from Canalco.
  - Sodium dodecyl sulfate, acrylamide, N,N'-methylene bisacrylamide, and Dowex AG50W-X8 were from Bio-Rad.

**Methods**

**Construction of T73,5,6,a::w**

T73,5,6,a::w was crossed with T7 LG12 as described by Studier (3). Except that KCN was omitted, the plates were incubated at 30°C and the colonies were picked, grown on E. coli 011B1 and the resulting plaques were tested for the presence of amber mutations in genes 3, 5, and 6 by complementation against phages containing individual amber mutations (3). Approximately 10% of the plaques contained T73,5,6,a::w.

**Purification of NTP**

The dNTPs were treated with NaI to destroy rNTPs and were then chromatographed on DEAE-Sephadex A25 as described by Wu (28).

**Enzyme Assays**

**Complementation Assay for Gene 4 Protein** — The standard complementation assay for gene 4 protein measures the stimulation of DNA synthesis in an extract prepared from cells infected with T7 carrying an amber mutation in gene 4 and was carried out as previously described (7).

**Stimulation of T7 DNA Polymerase on Duplex DNA** — The gene 4 protein is required for DNA synthesis by the T7 DNA polymerase on duplex DNA (7).

**N2 DNA Containing One Nick per Molecule**

A nick refers to a single phosphodiester bond interruption in one of the two strands of a duplex DNA molecule. A nick can be created by the limited action of an endonuclease such as pancreatic DNase.
Polyacrylamide Gel Electrophoresis

Electrophoresis in the presence of 0.1% sodium dodecyl sulfate was performed according to Weber and Osborn (37). Electrophoretic analysis under native conditions was performed in the Tris system of Jovin et al. (38). Gels were stained with Coomassie brilliant blue (39), and the protein was quantitated by scanning with a Joyce Loebl recording microdensitometer.

Other Methods

Protein was determined by the method of Lowry (40) using bovine serum albumin as a standard. All pH measurements were made at room temperature at a buffer concentration of 0.05 M.

Purification of T7 Gene 4 Protein

An earlier procedure for the purification of the gene 4 protein from T7,5,,di,-infected Escherichia coli resulted in a preparation that was approximately 25% pure as judged by polyacrylamide gel analysis (7). A major contaminant was the T7 DNA ligase that represented over 50% of the protein in the most highly purified fraction. For this reason we constructed the T7,5,,di,- phage carrying a deletion of gene 1.3, the ligase gene (see "Experimental Procedures"). The use of this phage for the preparation of the infected cells eliminates T7 DNA polymerase and DNA ligase activities during purification.

The following procedure, a modification of that described by Hinkle and Richardson (7), has been used to purify the T7 gene 4 protein from T7,5,,di,-infected E. coli D110. A summary of a typical purification from 400 g of infected cells is presented in Table I. Unless indicated otherwise, all steps were performed at 4°C, and centrifugation was at 8000 rpm for 30 min in a Sorvall GS3 rotor.

Growth of Phage-infected Cells—E. coli D110 was grown and infected with T7,5,,di,- as previously described (7). The cell paste was suspended in 0.05 M Tris (pH 7.5)/10% sucrose at a final volume of 4 ml/g of cells. Aliquots of the suspended cells (67 ml) were distributed into Spinco 45-Ti rotor tubes, frozen in liquid nitrogen, and stored at −80°C.

Preparation of Cell Extract—Twenty-four tubes of suspended frozen cells (67 ml/tube) from 400 g of cells were thawed overnight on ice, and 1.5 ml each of 5 M NaCl and 10 mg/ml of lysozyme, 50 mM Tris (pH 7.5), 10% sucrose were added to each tube. After 45 min at 0°C, the tubes were transferred to a 37°C water bath, heated to 20°C with stirring, and then chilled to 5°C in an ice bath with stirring. The lysates were then centrifuged at 40,000 rpm for 45 min in a Spinco 45-Ti rotor. The supernatant fluids were decanted and saved; the loose upper layer of the pellets was removed, added to 200 ml of 10% sucrose, 100 mM NaCl, 50 mM Tris (pH 7.5), and the mixture was centrifuged at 10,000 rpm for 30 min in a Sorvall GSA rotor. The supernatant fluids were pooled and adjusted to a final volume of 1508 ml (Fraction I).

Streptomycin and Ammonium Sulfate Fractionation—To 1508 ml of Fraction I were added 151 ml of a freshly prepared solution of 30% (w/v) streptomycin sulfate over a 30-min period with stirring. After stirring for an additional 30 min, the precipitate was removed by centrifugation (Fraction II). Ammonium sulfate (499 g) was then added to the supernatant fluid (1595 ml) with stirring over a 30-min period. After

| Fraction | Step | Units | Milligrams of protein | Units/mg | Recovery % |
|----------|------|-------|----------------------|----------|------------|
| I        | Extract | 256,400 | 26,500 | 9.7 | 100 |
| II       | Streptomycin sulfate | 299,900 | 21,500 | 14 | 117 |
| III      | Ammonium sulfate | 248,000 | 8,000 | 30.9 | 97 |
| IV       | DEAE-cellulose | 82,800 | 347 | 239 | 32 |
| V        | Phosphocellulose | 20,700 | 8.1 | 2,440 | 8.1 |
| VI       | Sephadex G-150 concentrate | 11,250 | 1.13 | 9,900 | 4.4a |

* Only one-fifth of Fraction V was subjected to gel filtration through Sephadex G-150. The 4.4% overall yield shown is corrected for this fact.

DDE-Cellulose Chromatography—A column of Whatman DE52 DEAE-cellulose (62 cm2 × 33 cm) was prepared and washed with 12 liters of Buffer A containing 0.1 M NaCl. The concentration of (NH4)2SO4 in Fraction III was determined by measuring its conductivity. The fraction was diluted to 4 liters with Buffer A to reduce the (NH4)2SO4 concentration to 30 mM and then applied to the column at a rate of 900 ml/h. The column was washed with 2 liters of Buffer A containing 0.1 M NaCl, and the proteins were eluted with 16 liters of a linear gradient from 0.1 to 0.4 M NaCl in Buffer A applied at 900 ml/h. Gene 4 complementing activity eluted at approximately 0.24 M NaCl. Fractions having a specific activity of 80 units/mg or greater were pooled (1850 ml), and the protein was precipitated with (NH4)2SO4 (350 g/liter). The precipitate was collected by centrifugation, dissolved in 40 ml of 20 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol (Buffer A) (Fraction III).

Phosphocellulose Chromatography—A column of Whatman P11 phosphocellulose (4.9 cm2 × 30 cm) was prepared and washed with 2 liters of 20 mM potassium phosphate buffer (pH 6.5), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, and dialyzed immediately against 2 liters of the same buffer for 12 h (Fraction IV).

Sephadex G-150—A column of Sephadex G-150 (4.9 cm2 × 100 cm) was prepared and washed with 1 liter of 20 mM Tris (pH 7.5), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM NaCl, 20% (v/v) glycerol at a hydrostatic pressure of 20 cm. Five milliliters of Fraction V were diluted with 5 ml of 20 mM Tris (pH 7.5), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM NaCl, layered on the column, and eluted with 20 mM Tris
This activity has been discussed in detail elsewhere (13). The gene 4 protein alters the surface tension of solutions. A drop causing a decrease in fraction size if fractions are collected by drop counting. A similar effect also occurs in solutions of gene 4 protein containing 0.5 mg/ml of bovine serum albumin. Apparently the gene 4 protein exists as a single-stranded DNA-dependent nucleoside triphosphatase. Incubation of 2 µg of gene 4 protein with native T7 DNA, heat-denatured T7 DNA, or φX viral DNA under conditions of the T7 DNA polymerase stimulation assay (minus T7 DNA polymerase) resulted in no detectable incorporation (<2 pmol) of [3H]dTTP into acid-insoluble material. Similarly, incubation of 1 µg of gene 4 protein in the standard T7 DNA polymerase assay (11), in which denatured salmon sperm DNA is the template, resulted in no detectable incorporation (<2 pmol) of [3H]dTTP into acid-insoluble material.

**RNA Polymerase Activity**—Although the gene 4 protein, under appropriate conditions, can itself catalyze the polymerization of rNTPs to yield short oligoribonucleotides (9), the gene 4 preparation is not contaminated with either the E. coli or T7 RNA polymerase.

Incubation of 2 µg of gene 4 protein with native T7 DNA, heat-denatured T7 DNA, or φX viral DNA under conditions of the T7 DNA polymerase stimulation assay (minus T7 DNA polymerase) resulted in no detectable incorporation (<4 pmol) of [3H]UTP or [3H]ATP, UTP, CTP, and GTP into acid-insoluble product.

**Nuclease Activities**—The purified gene 4 protein (Fraction VI) contains no detectable exonuclease or endonuclease activities. Incubation of 1 µg of gene 4 protein with 6 nmol of native or denatured T7 [3H]DNA for 20 min at 30° under the conditions of the T7 DNA polymerase stimulation assay (minus T7 DNA polymerase) produced less than 2 pmol of acid-soluble nucleotides. Analysis of the treated DNA by band sedimentation through alkaline sucrose gradients showed no detectable breakdown (<10%) of the DNA.

**DNA Ligase**—In contrast to the earlier purification of gene 4 protein from T7,λ-infected E. coli in which more than 50% of the protein was the 40,000-dalton T7 DNA ligase (7), Fraction VI, purified from T7,λ-infected cells, has no detectable 40,000-dalton protein contaminant (Fig. 2); neither is there any detectable activity (<0.002 unit/mg) (31).

**Molecular Weight**

The mobility of the gene 4 protein relative to bromphenol blue on 5% polyacrylamide gels containing sodium dodecyl sulfate (Fig. 2a) shows a molecular weight of 53,000 ± 2,000 that is in good agreement with that of 58,000 ± 5,000 that was determined for the denatured and reduced gene 4 polypeptide chain and indicates that the gene 4 protein exists as a monomer.
**Bacteriophage T7 Gene 4 Protein**

**FIG. 2.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the gene 4 protein. 
(a) Gene 4 protein (Fraction VI, 5 µg) was denatured and reduced as described under "Experimental Procedures" and subjected to electrophoretic analysis in the presence of sodium dodecyl sulfate on gels of 5% polyacrylamide. After staining with Coomassie blue, the gels were scanned on a recording microdensitometer. Similar patterns were obtained when 10 and 15 µg of gene 4 protein were analyzed. 
(b) The mobility of the gene 4 protein compared to the mobilities of proteins of known molecular weight. Mobilities are relative to the tracking dye, bromphenol blue. BSA, bovine serum albumin.

**FIG. 3.** Determination of the Stokes radius and sedimentation coefficient for the gene 4 protein. 
(a) The Stokes radius was determined by gel filtration of the gene 4 protein (Fraction VI) on a column (0.43 cm² × 50 cm) of Sephadex G-200. The column was run in 0.09 M Tris (pH 7.5), 10 mM 2-mercaptoethanol, 10 mM NaCl, 30% (w/v) glycerol with a hydrostatic head of 4 cm. Three-drop fractions were collected. 
(b) The sedimentation coefficient of the gene 4 protein (Fraction VI) was determined using a 10 to 30% (w/v) linear glycerol gradient containing 0.02 M Tris (pH 7.5), 10 mM 2-mercaptoethanol, 10 mM NaCl. Centrifugation was for 27 h at 40,000 rpm at 2°C in a Spinco SW 41 type rotor. Four-drop fractions were collected. The gene 4 protein activity was measured in the complementation assay and the T7 DNA polymerase stimulating assay (see "Experimental Procedures"). BSA, bovine serum albumin; ADH, alcohol dehydrogenase.

**Stimulation of T7 DNA Polymerase on Duplex Templates**

**Gene 4 Protein and T7 DNA Polymerase Together Can Use Duplex DNA Templates**—Purified T7 DNA polymerase alone has little or no activity on duplex DNA templates such as T7 DNA (7, 11, 12). However, earlier studies with a partially purified gene 4 protein provided strong evidence that the gene 4 protein is essential for DNA synthesis catalyzed by T7 DNA polymerase on duplex DNA templates (7), a property also reported by Scherzinger and Klotz (10). As shown in Fig. 4, the addition of purified gene 4 protein (Fraction VI) to a reaction mixture containing T7 DNA polymerase and native T7 DNA effects a marked stimulation of DNA synthesis. In the experiment shown in Fig. 4, maximum stimulation (greater than 50-fold) occurs at a ratio of 10 gene 4 protein molecules per polymerase molecule. Increasing the amount of T7 DNA polymerase reduces the relative amount of gene 4 protein required for maximum activity (data not shown).

**Complementation Activity and T7 DNA Polymerase-stimulating Activity Reside in Gene 4 Protein Molecule**—As previously reported (7), the complementing activity and the T7 DNA polymerase stimulating activity purify together during the last stages of the purification described in Table I. During the determination of the sedimentation coefficient of the gene 4 protein described in Fig. 3b, the ratio of the two activities in the protein peak of the sucrose gradient was constant. Finally, as shown in Fig. 1, the two activities migrate together during electrophoresis through polyacrylamide gels under nondenaturing conditions. The ratio of the complementing activity to T7 DNA polymerase-stimulating activity is identical with the ratio of these two activities in Fraction VI.

**Synthesis on Duplex DNA is Specific for T7 DNA Polymerase**—As previously reported (7) for the less pure gene 4 protein, Fraction VI described here will stimulate only T7...
DNA polymerase activity on duplex DNA; gene 4 protein has no effect (<5%) on T4 DNA polymerase or E. coli DNA polymerases I, II, or III.

**Specificity for Duplex DNA** — Whereas duplex T7 DNA, isolated from the phage, is a good template for T7 DNA polymerase and gene 4 protein, covalently closed circular duplex PM2 DNA is not (Table II). However, PM2 containing a nick, while not an effective template for the T7 DNA polymerase alone, supports synthesis at a rapid rate in the presence of T7 DNA polymerase and gene 4 protein. DNA synthesis on denatured T7 DNA is not dependent on gene 4 protein nor is there any significant stimulation upon the addition of the gene 4 protein (Table II). Although the T7 DNA polymerase alone is more active on linear single-stranded T7 DNA than on duplex DNA, the rate of DNA synthesis, even with gene 4 protein present, is at most only 20% of that catalyzed by the two proteins together on duplex T7 DNA (Table II). The circular single-stranded DNA of φX174, lacking ends, does not support synthesis by T7 DNA polymerase either in the presence or absence of gene 4 protein.

**Requirement for Single Strand Interruptions in Duplex Templates**

The results presented in the preceding section show that the T7 DNA polymerase alone can synthesize DNA using linear single-stranded templates, but not circular single-stranded templates or any duplex template. Presumably, the synthesis observed on single-stranded linear DNA reflects the ability of the T7 DNA polymerase to use the 3'-terminus of the polynucleotide strand as a primer and the remainder of the DNA as a template (43). The ability of the T7 DNA polymerase, in conjunction with the gene 4 protein, to use PM2 DNA containing nicks as a template suggests that, like the reaction catalyzed by E. coli DNA polymerase I (43), DNA synthesis by these two proteins requires nicks in the DNA. In this section, we show that DNA synthesis, not only on PM2 DNA but also on duplex T7 DNA, requires nicks in the DNA.

**Inhibition of DNA Synthesis on Duplex T7 DNA by Repair of Template** — In order to determine whether nicks displaying 3'-hydroxyl and 5'-phosphoryl groups in the duplex T7 DNA were contributing to the template activity, we incubated the T7 template DNA with T7 polynucleotide ligase prior to the addition of T7 DNA polymerase and gene 4 protein (Fig. 5). This treatment inhibited the rate of synthesis by 60%.

In control experiments, heat inactivation of the ligase prior to the addition of T7 DNA polymerase and gene 4 protein did not alter the results shown in Fig. 5. When ligase was added

**Table II**

| DNA synthesis | Template | –Gene 4 protein| +Gene 4 protein |
|---------------|----------|----------------|----------------|
| pmol dTMP incorporated | Native T7 DNA | 2 | 90 |
| | Closed, circular PM2 DNA | 2 | 3 |
| | PM2 DNA with nicks | 2 | 130 |
| | Denatured T7 DNA | 8 | 12 |
| | φX174 DNA | 2 | 2 |

**Fig. 4.** Stimulation of T7 DNA polymerase by gene 4 protein. Reactions were carried out as described under "Experimental Procedures."
Burk plot. The $K_{\text{m}}$ for nicking is $3 \times 10^{-9}$ M, with nicking and is shown in Fig. 6b in the form of a Lineweaver-Burk plot increased with increasing amounts of template DNA mixture containing saturating amounts of gene 4 protein.

In the presence of nicking DNA molecules containing one nick per molecule were mixed with covalently closed circular PM2 DNA molecules with various amounts of PM2 DNA molecules that contained one nick per molecule. 6. Lineweaver-Burk plot of the above data. $\circ - \circ$, 0.1 unit of T7 DNA polymerase and 1.3 units of gene 4 protein per reaction; $\bullet - \bullet$, 0.3 unit of T7 DNA polymerase and 3.9 units of gene 4 protein per reaction. Molecular weights of 6, 10 for PM2 DNA (3D) and 31 for an average nucleotide were used to calculate the molarity of single strand breaks in a given reaction mixture.

3 min or more after the addition of T7 DNA polymerase and gene 4 protein, no inhibition was observed.

Introduction of Nicks into Duplex DNA — Covalently closed circular PM2 DNA will not serve as a template for T7 DNA polymerase and gene 4 protein (<1%), while PM2 DNA containing nicks is an efficient template (Table II). PM2 DNA molecules containing one nick per molecule were mixed with covalently closed PM2 molecules in various proportions and then used as template DNA in reactions with gene 4 protein and T7 DNA polymerase (Fig. 6a). The rate of DNA synthesis increased with increasing amounts of template DNA with nicks and is shown in Fig. 6b in the form of a Lineweaver-Burk plot. The $K_{\text{m}}$ for nicks is $3 \times 10^{-9}$ M. Assuming that the T7 DNA polymerase is 85 to 90% pure (22) and that all of the DNA polymerase molecules are active, 560 nucleotides are polymerized per min per polymerase molecule in a reaction mixture containing saturating amounts of gene 4 protein.

**DISCUSSION**

The development of in vitro systems for the replication of bacteriophage T7 DNA has made possible the purification of the gene 4 protein since T7 DNA replication in these systems is dependent on the presence of the gene 4 protein as is replication *in vivo*. We, as well as others, have used such complementation assays to purify the gene 4 protein and to study some of its properties (6-9, 13). Although the gene 4 protein can be purified from wild type *Escherichia coli* infected with wild type T7 phage, we have encountered several problems using this approach.

First, if T7 DNA polymerase or *E. coli* DNA polymerase 1 is present in gene 4 fractions, there is some stimulation of DNA synthesis in the complementation assay due to the addition of DNA polymerase alone. To circumvent this problem, an *E. coli* host carrying a *polA* mutation and a T7 phage carrying an amber mutation in gene 5 were used to prepare the phage-infected cells in order to eliminate *E. coli* DNA polymerase I and T7 DNA polymerase activity from the extract.

Second, on several occasions when T7 DNA polymerase was present during purification of the gene 4 protein, we have observed a peak of activity during chromatography that contains both gene 4 protein and T7 DNA polymerase. In fact, the two purified proteins, under appropriate conditions, can be shown to form a complex that can be detected by gel filtration. Again, the removal of T7 DNA polymerase by a mutation solves this problem.

Third, a major component at all steps of our earlier purification procedure was the T7 DNA ligase. By deleting gene 1.3, the structural gene for the ligase, we have eliminated this protein and its activity. It seems likely that the association of T7 DNA polymerase and DNA ligase with the gene 4 protein during purification is not fortuitous, but further studies on the functional association between these proteins are best carried out with homogeneous proteins. The use of the T7, D10, D11, E. coli D110 polA1 endA and a modified purification procedure to prepare the gene 4 protein has enabled us to obtain a nearly homogeneous protein.

The native gene 4 protein we have purified is a single polypeptide chain with a molecular weight of 58,000. Analysis carried out by Studier (1) of proteins synthesized after T7 infection indicates that two proteins are altered by amber mutations in the gene 4. a major species with a molecular weight of 58,000 and a minor species with a molecular weight of 66,000. Thus, the gene 4 protein we have purified corresponds to the major species produced in a T7 infection. The gene 4 preparation described by Scherzinger et al. (9) contains both the 58,000- and 66,000-dalton components, and they discuss evidence that the two forms have related amino acid sequences. Thus, the 66,000-dalton protein could be a precursor of the 58,000-dalton protein, the latter arising by proteolysis. At present, it is not known whether the 66,000-dalton protein has any of the activities shown to be associated with the 58,000-dalton protein described here. It cannot be ruled out that the larger form of the gene 4 protein performs an as yet unknown role in T7 DNA replication.

The stimulation of T7 DNA polymerase by the gene 4 protein reported here occurs only with duplex DNA templates containing nicks. It should be emphasized here that the requirement for duplex DNA templates occurs only in the absence of rNTPs. In the presence of rNTPs, the gene 4 protein can catalyze the synthesis of oligoribonucleotides on single-stranded linear or circular DNA molecules (9). Hence, the gene 4 protein can also stimulate synthesis by T7 DNA polymerase on single-stranded DNA by synthesizing oligoribonucleotide primers.
In order to observe stimulation of synthesis on duplex DNA, the DNA must contain nicks. The $K_m$ for nicks in a reaction using PM2 DNA molecules with a defined number of nicks is $3 \times 10^{-10}$ M. Although only 1 out of 10 T7 DNA molecules used in these studies contains single strand interruptions, the concentration of $7 \times 10^{-10}$ M single strand interruptions in the reaction mixture is sufficient for the observed rate of synthesis. Such a requirement suggests that DNA synthesis by the T7 DNA polymerase and gene 4 protein must originate at these interruptions. In the accompanying paper (16), we show that this is indeed the case. Again, in order to address the role of nicks in the DNA and to characterize the role of gene 4 protein in the elongation of polynucleotide chains in the synthetic reaction, we have omitted rNTPs from the reaction. By doing so, we prevent the synthesis of oligoribonucleotide primers by the gene 4 protein on the displaced single strands of template DNA that are formed during DNA synthesis and restrict our studies to the role of the gene 4 protein in elongation of the polynucleotide chain.

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