Bacteriophage T7 Deoxyribonucleic Acid Replication in Vitro

PURIFICATION AND PROPERTIES OF THE GENE 4 PROTEIN OF BACTERIOPHAGE T7*

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The T7 gene 4 protein, a protein known from genetic analysis to participate in phage DNA replication in vivo, has been purified approximately 500-fold with an in vitro complementation assay. The protein, purified from cells infected with a T7 gene 4 temperature-sensitive mutant, is thermolabile, establishing that the complementation activity is in the protein product of the phage gene 4. The purified protein has no detectable nuclease, DNA polymerase, or RNA polymerase activity. However, in addition to stimulating the rate of DNA replication in crude extracts of T7 gene 4 mutant-infected cells, the gene 4 protein effects a marked stimulation of DNA synthesis by the purified T7 DNA polymerase when duplex T7 DNA is used as template. This effect is not observed when denatured T7 DNA is used as template, or when phage T4 DNA polymerase or Escherichia coli DNA polymerase I, II, or III is substituted for the T7 enzyme. Analysis of the DNA synthesized by the T7 DNA polymerase in the presence of the gene 4 protein indicates that much of the product is in short DNA chains which are not covalently attached to the template. This result suggests a novel mechanism for the initiation of DNA chains in this reaction.

As summarized in a preceding paper (1), T7 DNA replication in vivo requires at least six phage proteins and two host proteins. We have developed (2, 3), as have others (4, 5), a cell-free system in which DNA synthesis retains many of the properties of the in vivo DNA replication reaction. In particular, DNA synthesis in vitro requires both the T7 DNA polymerase and the T7 gene 4 protein. In the accompanying papers (1, 6) the properties of the T7 DNA polymerase have been examined. The enzyme has been shown to consist of two subunits: one is the T7 gene 5 protein; the other is a host protein which is either missing or altered in the bacterial tsnC mutants. Since the T7 gene 4 protein is also required for DNA synthesis in the crude in vitro system, we have used a complementation assay to partially purify and characterize the T7 gene 4 protein. Stratling and Knippers (7) have also reported the partial purification of this protein.

EXPERIMENTAL PROCEDURE

Assay for Gene 4 Protein—The 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. Reactions (final volume 0.1 ml) contained 10 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol, 20 mM MgCl₂, 0.3 mM each dNTP, and 0.3 mM each dNTP, with one of the dNTPs labeled with ³H or ²³P, and 6 nmol of T7 DNA. Twenty microliters of an extract (Fraction I) prepared from T7 ts, 1.2-infected Escherichia coli DllO (3) and from 0.1 to 0.5 unit of gene 4 protein, diluted in 10 mM Tris-HCl (pH 7.5)/10 mM 2-mercaptoethanol/1 mg/ml of bovine serum albumin were added to the reaction at 0°, and DNA synthesis was initiated by placing the reaction at 30°. After incubation at 30° for 20 min, acid-insoluble radioactivity was determined as described previously (3).

One unit of activity is defined as the amount of gene 4 protein which causes an increase in the rate of DNA synthesis equivalent to the incorporation of 1 nmol of radioactive nucleotide during the 20-min incubation.

Enzymes—E. coli DNA polymerase I was prepared by the procedure of Jovin et al. (8). Phage T4 DNA polymerase was the hydroxylapatite fraction purified as described by Goulian et al. (9); and phage T7 DNA polymerase was the DNA-cellulose fraction of Modrich and Richardson (6). Each enzyme was estimated to be at least 85% pure by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (10). E. coli DNA polymerase III was Fraction VIII of Livingston et al. (11). E. coli DNA polymerase II and E. coli DNA-binding protein were gifts from Ian Molineux and M. Gefter. Phage T7 DNA-binding protein was a gift from Roberta Reuben.

Other Materials and Methods—T7 [¹⁵N,¹⁴C,³H]DNA was a gift from Warren Masker. All phage strains were obtained from F. W. Studier. All other materials and methods were as described previously (3).

RESULTS

Complementation Assay for Gene 4 Protein

Since the T7 gene 4 protein is required for extensive DNA synthesis in extracts of T7-infected Escherichia coli (3, 4), we have been able to purify the gene 4 protein using an in vitro complementation assay. The effect of the purified gene 4 protein on the rate of DNA synthesis in extracts prepared from cells infected with T7 bearing an amber mutation in gene 4 is shown in Fig. 1. The addition of the purified protein to these extracts resulted in a stimulation of the rate of DNA synthesis

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that was initially proportional to the amount of gene 4 protein added to the reaction. The maximum stimulation of 5- to 6-fold represents almost a full restoration of DNA synthesis activity. (Extracts prepared from cells infected with T7 bearing a wild type gene 4 incorporate about 1 nmol of dAMP during the 20-min incubation (3).)

**Purification of Gene 4 Protein**

All procedures were carried out at 0-4° unless otherwise indicated. The results of a typical purification are shown in Table I.

**Growth of Phage-infected Cells**—E. coli D110 was grown at 30° in a 100-liter fermentor (New Brunswick) in L-broth (10 g/liter of Bacto-tryptone, 5 g/liter of yeast extract, 10 g/liter of NaCl) supplemented with 1 g/liter of glucose and 10 mg/liter of thymine. At a cell density of 10^9/ml (A_660 = 2.0), T7, x, s phage was added at a multiplicity of 3 to 5, and 17 min after infection the culture was quickly chilled to 4° by the addition of crushed ice. The cells were harvested, and the cell paste (200 g) was resuspended in 800 ml of 50 mM Tris-HCl (pH 7.5)/10% sucrose, and 200-ml aliquots in 250-ml polycarbonate bottles were frozen in liquid nitrogen.

**Preparation of Cell Extract**—Frozen cells were thawed overnight at 4°, and 20 ml each of 5 NaCl and 10 mg/ml of lysozyme were added. After 45 min at 0° the solution was placed in a 37° water bath, stirred gently for 10 min to bring the temperature to 20°, and then transferred to an ice bath and stirred until the temperature reached 5°. The lysate was then centrifuged for 1½ hours at 19,000 rpm in an International A54 rotor. The supernatant fluid was recovered and adjusted to A_660 = 200 by addition of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 10% sucrose (Fraction I).

**Streptomycin Sulfate and Ammonium Sulfate Fractionation**—To 900 ml of Fraction I were added, 90 ml of freshly dissolved 30% (w/v) streptomycin sulfate. The solution was stirred for 30 min, and the precipitate was removed by centrifugation. Ammonium sulfate (288 g) was added to 920 ml of the supernatant fluid. The solution was stirred for 45 min, and the precipitate was collected by centrifugation and redissolved in 1 liter of 20 mM Tris-HCl (pH 7.5)/0.1 mM EDTA/10 mM 2-mercaptoethanol/10% glycerol (Buffer A) (Fraction III).

**DEAE-cellulose Chromatography**—A column of Whatman DE-52 DEAE-cellulose (28 cm x 36 cm) was prepared and washed with 5 liters of Buffer A. The concentration of (NH_4)_2SO_4 in Fraction III was determined by measuring the conductivity. The fraction was diluted to 2 liters with Buffer A to reduce the (NH_4)_2SO_4 concentration to 30 mM and then was applied to the column. The resin was washed with 1 liter of Buffer A containing 0.1 mM NaCl, and proteins were then eluted with an 8-liter linear gradient from 0.1 to 0.4 NaCl in Buffer A. Gene 4 complementation activity eluted at about 0.28 mM NaCl. Fractions containing the major portion of the activity were pooled (1.3 liters), and the protein was precipitated with (NH_4)_2SO_4 (390 g/liter). The precipitate was collected by centrifugation, dissolved in 20 ml of 90 mM potassium phosphate buffer (pH 7.0)/0.1 mM EDTA/10 mM 2-mercaptoethanol/10% glycerol (Buffer B). Fraction IV (27 ml) was adjusted to pH 6.5 by the addition of an equal volume of 20 mM KH_2PO_4/0.1 mM EDTA/10 mM 2-mercaptoethanol/10% glycerol and was applied to the column. The resin was washed with 120 ml of Buffer B, and the proteins were eluted with a 500-ml linear gradient from 0.0 to 0.5 mM KCl in Buffer B. Gene 4 complementation activity eluted at about 0.18 mM KCl. Fractions containing the activity were pooled (123 ml), concentrated 5-fold by dialysis against dry polyethylene glycol for 6 hours, and then dialyzed overnight with 500 ml of Buffer B containing 0.15 mM NaCl (Fraction V).

**DEAE-Sephadex Chromatography**—A column of DEAE-Sephadex A-50 (0.8 cm x 11 cm) was prepared and washed with 100 ml of Buffer B containing 0.16 mM NaCl. Fraction V was applied to the column, and the resin was washed with 10 ml of Buffer B containing 0.2 mM NaCl. Protein was then eluted with a 100-ml linear gradient from 0.2 to 0.5 mM NaCl in Buffer B. Gene 4 complementation activity eluted at 0.3 mM NaCl at the leading edge of the major protein peak (Fig. 2). Fractions containing greater than 1,000 units/mg of gene 4 activity were pooled, concentrated approximately 2-fold by dialysis against dry polyethylene glycol, and then dialyzed overnight against 10 mM sodium phosphate buffer (pH 7.1), 10 mM 2-mercaptoethanol/0.1 mM EDTA/60% (w/v) glycerol (Fraction VI). The concent-

![FIG. 1. Complementation assay for gene 4 protein. Reactions were carried out as described under "Experimental Procedure" using Fraction VI of gene 4 protein. Reactions contained either 10 μl or 20 μl of extract.](http://www.jbc.org/)

**TABLE I**

| Fraction | Step             | Units | Milligrams of protein | Units/mg |
|----------|------------------|-------|-----------------------|----------|
| I        | Extract          | 63,000| 13,500                | 4.7      |
| II       | Streptomycin sulfate| 92,000| 9,300                 | 10       |
| III      | Ammonium sulfate | 70,000| 5,600                 | 12.5     |
| IV       | DEAE-cellulose   | 30,000| 360                   | 84       |
| V        | Phosphocellulose | 14,500| 20                    | 725      |
| VI       | DEAE-Sephadex    | 3,000 | 1.2                   | 2,500    |

Procedures are described in the text.

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FIG. 2. DEAE-Sephadex chromatography of gene 4 protein. Chromatography was carried out as described in the text.

treated protein was stored at \(-15^\circ\) for several months without significant loss of activity.

Evidence That Complementation Activity Resides in Product of T7 Gene 4

The assay employed for the purification of the gene 4 protein is not completely specific for the product of the phage gene 4. The addition of highly purified T7 DNA polymerase to these extracts can also result in significant stimulation of DNA synthesis. Moreover, it seemed likely that a number of nucleases might also bring about this effect. However, two lines of evidence argue that the complementation activity that we have purified is indeed the product of the T7 gene 4. First, when a “mock” purification was carried out through the DEAE-cellulose step using cells infected with a T7 gene 4 amber mutant, no complementation activity corresponding to the gene 4 protein could be identified. A small amount of stimulatory activity was observed in fractions from the DEAE-cellulose column, but this activity was eluted coincident with the T7 DNA polymerase at a lower salt concentration than the gene 4 protein. Second, a thermolabile activity has been purified through DEAE-cellulose from cells infected with T7ts101, a T7 gene 4 temperature-sensitive mutant. The thermolability of this protein is compared with that purified from a T7+ infection in Table II. When the complementation assay was carried out a 42° instead of the standard 30°, the protein purified from the T7ts101, infection appeared to be only slightly more thermolabile than the wild type protein. However, if the gene 4 protein was first incubated in buffer at 42° and then assayed at 30°, the wild type protein lost only 15% of its complementation activity, while the mutant protein suffered a greater than 95% loss of activity. An attempt to purify the temperature-sensitive protein further resulted in a rapid loss of activity, presumably because of its increased lability.

Physical Properties

Purity—Analysis of Fraction VI of the gene 4 protein by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed several protein bands (Fig. 3). However, analysis of separate fractions from the DEAE-Sephadex column revealed that only the 57,000- and the 66,000-dalton proteins were eluted from the column coincident with gene 4 complementation activity. The other major proteins were found to elute from the column just after the gene 4 activity along with the major protein peak.

Studier (12) has reported that gel electrophoresis of radioactive proteins synthesized by cells infected with T7+ and T7 gene 4 amber mutants indicates that two polypeptides are altered in the mutant infections. We have confirmed this observation (data not shown) and find that the 57,000- and the 66,000-dalton proteins present in the purified gene 4 protein run coincident with the radioactive polypeptides which are absent in T7 gene 4 amber mutant infections during electrophoresis in the slab gel system described by Studier (13). Assuming, therefore, that these two proteins are the product of the T7 gene 4, one perhaps being a proteolytic cleavage product of the other, then Fraction VI is approximately 25% pure.

Sedimentation Coefficient of Gene 4 Protein—Fraction V of gene 4 protein (20 units) was analyzed by zonal sedimentation through a 10 to 30% glycerol gradient containing 20 mM Tris-HCl buffer (pH 7.5)/0.1 M NaCl. A single peak containing 70% of the applied activity was recovered from the gradient. The \( s_{20,w} \) was calculated to be 4.7 S using bovine serum albumin (4.4 S) as a standard. Assuming that the gene 4 protein is globular and has a \( \bar{V} = 0.73 \), this sedimentation coefficient would correspond to a protein with a molecular
weight of 50,000 to 60,000 (14). This molecular weight is in reasonable agreement with that of either of the two polypeptides that have been identified as the products of gene 4, and suggest that, at least under the conditions of the analyses, the gene 4 protein is not in a complex with other polypeptides.

Absence of Nuclease Activities—The purified gene 4 protein (Fraction VI) contains no detectable exonuclease activity. Incubation of 4 μg of the purified protein with 1 nmol of either native or denatured T7 [3H]DNA for 20 min at 30°C under the conditions used for the complementation assay produced less than 1 pmol of acid-soluble radioactive nucleotides. In addition, analysis of the treated DNA by zonal sedimentation in an alkaline sucrose gradient (data not shown) shows no detectable increase in the number of internal breaks in the DNA, indicating that the purified protein is also free of endonuclease contamination.

Absence of DNA and RNA Polymerases—Incubation of up to 4 μg of the purified gene 4 protein (Fraction VI) in a standard complementation assay from which the extract was omitted resulted in no detectable incorporation (<1 pmol) of [3H]dTTP or [α-32P]ATP into an acid-insoluble product during the 20-min incubation. Similarly, incorporation of [3H]UTP into an acid-insoluble product could not be detected (<10 pmol). A small amount of [α-32P]ATP (10 pmol) was found in an acid-insoluble product after the standard incubation, but this could be caused by the adenylation of T7 DNA ligase which is present in Fraction VI (see below).

Contamination with T7 DNA Ligase—In the process of testing the purified gene 4 protein for a variety of enzymatic activities we found that Fraction VI contained an activity catalyzing the exchange of pyrophosphate into either dATP or rATP. In a reaction containing 1 mM [32P]PPI and 0.3 mM rATP or dATP, the specific exchange activity was 63 nmol/mg/min for exchange into rATP, and 300 nmol/mg/min for exchange into dATP. No significant exchange into any of the other dNTPs or rNTPs was observed when each was tested separately. The exchange activity was not DNA-dependent. From these results we suspected that Fraction VI might be contaminated with T7 DNA ligase (15, 16). Indeed the major contaminant in Fraction VI is a protein of molecular weight 40,000, the same molecular weight as that reported for the T7 DNA ligase (12). That Fraction VI contains a DNA ligase activity was confirmed using the assay of Weiss et al. (17). Furthermore, the specific activity was 1,200 units/mg of protein, approximately the same as that of the most purified fractions of T4 DNA ligase, suggesting that it is present as a major contaminant in the gene 4 preparation. In subsequent studies the DNA ligase activity was found to elute from DEAE-Sephadex coincident with the major peak of protein, lending further support to the idea that this activity is not associated with the gene 4. The fact that Fraction VI carried out an exchange of pyrophosphate into either ATP or dATP suggests that perhaps both of those nucleotides can be used as cofactors by the T7 DNA ligase.2

Enzymatic Properties

Stimulation of T7 DNA Polymerase by Gene 4 Protein—We have not yet identified a catalytic activity for the gene 4 protein. However, in addition to stimulating the rate of DNA synthesis in extracts of T7 gene 4 amber mutant-infected E. coli, the purified protein effects a marked stimulation of DNA synthesis by the T7 DNA polymerase when duplex T7 DNA is used as template (Fig. 4). As previously reported (19, 20), T7 DNA polymerase is essentially inert when given duplex T7 DNA as template. The addition of purified gene 4 protein to these reactions results in a 10- to 20-fold increase in the rate of DNA synthesis. A maximum rate of DNA synthesis is obtained when the weight ratio of gene 4 protein (Fraction VI) to DNA polymerase is about 10. Since this preparation of gene 4 protein is estimated to be only about 25% pure, this suggests that the two proteins may be required in approximately stoichiometric amounts.

DNA synthesis in the presence of these two protein fractions proceeds at a constant rate of at least 30 min and the total amount of DNA synthesized in the reaction can represent at least 20% the amount of template DNA added to the reaction. The maximum rate of synthesis in this purified system is at most only 10% that observed in the crude in vitro reaction, suggesting that additional stimulatory factors are present in the crude extract.

While we have not yet established that the activity responsible for the stimulation of DNA synthesis by the T7 DNA polymerase resides in the gene 4 protein, this activity purifies together with gene 4 complementation activity through phosphocellulose and DEAE-Sephadex chromatography. Furthermore, as will be discussed below, DNA synthesis catalyzed by the T7 DNA polymerase in the presence of Fraction VI of gene 4 protein resembles in several ways the reaction carried out in the crude in vitro system. In particular, our preliminary

![Fig. 4. Stimulation of T7 DNA polymerase by gene 4 protein.](http://www.jbc.org/)
evidence suggests that de novo initiation of DNA chains occurs in this reaction.

*Specificity for T7 DNA Polymerase*—DNA synthesis in extracts prepared from T7-infected cells requires the presence of the phage-induced DNA polymerase. Activity in extracts prepared from cells infected with a T7 gene 5 (subunit for DNA polymerase) amber mutant can be restored only by the T7 DNA polymerase and not by a variety of other DNA polymerases tested (6). DNA synthesis with the purified gene 4 protein exhibits the same specificity for the T7 DNA polymerase (Table III). The rate of DNA synthesis on duplex T7 DNA by phage T4 DNA polymerase and by the E. coli DNA polymerases I, II, and III was not detectably increased by the addition of up to 0.4 μg of gene 4 protein.

As shown in Table III, the effect of gene 4 protein on DNA synthesis also requires that duplex T7 DNA be used as a template. When denatured T7 DNA was substituted as a template, gene 4 protein had no detectable effect on the rate of DNA synthesis by the T7 DNA polymerase. This latter observation distinguished the stimulatory effect of gene 4 protein from that observed with the E. coli and the T7 DNA polymerases. These proteins also stimulate DNA synthesis by the T7 DNA polymerase, but their effect is greater with denatured DNA than it is with a duplex DNA template (21).

*Effect of rNTPs*—In the *in vitro* T7 DNA replication system the rate of DNA synthesis in the absence of the four rNTPs is only about 15% that observed in their presence (3). ATP alone has no effect on the rate of synthesis. Addition of the four rNTPs to the reaction catalyzed by the purified gene 4 and gene 5 proteins results in only a 2-fold stimulation in the rate of DNA synthesis. As in the crude system, however, ATP alone has no stimulatory effect (less than 10%). To determine whether ribonucleotides are required for the initiation of new DNA chains by the T7 gene 4 and 5 proteins we have carried out pycnographic analysis of the products synthesized both in the presence and in the absence of the four rNTPs.

*Pycnographic Analysis of Product*—For pycnographic analysis, DNA was synthesized in reactions containing T7 [3H, 13C, 15N]DNA as template and [α-32P]dATP to label the product. The CsCl density gradient centrifugation was then carried out under both neutral and alkaline conditions (Fig. 5). It is apparent that the 3H-labeled template DNA is not significantly degraded during incubation with the purified gene 4 and 5 proteins. While the analysis in alkaline sucrose density gradients indicates that a fraction of the template DNA molecules contained single strand breaks, the sedimentation profiles shown in Fig. 5 are not detectably different from that obtained when the template DNA was analyzed before addition to the DNA synthesis reaction.

The 32P-labeled product shows a broad distribution of sedimentation coefficients under neutral conditions. The sedimentation profile is very similar that to which we have obtained in

![Fig. 5. Pycnographic analysis of DNA synthesized by T7 DNA polymerase in the presence of gene 4 protein. Reactions were carried out in 0.1 ml containing 20 mM Tris-HCl buffer (pH 7.5), 20 mM MgCl2, 10 mM 2-mercaptoethanol, 0.3 mM each dGTP, dCTP, TTP, and [α-32P]dATP (104 cpmp/nmol), 6 nmol of [3H, 13C, 15N]DNA (3.3 cpm/pmol), 0.15 mg of T7 DNA polymerase, 0.80 μg of Fraction VI gene 4 protein and, where indicated, 0.3 μg each rNTP. After incubation at 30° for 20 min, reactions were diluted with 1 ml of 10 mM Tris-HCl buffer (pH 8.0)/10 mM NaCl/5 mM EDTA, and were divided into equal aliquots for pycnographic analysis under neutral and alkaline conditions. Eight grams of CsCl, and for alkaline gradients 0.7 ml of 1 M potassium phosphate (pH 12.5), were added to each sample in a tared polyalomer tube, and each gradient was then brought to 14.0 g by the addition of 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 1 mM EDTA. Centrifugation was for 90 hours at 33,000 rpm in a Spinco 40 angle rotor at 25°. Fractions were collected, and acid-insoluble radioactivity was determined as described previously (3). The *arrows* mark the approximate banding positions of [13C, 15N]DNA (L, LL) and [3H, 15N]DNA (H, HH).
analyzing the product synthesized after a short incubation with Fraction I or Fraction II prepared from T7-infected E. coli (3). However, it is important to note that, while extensive DNA synthesis is achieved in the crude in vitro reactions, in the experiments shown in Fig. 6 the total amount of DNA synthesized represents only 10 to 20% the amount of template DNA added to the reaction.

Under alkaline conditions much of the product is of low molecular weight (10 to 20 S), although some of the 32P-labeled DNA sediments at the position expected for an intact single strand of T7 DNA. Since the experiments shown in Fig. 5 suggest that some of the product DNA may be covalently attached to the template, we cannot rule out the possibility that the high molecular weight 32P-labeled DNA observed in the alkaline sucrose gradients is the product of repair synthesis in which the product is covalently attached to the long strands of template DNA. In this regard it is interesting that the addition of the four rNTPs to the DNA synthesis reaction appears to preferentially stimulate the synthesis of low molecular weight DNA chains.

Effect of T7 and E. coli DNA-binding Proteins—The rate of DNA synthesis by the T7 DNA polymerase is increased by the addition of either the T7-induced or the E. coli DNA-binding protein (21). This stimulation is greatest when single-stranded DNA is used as template-primer and when the reaction is carried out at low temperatures. Since no mutations in the E. coli or T7 DNA-binding proteins have been isolated, it is not known whether either of these proteins plays a role in T7 DNA replication in vivo. In the case of phage T4, however, the gene 32 DNA-binding protein is essential for phage DNA replication (22).

We have tested the effect of both the T7 and the E. coli DNA-binding proteins (generously supplied by Roberta Reuben and Ian Molineux) on the rate of DNA synthesis in the in vitro reaction. Under our conditions the rate of DNA synthesis by the T7 DNA polymerase, using native T7 DNA as template, is stimulated up to almost 10-fold by the addition of either DNA-binding protein (Fig. 7). Thus, the maximum rate of DNA synthesis achieved by the addition of DNA-binding protein is essentially the same as that obtained by the addition of the purified gene 4 protein. However, to obtain this rate of synthesis it is necessary to add about 500 times as many molecules of DNA-binding protein as of DNA polymerase, while approximately stoichiometric amounts of the gene 4 protein are sufficient to give maximum stimulation. When either the T7 or the E. coli DNA-binding protein was added to a reaction containing both the T7 DNA polymerase and the gene 4 protein, the rate of DNA synthesis was increased by at most a factor of 2 (Fig. 7).

**DISCUSSION**

Extracts prepared from T7-infected cells catalyze DNA synthesis in vitro that is dependent upon the protein product of the phage gene 4 (3, 4). Using a complementation assay we have purified this protein, as have Strätling and Knippers (7).

The T7 gene 4 protein is also essential for phage DNA replication in vivo (19). At least two in vivo studies suggest that the gene 4 protein may function in the initiation of "Okazaki fragment" synthesis. Strätling and Knippers (7) have reported that the small amount of DNA synthesized in the absence of the gene 4 protein is synthesized only from the r strand of the template, and have suggested that the gene 4 protein is required for the initiation of l strand replication.
fragments requires the gene 4 protein. In studies using a temperature-sensitive gene 4 mutant, Nossal and Dressler (23, 24) have shown that, after shifting to the nonpermissive temperature, large gaps appear on one side of each growing fork, further supporting the idea that the gene 4 protein functions in the initiation of "Okazaki fragments."

Thus, studies with the purified gene 4 protein should elucidate the mechanism of DNA chain initiation during DNA replication. After a 500-fold purification, the gene 4 protein is estimated to be at most 25% pure. The preparation has no detectable nuclease activities, but contains T7 DNA ligase as a major contaminant. We have not identified catalytic activity for the purified gene 4 protein. However, the protein appears to stimulate DNA synthesis significantly by the T7 DNA polymerase when native T7 DNA is used as template. While we have not firmly established that this stimulatory activity resides in the gene 4 protein, it purifies with the gene 4 complementation activity. Furthermore, DNA synthesis by the purified gene 4 and 5 proteins shares many features in common with the reaction catalyzed in our crude in vitro system (3). Stimulation specifically, and also stimulation is not observed when denatured T7 DNA is used as a template-primer. The rate of synthesis is increased by the addition of the four rNTPs to the reaction; rATP alone has no effect. The ratio of gene 4 protein to DNA polymerase required for maximal activity is approximately the same in the purified system as is observed in the complementation of crude extracts. Finally, pucrycographic analysis of the product synthesized using T7 [13C,14N]DNA as template suggests that a major portion of the product DNA is not covalently attached to the template DNA. While we cannot rule out the possibility that short pieces of the template-primer are attached to this product, it is possible that some novel mechanism for the initiation of DNA chains functions in this reaction.

The addition of the four rNTPs to the reaction appears to stimulate preferentially the production of short DNA chains that are not attached to the template DNA. Okazaki and his mechanism for the initiation of DNA chains functions in this reaction. The rate of DNA synthesis in a reaction containing the purified gene 4 and 5 proteins is at most 10% that obtained when the same amount of these two proteins is added as a crude extract prepared from T7-infected cells. Apparently the extract contains a factor or factors which increase the rate of DNA synthesis by these proteins. The E. coli and T7 DNA-binding proteins both significantly increase the rate of DNA synthesis by the T7 DNA polymerase using either denatured or native T7 DNA as template. However, addition of the DNA-binding proteins to a reaction containing the gene 4 and 5 proteins resulted in only a 2-fold increase in the rate of DNA synthesis. Nossal (28) has recently characterized the DNA synthesized with native T7 DNA template-primer using T4 DNA polymerase and T4 DNA-binding protein. The DNA synthesized in this reaction was found to contain a high content of the alternating copolymer, d(A-T). An analogous reaction may be carried out by the T1 DNA polymerase in the presence of a DNA-binding protein. However, from our pucrycographic analysis we conclude that significant amounts of d(A-T) copolymer are not produced in the reaction catalyzed by the gene 4 and 5 proteins. Further studies are required to elucidate the mechanism by which the gene 4 protein enables the T7 DNA polymerase to make use of a native T7 DNA template.

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