DNA Synthesis on a Double-stranded DNA Template by the T4 Bacteriophage DNA Polymerase and the T4 Gene 32 DNA Unwinding Protein

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SUMMARY

T4 DNA polymerase cannot use a nicked duplex DNA molecule as a template-primer for DNA synthesis, apparently because it is unable to displace the 5' end of the strand paired to the strand serving as the template. In addition, the T4 phage gene 32 DNA unwinding protein (32-protein) facilitates strand displacement and allows the T4 DNA polymerase to synthesize DNA using nicked duplex T7 DNA as the template-primer. The first product of this reaction is a rapidly renaturable DNA which is found to contain a few large branches when examined by electron microscopy. Later in the reaction there is also synthesis of a product containing predominantly alternating residues of dAMP and dTMP.

The DNA polymerase encoded by T4 bacteriophage gene 43 and the DNA unwinding protein encoded by the phage gene 32 (32-protein) are both absolutely required for T4 DNA synthesis in vitro (1-4). In vitro the 32-protein lowers the melting temperature of double-stranded DNA helices and also facilitates the renaturation of single-stranded DNA, apparently by melting regions of intrastrand hydrogen bonding which interfere with the annealing of complementary single strands. Although the 32-protein does not promote substantial denaturation of helical DNA under physiological salt conditions at 37° as judged by hyperchromic shift measurements (5), the protein does bind to some A-T-rich regions of the helical DNA under these conditions, resulting in local areas of denaturation observable by electron microscopy of the glutaraldehyde-fixed DNA 32-protein complex (6).

In vitro the T4 DNA polymerase can copy single-stranded regions of DNA, using as a primer either the 3'-hydroxy terminus of the opposite strand in the case of duplex DNA partially degraded with a 3' to 5' exonuclease, or the 3'-hydroxyl end of a single strand which has formed a hairpin by annealing with a fortuitously complementary section of the same strand (7). The 32-protein has been shown by Huberman et al. (8) to stimulate the reaction of the T4 DNA polymerase with each of these types of template. The effect of the 32-protein was greatest under conditions which promoted intrastrand base-pairing (low temperature, high salt), suggesting that the 32-protein was melting regions of intrastrand hydrogen bonding in the single strand that was acting as the template.

While T4 phage DNA replication in vivo requires the copying of a linear duplex DNA, the purified T4 DNA polymerase cannot use an intact or nicked duplex DNA molecule as a template-primer (7, 9). T4 DNA polymerase can bind to duplex DNA, however, since the 3' to 5' exonuclease of this enzyme can begin hydrolysis at the 3' hydroxyl chain end of duplex DNA (10, 11) or at a single-stranded nick in such a duplex (12). Furthermore, the T4 polymerase can repair a gap created by 3' to 5' hydrolysis at a nick in duplex DNA (12). It thus appeared that the failure of the T4 DNA polymerase to begin synthesis at a nick or at an end of duplex DNA results from the inability of the phage enzyme to displace the strand which is annealed to the template strand.

The studies reported here were undertaken to determine whether the T4 DNA polymerase could use a duplex template in the presence of the gene 32 DNA unwinding protein, which would be expected to promote the displacement of the strand paired to the strand serving as the template. The present study shows that nicked linear duplex molecules of T7 DNA do serve as a template for the T4 DNA polymerase if the 32-protein is also present. The first product formed is a DNA which is rapidly renaturable and is found to contain a few large branches when examined by electron microscopy. Later in the reaction there is also extensive synthesis of a polymer containing predominantly alternating residues of dAMP and dTMP.

MATERIALS AND METHODS

DNA Unlabeled T7 DNA was prepared as described previously (10). To prepare labeled T7 DNA, Escherichia coli 011' (13) was grown overnight in M9 medium (13) containing 1.0 µg per ml of thymine, diluted to 1.0 X 10^6 cells per ml in M9 containing ^3H] or ^3H]thymine, grown to 5 X 10^6 cells per ml and then infected with T7 bacteriophage at a multiplicity of 0.5. T7 DNA was denatured with alkali as described by Studier (14).

E. coli DNA containing either [H]thymine or [H]bromouracil was prepared as described by Hanawalt (15, 16), using a thymine auxotroph, E. coli 70 U3 (from T. Breitman, National Institutes of Health). [H]Poly[d(A-T)] prepared using E. coli polymerase I was the gift of M. Gellert (National Institutes of Health). Polymer concentrations are given as that of nucleotide phosphorus.
Nucleotides—^{4}C, ^{3}H, and ^{3}P-labeled deoxyribonucleoside triphosphates, obtained from New England Nuclear, and unlabeled nucleotides from various commercial sources, were purified if necessary by DEAE-cellulose chromatography as previously described (10). BrdUTP was prepared by bromination of dUTP (Sigma) with hydrobromic acid (17) and purified by DEAE-cellulose chromatography. Note that the correct Br concentration for this method is 0.92 mM rather than 0.92 mM given in the original description.

**Enzymes—**32-Protein was purified from E. coli EK 21, a DNA endonuclease I-deficient derivative of E. coli B obtained from J. Eigner (18). The cells were suspended in a buffer containing 1.7 M NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 20% sucrose in 0.1 M sodium phosphate, pH 7.0, infected with bacteriophage T4 (19, 20). After lysis, samples were centrifuged, and the nuclear acids were removed by polyethylene glycol precipitation (10% final concentration) as described by Alberts and Herrick (21). The supernatant solution was dialyzed and chromatographed on a DNA-cellulose column (100 ml packed volume) and then on DEAE-cellulose as described (5).

The supernatant solution was dialyzed and chromatographed on a DNA-cellulose column (100 ml packed volume) and then on DEAE-cellulose as described (5). Forty per cent of the 32-protein after DEAE-cellulose chromatography (9 mg) was loaded onto a 17-ml column of hydroxylapatite which had been equilibrated with 20 mM Tris-HCl, pH 7.4, 0.1 M KCl, 10 mM glycerol, and 1 mM β-mercaptoethanol. A gradient formed from 20 ml of this buffer and 20 ml of the same buffer containing 10% saturated ammonium sulfate was used to elute some contaminating nucleases, and the ammonium sulfate was then removed by washing the column with 20 ml of the equilibration buffer. The 32-protein was then eluted with a gradient formed from 50 ml of 0.04 M and 50 ml of 0.4 M potassium phosphate, pH 7.0, each containing in addition 10% glycerol and 1 mm β-mercaptoethanol (6). There was a shoulder of nuclease activity overlapping the earliest fractions of 32-protein eluted from this column. Only the fractions from the second half of the 32-protein peak, which were free of DNA exonuclease activity measured with denatured, partially digested E. coli [γ-32P]DNA and of DNA endonuclease as measured on neutral and alkaline sucrose gradients with T7 DNA were used in the experiments reported here. These fractions gave a single band on disc gel electrophoresis in the presence of sodium dodecyl sulfate (20).

32-Protein concentrations are given in terms of the monomer subunit with a molecular weight of 35,000 (5, 22).

T7 DNA Polymerase—The T7 DNA polymerase is eluted from the DNA-cellulose column used in the 32-protein purification by stepwise elution with 0.15 and 0.5 M NaCl. The pooled polymerase fractions were applied directly to a hydroxylapatite column (20 ml packed column) as described previously (20) and then purified by phosphocellulose chromatography as described previously for the polymerase from a temperature-sensitive mutant (23). The final preparation gives a single band on disc gel electrophoresis in the presence of sodium dodecyl sulfate and is free of endonuclease activity with native or alkali-denatured λ or T7 DNA.

It had a specific activity of 15,300 units per mg (20).

Aspergillus Nucleases—Aspergillus nuceae S1, was purified in collaboration with T. Vogel (National Institutes of Health), by the method of Vogt (24) through the DEAE-cellulose chromatography unit as described (20). The unit solubilized 10-μg unit denatured calf thymus DNA in 10 min at 45°, under the conditions described by Vogt (24).

**Enzyme Assay—**Unless noted otherwise, polymerase reactions contained 4 mM MgCl₂, 9 μM EDTA, 3 mM ammonium bicarbonate buffer, pH 8.0, and bovine serum albumin, 60 μg/ml, in addition to the deoxyribonucleoside triphosphates, DNA, and enzymes given in the individual figures. Radioactivity in trichloroacetic acid-insoluble polymer was measured by a modification of the method of Furano (25). A glass fiber filter (Whatman GF/C) of 12.5 cm diameter was rinsed into a grid of 25 squares of 1.8 cm sides with a No. 1 pencil. The filter was wetted with 10% trichloroacetic acid containing 10⁻⁴ M Na pyrophosphate and then air-dried. Aliquots of up to 25 μl of the reaction mixtures were pipetted onto successive squares. After all the samples had been applied, the filter was washed successively with 50 ml of cold 10% trichloroacetic acid, three times with 50 ml of ice-cold distilled water, and once with 20 ml of 95% ethanol, dried briefly under a heat lamp (10 min), and cut into squares which were counted in a toluene-based scintillation solution (26). Simultaneous measurement of the incorporation of deoxyribonucleoside triphosphate into polymer and the DNA-dependent formation of deoxyribonucleotide monophosphate from deoxyribonucleotide triphosphate was determined by thin layer chromatography on polyethyleneimine cellulose (PEI-cellulose) as previously described (10).

**Sucrose Density Gradient Centrifugation—**Neutral gradients were formed from 1.8 ml each of 5 and 20% sucrose in 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1% sodium dodecyl sarcosinate. Alkaline gradients were formed from 1.8 ml of 5 and 20% sucrose in 1 M NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sarcosinate, with the pH of each solution adjusted to 12.2 with NaOH using a low sodium error glass electrode. After centrifugation for 100 min at 54,000 rpm in the SW 56 rotor, samples were collected by inserting a 20-μl disposable pipette through the gradient and then pumping from the bottom of the tube with an LKB peristaltic pump. Fractions flowing for equal time intervals were collected onto thin glass fiber filters (Whatman GF/B) which had been prepared as described for the GF/C filters used in the polymerase assay. A grid of 10 squares of 2.2 cm per side absorbed up to 0.2 ml of fluid per square. Acid-insoluble radioactivity was determined as described for the polymerase assay, except that four washes with water were used.

**DNA Strand Separation—**DNA strand separation by CsCl centrifugation was carried out as described by Englund (27), using poly(U,G) (1:1) obtained from Miles Laboratories. Fractions were collected as described for sucrose gradients.

**Asymmetrical Neighbor Analysis—**This analysis was carried out as described by Englund (27).

**Electron Microscopy—**Electron microscopy was generously carried out by Michael Gottlesman (National Institutes of Health) using the formamide-spreading technique as described by Davis et al. (28).

DNA from the neutral CsCl density gradient of the undenatured reaction mixture shown in Fig. 7C (renaturable T4 DNA, Fraction 20; AT-rich product, Fractions 25 and 26) was dialyzed as described in the legend of Fig. 6. The DNA was diluted to give a final concentration of 0.1 to 0.2 μg per ml in 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA, 40 μg per ml of cytochrome c, and 40% formamide. In some cases the dialyzed DNA fractions were heated for 3 min at 85° and then cooled quickly by dilution into the solution of Tris-HCl, EDTA, and cytochrome c, followed by the addition of formamide. The DNA solutions were layered on a hypophase of 0.01 M Tris-HCl, pH 8.0, and 1 mM EDTA in 10% formamide. The DNA monolayers were picked up on paraffin-coated copper grids, multidirectionally shadowed with platinum, observed in a Phillips 200 electron microscope, and photographed on glass plates. Molecular lengths were determined with a map measurer as described by Davis et al. (28), using circular E. coli DNA molecules for length calibration. Complexes of the 32-protein and DNA are dissociated by this technique. They can be visualized only if they are fixed with glutaraldehyde before the addition of formamide (6).

**RESULTS**

**Stimulation of DNA Synthesis by 32-Protein**

The DNA unwinding protein stimulates DNA synthesis catalyzed by T4 DNA polymerase using single-stranded T7 DNA (Fig. 1A) as shown previously by Huberman et al. (8). In the experiment shown the 32-protein to DNA ratio was 0.65, assuming that one monomer of 32-protein covers 10 nucleotides (5), and the temperature was 37°. At lower temperatures less DNA was made, but the percentage stimulation by the 32-protein was

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1. H. T. Miles, personal communication.
2. Unpublished experiments.
Fig. 1. 32-Protein stimulation of the incorporation of dATP into polymer with alkali-denatured T7 DNA and duplex T7 DNA. Reaction mixtures (25 μl) contained 1.7 nmoles of T7 DNA, 1.25 pmole of T4 DNA polymerase, 110 pmoles of 32-protein, and 15 nmoles of each deoxynucleoside triphosphate ([3H]dATP, 4 cpm per pmole) in addition to the components given under "Materials and Methods." After the indicated times at 37°, 5-μl aliquots were precipitated on GF/C filters as described under "Materials and Methods." 0, omit 32-protein; 1, plus 32-protein.

Table I
DNA synthesis with T7 DNA requires 32-protein
Polymerase activity was measured as described under "Materials and Methods" in reaction mixtures (20 μl) containing 4 nmoles of each of the four deoxynucleotide triphosphates and DNA and enzymes as given below. 32-Protein was added before the polymerase. Incubation was for 60 min at 37°.

<table>
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<th>T7 DNA</th>
<th>32-Protein</th>
<th>T4 DNA polymerase</th>
<th>dATP incorporated</th>
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<td>pmoles</td>
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<td>pmoles</td>
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<td>1360</td>
<td>45</td>
<td>0</td>
<td>&lt;4</td>
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greater (data not shown) in agreement with the findings of Huberman et al. (8).

With duplex T7 DNA as the template-primer, DNA synthesis occurs only if the 32-protein is present, in addition to the T4 DNA polymerase (Fig. 1B). DNA synthesis increases with increasing concentration of the 32-protein (Table I) and requires the T4 DNA polymerase (Table II). The rate of synthesis increased 60% if the 32-protein was added 5 min before the addition of magnesium and the polymerase. There was no further increase by prolonging the period before the addition of magnesium and polymerase.

Although no net synthesis of polymer is observed with duplex T7 DNA in the absence of the DNA unwinding protein, there is extensive DNA-dependent conversion of dATP to dAMP (Fig. 2A) as previously reported (10, 11). The 32-protein stimulates this formation of deoxynucleoside monophosphate, in addition to allowing stable incorporation of deoxynucleoside triphosphate into polymer (Fig. 2, B and C).

Characterization of Product with Duplex T7 DNA

Density Label—The product of the synthesis using duplex T7 [3H]DNA with [32P]dGTP and unlabeled dUTP is shown in Fig. 3. After equilibrium centrifugation in neutral CsCl, newly synthesized polymer (Fig. 3A) bands at the density of heavy DNA and at intermediate densities between the heavy and light DNA markers. E. coli DNA which has approximately the same GC content as T7 DNA was used as a density marker (Fig. 3B) (29). Most of DNA serving as template-primer bands at a density between that of the light and hybrid DNA markers. E. coli DNA which has approximately the same GC content as T7 DNA was used as a density marker (Fig. 3D) (29). Most of DNA serving as template-primer bands at a density between that of the light and hybrid DNA markers, showing that it has become associated with newly formed product (Fig. 3A). There is no synthesis or change in the banding position of the template-primer DNA in the absence of either the T4 DNA polymerase (Fig. 3B) or the 32-protein (Fig. 3C). In alkaline CsCl, some of the product bands at the position of heavy dATP incorporation.

Table II
Deoxynucleotide triphosphate requirement for synthesis with T7 DNA
Polymerase reaction mixtures (20 μl) containing four deoxynucleotide triphosphates (12 nmoles each), T7 DNA, 1.7 nmoles, 32-protein, 44 pmoles, and T4 polymerase, 2.5 pmole, in addition to the components listed under "Materials and Methods," were incubated for 105 min at 37°.

<table>
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<th>dTTP incorporated</th>
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<tr>
<td>pmoles</td>
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<tr>
<td>Omit 32-protein</td>
</tr>
<tr>
<td>Omit dATP, dCTP, dGTP</td>
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<td>Omit dGTP, dCTP</td>
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FIG. 3. Neutral and alkaline CsCl equilibrium density gradient centrifugation of the product made from [32P]dGTP and BrdUTP with duplex T7 DNA as the template-primer. The polymerase reaction mixture (63 μl) contained the constituents listed under "Materials and Methods." 8.4 nmoles of T7 [3H]DNA (12 cpm per pmole), 15 nmoles each of dATP, dCTP, and [32P]dGTP (46 cpm per pmole), 30 nmoles of BrdUTP, 520 pmoles of 32-protein, and 15 pmoles of T4 DNA polymerase. Magnesium chloride and polymerase were added after 5 min at 37°. After 2 hours at 37°, the reaction was terminated by the addition of 25 μl of 0.1 M EDTA. Chromatography of a 5-μl aliquot on PEI-cellulose thin layer showed that 1.0 nmole of dGTP was converted to polymer, and 2.7 nmoles were converted to dGMP. Neutral CsCl gradients contained 25 μl of the reaction mixture, 5.97 ml of a solution of 10 mM NaCl, 1 mM EDTA, and 1 mM Tris-HCl, pH 8.0, and 8.0 g of CsClz (Harshaw, optical grade), giving a refractive index of 1.4036 at 25°. For alkaline CsCl gradients, 25 μl of the reaction mixture were added to 0.6 ml of 0.4 M potassium phosphate buffer, pH 12.5. After 10 min at room temperature, 5.38 ml of a solution of 10 mM NaCl, 1 mM EDTA, and 1 mM Tris-HCl, pH 8.0, and 8.9 g of CsCl were added, giving a refractive index of 1.4090. Heavy (HH) and hybrid (HL) DNA from Escherichia coli grown with bromo[3H]uracil (Schwarz-Mann) and light (LL) DNA from E. coli grown in [3H]thymine prepared and isolated by CsCl equilibrium centrifugation as described by Hanawalt (15, 16) were used as density markers (Panel D). Mineral oil was added to fill the polyallomer tubes, and the solutions centrifuged for 66 hours at 37,000 rpm in the Spinco 50 Ti rotor at 20°. Fractions of 0.15 ml were collected and 0.1 ml aliquots precipitated as described under "Materials and Methods." The CsCl concentration of the fractions was determined from the refractive index at 25°.

single-stranded DNA; the remainder band at a density characteristic of single strands with both heavy and light residues. The template-primer DNA bands in the light and mixed heavy and light regions of the gradient.

The presence in the alkaline gradients of both product and template label in the position between the heavy and light marker strands is evidence for covalent attachment of at least some of the product to the template-primer. The apparent absence of template label with the product banding in the heavy position does not indicate de novo initiation of new strands since short template strands arising from uicks near the ends of the T7 DNA could act as primers without introducing enough radioactive or density label to be detected under these conditions.

Size and Renaturability of Product—The two strands of T7 DNA can be easily separated by equilibrium centrifugation in neutral CsCl after heat denaturation and annealing with poly(U,G) (30). When the reaction mixture from synthesis with duplex T7 DNA as the template-primer was examined under these conditions, only a small part of the product banded with the separated T7 strands (Fig. 4A). Most of the product banded at the density of duplex T7 DNA (Peak I, Fig. 4A), T7 DNA with both helical and single-stranded regions (Fractions 17 to 22), or poly[d(A-T)] (Peak II, Fig. 4A). The T7 [14C]DNA
serving as the template-primer banded as separated T7 strands as well as in the region where duplex T7 DNA or T7 DNA with both duplex and denatured regions would band. This change in the banding pattern of the template-primer is dependent on new DNA synthesis since in the absence of either the polymerase (Fig. 4C) or the 32-protein (Fig. 4D), there was no new synthesis and the template DNA banded as expected as the two separate strands of T7 DNA. The two peaks of product with light density (Peaks I and II) were also found when the reaction mixture was heated and cooled quickly in the absence of the poly(U,G) (Fig. 4B). The length of the strands containing newly synthesized product was examined by neutral and alkaline sucrose density gradient centrifugation of aliquots of the same reaction mixtures (Fig. 5). Fig. 6 shows sucrose density centrifugation of Peaks I and II from the CsCl centrifugation of Fig. 4A. The presence of nicks in the duplex T7 DNA used as the template-primer is evident from the alkaline sucrose gradient shown in Fig. 6C.

Since the banding position of DNA in neutral CsCl depends on both the base composition and the extent of single-strandedness, the results shown in Fig. 4 suggested that Peak I was a renaturable DNA with a base composition similar to that of T7, while Peak II was a polymer rich in A and T. This interpretation was supported by the resistance of the products to hydrolysis by the single strand-specific S1 nuclease, and by the base composition of the isolated peaks.

The S1 nuclease degrades single-stranded heat-denatured T7 DNA, but not duplex T7 DNA, at both 19° and 37° (Table III). Poly[d(A-T)] is relatively resistant to hydrolysis at 19° where there is extensive self-annealing, but is degraded to a large extent at 37°, which is close to its Tm. After heat denaturation, Peak I is hydrolyzed at both 19° and 37° to an extent which is greater than that of duplex DNA but less than heat-denatured T7 DNA, as expected for a DNA with both helical and single-stranded regions. Peak II is similar to poly[d(A-T)] in that it is hydrolyzed to a much greater extent at 37° than at 19°.

**Base Composition**—Separate reaction mixtures each containing two labeled and two unlabeled deoxynucleoside triphosphates and unlabeled T7 DNA were used to determine the base composition of the product. Fig. 7 shows neutral CsCl equilibrium centrifugation of the untreated reaction mixtures, and of the reaction mixtures after heat denaturation in the presence of poly(U,G). The undenatured product banding at the same position as duplex T7 DNA contains the four newly incorporated nucleotides in equimolar amounts. In other experiments (data not shown) all of the label from T7 [14C]DNA used as template was also found to band in this position. After heat denaturation in the presence of poly(U,G), there is equimolar incorporation of the four nucleotides in bands corresponding to the separated T7 DNA strands. Most of the newly incorporated dCTP and dGTP, however, bands with duplex T7 DNA (Peak I) or between the positions

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**Fig. 5.** Neutral and alkaline sucrose density gradient centrifugation of the product of the synthesis with a duplex T7 DNA template. Aliquots (25 µl) of the reaction mixture described in Fig. 4 were mixed with 25 µl of 0.1 M EDTA and 50 µl of H2O before layering on neutral sucrose gradients as described under "Materials and Methods." Aliquots (25 µl) of the same reactions were mixed with 25 µl each of 0.1 M EDTA, 2 M NaOH, and 4 M NaCl for 5 min at room temperature before layering on alkaline sucrose gradients.

**Fig. 6.** Neutral and alkaline sucrose density gradient centrifugation of the products isolated by CsCl density gradient centrifugation. The fractions comprising Peaks I and II in Fig. 4A were pooled and dialyzed against a solution of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 1 mM EDTA. Samples for the neutral gradients contained 75 µl of the dialyzed fraction and 25 µl of 0.1 M EDTA. Samples for the alkaline gradients which contained 75 µl of the dialyzed fractions, 25 µl of 0.2 M EDTA, and 50 µl each of 2 M NaOH and 4 M NaCl were incubated for 5 min at room temperature before layering on the gradients.
occupied by duplex and single-stranded T7 DNA in agreement with the experiment shown in Fig. 4. The lightest peak found both before and after heat denaturation contains predominantly newly incorporated dATP and dTTP, and very little dCTP or dGTP (Fig. 7, A, B, and C). Its bonding position is similar with that of polymer disulfate (29) which absorbs at 6.78 g per cm$^3$ (31). Nearest neighbor analysis of this product (Table IV) shows that it is composed almost entirely of alternating adenine and thymine residues. The formation of this product is a late event, since it is not present after a 15-min reaction (Fig. 7A). It is not formed in the absence of the T7 DNA template-primer (data not shown).

Electron Microscopy—The products of the reaction with T7 DNA, the T4 polymerase, and the 32-protein were examined in the electron microscope by Michael Gottesman after fractionation of the reaction mixture by centrifugation in neutral CsCl. Fraction 20 from the centrifugation of the undenatured reaction mixture (Fig. 7C) which contained 60% T7 DNA template, 38 pmol of T4 polymerase, and 20 units of S1 nuclease was used. The per cent solubilization was determined by comparing the recovery of acid-soluble DNA in the presence and absence of the S1 nuclease.

Table III

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Table IV

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Fig. 7. CsCl equilibrium density gradient centrifugation of the product of the incorporation of each of the four deoxynucleoside triphosphates with duplex T7 DNA template. The four reaction mixtures (75 µl) contained the constituents shown under "Materials and Methods," 13.4 moles of T7 DNA, 205 pmol of 32-protein, 38 pmol of T4 polymerase, and 20 nmol of each deoxynucleoside triphosphate including [PH]dTTP (19 cpm per pmole) and either [3H]dGTP (6.3 cpm per pmole) (Panel A), [3H]dATP (5.9 cpm per pmole) (Panel B), or [3H]dCTP (8.4 cpm per pmole) (Panels C and D), as indicated in the figure. Magnesium chloride and polymerase were added after 5 min at 37$^\circ$. After 120 min at 37$^\circ$, 3.2 nmol of dGTP and 5.9 nmol of dITP had been incorporated in Reaction A; 5.5 nmol of dATP and 5.6 nmol of dTTP in Reaction B; and 3.0 nmol of dCTP and 6.5 nmol of dTTP in Reaction C. After 15 min 1.0 nmol of dCTP and 1.3 nmol of dTTP had been incorporated in Reaction D. An aliquot (25 µl) of each reaction mixture was denatured with poly(U,G) as described in the legend to Fig. 4. Another aliquot (25 µl) was mixed with 10 µmol of EDTA and 10 µl of 30% sodium dodecyl sarcosinate in a total volume of 1.2 ml. Saturated CsCl (5.3 ml) was added to both the native and denatured samples (refractive index 1.491), which were centrifuged at 10$^\circ$ for 34.5 hours at 40,000 in the Spinco 50 Ti rotor. Incorporation of separated T7 strands as shown in Panel E, I and II designates the peaks corresponding to those in Fig. 4.

Table III

Renaturability of product measured by resistance to S1 nuclease

DNA was denatured in 0.015 M NaCl (70 µl) by heating for 3 min at 95$^\circ$ and cooling quickly in an ice bath. Hydrolysis by the S1 nuclease at 19$^\circ$ was measured in reaction mixtures (0.1 ml) containing either T7 [3H]DNA, 500 pmol, [PH]poly[d(A-T)], 450 pmol, or the dialyzed fractions (see Fig. 6) from Peaks I or II in Fig. 4, 5 µl, in addition to 50 mM sodium acetate, pH 4.6, 0.1 M NaCl, 1.5 mM ZnSO$_4$. 50% glycerol, 5 µg of calf thymus DNA (sonicated, then heated and cooled quickly), and 20 units of S1 nuclease. After 90 min at 19$^\circ$, the entire reaction mixture was transferred to a GF/B filter, which was treated as described under "Materials and Methods." Degradation at 37$^\circ$ was measured for 60 min with 12 units of S1 nuclease. The per cent solubilization was determined by comparing the recovery of acid-soluble DNA in the presence and absence of the S1 nuclease.

FIG. 7. CsCl equilibrium density gradient centrifugation of the product of the incorporation of each of the four deoxynucleoside triphosphates with duplex T7 DNA template. The four reaction mixtures (75 µl) contained the constituents shown under "Materials and Methods," 13.4 moles of T7 DNA, 205 pmol of 32-protein, 38 pmol of T4 polymerase, and 20 nmol of each deoxynucleoside triphosphate including [PH]dTTP (19 cpm per pmole) and either [3H]dGTP (6.3 cpm per pmole) (Panel A), [3H]dATP (5.9 cpm per pmole) (Panel B), or [3H]dCTP (8.4 cpm per pmole) (Panels C and D), as indicated in the figure. Magnesium chloride and polymerase were added after 5 min at 37$^\circ$. After 120 min at 37$^\circ$, 3.2 nmol of dGTP and 5.9 nmol of dITP had been incorporated in Reaction A; 5.5 nmol of dATP and 5.6 nmol of dTTP in Reaction B; and 3.0 nmol of dCTP and 6.5 nmol of dTTP in Reaction C. After 15 min 1.0 nmol of dCTP and 1.3 nmol of dTTP had been incorporated in Reaction D. An aliquot (25 µl) of each reaction mixture was denatured with poly(U,G) as described in the legend to Fig. 4. Another aliquot (25 µl) was mixed with 10 µmol of EDTA and 10 µl of 30% sodium dodecyl sarcosinate in a total volume of 1.2 ml. Saturated CsCl (5.3 ml) was added to both the native and denatured samples (refractive index 1.491), which were centrifuged at 10$^\circ$ for 34.5 hours at 44,000 in the Spinco 50 Ti rotor. Incorporation of separated T7 strands as shown in Panel E, I and II designates the peaks corresponding to those in Fig. 4.
with itself is shown clearly in electron micrographs made after heat denaturation and rapid cooling of the DNA. Under conditions where the template T7 DNA was quantitatively converted to single strands, 45 out of 99 molecules of denatured Peak I were single strands, 27 were linear double strands (hairpins), 10 were linear molecules with single- and double-stranded regions, and 17 were complex branched structures such as those shown in Fig. 9, C and D. The lightest peak from the same gradient (Fractions 25 and 26) which contained predominantly newly synthesized poly[d(A-T)], was highly branched when examined before or after heating (Fig. 9B). Partial denaturation in 82% formamide has been used to identify easily melted, A-T-rich, regions in duplex DNA (32). Under these conditions, T7 DNA and Fraction 20 had small regions of denaturation, while the product in Fractions 25 and 26 was entirely single-stranded, as expected from its base composition (not shown).

**DISCUSSION**

The T4 DNA polymerase is one of several proteins which are absolutely required for T4 phage DNA synthesis (1-3). While replication in *vivo* requires the copying of the linear duplex phage DNA, the purified polymerase itself is unable to use such a template *in vitro* (7, 9). This defect is not due to an inability of the enzyme to bind to double-stranded DNA, since the T4 enzyme catayzes extensive hydrolysis and reassembly of the 3'-hydroxyl ends and nicks of duplex DNA (10-12, 27). The polymerase synthesizes DNA by the addition of nucleotides to the 3'-hydroxyl end of the chain serving as the primer. Therefore, synthesis on a duplex template requires that the 5'-hydroxyl chain end of the strand hydrogen bonded to the strand serving as template be removed either by physical displacement or by enzymatic degradation. The T4 DNA polymerase lacks a 5'- to 3'-exonuclease which could hydrolyze the opposing chain (12, 33). It is apparently also unable to physically displace this strand, since the enzyme cannot begin synthesis at the end or at an internal nick in a duplex DNA (7, 9). The phage enzyme differs in this respect from *E. coli* polymerase I which can both remove the 5' end of the complementary chain with its 5'- to 3'-exonuclease activity, and physically displace it (9, 34). The 5'-exonuclease is not required for synthesis at a nick, since polymerase I from which the 5'-exonuclease activity has been removed by limited proteolysis (9) or by mutation (35) can still begin synthesis at an internal nick in duplex DNA.

The DNA unwinding protein encoded by the T4 phage gene 32 is apparently able to perform strand displacement both in *vivo* and *in vitro*. In *vivo*, it has been found that in the absence of the 32-protein there are fewer branched recombinant DNA molecules whose formation requires displacement of part of one strand of each of the two duplex DNAs and annealing of complementary regions of the 2 recombining molecules (36-39). Furthermore, the 32-protein may also be temporarily displacing the strand complementary to the template strand at each growing point during DNA synthesis (5), since phage DNA replication in *vivo* requires that the 32-protein be present throughout the period when DNA is being made (4) in quantities such that there are approximately 170 monomer units of 32-protein per growing point on the phage DNA (5, 40). *In vitro* the 32-protein binds to A-T-rich regions of duplex DNA, as shown by local areas of denaturation in electron micrographs of the glutaraldehyde-fixed DNA 32-protein complexes (6).

The 32-protein increases the rate at which the T4 DNA polymerase copies single-stranded DNA and repairs gaps created at the 3' end of duplex DNA under conditions which suggest that it acts by interfering with intrastand hydrogen bonding in the strand serving as the template (8). The 32 protein does not stimulate *E. coli* polymerases I (8), II (41), or III (41), which may indicate a specific interaction of the 32-protein and the T4 DNA polymerase. However, the possibility that this specificity is due to different template requirements for the host and phage enzymes has not yet been completely ruled out.

The studies reported here show that the 32-protein does enable the T4 polymerase to use a double-helical template (Fig. 1). The products of the reaction are a rapidly renaturable DNA and a polymer consisting primarily of alternating adenylate and thymidylicate residues. Most of the T7 DNA molecules present in the reaction do function as a template-primer, as shown by the density shift of labeled T7 DNA template-primer to the hybrid region of a neutral CsCl density gradient after synthesis with 32P dUTP (Fig. 3). In this experiment new product was measured by the incorporation of radioactive dGTP, so that the DNA, but not the A-T rich product, was followed. In alkaline CsCl, part of the product bands between the heavy and light single-stranded markers, suggesting covalent attachment of at least some of the product to the primer. The absence of template label in the product that bands with the heavy DNA in alkaline CsCl does not indicate new chain initiation since the heavy product may be covalently attached to primer strands too short to contribute radioactive or density labels. The experiments reported do not exclude the possibility that there is some chain initiation in this system. However, the T4 DNA polymerase cannot initiate chains using poly(dT) as template (42). Furthermore, there was no effect on the synthesis with duplex

**Fig. 8.** Length distribution of the product of the synthesis using duplex T7 DNA with T4 DNA polymerase and 32-protein and of the T7 DNA template. Electron micrographs of renaturable DNA (Fraction 20 from Fig. 7C (native)) and of duplex T7 DNA were made and measured as described under "Materials and Methods."
FIG. 9. Electron micrographs of the products of synthesis with duplex T7 DNA. The samples from Fig. 7C (native) were prepared and photographed as described under "Materials and Methods." All samples are shown at the same magnification. A, renaturable DNA (Fraction 20); B, d(A-T)-rich product (Fractions 25 and 26); C and D, renaturable DNA (Fraction 20), heat-denatured and rapidly cooled. The intact circles in A are col E DNA added as a length standard. The arrows point to the ends of a molecule the length of T7 DNA.

T7 DNA of E. coli RNA polymerase, or the ribonucleoside triphosphates (data not shown), or both, although the synthesis of RNA may be involved in the initiation of T4 DNA synthesis in vivo (43).

The rapid renaturability of the DNA product of the reaction with T4 DNA polymerase and the 32-protein was shown by its banding position in neutral CsCl after heat denaturation (Fig. 4), its resistance to S1 nuclease after heat denaturation (Table III), and its branched appearance in electron micrographs before denaturation (Figs. 8 and 9A), as well as its appearance as branched and hairpin molecules in electron micrographs made after heating and rapid cooling of the DNA (Fig. 9, C and D). Such renaturable structures result when the polymerase copies either the newly synthesized product or the displaced strand rather than the strand originally serving as the template, as shown in Fig. 10. Similar products are formed by E. coli polymerase I using either linear duplex T7 DNA containing many internal nicks (34, 44) or double-circular DNA containing nicks (9, 35). Detailed schemes for generating such molecules have been presented previously (9, 44). The importance of copying the displaced strand in generating these renaturable structures is suggested by the observation that a mutant E. coli polymerase I which is unable to hydrolyze the displaced strand, gives a more highly branched product than the wild type enzyme (35).

The product containing predominantly alternating adenine...
and thymine residues was characterized by its banding position in neutral CsCl before or after heat denaturation (Figs. 4 and 7), base composition (Fig. 7), greater resistance to S1 nuclease digestion at 19° versus 37° (Table III), nearest neighbor analysis (Table IV), and the appearance of highly branched molecules in electron micrographs made before or after heat denaturation (Fig. 9) which were converted to single strands in 52% formamide. This product contains small, but significant amounts of dGMP and dCMP (Table IV) and it is not made in the absence of dGTP or dCTP (Table II), or in the absence of the 32-protein (Fig. 4) or the T7 DNA template (data not shown). It probably results from the creation of a poly[d(A-T)] template by continuous strand slippage (45, 46) after the copying of a region of the T7 DNA with a short stretch of alternating adenylate and thymidylate residues. The poly[d(A-T)] template appears late in the reaction (Fig. 7, C and D), and the quantity of this product compared with the renaturable DNA varied from experiment to experiment, and with different preparations of this product.

The strand slippage required for poly[d(A-T)] synthesis may be facilitated by the 32-protein. In the absence of this unwinding protein T4 DNA polymerase does not synthesize poly[d(A-T)] de novo, in contrast to E. coli polymerase I and micrococal DNA polymerase (7). When poly[d(A-T)] is used as the template-primer, the phage enzyme makes less than a single copy before both the template and new product are hydrolyzed by the 3'-exonuclease associated with the T4 DNA polymerase (7). The addition of the 32-protein to the T4 DNA polymerase greatly increases both the rate and extent of synthesis with a poly[d(A-T)] template.4

Although the 32-protein allows the T4 polymerase to use a duplex template, the product is not a complete copy of the template. At some point after synthesis begins, the polymerase stops copying the strand complementary to the primer strand and begins instead to copy either the primer strand itself or the displaced strand, producing a highly renaturable hairpin structure (Fig. 10). Presumably this is prevented in vivo by one or more of the other protein factors required for T4 DNA replication.

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