Deoxiribonucleic Acid Polymerase III of Escherichia coli

PURIFICATION AND PROPERTIES*

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SUMMARY

DNA polymerase III has been purified 4,500-fold from the Escherichia coli mutant, HMS83, which lacks DNA polymerases I and II. When subjected to disc gel electrophoresis, the most purified fraction exhibits a single major protein band from which enzymatic activity may be recovered. Polyacrylamide gel electrophoresis under denaturing conditions produces two protein bands with molecular weights of 140,000 and 40,000. The sedimentation coefficient of the enzyme is 7.0 S, and the Stokes radius is 62 Å. Taken together, these two parameters indicate a native molecular weight of 180,000. Purified DNA polymerase III catalyzes the polymerization of nucleotides into DNA when provided with both a DNA template and a complementary primer strand. The newly synthesized DNA is covalently attached to the 3' terminus of the primer strand. Because the extent of polymerization is only 10 to 100 nucleotides, the best substrates are native DNA molecules with small single-stranded regions. The most purified enzyme preparation is devoid of endonuclease activities.

In addition to the two exonuclease activities described in the accompanying paper, purified polymerase III also catalyzes pyrophosphorolysis and the exchange of pyrophosphate into deoxynucleoside triphosphates. DNA polymerase III has also been isolated from wild type E. coli containing the other two known DNA polymerases. Furthermore, the enzyme purified from three different polC mutants exhibits altered polymerase III activity, confirming that polC is the structural gene for DNA polymerase III (Gefter, M., Hirota, Y., Kornberg, T., Wechsler, J., and Barnoux, C. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 3150-3153).

The isolation and characterization of a mutant strain of Escherichia coli (polA-) deficient in DNA polymerase I (1)

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suggested that the enzyme was not solely responsible for chromosome replication. In addition, the reduced levels of polymerase I in cellular extracts of the mutant facilitated the detection of other polymerase activities and made possible the identification of DNA polymerase II (2-5) and DNA polymerase III (6). Neither of the two mutants of E. coli (polB-) isolated so far, which lack polymerase II activity in vitro (7, 8), have defects in DNA replication, making it probable that polymerase II is dispensable for chromosome replication.

In contrast, the central role of polymerase III during replication has been established by Gefter et al. (9) who showed that altered polymerase III activities resulted from mutations at the dnaE, now termed polC, locus of the E. coli genetic map. All of the known polC mutations are temperature-sensitive, conditional lethal mutations (10). At the nonpermissive temperature, polC mutant strains quickly stop DNA synthesis (10). These results indicate that polymerase III is essential for chromosome replication.

In order to gain insight into the mechanism of chromosome replication we have examined the enzymological properties of polymerase III as have others (11-15). In this paper we report the extensive large scale purification of polymerase III and some of its physical and enzymological properties. In the accompanying paper (16) we examine the properties of two exonuclease activities associated with the purified polymerase.

EXPERIMENTAL PROCEDURE

Materials

Nucleotides and Synthetic Polymers—Deoxynucleoside 5'-triphosphates were purchased from Schwarz BioResearch; [3H]dATP (18 Ci per mmol) and [α-32P]dATP (5 Ci per mmol) from New England Nuclear. Poly(dA) was obtained from Miles Laboratories. 5-Bromodeoxyuridine 5'-triphosphate and d(T)11 were from P-L Biochemicals.

Enzymes—Phage T4 DNA polymerase was the hydroxylapatite fraction purified as described by Goulian et al. (17). Exonuclease I was purified through the DEAE step (18), and exonuclease III was prepared as previously described (19).

Nucleic Acids—Bacteriophage T7 [3H]DNA was prepared as previously described (20). The ϕX174 RF I [3H]DNA and the ϕX174 [3H]DNA were prepared by the methods of Francke and Ray (21) and Sinsheimer (22), respectively. Salmon sperm DNA was from Sigma Chemical Co.

Chemicals and Reagents—Bovine serum albumin (Fraction V) was obtained from Pentex. Dithiothreitol was purchased from...
Calbiochem. Streptomycin sulfate (740 mg of activity per g) was the product of Pfizer. DEAE-cellulose (DE52) and phosphocellulose (PII) were purchased from Whatman, the Sepharose 6B, DEAE-Sephadex A-50, and CM-Sephadex C-25 from Pharmacia, and the hydroxylapatite (Bio-Gel HTP) from Bio-Rad.

Methods

DNA Polymerase III Assay—Reaction mixtures (0.3 ml) contained 20 mM Tris-HCl buffer (pH 7.2), 13.3 mM MgCl₂, 2 mM dithiothreitol, 33 μM each dATP, dCTP, dGTP, and [3H]dTMP (50 cpm per pmol), 7.5 μmol of salmon sperm DNA, and 0.001 to 0.01 unit of enzyme. The enzyme was diluted in polymerase III buffer (20 mM Tris-HCl buffer (pH 7.2), 2 mM dithiothreitol, and 0.5 mg per ml of bovine serum albumin). Reactions were initiated by the addition of enzyme. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 2 ml of 1 N HCl-0.1 % sodium pyrophosphate, and the DNA was collected on a filter as previously described (23). Radioactivity on the dried filter was determined in a liquid scintillation counter using a toluene-based solvent.

Preparation of Exonuclease III-treated T₇ [³H]DNA—Substrates for DNA polymerase were produced by hydrolyzing T₇ [³H]DNA to varying extents with exonuclease III. In some cases the T₇ [³H]DNA was first degraded by sonic irradiation 5- to 10-fold higher than on the same DNA dissolved in toluene-based solvent. One unit of DNA polymerase III activity is defined as the amount catalyzing the incorporation of 10 nmol of [³H]DNA substrate unless otherwise noted. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 3 ml of 1 M NaCl and 10 mM EDTA.

When molecules with varying amounts of single-stranded regions were used, larger reaction mixtures were used, and aliquots were removed at various times, made 3 M in EDTA, and incubated at 65°C for 5 min. A small portion was then removed, brought to 0.15 ml with water, and the actual amount of acid-soluble nucleotides was determined as described above.

Polynucleoside Gel Electrophoresis—Polynucleoside disc gel electrophoresis was carried out according to the method of Davis (26). Electrophoresis in gels containing sodium dodecyl sulfate was by the procedure of Weber and Osborn (27). The gels were stained with Coomassie brilliant blue and scanned on a Joyce-Loebl microdensitometer.

Other Methods—Protein was determined by the method of Lowry et al. (28) after precipitation with trichloroacetic acid and using bovine serum albumin as a standard. DNA concentrations are expressed as equivalents of nucleotide phosphorus. All pH measurements were made at ambient temperature at a buffer concentration of 0.05 M. DNA was denatured by boiling for 2 min and subsequent rapid cooling in an ice bath. Salt concentrations were determined using a conductivity bridge.

RESULTS

Purification

The purification is designed for 1 kg or more of cell paste. All operations were performed at 4°C. The flow rate of all columns was maintained at one column volume per hour, and fractions totaling one-fifth column volume were collected unless otherwise noted. The results of the purification are shown in Table 1.

Cell Growth—E. coli HMS83 was grown by the Grain Processing Corp., Muscatine, Iowa, in a 1000-liter fermentor at 37°C at pH 7.0 in a broth containing: 0.5% (w/v) yeast extract, 4% (w/v) Casamino acid, 0.5% (w/v) KH₂PO₄, 1% (w/v) glucose, 1% (w/v) tryptone, and 20 μg per ml of thymine. The cells were harvested in late log phase. Strains bearing polC mutations were grown in a Fernandez (New Brunswick) at 30°C in 100 liters of LB broth (1% (w/v) Bacto-tryptone, 1% NaCl, 0.5% yeast extract) supplemented with 0.1% (w/v) glucose and 10 μg per ml of thymine. Rapid aeration was maintained during growth, and the culture was kept at pH 7.0. The cells were harvested in late log phase (A₅₆₀ = 3) by centrifugation in a refrigerated Sharples continuous flow centrifuge and were stored at -30°C until use.

Preparation of Extracts—Two volumes (2 liters) of Buffer A (0.02 M potassium phosphate buffer, pH 6.8, 10% (w/v) glycerol, and 10 mM β-mercaptoethanol) were added to 1 kg of cell paste. The paste was homogenized in a Waring Blender, passed twice through a mechanical press (Almount-Ganlin) at 6000 p.s.i., and collected in an ice-jacketed container (Fraction I).

Preparation of Exonuclease III-treated T₇ [³H]DNA—The extract was diluted with Buffer A to A₅₆₀ = 400. A freshly prepared solution of streptomycin sulfate (60%, w/v) was added to the extract over a 30-min period to a final concentration of 6% (w/v). The solution was stirred for an additional 30 min, centrifuged for 45 min at 15,000 × g to yield a clear supernatant solution (Fraction II); the pellet was discarded.

Ammonium Sulfate Precipitation—Ammonium sulfate (243 g per liter) was added to Fraction II (5 liters), and the solution was stirred for 30 min. The precipitate was collected by centrifugation at 27,000 × g for 30 min and was suspended in 1 liter of 1.3 M (NH₄)₂SO₄ dissolved in Buffer A. The solution was stirred for 30 min, and the precipitate was again collected by centrifugation and was dissolved in 1 liter of Buffer B (0.02 M potassium phosphate buffer, pH 6.5, 25% (w/v) glycerol, and 10 mM β-mercaptoethanol) (Fraction III).

DEAE-cellulose Chromatography—A column of DEAE-cellulose (100 cm² × 30 cm) was prepared and equilibrated with Buffer B. Fraction III was then diluted in Buffer B until
measurement of the conductivity indicated that there was less than 0.03 M (XH₂)₂SO₄ (total volume 3 liters) and was applied to the column. The column was washed with 6 liters of Buffer B containing 0.1 M KCl, and the enzyme was eluted with Buffer B containing 0.25 M KCl. Fractions containing polymerase activity were pooled, and the protein was precipitated with ammonium sulfate (313 g per liter). The precipitate was collected by centrifugation and was dissolved in 200 ml of Buffer B (Fraction IV).

**Phosphocellulose Chromatography**—A phosphocellulose column (33.3 cm² x 30 cm) was prepared and equilibrated with Buffer B. After dilution to 600 ml with Buffer B to reduce the concentration of ammonium sulfate to <0.015 M, Fraction IV was applied to the column. The resin was washed with 2 liters of Buffer B, and proteins were eluted with a linear gradient (20 liters) of potassium phosphate (pH 6.5) from 0.02 M to 0.20 M containing 25%/ (w/v) glycerol and 10 mM β-mercaptoethanol. The enzyme elutes at 0.15 M potassium phosphate. Fractions containing the polymerase activity were pooled, and the protein was precipitated with ammonium sulfate (313 g per liter). The precipitate, collected by centrifugation, was dissolved in 10 ml of Buffer B (Fraction V).

**Gel Filtration**—A Sephrose 6B column (5 cm² x 40 cm) was washed with Buffer B plus 0.1 M KCl. Fraction V was layered onto the column and eluted with Buffer B plus 0.1 M KCl at a flow rate of 40 ml per hour. Four-milliliter fractions were collected. The fractions with enzymatic activity were pooled to a total of 40 ml (Fraction VI).

**DEAE Sephadex**—A column (1 cm² x 20 cm) of DEAE-Sephadex A-50 was equilibrated in Buffer B containing 0.1 M KCl. After Fraction VI was applied, the column was washed with 40 ml of Buffer B plus 0.1 M KCl and then developed with a linear gradient of 200 ml of KCl (0.1 to 0.4 M) in Buffer B. The enzyme eluted at 0.25 M KCl. The pooled fractions (25 ml) containing enzyme activity were diluted with 80 ml of Buffer B and applied to a second DEAE-Sephadex column (1 cm² x 2 cm) equilibrated in Buffer B. The enzyme was eluted with Buffer B plus 0.3 M KCl. The enzyme fraction (Fraction VII) was dialyzed against 20 mM Tris-HCl buffer (pH 7.2), 2 mM di-thiotreitol, and 10% (w/v) glycerol and stored in liquid nitrogen. Samples thus prepared lost less than 20% of their activity in 6 months.

**Hydroxyapatite Chromatography**—A portion (2400 units) of Fraction VII was dialyzed against 2 liters of Buffer B and then applied to a hydroxyapatite column (5 cm² x 10 cm) previously equilibrated with Buffer B. After 100 ml of Buffer B had been pumped through the column, a linear 500 ml of potassium phosphate buffer (pH 6.5) gradient with a phosphate concentration of 0.02 M to 0.20 M and containing 25%/ (w/v) glycerol-10 mM β-mercaptoethanol were applied. The enzyme fraction with the highest activity had a potassium phosphate concentration of 0.08 M.

**CM-Sephadex Chromatography**—The enzyme pool (150 ml) from the hydroxyapatite column was dialyzed against 2 liters of Buffer C (0.02 M potassium phosphate buffer, pH 6.0, 25%/ (w/v) glycerol, and 10 mM β-mercaptoethanol) for 8 hours. A column of CM-Sephadex (1 cm² x 20 cm) was prepared in Buffer C. The protein was applied, 40 ml of Buffer C were pumped through the column, and then a linear gradient (200 ml) of KCl (0.00 to 0.30 M) in Buffer C was applied. The flow rate was maintained at 10 ml per hour. The enzyme eluted at 0.1 M KCl.

For the enzymological studies described in this and the accompanying paper (16) concentrated aliquots of Fractions VIII and IX were used. These fractions were concentrated on phosphocellulose columns (1 cm² x 2 cm) which were prepared according to the method described under "Phosphocellulose Chromatography." The enzyme was eluted with 0.20 M potassium phosphate buffer (pH 6.5), 25% (w/v) glycerol, and 10 mM β-mercaptoethanol, and brought to 50% (v/v) with glycerol for storage at -20°. The enzyme lost less than 50% of its activity in 6 months when stored in this fashion.

**Physical Properties**

**Homogeneity**—When 8 µg of Fraction IX are analyzed by polyacrylamide gel electrophoresis at pH 8.7, one major protein band is observed after staining with Coomassie blue (Fig. 1A). When an identical gel is run and sliced, DNA polymerase activity can be eluted from the region corresponding to this major protein band (Fig. 1B).

**Absence of Endonuclease and Double-stranded Exonuclease Activities**—Purified polymerase III (Fraction VIII) contains no detectable endonuclease activity on either double- or single-stranded DNAs. To detect double-stranded activity, 1 unit of enzyme was incubated with 3.6 nmol of φX174 RFI [³H]DNA. As shown in Fig. 2, less than 10% of the DNA was converted to a form containing single strand interruptions. This represents less than 0.04 pmol of phosphodiester bond cleavages during the incubation. Similarly, when 1.8 nmol of single strand circular φX174[³H]DNA are incubated with 1 unit of enzyme, less than

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**Fig. 1.** A, polyacrylamide gel electrophoresis of DNA polymerase III. DNA polymerase III (8 µg) Fraction IX, was layered on a 5% polyacrylamide gel subjected to electrophoresis, stained, and scanned according to the procedures described under "Experimental Procedure." B, a gel identical with the one shown in A was sliced into 1-mm sections and each slice was permitted to stand at 60° in 100 µl of polymerase III diluent. A sample was removed and assayed for polymerase activity as described under "Experimental Procedure." By this procedure 1 to 10% of the activity could be recovered.

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**Table 1**

<table>
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**Table 2**

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<tr>
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<td>IX</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>IX</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>IX</td>
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</table>

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**Figure 2**

A, polyacrylamide gel electrophoresis of DNA polymerase III. DNA polymerase III (8 µg) Fraction IX, was layered on a 5% polyacrylamide gel subjected to electrophoresis, stained, and scanned according to the procedures described under "Experimental Procedure." B, a gel identical with the one shown in A was sliced into 1-mm sections and each slice was permitted to stand at 60° in 100 µl of polymerase III diluent. A sample was removed and assayed for polymerase activity as described under "Experimental Procedure." By this procedure 1 to 10% of the activity could be recovered.

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**Experimental Procedure**—Purified polymerase III (Fraction VIII) contains no detectable endonuclease activity on either double- or single-stranded DNAs. To detect double-stranded activity, 1 unit of enzyme was incubated with 3.6 nmol of φX174 RFI [³H]DNA. As shown in Fig. 2, less than 10% of the DNA was converted to a form containing single strand interruptions. This represents less than 0.04 pmol of phosphodiester bond cleavages during the incubation. Similarly, when 1.8 nmol of single strand circular φX174[³H]DNA are incubated with 1 unit of enzyme, less than...
Solutions contained 0.3 N NaOH, 0.7 M NaCl, 0.1 mM EDTA and addition of 1 unit of polymerase III and incubated for 30 min at 20°C in a 20% sucrose gradients (4.6 ml) formed in cellulose nitrate tubes. NaOH and 0.7 M in NaCl. The samples were layered on linear 5-80% RFI and 20% RFII). The reaction was initiated with the addition of 1 unit, and the amount of polymerase III was 1 unit. The amount of EDTA, and then 0.3 M in NaOH and 0.7 M in NaCl. The samples were layered on linear 5 to 20% sucrose gradients (4.6 ml) formed in cellulose nitrate tubes. Solutions contained 0.3 N NaOH, 0.7 M NaCl, 0.1 mM EDTA and 0.015% (w/v) Sarkosyl (Geigy Industrial Chemicals). After centrifugation in a Spinco type 50.1 rotor for 110 min at 49,000 rpm at 4°C, fractions were collected by puncturing the bottom of the tube. The fractions were neutralized with 0.3 N HCl, and the radioactivity was determined in a toluene-based scintillation fluid containing Triton X-100.

**TABLE II**

Absence of endonuclease activity on single-stranded circular DNA

The standard nuclease assay was used (see “Experimental Procedure”) with 1.8 nmol of φX174 [3H]DNA (specific activity, 41 cpm pmol). When present, the amount of exonuclease I was 1 unit, and the amount of polymerase III was 1 unit. The amount of exonuclease I was sufficient to degrade all single-stranded molecules. Thus, because φX174 DNA contains 5000 nucleotides, each picomole of endonucleolytic cleavage would result in 5000 pmol of acid-soluble material.

<table>
<thead>
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<th>Enzyme</th>
<th>Acid-soluble radioactivity (pmol %)</th>
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<td>None</td>
<td>0.26 0.02</td>
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<tr>
<td>Exonuclease I</td>
<td>8.15 0.47</td>
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<td>Polymerase III + exonuclease I</td>
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0.05% of the DNA is converted to a linear form susceptible to degradation by exonuclease I (Table II). Fraction VIII does not contain any detectable nuclease activity on linear double-stranded T7 [3H]DNA. After incubation of 1.8 nmol of T7 [3H]DNA with 2 units of enzyme, no change in the alkaline sedimentation velocity of the DNA could be detected.

Purified fractions of polymerase III are able to degrade linear single-stranded DNAs either from the 3' or the 5' termini. These two exonuclease activities appear to reside in the same protein molecule as the polymerizing activity, and will be described in the accompanying paper (16).

**Molecular Weight**—The molecular weight of DNA polymerase III has been determined from the sedimentation coefficient (29) and the Stokes radius by the method of Siegel and Monty (30). The sedimentation coefficient of 7.0 S (Fig. 3B) and the Stokes radius of 62 A (Fig. 3A) indicate an oblong rather than a spherical molecule. From these data a molecular weight of 180,000 is calculated (30).

When 8 μg of Fraction IX are denatured and analyzed on polyacrylamide gels containing sodium dodecyl sulfate, two major protein bands are observed (Fig. 4A). The molecular weights of these proteins are estimated from protein standards of known molecular weight to be 140,000 and 40,000, respectively. Thus, the 180,000-dalton native enzyme might be composed of a 140,000-dalton and a 40,000-dalton subunit. However, the intensity of staining by Coomassie blue were proportional to the mass of the protein. Fraction IX contains 4 mol of the 40,000-dalton protein for every mole of the 140,000-dalton protein. Furthermore, analysis of the protein recovered from a sliced native polyacrylamide gel indicates that the stoichiometric relationship of the two proteins is not constant across the enzyme peak. While the 140,000-dalton protein corresponds exactly to the peak of enzymatic activity, the 40,000-dalton protein is present in largest amounts on the leading edge of the activity peak. These results indicate that either the 40,000-dalton protein is not an integral part of the enzyme, or that the enzyme can exist in multiple forms containing different ratios of the two protein components.

**Characterization of Polymerase Activity**

**Requirements for Activity**—Maximal activity depends on the presence of the four deoxyribonucleoside triphosphates, DNA, Mg++, and dithiothreitol. There is no detectable activity in the absence of DNA or Mg++. When dithiothreitol is omitted...
from the incubation mixture, the reaction proceeds at only 10% of the optimal rate, and the addition of 7 mM N-ethylmaleimide to the incubation mixture results in a complete loss of activity. Maximal activity is obtained in Tris-Cl buffer at pH 8.5. At pH 8.5 in Tris-Cl buffer or at pH 6.5 in potassium phosphate buffer the reaction proceeds at half the optimal rate. The reaction is sensitive to ionic strength and the addition of 20 mM KCl to the standard incubation mixture results in a 50% inhibition.

Requirement for Primer-Template—DNA polymerase III has no detectable activity in the absence of DNA. Of several DNAs tested salmon sperm DNA incubated in MgCl₂ supports the highest rate of DNA synthesis (Table III). Incubation with MgCl₂ appears to “activate” the DNA since such a DNA preparation proved to be superior as a primer-template to DNA dissolved in EDTA, and is comparable to DNA treated with either pancreatic DNAse or exonuclease III.

Double-stranded DNA does not serve as a primer-template for polymerase III. The double-stranded circular φX174 RF DNA will not support synthesis by DNA polymerase III even after it has been treated with a small amount of pancreatic DNAse to introduce several single-stranded phosphodiester bond interruptions (Table III). Similarly, duplex T7 DNA is a very poor template for the enzyme.

The template activity of T7 DNA is increased when single-stranded regions are produced by treatment with exonuclease III (Table III). If the T7 DNA is fragmented by sonic irradiation prior to exonuclease III treatment, the DNA becomes an even better primer-template. With this primer-template the initial rate of polymerization varies with DNA concentrations. At the maximal concentration employed (67 μM) the rate of synthesis is approximately one-third of that found with salmon sperm DNA; the latter is present in standard assays at nearly four times this concentration. These results suggest that DNA with single-stranded regions serves as an effective primer-template. This hypothesis is further supported by the fact that the single-stranded homopolymer, poly(dA), does not support synthesis unless it is made partially double-stranded by the addition of the complementary oligonucleotide, d(T)ₙₙ (Table III).

These results suggest that, like the other DNA polymerases that have been characterized, polymerase III carries out a “repair” reaction in which a primer strand is elongated by the sequential addition of nucleotides to its 3’ terminus with the base sequences of the product determined by pairing with a template strand.

Covalent Attachment of Product to Primer—In order to demonstrate that the product synthesized by polymerase III is covalently attached to a DNA primer, we have carried out the experiment shown in Fig. 5. T7 [³H]DNA, which had been partially degraded with exonuclease III, was repaired by polymerase III in a reaction containing [α-³²P]dATP and, in place of TTP, 5-bromodeoxyuridine 5'-triphosphate. Pycnographic analysis indicates that both before and after denaturation, all of the ³²P-labeled product bands in CsCl at the density of the fully light primer-template [³H]DNA. None of the product is found at the position expected for denatured T7 DNA which has been fully substituted with 5-bromodeoxyuridine (1.826 g per cc), indicating that the product is covalently attached to the primer DNA.

Direction of Synthesis—In an effort to establish that the product DNA is attached to the 3’ terminus of the primer, we have measured the kinetics of degradation by exonuclease III of the newly synthesized DNA polymerized on partially single-stranded DNA (Fig. 6). It is apparent that all of the ³²P-labeled product is hydrolyzed before a detectable fraction of the ³H-labeled template-primer has been made acid-soluble. Since exonuclease III degrades DNA in the 3’ → 5’ direction, our results indicate that the product is attached to the 3’ terminus.
of polymerization never equals the extent of exonuclease III-treated DNA serves as a primer-template reveal that the extent placed in an exonuclease III reaction mixture (see "Experimental Procedure") using 33 μM each dCTP, dGTP, [α-32P]dATP (1000 cpm per pmol), and 5-bromodeoxyuridine 5'-triphosphate. The substrate concentration was 37 μM, and 1 unit of polymerase III was added. After incubation, the reaction was made 66 mM in EDTA and analyzed by isopycnic centrifugation as previously described (31). After the fractions were collected, 50 μl of salmon sperm DNA (0.5 mg per ml) were added and the DNA was precipitated and collected on a filter as described under "Experimental Procedure." The recovery of acid-precipitable DNA from the gradient was >90% of that applied.

**Fig. 5.** Covariant attachment of newly synthesized DNA to primer. T7 [32P]DNA was hydrolyzed 4.2% by the action of exonuclease III. A standard polymerase III reaction was carried out (see "Experimental Procedure") using 33 μM each dCTP, dGTP, [α-32P]dATP (1000 cpm per pmol), and 5-bromodeoxyuridine 5'-triphosphate. The substrate concentration was 37 μM, and 1 unit of polymerase III was added. After incubation, the reaction was made 66 mM in EDTA and analyzed by isopycnic centrifugation as previously described (31). After the fractions (30 per tube) were collected, 50 μl of salmon sperm DNA (0.5 mg per ml) were added and the DNA was precipitated and collected on a filter as described under "Experimental Procedure." The recovery of acid-precipitable DNA from the gradient was >90% of that applied.

**Fig. 6.** DNA polymerase III initiates synthesis at the 3' terminus. Exonuclease III was used to degrade native T7 [3H]DNA (3350 cpm per pmol) to 3.9% acid-soluble material. The DNA was then used in a standard polymerase assay with [α-32P]dATP (1000 cpm per pmol), 90 μM DNA, and 1 unit of polymerase III. After incubation the 1DNA was purified on a small Sephadex G-50 column (0.50 cm2 X 4 cm) prepared with 10 mM Tris-HCl, 0.14 mM EDTA, and 0.1 mM dATP (1000 cpm per pmol) was substituted for the [3H]dATP. The values given for the extent of the T4 polymerase reaction represent the addition of 0.1 unit of enzyme, for the extent of the polymerase III reaction the addition of 0.2 unit of Fraction VIII, and for the initial rate of the polymerase III reaction 0.012 unit of enzyme. The initial rate was determined by stopping the reactions 2 and 4 min after the addition of the enzyme. All other conditions are those listed under "Experimental Procedure." The extent of synthesis is expressed relative to the DNA present prior to exonuclease III treatment as previously described (23).

<table>
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<tr>
<th>Extent of polymerization</th>
<th>Initial rate of polymerization by polymerase III</th>
<th>T4 polymerase</th>
<th>Polymerase III</th>
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<tr>
<td>%</td>
<td></td>
<td>T4</td>
<td>Polymerase III</td>
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**Table IV**

Repair synthesis: extent and rate

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<th>Extent of synthesis</th>
<th>Extent of polymerization</th>
<th>Initial rate of polymerization by polymerase III</th>
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<tr>
<td>Extent of polymerization</td>
<td>Initial rate of polymerization by polymerase III</td>
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<tr>
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<td>25</td>
<td>13.0</td>
<td>3.9</td>
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</table>

Each reaction mixture contained a total of 150 μl. The concentrations of all chemicals were the same as those listed under "Experimental Procedure" except that 11 μM exonuclease III-degraded, sonically irradiated T7 [3H]DNA was substituted for the salmon sperm DNA, and 33 μM [α-32P]dATP (1000 cpm per pmol) was substituted for the [3H]dATP. The values given for the extent of the T4 polymerase reaction represent the addition of 0.1 unit of enzyme, for the extent of the polymerase III reaction the addition of 0.2 unit of Fraction VIII, and for the initial rate of the polymerase III reaction 0.012 unit of enzyme. The initial rate was determined by stopping the reactions 2 and 4 min after the addition of the enzyme. All other conditions are those listed under "Experimental Procedure." The extent of synthesis is expressed relative to the DNA present prior to exonuclease III treatment as previously described (23).

In order to explore this point further, sonically irradiated T7 [3H]DNA has been hydrolyzed to varying extents with exonuclease III. With the primer-templates examined the extent of synthesis does not depend on the length of the single-stranded region (Table IV). While T7 DNA polymerase shows progressively increasing extents of repair with the increasing length of the single-stranded region, the extent of synthesis with polymerase III remains constant. Since the average length of the T7 [3H]DNA is 300 nucleotides, the average extension of its ends by polymerase III can be calculated to be approximately 12 nucleotides. Introduction of additional enzyme after the initial reaction period does not result in further polymerization. Although the 3' → 3' hydrolytic activity of polymerase III, to be described in the accompanying paper (16), could reduce the size of the single-stranded region, the recovery of primer-template indicates that this has not occurred. Furthermore, the initial rate of polymerization decreases with the increased length of single-stranded region (Table IV). These results suggest that the rate of initiation of synthesis and the extent of synthesis may be sensitive to the secondary structure of the template.

**Pyrophosphate Exchange and Pyrophosphorolysis**

Polymerase III catalyzes the exchange of pyrophosphate with nucleoside triphosphates in the presence of DNA (Table V). The rate of exchange is maximal when a single nucleoside triphosphate is present, indicating that, during polymerization, exchange is reduced in comparison to that observed when polymerization is limited by the presence of a single nucleoside triphosphate. When poly(dA)-oligo(dT) is used as the template-primer, there is exchange into dTTP, but not into dCTP or dGTP. Thus, exchange is dependent on a suitable DNA concentration of all chemicals were the same as those listed under "Experimental Procedure" except that 11 μM exonuclease III-degraded, sonically irradiated T7 [3H]DNA was substituted for the salmon sperm DNA, and 33 μM [α-32P]dATP (1000 cpm per pmol) was substituted for the [3H]dATP. The values given for the extent of the T4 polymerase reaction represent the addition of 0.1 unit of enzyme, for the extent of the polymerase III reaction the addition of 0.2 unit of Fraction VIII, and for the initial rate of the polymerase III reaction 0.012 unit of enzyme. The initial rate was determined by stopping the reactions 2 and 4 min after the addition of the enzyme. All other conditions are those listed under "Experimental Procedure." The extent of synthesis is expressed relative to the DNA present prior to exonuclease III treatment as previously described (23).
TABLE V
Pyrophosphate exchange and pyrophosphorolysis catalyzed by DNA polymerase III

<table>
<thead>
<tr>
<th>Conditions</th>
<th>%P Norit-adsorbable (nmol/30 min/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−DNA + 4dNTP</td>
<td>0.01</td>
</tr>
<tr>
<td>+DNA + 4dNTP</td>
<td>0.24</td>
</tr>
<tr>
<td>+DNA + dATP, dCTP, dGTP</td>
<td>0.33</td>
</tr>
<tr>
<td>+DNA + dTTP</td>
<td>1.29</td>
</tr>
<tr>
<td>+DNA − 4dNTP</td>
<td>0.63</td>
</tr>
</tbody>
</table>

The enzyme also catalyzes the pyrophosphorolysis of DNA in a reversal of the polymerization reaction. When the four nucleotide triphosphates are replaced by pyrophosphate in a standard polymerization reaction, the rate of pyrophosphorolysis is approximately 6% the rate of polymerization.

**Purification from Wild Type and PolCt Strains**

Although our purification procedure was developed using E. coli HMS83 which lacks DNA polymerases I and II, we have also been able to use this procedure to examine the relative amounts and physical properties of DNA polymerase III in other strains.

**Purification from E. coli W3110—**

E. coli W3110 is a wild type E. coli K-12 strain containing normal levels of DNA polymerases I and II. In order to examine whether or not DNA polymerase III is present at the same level in wild type cells as in HMS83, we fractionated polymerase III through Fraction V from W3110. The phosphocellulose chromatogram of DNA polymerase III from this strain is shown in Fig. 7. The addition of streptomycin sulfate to the extract (Fraction I) precipitates approximately 90% of the polymerase I activity and leaves all of the polymerase III in the supernatant solution. Fractionation with ammonium sulfate (0 to 40%) removes 90% of the remaining polymerase I. Although we do not know whether polymerase II is removed during streptomycin or ammonium sulfate precipitation, approximately 90% is removed during these two steps of purification. The remainder is easily separated from polymerase III by chromatography on phosphocellulose. The small amount of polymerase I remaining elutes from phosphocellulose at 0.18 M potassium phosphate and is easily distinguished from polymerase III by its insensitivity to N-ethylmaleimide and its ability to use poly[d(AT)] as a template. The yield of polymerase III activity from E. coli W3110 is similar to that of E. coli HMS83, indicating that polymerase III most likely is not the result of mutations affecting polymerases I and II.

**Purification from E. coli JW108, E613, and BT1026—**

Gefer et al. (9) have previously shown that polC is the structural gene for polymerase III. Only one of three polymerase preparations partially purified from three genetically distinct temperature-sensitive polC strains shows unusual heat lability. With the
procedure described above we have purified polymerase III free of polymerases I and II from these three polC strains and have confirmed the early reports. The enzyme (Fraction V), purified from E. coli JW108, is no more thermolabile than that purified from wild type (Fig. 8A). No activity is detected at any temperature in extracts of E. coli E613 (Fig. 8B). Purified enzyme from E. coli BT1026 is active at 30°, but inactive at 45° (Fig. 8C).

**Discussion**

In this paper we describe the 4500-fold purification of DNA polymerase III from E. coli. Although the purification described is for 1 kg of cell paste, we have carried out fractionations of quantities of cells up to 10 kg. More than 99% of DNA polymerase I and 90% of the DNA polymerase II are removed during the first three steps of our procedure when the purification is carried out with wild type E. coli. The removal of polymerases I and II during the first few isolation steps has simplified the purification of polymerase III from strains bearing polC mutations without genetically manipulating these mutations into a polA strain.

The enzyme is nearly homogeneous, yielding a single major band of protein on a nondenaturing polyacrylamide gel and two protein bands on a polyacrylamide gel under denaturing conditions. A degree of heterogeneity on native gels is indicated by the presence of a shoulder with an Rf higher than that of the peak of enzymatic activity. Such electrophoretic heterogeneity could result from multiple forms of the enzyme, from reassociation of subunits, or from limited proteolysis.

Our purest preparation has a specific activity of 2500 units per mg of protein, a value approximately 10-fold lower than that obtained by Kornberg and Gefter (11) and 5-fold higher than that obtained by Hurwitz et al. (33) using a modification of the procedure of Kornberg and Gefter. Although our assay procedure does not employ activated DNA or ethanol as does that of Kornberg and Gefter (11), differences in assay conditions probably do not account for the 10-fold difference in activity. One possible explanation for the variation in specific activity lies in the difference in the methods used to measure protein concentration. Kornberg and Gefter (11) have used the turbidimetric method of Bucher (34), while we determined protein by the spectrophotometric method of Lowry et al. (28). At the low protein concentrations (less than 0.01 mg per ml) and with the nearly homogeneous protein solutions used, such a difference in method could result in a large difference in the measured values of protein concentration and correspondingly in specific activity.

We have calculated a molecular weight of 180,000 for the native enzyme from measurements of the Stokes radius and the sedimentation coefficient. Acrylamide gel electrophoresis of our purest fraction in the presence of sodium dodecyl sulfate reveals two proteins with molecular weights of 140,000 and 40,000. An enzyme comprised of one subunit each of molecular weight 140,000 and 40,000 would yield a species with a molecular weight of 180,000, corresponding to the value calculated for the native enzyme. However, the stoichiometry of the two protein bands shown in Fig. 4 indicates a ratio of 4 molecules of molecular weight 40,000 to every molecule with a molecular weight of 140,000. The finding that the 140,000-dalton protein is present throughout the peak of enzymatic activity on a native polyacrylamide gel suggests that this species is an integral part of the enzyme.

The enzymatic properties of our polymerase III preparation are similar to those described by Kornberg and Gefter (11) and by Otto et al. (12). DNA polymerase III cannot synthesize polyolymidyinosine triphosphate chains de novo; a template as well as a complementary primer with a free 3'-hydroxyl group is necessary for activity. Newly synthesized DNA is covalently attached to the primer, and the direction of synthesis is in the 5' → 3' direction. Like DNA polymerase I (33) polymerase III carries out only a limited reaction. In our experiments polymerase III extends the primers by only 12 to 60 nucleotides even when longer stretches of single-stranded template are available. The length of available template affects the rate of polymerization; the longer the single-stranded region becomes, the slower the initial rate of the reaction. Thus, polymerase III appears to be sensitive to the secondary structure of the template. Polymerase III catalyzes the exchange of pyrophosphate into nucleoside triphosphates as well as the pyrophosphorylation of DNA. Because the exchange reaction is completely dependent on the presence of DNA and a nucleoside triphosphate which can base pair with the template, the transition state during which exchange can occur results from the discrimination of a proper base pair by polymerase III.

Wickner et al. (13) and Wickner and Kornberg (14) have isolated two different forms of DNA polymerase III by assaying for synthesis on single-stranded DNA with a short RNA primer. One form, DNA polymerase III*, will synthesize on RNA-primed φX174 DNA in the presence of a second protein termed copolymerase III*. DNA polymerase III* is labile and decays to yield DNA polymerase III which is no longer able to support synthesis on φX174 DNA in the absence of copolymerase III*. Polymerase III*, like polymerase III, is thermolabile when isolated from a polC strain. A second form, polymerase III holoenzyme, seems to be a complex composed of polymerase III and copolymerase III*. It will synthesize on RNA-primed φX174 DNA in the absence of other protein factors. When chromatographed on phosphocellulose, polymerase III holoenzyme separates into polymerase III* and copolymerase III*.

Hurwitz and Wickner (15) also have found that polymerase III will not catalyze polymerization on RNA-primed φX174 DNA without added protein factors. They have found that two factors are necessary: Factor I, a protein presumed to be the same as copolymerase III*, and Factor II. They suggest (15) that polymerase III* as isolated by Wickner et al. (13) might be composed of polymerase III plus Factor II, while Wickner and Kornberg (14) propose that Factor II might convert polymerase III into polymerase III*. The models of Wickner et al. (13) and Hurwitz and Wickner (15) differ in that Wickner et al. (13) claim that polymerase III* is irreversibly converted to polymerase III, while Hurwitz and Wickner (15) are able to reverse the process by the addition of Factor II.

The enzyme preparation used in our studies seems to be largely in the form of polymerase III as designated by Wickner and Kornberg (13). Judged by their criteria for polymerase III, our enzyme preparation does not appear to be polymerase III* or polymerase III holoenzyme. Our preparation has a molecular weight smaller than β-galactosidase as determined by gel filtration on Sepharose 4B and fails to incorporate nucleotides on RNA-primed φX174 DNA. Synthesis on an oligo(dT)-poly(dA) template does take place, but at a low level which cannot be stimulated by ATP. We do, however, find incorporation on a single strand template when both stimulatory Factors I and II (15), provided by J. Hurwitz, are added (Table VI).

The subunit molecular weights we have determined differ
TABLE VI

Stimulation of polymerase III by Escherichia coli factors

<table>
<thead>
<tr>
<th>Components</th>
<th>Acid-insoluble [3H]TMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase III</td>
<td>5.45</td>
</tr>
<tr>
<td>Polymerase III + Factor I</td>
<td>7.26</td>
</tr>
<tr>
<td>Polymerase III + Factor II</td>
<td>4.50</td>
</tr>
<tr>
<td>Factor I + Factor II</td>
<td>5.31</td>
</tr>
<tr>
<td>Polymerase III + Factor I + Factor II</td>
<td>46.8</td>
</tr>
</tbody>
</table>

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