

Deoxyribonucleic Acid Synthesis in Cell-free Extracts

IV. PURIFICATION AND CATALYTIC PROPERTIES OF DEOXYRIBONUCLEIC ACID POLYMERASE III*

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SUMMARY

DNA polymerase III, an enzyme essential for DNA replication, has been purified more than 10,000-fold from cell-free extracts of *Escherichia coli*. The enzyme, judged to be 10 to 20% pure, requires all four deoxynucleoside 5'-triphosphates, Mg^{++} , ethanol, and native DNA for maximal activity. It is present in amounts sufficient to account for the *in vivo* rate of replication.

DNA polymerase III activity is inhibited by sulfhydryl inhibitors and by salt; it is not inhibited by antiserum directed against DNA polymerase I. The most active template for DNA polymerase III is DNA degraded partially by exonuclease III. Linear duplex DNA, single-stranded DNA, or DNA with single strand scissions is not used as template. The enzyme requires a primer to initiate synthesis and polymerization proceeds in the 5' to 3' direction by covalent extension of the primer.

The isolation of a mutant of *Escherichia coli*, (Pol A₁⁻), lacking detectable levels of DNA polymerase activity (DNA polymerase I) (1), stimulated several investigations into the nature of the DNA synthesis capacity of these cells (2-4). Several laboratories have described the purification and initial characterization of DNA polymerase II, an enzyme distinct from DNA polymerase I (5-10).

DNA polymerase II can be distinguished from DNA polymerase I by several criteria, including its sensitivity to thiol reagents, to ionic strength, and to antiserum directed against DNA polymerase I. Compared with DNA polymerase I, the specific activity of pure DNA polymerase II is less than 10% and there are fewer than 25% as many molecules per cell (6). DNA polymerase II does, however, bear strong resemblance to DNA polymerase I with regard to its catalytic properties. Like DNA polymerase I, DNA polymerase II catalyzes the template-directed synthesis of DNA in the 5' to 3' direction by covalent attachment of the product to the primer. DNA polymerase II

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also catalyzes the exonucleolytic degradation of single-stranded DNA from the 3' end; but unlike DNA polymerase I, hydrolysis of DNA from the 5' end is not observed (7, 10).

The physiological roles of both DNA polymerases I and II remain obscure. The availability of mutants defective in DNA polymerase I has not helped to elucidate the role of this enzyme in DNA metabolism (11-13). Evaluation of the biological significance of both DNA polymerases I and II must await further analysis.

During our investigations into the DNA synthetic capacity of Pol A₁⁻ cells, another DNA-synthesizing activity (DNA polymerase III) was observed (6). On the basis of its chromatographic behavior, inhibition by salt, thermal stability, and insensitivity to antiserum directed against DNA polymerase I, DNA polymerase III can be distinguished from DNA polymerases I and II. Furthermore, analysis of mutants temperature sensitive for DNA synthesis and for cell viability indicates that DNA polymerase III is essential for DNA replication (14, 15). In this report we describe the purification and the general catalytic properties of DNA polymerase III, and compare these properties with those of DNA polymerases I and II.

MATERIALS

Nucleotides and Polymers—Unlabeled deoxynucleoside 5'-triphosphates were purchased from Sigma and [³H]TTP (17.8 Ci per mmole) from Schwarz BioResearch. [γ -³²P]ATP (1 × 10⁶ cpm per pmole) was a gift from Dr. W. Sugden. Dr. F. Bollum kindly provided poly(dA) and poly(dC). The synthetic products, (dA-dC)₄, (dG-dT)_n, and (pdT)₁₀, were a gift from Drs. I. Molineux and H. G. Khorana. The oligonucleotide (dG)₁₂₋₁₆ was purchased from Collaborative Research.

Enzymes—DNase I, bacterial alkaline phosphatase, and micrococcal nuclease were products of Worthington. Polynucleotide kinase (1,140 units per mg) and terminal deoxynucleotidyltransferase from calf thymus (6,750 units per mg) (16) were generous gifts from Dr. P. Modrich. Dr. A. Kornberg donated *E. coli* DNA polymerase I (Fraction VII) (17) and anti-DNA polymerase I antiserum. *E. coli* DNA polymerase II (Fraction V, 270 units per mg) was prepared as previously described (6). Exonuclease III (180,000 units per mg) was isolated as previously described (6) and further purified by a method adapted from that of Richardson and Kornberg (18).

Miscellaneous—*E. coli* W3110 *thy*⁻, *rha*⁻, *lac*⁻, *str*⁻, Pol A₁⁻ was a gift from Dr. J. Gross. Calf thymus DNA was purchased

from Calbiochem; DEAE-cellulose (DE 23) and phosphocellulose (P11) from Whatman.

METHODS

DNA Polymerase III Assay—DNA polymerase was assayed in an incubation mixture (0.3 ml) containing 33 mM morpholinopropane sulfonic acid-KOH (pH 7.0), 13 mM MgCl₂; 50 mM 2-mercaptoethanol; 0.13 mM (each) dCTP, dATP, dGTP; 0.13 mM [³H]TTP (50 cpm per pmole); 32 nmoles of calf thymus DNA;¹ 10% ethanol (v/v), and enzyme. Incubations were for 5 min at 30°; nucleotide incorporation into acid-insoluble product was measured as described (5). One unit of enzyme is defined as the amount catalyzing the incorporation of 1 nmole of TTP into acid-insoluble product in 5 min at 30°.

Preparation of Template for DNA Polymerase III Reaction—Suitable template ("gapped" DNA) was prepared by the sequential action of DNase I and exonuclease III. The reaction mixture (10 ml) contained 5.1 M Tris-acetate (pH 8.2); 2 mM MgCl₂; 3 mM 2-mercaptoethanol; 40 μmoles of calf thymus DNA; and 0.3 μg of DNase I. After a 20-min incubation at 30°, DNase was inactivated by heating at 65° for 10 min. An appropriate amount of exonuclease III, titrated to give maximal DNA polymerase activity, was added and allowed to incubate for 5 min at 30°. The reaction was terminated by heating at 65° for 10 min.

Preparation of Nuclease-treated T-7 DNA—Digestion of T-7 DNA with DNase I was carried out in a reaction mixture (0.55 ml) containing 0.1 M Tris-acetate (pH 8.2); 2 mM MgCl₂; 4 mM 2-mercaptoethanol; 175 nmoles of T-7 DNA; and 2.5 ng of DNase I. Incubation was for 5 min at 30° followed by heating at 65° for 10 min. Digestion with micrococcal nuclease (2.5 ng) was in an identical reaction mixture except that 2 mM CaCl₂ replaced MgCl₂. Subsequent treatment with exonuclease III was performed in the reaction mixture as described for the assay of DNA polymerase III except that calf thymus DNA and ethanol were omitted. Exonuclease III (10 units) and 16 nmoles of "activated" T-7 DNA were added, and after 5 min at 30°, the reaction was terminated by heating for 10 min at 65°.

Preparation of [³²P]pdT(pdT)₉—Unlabeled, chemically synthesized (pdT)₁₀ was dephosphorylated with 0.2 unit of alkaline phosphatase in a reaction mixture (0.15 ml) containing 60 mM Tris-acetate (pH 8.2); and 50 nmoles of (pdT)₁₀. After 30 min at 65° the incubation mixture was cooled to 0° and made 10% in trichloroacetic acid by the addition of cold 50% trichloroacetic acid. After 30 min, the precipitate was removed by centrifugation and the supernatant extracted three times with ether. Phosphorylation was carried out in a reaction mixture (0.6 ml) containing 85 mM Tris-acetate (pH 7.5); 17 mM MgCl₂; 17 mM 2-mercaptoethanol; 8.5 mM potassium phosphate (pH 7.5); 17 mM [³²P]ATP (5 × 10⁸ cpm per pmole); and 5.7 units of polynucleotide kinase. After 30 min at 37°, an additional 5.7 units of polynucleotide kinase were added. After an additional 30 min, the incubation mixture was heated at 100° for 5 min and applied to a column of Sephadex G-50 (1 × 110 cm) at 65°. [³²P]-pdT(pdT)₉ was eluted from the column after collecting 25% of the bed volume.

General—Protein was determined by the method of Bucher (19), with bovine serum albumin as the standard. Salt concentration was measured using a conductivity bridge, with potassium phosphate as the standard. Deoxynucleoside triphosphates were neutralized with M Tris buffer.

¹ Concentrations of DNA are expressed as total nucleotide.

TABLE I
Purification of DNA polymerase III

Fraction	Units	Protein	Specific activity	Yield
		mg/ml	units/mg protein	
I. S100.....	5,440	11.0	1.16	100 ^a
II. DEAE-cellulose I....	6,048	3.4	4.2	110
III. Ammonium sulfate...	3,080	10.0	14.4	57
IV. DEAE-cellulose II...	1,320	0.05	120	24
V. Phosphocellulose.....	220	<0.01	>12,000	4

^a Polymerase activity in the S100 measures DNA polymerase III. Mutants defective in DNA polymerase III with normal amounts of DNA polymerase II (14) have no measurable polymerase activity in the S100 fraction.

RESULTS

Purification

All steps were performed at 4° and all buffers contained 50 mM 2-mercaptoethanol and 20% glycerol (v/v). The purification is designed for 100 g of cell paste. A summary of the purification is given in Table I.

S100—Preparation of the S100 cell-free extract was described (6). The S100 (200 ml) was brought to 20% glycerol by the addition of glycerol (50 ml) and brought to 400 ml by the addition of 0.01 M potassium phosphate buffer, pH 6.5.

DEAE-cellulose I—This procedure was performed as previously described (6). The diluted S100 was brought to 0.2 M (NH₄)₂SO₄ by the dropwise addition of saturated ammonium sulfate, previously neutralized with NH₄OH. The sample was applied to a column of DEAE-cellulose (7.1 × 10 cm) previously equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, containing 0.2 M (NH₄)₂SO₄. The protein not adhering to the column was collected in a single fraction (420 ml).

Ammonium Sulfate—The DEAE-cellulose fraction was brought to 35% saturation by the addition of solid ammonium sulfate (73.5 g) over a 30-min period. After an additional 30 min, the precipitate was removed by centrifugation at 17,000 × g. To the supernatant, solid ammonium sulfate (38.7 g) was added over a 30-min period. After an additional 30 min, the precipitate was collected by centrifugation. The precipitate was washed once with 50% saturated ammonium sulfate and then dissolved in 0.02 M potassium phosphate buffer, pH 6.5 (Buffer A). The ammonium sulfate fraction (21.4 ml) was dialyzed for 10 hours against 1 liter of Buffer A and the dialysis buffer was changed once.

DEAE-cellulose II—The dialyzed ammonium sulfate fraction was applied to a column of DEAE-cellulose (4.1 × 30 cm) previously equilibrated with Buffer A. A linear gradient, 0.02 to 0.3 M potassium phosphate, pH 6.5 with a total volume of 2 liters, was applied immediately and 20 ml fractions were collected. DNA polymerase activity eluted in two peaks. The first peak, DNA polymerase II, eluted at a salt concentration of 0.1 M.² DNA polymerase III emerged from the column at 0.15 M salt. Fractions containing DNA polymerase III were pooled and dialyzed against 2 liters of Buffer A for 10 hours.

Phosphocellulose—The dialyzed DEAE-cellulose fraction (220 ml) was applied to a column of phosphocellulose (2.2 × 30

² The sensitivity to thiol reagents, salt, anti-DNA polymerase I antiserum, and chromatographic behavior of this enzyme activity is consistent with known properties of DNA polymerase II (6).

TABLE II
Properties of DNA polymerase III

The reaction mixture and the details of the assay are described under "Methods."

Reactants	Incorporation
	<i>pmoles</i>
Complete system.....	56
+ Ethanol (10% v/v).....	112
- DNA.....	<2
- dATP, dGTP, dCTP.....	5.6
- Mg ⁺⁺ , + EDTA (3 mM).....	<2
- 2-Mercaptoethanol + <i>N</i> -ethylmaleimide (10 mM).....	<2
+ KCl (0.15 M).....	<2
+ DNase (15 μg per ml).....	<2

cm) previously equilibrated with Buffer A. A linear gradient 0.02 to 0.2 M potassium phosphate, pH 6.5, with a total volume of 2 liters was applied immediately and 20-ml fractions were collected. DNA polymerase activity was eluted in a single peak at 0.1 M potassium phosphate.³ Fractions (about one-third of the activity) were pooled, dialyzed against 0.04 M potassium phosphate (Buffer B), and concentrated by adsorption to a column of phosphocellulose (1 × 4 cm) previously equilibrated with Buffer B. DNA polymerase III was eluted with 0.2 M potassium phosphate, pH 6.5. Fractions of 0.5 ml were collected and fractions representing about half of the activity applied to the column were pooled (1.5 ml). This fraction, Fraction V, after dialysis for 10 hours against 100 ml of Buffer B, was used for all experiments to be described.

DNA polymerase III purified by this procedure yielded preparations purified greater than 10,000-fold with respect to the S100 fraction. The yield of enzyme activity varied from 3 to 10%. Preparations stored for three months at 0° lost 50% of their original activity.

General Properties of DNA Polymerase III

Fraction V is devoid of detectable deoxyribonuclease activity directed against native DNA capable of generating acid-soluble DNA. Incubation of native, *E. coli* [³H]DNA with an amount of Fraction V sufficient to catalyze the incorporation of 60 nmoles of nucleotide resulted in the appearance of less than 1 pmole of acid-soluble material. Incubation of 5' terminally labeled (pT)₁₀ with enzyme (1.2 units) did not result in the appearance of inorganic phosphate. Thus Fraction V does not appear to contain phosphatase activity.

The general requirements for DNA polymerase III-catalyzed synthesis are summarized in Table II.

pH Dependence of Reaction Rate—Maximal activity was observed at pH 7.0 in morpholinopropane sulfonic acid buffer. The rate of polymerization was 85% of the optimal value at pH 6.5 in morpholinopropane sulfonic acid buffer or at pH 7.5 in Tris buffer.

Divalent Metal Requirement—DNA polymerase III requires Mg⁺⁺ for optimal activity. In the absence of Mg⁺⁺ and in the presence of EDTA, (3 mM), no detectable activity was observed. Under the conditions of the standard assay, 6 to 16 mM MgCl₂ was optimal. MnCl₂ at 1 mM was 37% as efficient.

³ This purified preparation of DNA polymerase III chromatographs on phosphocellulose in a manner identical with that previously reported (6).

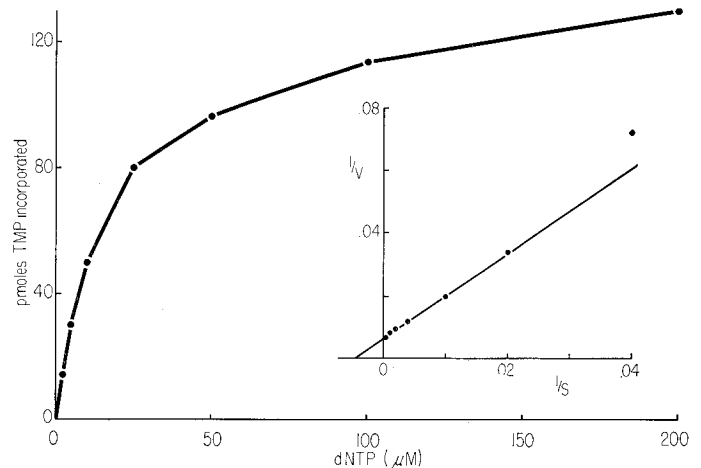


FIG. 1. Dependence of the velocity of DNA polymerase III on the deoxyribonucleoside triphosphate concentration. The reaction conditions are those described under "Methods" except that the concentration of deoxynucleoside triphosphates was varied as indicated.

Sulfhydryl Requirement—DNA polymerase III requires 2-mercaptoethanol or dithiothreitol for maximal activity. The optimum concentration of either compound was 50 mM; in their absence and with *N*-ethylmaleimide (10 mM) present, all activity was abolished (Table II).

Requirements for Deoxynucleoside Triphosphates—All four deoxynucleoside 5'-triphosphates are required for the enzyme to utilize calf thymus DNA as template. A reciprocal plot of substrate concentration versus velocity indicates an apparent K_m for deoxynucleoside triphosphates of 2×10^{-5} M (Fig. 1). A concentration of 1.3×10^{-4} M of triphosphates was sufficient to support the maximal rate of synthesis by DNA polymerase III. Addition of ATP to the reaction mixture had no effect.

Effect of Ionic Strength on Rate of Reaction—DNA polymerase III activity is sensitive to the ionic strength of the reaction mixture. Activity was maximal at low ionic strength in a reaction mixture including the minimum amount of buffer required for pH maintenance, the minimum of base required to neutralize deoxynucleoside triphosphates, and ethanol (10% v/v). K⁺, NH₄⁺, and Na⁺ salts strongly inhibited DNA polymerase III activity at concentrations above 100 mM; 50 mM KCl reduced the rate of polymerization by 50%. Ethanol relieved the salt inhibition and can restore enzyme activity partially reduced by the presence of salt. As shown in Fig. 2, the response to salt and to ethanol distinguishes DNA polymerase III from DNA polymerases I and II.

Template Requirement of DNA Polymerase III

DNA polymerase III requires the presence of a DNA template, and the incorporation of nucleotide residues is abolished by DNase (Table II). The rate of nucleotide incorporation by DNA polymerase III is also sensitive to the nature of the template. Linear, duplex T-7 DNA, and denatured T-7 DNA are inert as templates. Limited digestion of native T-7 DNA with either pancreatic DNase or micrococcal nuclease does not render the DNA active as a template. Thus, synthesis cannot initiate at the site of single-stranded scissions in native DNA. The sequential action of DNase I or micrococcal nuclease and exonuclease III removes nucleotides from the 3' end of a single-stranded scission, leaving a 3'-hydroxyl-terminated, single-

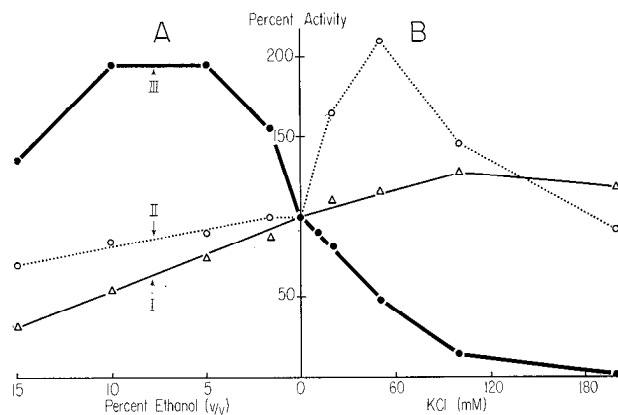


FIG. 2. *A*, effect of ethanol on reaction rate. Ethanol was added to standard reaction mixtures, unsupplemented with ethanol and containing 0.4 unit of DNA polymerase I (Δ — Δ), 0.04 unit of DNA polymerase II (\circ — \circ), or 0.1 unit of DNA polymerase III (\bullet — \bullet). Incubations were performed under standard conditions. *B*, effect of KCl on reaction rate. KCl was added to standard reaction mixtures, unsupplemented with ethanol, containing DNA polymerase I (Δ — Δ), DNA polymerase II (\circ — \circ), or DNA polymerase III (\bullet — \bullet) as above.

TABLE III

Template requirements of DNA polymerase III

The details of the assay are described under "Methods." The amounts of DNA present were calf-thymus DNA (32 nmoles) or T-7 DNA (16 nmoles).

Template DNA	Incorporation <i>p</i> moles TMP
"Gapped" (calf thymus).....	140
Native (T-7).....	<2
Denatured (T-7).....	4
DNase I-digested (T-7).....	<2
DNase I-digested, exonuclease III-digested (T-7).....	24
Micrococcal nuclease-digested (T-7).....	<2
Micrococcal nuclease-digested, exonuclease III-digested (T-7).....	57

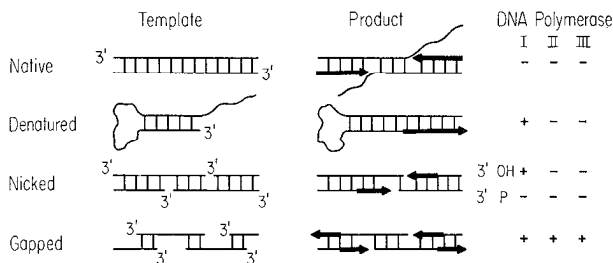


FIG. 3. Template requirements of DNA polymerases I, II, and III.

stranded "gap" in native DNA. "Gapped" T-7 DNA effectively supports synthesis by DNA polymerase III (Table III). Gaps initiated by DNase I yield 5'-phosphoryl termini. Gaps with 5'-hydroxyl termini result from sequential digestion with micrococcal nuclease and exonuclease III. Both templates are equally active, indicating that neither a 5'-phosphoryl nor a 5'-hydroxyl is mandatory. Thus DNA polymerase III is capable of "repair" type synthesis using templates with single-stranded regions and 3'-hydroxyl-terminated primer strands. Fig. 3 gives a schematic representation of these results and compares

the template-primer requirements of DNA polymerase III to the known properties of DNA polymerases I (20) and II (6, 10).

The sequential digestion of DNA by DNase I and exonuclease III yields the most active template-primer for synthesis studied to date.⁴ DNA polymerase III performs the same "repair" reaction on DNA extensively degraded by DNase I, although this template is less than 40% active relative to the best "gapped" templates studied.

Kinetics of Synthesis

Under the conditions of the stranded assay, DNA polymerase III catalyzes a "repair" type reaction. Both the initial rate and the final extent of the reaction are determined by the template. When an excess of "gapped" DNA was used, the initial rate of incorporation of TMP residues was directly proportional to enzyme concentration (0.006 to 0.24 units). However, under standard assay conditions (30°), synthesis was linear with time for only 10 min and continued at a decreasing rate thereafter. At 37° all synthesis ceased after 10 min of incubation. Addition of more DNA is without effect, while addition of more enzyme resulted in the resumption of synthesis. These results indicate that curtailment of synthesis can be independent of DNA concentration, and is not due to the saturation of available substrate. These observations suggest that the enzyme is labile in the reaction mixture. Sensitivity of DNA polymerase III activity to incubation was not overcome by the addition of bovine serum albumin (1 mg per ml) or by the addition of a sulfhydryl-reducing agent. The proposed lability of DNA polymerase III is further substantiated by the observation that at 23°, in the presence of 20% glycerol and excess substrate, incorporation was linear for at least 2½ hours (Fig. 4).

As shown in the *inset* to Fig. 4, the initial rate of the reaction increases with temperature between 15° and 37°. A 1.5-fold increase in the rate of incorporation at 37° relative to 30° was observed.

Primer Requirement

As shown in Table III, DNA polymerase III is most effective in repairing single-stranded regions generated by exonuclease III digestion. Can DNA polymerase III initiate synthesis *de novo*, in the absence of a primer strand? Studies with the single-stranded, synthetic polydeoxynucleotides (dA)_n, (dC)_n, (dT-dG)_n, and single-stranded, circular M13 DNA indicate that single-stranded DNA does not support synthesis by DNA polymerase III. The failure of DNA polymerase III to utilize the polymers as template suggests that DNA polymerase III is incapable of *de novo* chain initiation with any of the four common deoxynucleoside triphosphates. That single-stranded circles are inactive as well indicates that the failure of DNA polymerase III to utilize single-stranded polymers as template is not a consequence of exonucleolytic degradation.⁵ The synthetic polymers can be

⁴ The number of nucleotides released upon exonuclease III treatment strongly influences the ability to prime DNA polymerase III directed synthesis. The influence of gap size on the ability to prime synthesis by DNA polymerase II has been reported (6, 10) and initial rate studies indicate that there is an optimal gap size for both DNA polymerases II and III. DNA polymerase III, like DNA polymerase II, does not efficiently repair large gaps created by extensive exonuclease III digestion.

⁵ DNA polymerase III preparations show the capacity to degrade single-stranded DNA exonucleolytically from the 3' end (data to be presented elsewhere). Thus the possibility exists that the failure to utilize single-stranded DNA as template could be due to the exonucleolytic degradation of the polymers.

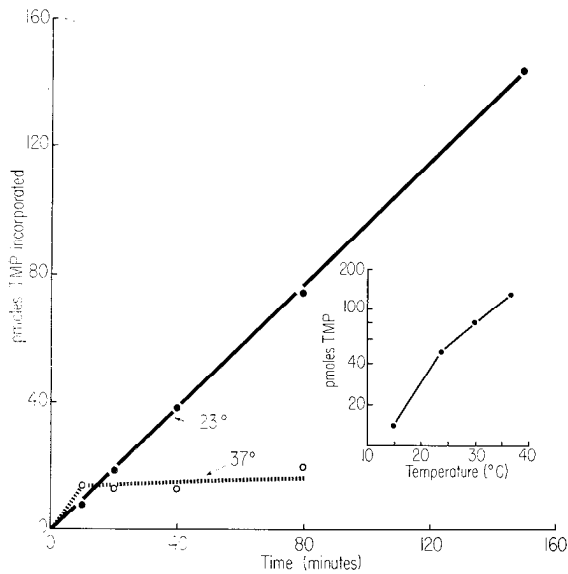


Fig. 4. Kinetic analysis of enzyme activity. Reaction mixtures (0.9 ml) contained 33 mM morpholinopropane sulfonic acid (pH 7.0), 13 mM MgCl₂, 50 mM 2-mercaptoethanol; 0.13 mM (each) dATP, dCTP, and dGTP; 0.13 mM nmoles of [³H]TTP (50 cpm per pmole); ethanol, 10% (v/v); glycerol, 29% (v/v); 384 nmoles of "gapped" DNA; and 0.01 unit of enzyme. Aliquots of 0.1 ml were withdrawn at the indicated times, precipitated with 5% trichloroacetic acid, and radioactivity determined. Values in figure are corrected to that of standard 0.3 ml reaction mixture. *Insert*, dependence of the initial rate on temperature. DNA polymerase III (0.08 unit) was incubated for 5 min under standard conditions at the indicated temperatures.

TABLE IV

Primer requirement of DNA polymerase III

The reaction mixture (0.3 ml), containing 33 mM morpholinopropane sulfonic acid (pH 7.0); 13 mM MgCl₂; 15 mM 2-mercaptoethanol; 0.13 mM (each) of dATP, dCTP, and dGTP; and [³H]TTP (50 cpm per pmole), was used to assay DNA synthesis with calf thymus DNA. [³H]TTP (50 cpm per pmole) was used to assay synthesis with poly(dA) and (pdT)₁₀, [³H]dGTP (50 cpm per pmole) for poly(dC) and (dG)₁₂₋₁₆, and 0.13 mM dATP and [³H]-dCTP for poly[(dG-dT)_n] and (dA-dC)₄. (Morpholinopropane sulfonic acid buffer was replaced by 33 mM Tris-HCl (pH 7.5) in assays measuring incorporation with poly(dC) and (dG)₁₂₋₁₆. The presence of morpholinopropane sulfonic acid resulted in the complete inhibition of enzyme activity.) In all cases 4 nmoles of polymer and 2 nmoles of oligonucleotide were used. The reaction mixture was preheated at 100° for 5 min before addition of enzyme (0.12 unit). Incubation was for 20 min at 25°. The reaction was terminated by the addition of cold 5% trichloroacetic acid and incorporation determined.

Template	Incorporation of ³ H nucleotide
"Gapped" calf thymus DNA	122
(dA) _n	<1
(pdT) ₁₀	<1
(dA) _n + (pdT) ₁₀	160
(dC) _n	<1
(pdG) ₁₂₋₁₆	<1
(dC) _n + (pdT) ₁₀	<1
(dC) _n + (pdG) ₁₂₋₁₆	90
(dT-dG) _n	8.0
(dC-dA) ₄	5.6
(dT-dG) _n + (dC-dA) ₄	112

TABLE V

Covalent attachment of product to primer

The reaction mixture (0.6 ml) contained 33 mM morpholinopropane sulfonic acid (pH 7.0); 13 mM MgCl₂; 50 mM 2-mercaptoethanol; 0.13 mM [³H]TTP (200 cpm per pmole); 4 nmoles of (dA)_n; 1.6 nmoles of [³²P](dT)₁₀ (1000 cpm per pmole); 60 μl of ethanol; and enzyme (1.2 units). The reaction mixture was previously heated at 100° for 5 min before addition of enzyme. Incubation was at 25° and 10-μl aliquots were withdrawn at the indicated times. Aliquots were chilled by addition to 200 μl H₂O, cold trichloroacetic acid was added, and acid-insoluble radioactivity was determined. The values reflect the total amount of product accumulated in the 0.6-ml reaction mixture.

Time	Incorporation	
	³² P	³ H
	pmoles	
At 0 min.	14.4	4.5
At 20 min.	74.4	480
At 40 min.	78.6	765
At 80 min.	82.2	1062

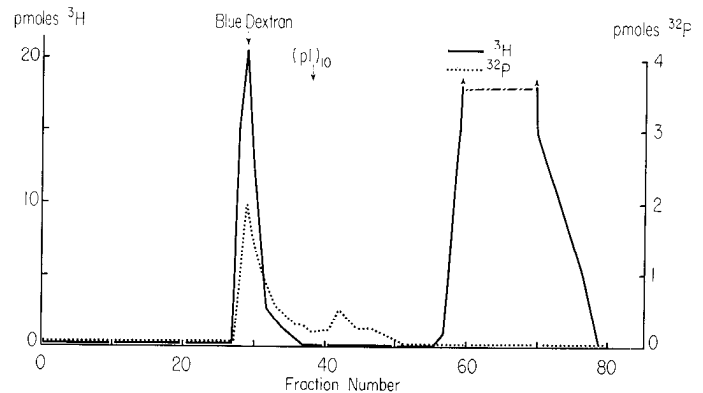


Fig. 5. Sephadex filtration of poly(dA)-directed product. The oligonucleotide (pdT)₁₀ labeled at the 5' terminus with ³²P was used to prime poly(dA)-directed synthesis of (pdT)_n. The details of the reaction are given in the legend to Table V. After 80 min of incubation the reaction mixture (0.6 ml) was heated to 100° for 5 min and subjected to filtration at 65° on a column of Sephadex G-50 (1 × 110 cm). A portion (0.1 ml) was analyzed for ³H (—) and for ³²P (---). The arrows mark the position of Blue Dextran and [³²P]pdT(pdT)₉, determined prior to application of the sample.

rendered active as templates if a complimentary oligonucleotide primer is provided. Primed synthetic polymers direct complementary synthesis by DNA polymerase III. These results are summarized in Table IV.

Mechanism of Chain Elongation; Direction of Synthesis

The role of the complimentary oligonucleotide primer in polymer-directed synthesis was assessed through the use of selectively labeled oligonucleotides. The oligonucleotide, [³²P]-pdT(pdT)₉, was prepared by sequential treatment of (pdT)₁₀ with alkaline phosphatase, and, using [³²P]ATP, with polynucleotide kinase. [³²P]pdT(pdT)₉ was used to prime poly(dA)-directed incorporation of [³H]TMP. (pdT)₁₀ is soluble in 5% trichloroacetic acid (see Table V) and was eluted at 25% of the bed volume on Sephadex G-50 (see Fig. 5). Polymer-directed synthesis using the oligonucleotide primer, [³²P]pdT(pdT)₉, rendered both ³²P and [³H]TMP acid insoluble. After 80 min of

TABLE VI

Characterization of product [^{32}P]pdT (pdT) $_9$ [^3H](pdT) $_{12}$

[^{32}P]pdT(pdT) $_9$ [^3H](pdT) $_{12}$ (61.6 pmoles) from fractions excluded from Sephadex G-50 was incubated at 65° for 20 min in an incubation mixture (0.2 ml) containing 0.1 M Tris (pH 8.2), 10 mM MgCl $_2$, and, where indicated, 2 μg of alkaline phosphatase. Acid-insoluble radioactivity was determined after the addition of 40 nmoles of calf thymus DNA and 5% trichloroacetic acid. Radioactivity adsorbable to Norit was removed by the addition of 2 drops of 25% Norit, 20 μl of 5% trichloroacetic acid, and 10 μl of saturated sodium pyrophosphate. After 5 min at 0°, the Norit was collected on a Millipore filter, and washed with 1 ml, 1% of trichloroacetic acid. Norit nonadsorbable material was collected and counted in Bray's solution.

Fraction	Label	No treatment	Treatment with alkaline phosphatase
		<i>cpm</i>	<i>cpm</i>
Norit-nonadsorbable	^{32}P	60	2643
Norit-nonadsorbable	^3H	27	24
Acid-insoluble	^{32}P	1831	57
Acid-insoluble	^3H	6708	6220

incubation 50% of the added primer was rendered acid insoluble; each acid-insoluble oligonucleotide was extended by approximately 12 residues. The product of this reaction was subjected to filtration at 65° on a column of Sephadex G-50. The oligonucleotide primer, which prior to incubation with DNA polymerase III was eluted at 25% of the bed volume, was now shown to be excluded from the gel as was a fraction of the ^3H (Fig. 5).

The product, [^{32}P]pdT(pdT) $_9$ [^3H](pdT) $_{12}$, excluded from the Sephadex gel, was completely insoluble in acid and adsorbable by Norit (see Table VI). Treatment of the product with alkaline phosphatase at 65° rendered the ^{32}P both acid soluble and Norit nonadsorbable. The ^3H remained insoluble in acid and adsorbable by Norit. Thus, ^{32}P initially present on the 5' end of the (pdT) $_{10}$ primer remained susceptible to phosphomonoesterase after the reaction, indicating that addition of TMP residues was to the 3' end of the primer.

Thus, the product of synthesis was covalently attached to the primer; the 5' end of the primer remained intact and the primer was extended by the addition of TMP residues to the 3' end.

The ability to quantitate the amount of primer active in chain elongation permits the examination of the mechanism of chain elongation by DNA polymerase III. In the experiment described above, 80 pmoles of oligonucleotide primer molecules were used in chain extension. Yet the 0.8 unit of enzyme present should not exceed 1 pmole of enzyme molecules. These results indicate that one enzyme molecule is capable of extending more than one DNA primer molecule under these conditions. We cannot, however, conclude from these results with what frequency the enzyme remains attached to the chain which it has just extended.

DISCUSSION

We have previously shown that DNA polymerase III performs an essential function in DNA replication (14). *E. coli* strains with thermosensitive mutations at the dnaE locus are temperature sensitive for DNA synthesis and for viability. DNA polymerase III is mutationally altered in these strains; both polymerases I and II are normal. We have concluded from these results that both DNA polymerase I and II are unrelated to DNA

polymerase III and that DNA polymerase III is essential for viability, independent of the presence of DNA polymerases I and II. The present communication represents an attempt to assess the distinctive functions of DNA polymerase III through an examination of both its general properties and its catalytic capabilities.

Perhaps the most distinctive feature of DNA polymerase III is the rate at which it can synthesize DNA. Contrary to previous reports that extracts from the Pol A $_1$ ⁻ mutant retain less than 1% the DNA-polymerizing capacity of wild type *E. coli* (1, 5-10), we now find that cell-free extracts of the Pol A $_1$ ⁻ mutant, assayed under conditions optimal for DNA polymerase III activity, possess DNA-synthesizing activity approximately equal to that amount measured in Pol⁺ cells. Purification of this activity permits the evaluation of the mechanism of its catalysis. Our most highly purified preparations of DNA polymerase III have a specific activity in excess of 60,000 units per mg of protein. From polyacrylamide gel analysis, we judge this preparation to be approximately 33% pure. Assuming that the purity is 33% and that the molecular weight is approximately 140,000,⁶ an approximate estimate can be made both of the rate of nucleotide incorporation per molecule of enzyme and of the number of DNA polymerase III molecules per *E. coli* cell. There are approximately 10 molecules of DNA polymerase III per bacterial cell; the rate of nucleotide incorporation at 30° is in excess of 15,000 nucleotides per min, per molecule of DNA polymerase III. (This measurement is limited to templates studied to date.) Relative to DNA polymerases I and II, of which there are approximately 400 (21) and 100 (6) molecules, respectively, per bacterial cell, the rate of synthesis by DNA polymerase III is greater than the rate of synthesis catalyzed by DNA polymerases I and II by a factor of 15 and 300, respectively. The total activity of DNA polymerase III is sufficient to account for the *in vivo* rate of replication.

Several groups of investigators have reported that a nonionic detergent was necessary to obtain DNA polymerase II from extracts of *E. coli* (8, 9). We have not observed such a requirement. We have previously reported that both DNA polymerases II and III can be obtained from French pressure cell lysates, and now report that this method yields DNA polymerase III activity in amounts sufficient to account for the *in vivo* rate of replication. Recently, Wickner *et al.* (10) have isolated DNA polymerase II from cells lysed by alumina grinding, confirming the observation that detergent treatment is not required for the isolation of DNA polymerase II. Thus, DNA polymerases II and III, like DNA polymerase I (21), can be obtained in soluble form without sonication or detergent treatment, suggesting that procedures disruptive to membranes are not required to yield soluble enzyme.

DNA polymerase III can be distinguished from DNA polymerases I and II by virtue of a low pH optimum, a requirement for high concentrations of sulfhydryl reagent, sensitivity to salt, and stimulation by ethanol. The apparent K_m for deoxynucleoside triphosphates is greater than the levels required for the saturation of DNA polymerases I and II.

DNA polymerase III closely resembles DNA polymerases I and II with regard to its catalytic properties. DNA polymerase III is not capable of *de novo* chain initiation using either single-stranded synthetic polynucleotides or single-stranded circular DNA as templates. Both can be rendered active if a primer (ribo or deoxyribo) with a free 3'-hydroxyl is provided. Synthesis can proceed in the 5' to 3' direction by covalent linkage of

⁶ T. Kornberg and M. L. Gefter, unpublished observations.

the product to the 3'-hydroxyl end of the primer. Although elongation of primers in the 5' direction has not been observed, we cannot conclude that DNA polymerase III is incapable of carrying out synthesis in the 3' to 5' direction. Such an event may require a triphosphate moiety at the 5' terminus (22), and this possibility is currently being explored.

As has been observed with DNA polymerases I and II, preparations of DNA polymerase III possess an associated nuclease which catalyzes the degradation of single-stranded DNA exonucleolytically from the 3' end. In studies which are not reported here, the nuclease activity was found to be inactive with double-stranded DNA; its catalytic requirements resembled those described for the polymerizing capacity of DNA polymerase III (*i.e.* sensitivity to sulfhydryl reagents and to salt). The rate of nucleotide removal is in excess of 5000 nucleotides per min per enzyme molecule.

The template requirements of DNA polymerase III suggest a strong similarity to DNA polymerase II. Neither enzyme, in contrast to DNA polymerase I, can utilize single-stranded natural DNA templates or DNA with single strand scissions. None of the polymerases can achieve the replication of native, duplex DNA. All perform a "repair" function with greatest efficiency.

Thus, the catalytic properties of DNA polymerase III do not establish its identity as a polymerase distinctly different from either DNA polymerase I or II, nor do they suggest the role of DNA polymerase III in DNA metabolism. The only properties which clearly differentiate DNA polymerase III from DNA polymerases I and II are its requirement for low ionic strength and ethanol as well as a rapid rate of nucleotide incorporation. Although DNA polymerase III has not been observed to initiate strands *de novo*, to replicate double-stranded DNA, or to carry out synthesis in the 5' direction, it may be naive to expect a single enzyme to do so. The distinctive features of this DNA polymerase may lie in its ability to cooperate with other proteins functioning in DNA replication.

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III

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