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Purification and DNA Synthesis in Cell-Free Extracts: Properties of DNA Polymerase II

THOMAS KORNBERG AND MALCOLM L. GEFTER

Department of Biological Sciences, Columbia University, New York, N.Y. 10027

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ABSTRACT **The major DNA-synthesizing enzyme present in Pol A_1^- *Escherichia coli* (DNA polymerase II) has been purified to homogeneity as judged by polyacrylamide gel electrophoresis. The enzyme requires all four deoxynucleoside triphosphates, Mg^{++}, NH_4^+, and native DNA for maximal activity. The enzyme activity is sensitive to sulfhydryl reagents and is insensitive to anti-DNA polymerase I antiserum. A second DNA-synthesizing enzyme, present in low amounts, has been identified in Pol A_1^- extracts. The relationship of this enzyme to DNA polymerases I and II is discussed.**

The isolation of a mutant of *Escherichia coli* lacking detectable levels of DNA polymerase activity (polymerase I) (1) has stimulated several investigations into the nature of the DNA-synthesis capacity of Pol A_1^- cells (2–6). We have previously reported (7) that Pol A_1^- cells contain a DNA polymerase activity (polymerase II) that can be distinguished from polymerase I on the basis of sensitivity to high ionic strength, sulfhydryl reagents, and insensitivity to anti-DNA polymerase I antiserum. We now report the purification to homogeneity of polymerase II, and show that the properties previously reported for the partially purified enzyme are retained by the pure enzyme. Partial characterization of the pure enzyme has also been reported by others (8). A second polymerase activity, distinct from polymerases I and II, has been observed in Pol A_1^- extracts; its properties are described.

MATERIALS AND METHODS

Materials

E. coli W3110 *thy*$^-$, *rha*$^-$, *lac*$^-$, Str$^-$, Pol A_1^- was obtained from Dr. J. Gross. Purified *E. coli* DNA polymerase I, anti-DNA polymerase I antiserum, and purified exonuclease III were a gift from Dr. A. Kornberg. Dr. C. Radding kindly provided purified λ-exonuclease. Unlabeled deoxynucleoside triphosphates were purchased from Sigma. [^{3}H]thymidine triphosphate was purchased from Schwarz BioResearch and was adjusted to a specific activity of 2×10^5 cpm/nmol. DEAE-cellulose (DE 23) and phosphocellulose (P11) were products of Whatman. Electrophoresis supplies were products of Canalco. Calf-thymus DNA was purchased from Calbiochem.

Methods

Polymerase was assayed in an incubation mixture (0.3 ml) containing 20 μmol Tris-acetate (pH 7.4); 2 μmol $MgCl_2$; 1 μmol 2-mercaptoethanol; 10 nmol (each) dCTP, dGTP, dATP; 10 nmol [^{3}H]TTP; 32 nmol (of nucleotide) calf-thymus DNA; and $(NH_4)_2SO_4$, titrated to give maximal activity (see Fig. 4). Incubations were for 5 min at 30°C; nucleotide incorporation into acid-insoluble product was measured as described (7). One unit of enzyme is defined as the amount catalyzing the incorporation of 1 nmol of TTP into acid-insoluble product. The rate of reaction is increased by 50% when incubations are at 36°C. Exonuclease III was assayed as described by Richardson and Kornberg (8). Tritiated *E. coli* DNA, with a specific activity of 350 cpm/pmol, was used as the substrate.

Suitable template was prepared by freezing and thawing calf-thymus DNA 5 to 10 times. This "frozen" DNA was further activated by incubation with exonuclease III for 5 min at 30°C, and was used for all activity measurements. The reaction mixture contained (7.0 ml) 0.07 M Tris-acetate (pH 8.2); 0.01 M $MgCl_2$; 0.02 M 2-mercaptoethanol; 4.5×10^3 units of enzyme; and 20 μmol of nucleotide. The reaction was terminated by heating at 65°C for 10 min.

Protein determination was according to the method of Bucher, with bovine serum albumin as the standard. Salt concentration was measured using a conductivity bridge, with $(NH_4)_2SO_4$ as the standard. Polyacrylamide gel disc electrophoresis was performed in 7.0% gels, using the "Canalco" protocol. Samples in 0.02 M phosphate buffer, pH 7.5, were applied in 30% sucrose; the gels were electrophoresed at 25°C at 4 mA/gel for 3 hr and stained for protein overnight with 0.25% Coomassie Brilliant Blue.

Purification of DNA polymerase II

All steps were performed at 4°C and all buffers contained 2 mM 2-mercaptoethanol; 0.5 mM EDTA; and 10% glycerol. The purification is designed for 100 g of cell paste. A summary of the purification is given in Table 1.

TABLE 1. *Purification of DNA polymerase II*

	Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg)	Total units
I.	S100	250.0	14.7	0.051	187
II.	Phosphocellulose 1	500.0	6.5	0.062	200
III.	DEAE-cellulose	550.0	3.3	0.151	275
IV.	Phosphocellulose 2	2.8	1.2	58.0	195
V.	Sephadex G-200*	46.0	0.019	133.0	115

* 50% of the enzyme preparation was passed through Sephadex G-200. The values in the table are corrected to 100%.

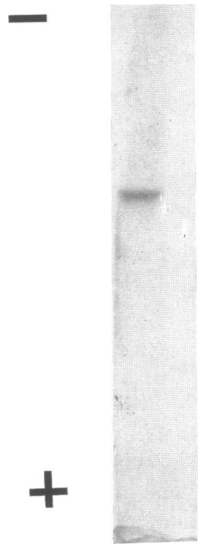

FIG. 1. Polyacrylamide gel electrophoresis of fraction V (see Table 1) enzyme (4.8 μg of protein). Fraction V was concentrated 20-fold by adsorption and elution from phosphocellulose.

S100. Preparation of the S100 cell-free extract (170 ml) was described (7). The S100 was brought to 250 ml, and a protein concentration of 13.1 mg/ml, by the addition of "lysis buffer", and was dialyzed against 10 volumes of 0.01 M potassium phosphate buffer, pH 6.5, for 2 hr.

Phosphocellulose 1. The dialyzed S100 was applied to a phosphocellulose column (13 cm^2 × 10 cm) that had been equilibrated with 0.01 M potassium phosphate buffer, pH 6.5. The flow rate was maintained at 2.5 ml/min and a single fraction (500 ml), containing all of the protein not adhering to the column, was collected.

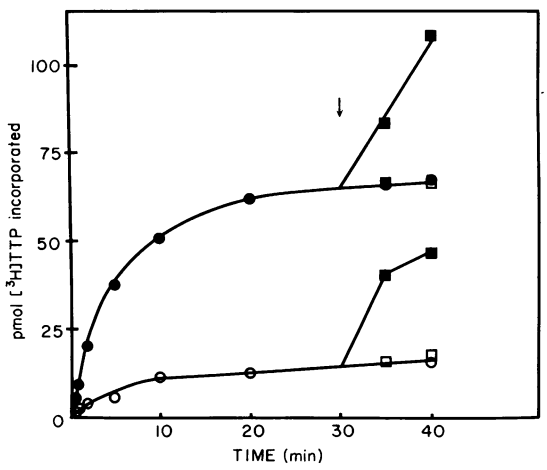

FIG. 2. Kinetic analysis of enzyme activity. Two reaction mixtures (4.5 ml each) were incubated with either exonuclease III-treated DNA (●——●) or "frozen" DNA (○——○), 225 nmol of nucleotide, and 0.75 units of fraction V enzyme. At the times indicated, samples (0.2 ml) were withdrawn and the acid-insoluble radioactivity determined. At 30 min (*arrow*), aliquots were removed and either exonuclease III-treated DNA (■——■) or 0.03 unit of enzyme (□—□) was added. Samples (0.2 ml) were withdrawn at 35 and 40 min.

DEAE-cellulose. The phosphocellulose eluate was brought to 0.2 M $(NH_4)_2SO_4$ by the dropwise addition of 0.01 M phosphate buffer, pH 7.5, containing $(NH_4)_2SO_4$ (50% of saturation). The sample was applied to a column of DEAE-cellulose (41 cm^2 × 10 cm) equilibrated with 0.01 M potassium phosphate, pH 7.5, containing 0.2 M $(NH_4)_2SO_4$. The protein not adhering to the column was collected in a single fraction (550 ml) and dialyzed against 10 volumes of 0.04 M potassium phosphate buffer, pH 6.5. There was one change of buffer, and dialysis was terminated when the sample reached 0.08 M salt (4 hr).

Phosphocellulose 2. The dialyzed DEAE-cellulose fraction was applied to a phosphocellulose column (4.1 cm^2 × 25 cm) previously equilibrated with 0.04 M potassium phosphate buffer, pH 6.5, at a flow rate of 36 ml/hr. The column was washed with 200 ml of 0.04 M potassium phosphate buffer, pH 7.5, and 20-ml fractions were collected. The enzyme was eluted with a linear gradient, 0.04–0.4 M potassium phosphate, pH 7.5 (total volume 2 liters). Polymerase activity eluted in two peaks: peak *A* emerged at 0.06 M and peak *B* at 0.18 M salt. Exonuclease III (specific activity, 180,000 units/mg) eluted between the two peaks, as shown in Fig. 3. Fractions were pooled as indicated, and peak *B* (140 ml) was dialyzed against 10 volumes of 0.04 M potassium phosphate buffer, pH 6.5, for 2 hr (0.1 M salt). It was then adsorbed to a column of phosphocellulose (1.3 cm^2 × 5 cm) that had been equilibrated with 0.04 M potassium phosphate buffer, pH 6.5. The flow rate was not allowed to exceed 10 ml/hr. The polymerase activity was eluted with 0.3 M potassium phosphate buffer, pH 7.5, and 0.5-ml fractions were collected. 80% of the activity applied was recovered. Fractions representing 60% of the applied activity were pooled (2.8 ml).

Sephadex G-200. A column of Sephadex G-200 (3 cm^2 × 50 cm), with an excluded volume of 45 ml, was equilibrated with 0.3 M potassium phosphate buffer, pH 7.5. One-half of the sample (1.4 ml) was loaded and 3-ml fractions were collected. 95% of the activity applied to the column eluted in a single

TABLE 2. *Properties of DNA polymerase II*

Additions	Incorporation (pmol)
Complete system*	25.5
− DNA	<0.5
− dATP, dGTP, dCTP	3.5
− Mg^{++} + EDTA (3 mM)	<0.5
− 2-mercaptoethanol + *N*-ethylmaleimide (10 mM)	4.0
− DNA + "activated" calf-thymus DNA	3.1
− DNA + calf-thymus DNA	5.3
− DNA + denatured DNA	1.1
− DNA + native T7-DNA	<0.5
+ DNase (75 μg/ml)	<0.5
+ KCl (0.2 M)	<0.5
+ Antiserum (20 μl) 5 min	17.5
+ Antiserum (20 μl) 0 min	18.0

* The components of the reaction mixture and the details of the assay are described in *Methods*. Reaction mixtures containing antiserum had reduced values of incorporation due to counting error. Antiserum added after the incubation period (5 min), and just prior to acid precipitation, served as a control.

peak after 72 ml. Fractions representing 60% of the applied activity were pooled (23 ml) and dialyzed for 2 hr against 10 volumes of 0.04 M potassium phosphate buffer, pH 6.5, until the salt concentration was 0.1 M.

RESULTS

Properties of DNA polymerase II

Enzyme from Sephadex G-200 has an absorbance ratio (280/260 nm) of 2.1, and shows a single band on polyacrylamide gels (Fig. 1). This fraction can be stored at 0°C for 1 month without loss of activity.

The pH optimum for the enzyme is 7.5 in Tris-acetate buffer. Optimal concentrations of magnesium ion and 2-mercaptoethanol are those used in the standard reaction mixture. The enzyme also requires all four deoxynucleoside triphosphates, and a suitable DNA as template, for maximal activity. The reaction can be inhibited by EDTA, *N*-ethylmaleimide, pancreatic DNase, and KCl. The reaction is unaffected by antiserum directed against DNA polymerase I. These properties are summarized in Table 2.

In the initial stages of purification (fraction III), "frozen" DNA is the most efficient template. After phosphocellulose chromatography, more than 50% of the activity applied was lost. The activity was recovered by the addition of an early-eluting fraction to the reaction mixture. The early-eluting fraction could be replaced by purified exonuclease III (see Fig. 3). Treatment of "frozen" DNA with exonuclease III renders this DNA 5 times as efficient as a template, in agreement with the results reported by Knippers (6). As shown in Fig. 2, both the initial rate and final extent of the reaction are determined by the template. Once synthesis has ceased, only the addition of fresh template leads to a resumption of synthesis; addition of more enzyme is without effect. When an excess of exonuclease III-treated DNA is used, incorporation of TTP is linear for up to 100 min of incubation, and the initial rate of the reaction is directly proportional to enzyme concentration (0.04–1.0 μg of fraction V).

Nondenatured, exonuclease III-treated, calf-thymus DNA is the most efficient template we have tested (see Table 2). Untreated, "frozen", or "activated" calf-thymus DNA is 5 to 8 times less efficient. Treatment of DNAs with alkaline phosphatase or λ-exonuclease has no effect on their respective template activities. Native or denatured T7 DNA is not active as a template. Native T7 DNA can be rendered active by treatment with pancreatic DNase and exonuclease III. However, it is not as effective as exonuclease III-treated calf-thymus DNA.

In addition to the separation of exonuclease III from polymerase II, phosphocellulose chromatography resolves a second polymerase activity (Fig. 3). All attempts to interconvert peaks *A* and *B* by rechromatography or salt treatment have failed. DEAE-cellulose chromatography can also resolve peaks *A* and *B* from fraction III. Peak *A* usually contains $^1/_3$ to $^1/_2$ of the activity present in peak *B*. With respect to requirements, peak *A* cannot be distinguished from peak *B*. Peak *A* is 3 times more sensitive to *N*-ethylmaleimide than peak *B* (polymerase I is 100% active in 10 mM *N*-ethylmaleimide), and both are totally insensitive to anti-DNA polymerase I antiserum.

The activity in peak *A* can be distinguished from the activity in peak *B* on the basis of its sensitivity to $(NH_4)_2SO_4$ (Fig. 4*A*). In contrast to peak *B*, which is maximally active at 7.5

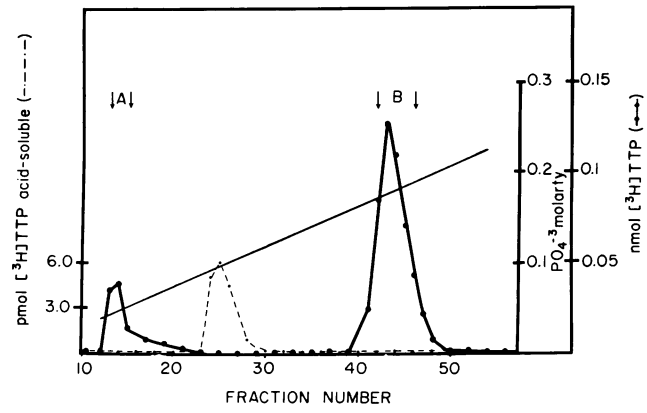

FIG. 3. Phosphocellulose chromatography of fraction III. Each fraction was assayed for DNA polymerase activity (●——●) and exonuclease III activity (- - -). Conditions were as described in *Methods*. Fractions bounded by arrows were pooled, and are referred to as peak *A* and peak *B* enzymes.

mM $(NH_4)_2SO_4$, peak *A* is only 35% active. $(NH_4)_2SO_4$ can be replaced by K_2SO_4, or other NH_4^+ or Na^+ salts; these, however, are less effective. (Because of the ionic-strength requirements for activity, each assay mixture is adjusted with $(NH_4)_2SO_4$ to give maximal activity.) Both peaks *A* and *B* are completely inhibited at salt concentrations above 75 mM. Because of their differential responses to salt, it is difficult to estimate the ratio of peak *A* to peak *B* in crude extracts. Polymerase I is relatively unaffected by salt. Peak *B* exhibits a 5-times greater thermal stability than peak *A* (Fig. 4*B*).

DISCUSSION

DNA polymerase II has been purified to homogeneity. The properties of the enzyme are in keeping with those previously reported by ourselves (7) and others (6, 9). Studies on tem-

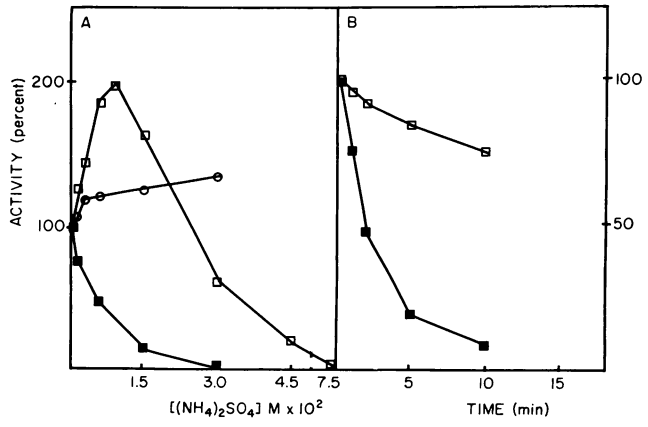

FIG. 4. *A*, Effect of salt on enzyme activity. $(NH_4)_2SO_4$ was added to standard reaction mixtures, unsupplemented with $(NH_4)_2SO_4$, containing 0.09 unit of DNA polymerase I (○——○); 0.025 unit of peak *A* enzyme (■——■); or 0.08 unit of peak *B* enzyme (□——□). Incubations were performed under standard conditions. *B*, Heat inactivation of enzyme activity. Peak *A* enzyme (■——■) and peak *B* enzyme (□——□) were adjusted to 0.5 mg of protein/ml with bovine serum albumin in 0.04 M phosphate buffer, and incubated at 45°C. At the times indicated, samples were withdrawn and assayed immediately for enzyme activity under standard conditions.

plate requirements suggest that the enzyme requires a 3′-hydroxyl group, and that synthesis occurs in the 5′- to 3′-direction. Studies using calf-thymus DNA treated with alkaline phosphatase and λ exonuclease suggest that 3′- or 5′-phosphoryl or 5′-hydroxyl groups are not sites for initiation of synthesis. We feel, however, that a sound conclusion concerning potential sites for initiation or *de novo* synthesis will require a more exhaustive study (now in progress) using natural templates.

During the course of purification, a second polymerase activity (peak *A*) present in Pol A_1^- extracts has been observed. On the basis of chromatographic behavior, salt inhibition, thermal stability, and insensitivity to anti-polymerase I antiserum, it can be clearly distinguished from polymerases I and II. All attempts to interconvert peak *A* and peak *B* (polymerase II) have failed. We must, however, be cautious in assuming that peak *A* is a distinct, new enzyme.

We have observed that reduction of salt from peak *B* preparations (dialysis or Sephadex filtration) to less than 0.04 M results in loss of activity and the appearance of an active form of the enzyme that will no longer adsorb to phosphocellulose. Preliminary experiments using Sephadex filtration and polyacrylamide gel electrophoresis indicate that the apparent molecular weight of the enzyme also varies with ionic strength. Given chromatographic and (probable) molecular weight variation of polymerase II, it is difficult to rule out the possibility that peak *A* is derived from peak *B*. We also cannot exclude the possibility that peak *A* is derived from peak *B* by proteolytic degradation, as has been observed with polymerase I (10).

The role of DNA polymerase II in DNA replication is still obscure. To date, no DNA synthesis-defective mutant that is defective in polymerase II has been reported. In addition, preliminary calculations have shown that there are approximately 100 molecules of polymerase II per cell, and that the rate of nucleotide incorporation per enzyme molecule is lower than polymerase I. (The rate measurement is, of course, limited to the templates studied to date.) Although we have measured DNA polymerase activity in crude extracts of Pol A_4^-, Rec A^- mutants, we have not determined if peak *A* or *B* enzyme is missing. Further work will be necessary to assess the role of polymerase II in replication.

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