DNA POLYMERASES OF ASCITES HEPATOMA CELLS

II. PURIFICATION AND PROPERTIES OF DNA POLYMERASES FROM NUCLEAR MEMBRANE-CHROMATIN FRACTION

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Summary. Two kinds of DNA polymerase (P-1 and P-2) have been purified from the nuclear membrane-chromatin fraction of rat ascites hepatoma cells. Polymerase P-1 differs from P-2 in several properties such as chromatographic behavior, sensitivity to p-chloromercuribenzoate, optimal conditions for the reaction, preference for template DNA and requirement of deoxyribonucleoside triphosphates.

A strain of Escherichia coli, pol A1 (Kornberg's enzyme negative) mutant, whose DNA synthesizing activity is located in the membrane-DNA fraction (1) has been isolated (2). DNA synthesis in this mutant seemed to proceed semiconservatively at the rates of chain growth comparable with replication in vivo (3). The polymerase activity was solubilized and purified, and the enzyme obtained was named DNA polymerase II (4). Furthermore, recent studies have revealed the presence of another DNA polymerase activity, DNA polymerase III, in this mutant (5). The mechanisms of chromosome replication in mammalian cells, however, still remained to be solved. Recently, mammalian chromosome replication has been reported to take place at the nuclear membrane (6, 7), but isolation and purification of DNA polymerase from the nuclear membrane-chromatin fraction has not been investigated. In this communication we describe the purification of DNA polymerases from nuclear membrane-chromatin fraction of ascites hepatoma cells. Two DNA polymerase activities P-1 and P-2 were partially purified and their properties were investigated.

MATERIALS AND METHODS

The assay of polymerase activity, and other materials and methods are

Table 1. Purification of DNA Polymerase from Nuclear Membrane-Chromatin Fraction of AH 108 A Cells

	Fraction	Total Units*	Specific Activity
		x 10^3	units/mg protein
I	Nuclear Membrane-Chromatin	3.9	66
II	DEAE-Sephadex Column	1.7	895
III	Phosphocellulose Column		
	P-1	0.55	4400
	P-2	0.70	3600

* A unit of polymerase activity is defined as the amount causing the incorporation of 1 mμmole of total nucleotides into acid-insoluble product in 60 min at 37°.

described in the preceding paper. The specific activity of the ^{3}H-TTP used was 30 mC/mmole.

RESULTS

Purification of polymerase

All operations were carried out at 0-2°. All buffers contained 10 mM 2-mercaptoethanol, 1 mM EDTA, and 20 % (v/v) ethylene glycol. The purification steps of the polymerase are summarized in Table 1.

Preparation of nuclear membrane-chromatin fraction. The nuclei of ascites cells were obtained according to Lynch et al. (8). The isolated nuclei were suspended in 12 ml of H-Solution (a solution containing 0.05 M potassium phosphate (pH 7.0), 0.15 M KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 1 M sucrose) and sonicated for 1-2 min, until all nuclei were lysed. The suspension was then centrifuged at 45,000 xg for 30 min. The precipitate was resuspended in 12 ml of H-Solution and centrifuged as above. The precipitate thus obtained was suspended in 5 ml of H-Solution and used as the nuclear membrane-chromatin fraction.

DEAE-Sephadex chromatography. To the suspension of nuclear membrane chromatin fraction was added 5 M NaCl solution to a final concentration of 1 M. After standing at 0° for 30 min, the suspension was centrifuged at 65,000 xg for 30 min. The precipitate showed no DNA polymerase activity. The supernatant

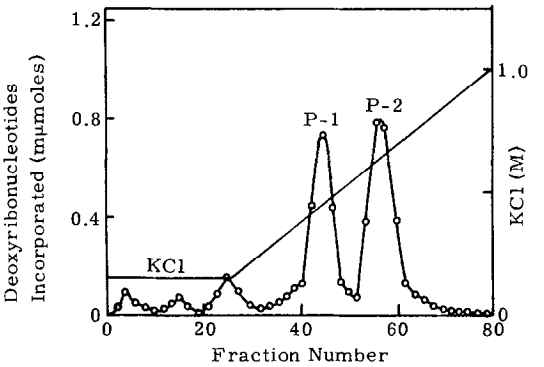

Figure 1. Phosphocellulose chromatography of DEAE-Fraction.

was diluted with 4 volumes of Dilution Buffer (0.02 M potassium phosphate (pH 7.0)) to lower the NaCl concentration and added 25 ml of DEAE-Sephadex (A-50) previously equilibrated with 0.05 M potassium phosphate buffer (pH 6.5) containing 0.2 M KCl. The suspension was then transferred to a column of DEAE-Sephadex (A-50)(3.14 cm^2 x 12 cm) which had been previously equilibrated with the above solution. The polymerase was eluted with a linear gradient of KCl (a total volume of 300 ml) from 0.2 to 1.2 M in 0.05 M potassium phosphate buffer (pH 6.5). Polymerase activity which was eluted out between 0.4-0.58 M KCl were pooled (DEAE-Fraction).

Phosphocellulose chromatography. DEAE-fraction was diluted 1:2 (v/v) with Dilution Buffer and applied to a column of phosphocellulose (1.12 cm^2 x 18 cm) previously equilibrated with 0.1 M potassium phosphate buffer (pH 6.8) containing 0.15 M KCl. A linear gradient elution with KCl (a total volume of 200 ml) from 0.15 to 1 M in 0.1 M potassium phosphate buffer (pH 6.8) was carried out. Two peaks of polymerase activity were eluted out separately at KCl concentration of 0.38-0.46 M and 0.56-0.65 M, respectively (Fig. 1). These two fractions were designated as P-1 and P-2. P-1 lost its activity by storage in liquid nitrogen for one week but P-2 maintained its full activity for more than 2 months under the same conditions.

Properties of Polymerase

Optimal conditions for the reaction. The incorporation of ^{3}H-TTP into DNA by P-1 and P-2 (each preparation contained 0.16 and 0.19 μg protein, respectively) proceeded linearly upto 60 minutes and the extent of incorporation was proportional to the amount of enzyme at least to 0.3 μg under standard reaction conditions. The enzymes, P-1 and P-2, showed several strikingly different properties from each other. Thus P-1 showed pH optimum at 6.3 in phosphate, 7.3 in Tri-HCl, and 8.5 in glycine-KOH buffer, while P-2 showed pH optimum at 9.8 in glycine-KOH buffer. P-1 revealed maximal incorporation of ^{3}H-TTP into DNA at Mg^{++} concentration of 3-5 mM in phosphate buffer (pH 6.5), while P-2 showed the maximum at 16 mM Mg^{++} in phosphate buffer (pH 7.3). As for the effect of the concentration of buffers for polymerase activities, the maximal activity for P-1 was observed in 40 mM potassium phosphate buffer (pH 6.5), and that for P-2 in 3 mM phosphate buffer (pH 7.3).

Requirements for polymerase reaction.

In Table 2, the effect of several compounds on DNA synthesis is summarized. Both DNA polymerase activities require Mg^{++} and template DNA. Neither Mn^{++} nor Ca^{++} can replace Mg^{++}. The decreases in the incorporation of ^{3}H-TTP into acid-precipitable material in these DNA polymerase reactions by addition of unlabeled TTP or sodium pyrophosphate indicate that the nucleoside triphosphates are the substrate for these reactions. p-Chloromercuribenzoate strongly inhibits the reaction of P-1 but weakly that of P-2.

These two polymerases show significant incorporation of ^{3}H-TTP into acid-insoluble product even in the absence of one to three kinds of deoxyribonucleoside triphosphates. In the case of P-2, 80 % of the control incorporation is observed in the absence of one triphosphate and 66 % of the control incorporation occurs even when three triphosphates other than TTP are omitted. This characteristic is similar to that of DNA polymerase of KB cells (9) and is different from the findings with the DNA polymerases from E. coli (10), Ehrlich

Table 2. Requirements for DNA Polymerase Activity

Condition	Relative Activity	
	P-1	P-2
Control	100	100
-DNA	0	0
-2-mercaptoethanol	100	98
-Mg^{++} + EDTA (8 mM)	0	0
-Mg^{++} + Mn^{++} (8 mM)	0	2.8
-Mg^{++} + Ca^{++} (8 mM)	0	2.4
+ATP (2 mM)	95	84
+TTP (0.8 mM)	7.3	16
+Sodium pyrophosphate (2 mM)	65	94
+p-Chloromercuribenzoate ($4x10^{-4}$M)	23	84
-dATP	29.5	77.6
-dATP, -dCTP	24.1	72.9
-dATP, -dCTP, -dGTP	22.0	66.3

Assay was performed by standard method with modifications indicated in the table. In the control reactions, 0.85 mμmoles and 0.8 mμmoles of total nucleotides were incorporated into acid-insoluble product by P-1 and P-2, respectively.

ascites cells (11), and soluble fraction of AH 108 A cells (preceeding paper).

Template efficiency.

As is shown in Table 3, both P-1 and P-2 enzymes preferentially utilize activated DNAs of E. coli, calf thymus, and ascites hepatoma cells as template. Native DNA, heat-denatured DNA or that treated with micrococcal nuclease does not serve as effective templates. It is noteworthy that P-2 enzyme showed extremely high preference for poly d(A-T).

DISCUSSION

As is described above, two kinds of DNA polymerase were purified from the nuclear membrane preparation of rat ascites hepatoma cells. Since chromosomal replication in mammalian cells has been reported to take place at their nuclear membrane (6, 7), and the DNA polymerases obtained this time were purified from a nuclear membrane fraction, there is the possibility that these enzymes are involved in the DNA replication in vivo.

Both polymerases showed several different properties from each other.

Table 3. Relative Template Efficiency of Various DNAs

Template DNA	Relative Efficiency (%)	
	P-1	P-2
Calf thymus, DNase I treated	100	100
E. coli, native	10	9
E. coli, heat denatured	22	4.5
E. coli, DNase I treated	280	142
E. coli, micrococcal nuclease treated	6	9
Ascites hepatoma cells, DNase I treated	47	99
Poly d(A-T), native	20	238
Poly d(A-T), DNase I treated	61	302

Assay was performed by standard method except that the DNAs indicated were used. When 600 μM of poly d(A-T) was used as DNA, 160 μM of dATP and TTP were added and dGTP and dCTP were omitted. In the reaction with DNase I treated calf thymus DNA, 0.5 mμmoles and 0.8 mμmoles of total nucleotides were incorporated into acid-insoluble product by P-1 and P-2, respectively.

P-2 utilized poly d(A-T) as a most effective template like E. coli DNA polymerase I (10). P-2 is clearly different from P-1 in this respect and this characteristic of P-2 also differs from that of DNA polymerases from soluble fraction of AH 108 A cells (preceeding paper), Ehrlich ascites cells (11), and KB cells (9). All four deoxyribonucleoside triphosphates were required for maximal incorporation of TTP (Table 2), but in the case of P-2 enzyme, the incorporation proceeded up to 66 % of the control reaction when only one triphosphate (^{3}H-TTP) was present. However, since the extent of replication of the template was very small, the reaction observed could be a form of very limited repair synthesis in which one or few thymidine monophosphate were being incorporated at many single strand breaks. An analogous interpretation has been reported in the case of KB cells DNA polymerase (9). We believe that P-2 does not have a type of terminal deoxynucleotidyl transferase activity as found in calf thymus gland (12) for three reasons as follows; (1) Terminal transferase incorporates one deoxyribonucleoside triphosphate at a maximal rate in the absence of the other three triphosphates. P-2 showed, however, high activity in the presence of the other three triphosphates. (2) Reaction velocity with terminal transferase is faster with dATP than with TTP (12). In the case of P-2, the same reaction

velocities were observed with dATP and TTP (unpublished data). (3) $(pT)_5$ is effective primer for terminal transferase (12), but no incorporation of triphosphate into acid-insoluble product was observed when $(pT)_5$ was used as a primer for P-2 (unpublished data).

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