DNA POLYMERASES OF ASCITES HEPATOMA CELLS

I. PURIFICATION AND PROPERTIES OF A DNA POLYMERASE FROM SOLUBLE FRACTION

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Summary: DNA polymerase from soluble fraction of ascites hepatoma cells has been purified about 490-fold. The polymerase requires template DNA, all four deoxyribonucleoside triphosphates, and magnesium ions for the reaction. Optimal activity was found at pH 7.0 - 7.5, with 3 - 8 mM magnesium chloride, and 20 - 40 mM potassium phosphate. The purified enzyme utilizes preferentially DNA treated with pancreatic DNase as template.

In prokaryotic cells, extensive studies on DNA polymerases have been carried out and several DNA polymerase activities have been characterized (1-4). On the other hand, in mammalian cells, the exact nature of the DNA polymerase activities has not yet been fully investigated because of their instability. Recently, however, achievements in the purification of DNA polymerases from several mammalian sources such as KB cells, HeLa cells, and Ehrlich ascites cells have been reported (5-7). In this paper we communicate the purification and some general properties of DNA polymerase from the soluble fraction of ascites hepatoma cells, AH 108A cells. The purification of the DNA polymerase from nuclear membrane-chromatin fraction of the same cells is reported in the following paper.

MATERIALS AND METHODS

Ascites hepatoma cells. Rat ascites hepatoma cells used in this experiment was a strain named AH 108A which was established in 1961 (8). Ascites fluid was harvested 10 days after intraperitoneal inoculation of AH 108A cells to the healthy female rats and the cells were collected as described previously (7).

Assay of polymerase. The standard assay mixture contained the followings in a total volume of 0.25 ml : 40 mM potassium phosphate buffer (pH 7.3), 8 mM magnesium chloride, 1 mM 2-mercaptoethanol, 600 μM* "activated" calf thymus DNA (9), 80 μM each of dATP, dGTP, dCTP, and ^{3}H-TTP (specific activity 10 mC / mmole) and enzyme. After incubation for 60 min at 37°, acid-insoluble radioactivity was measured as described previously (10).

Other methods. Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as standard. Activated DNAs were prepared according to Richardson et al. (9).

RESULTS

Purification of Polymerase

Unless otherwise indicated, all operations were carried out at 0-4° and all buffers contained 10 mM 2-mercaptoethanol, 1 mM EDTA, and 20 % (v/v) ethylene glycol. The purification procedure and results of a typical preparation are summarized in Table 1.

Preparation of extracts. The packed ascites cells (50 g) were suspended in 50 ml of H-Solution (a solution containing 0.05 M potassium phosphate (pH 7.0)

* molarity of DNA refers to concentration of nucleotide residues.

Table 1. Purification of DNA Polymerase from AH 108 A Cells

Fraction	Total units*	Specific Activity
	x 10^{-3}	units/mg protein
I Extract	52	19
II Supernatant I	46	23
III Supernatant II	50	40
IV Sephadex G-100 Column	21	70
V DEAE-Sephadex Column	20	1200
VI Phosphocellulose Column I	8.6	4500
VII Phosphocellulose Column II	4.8	9400

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

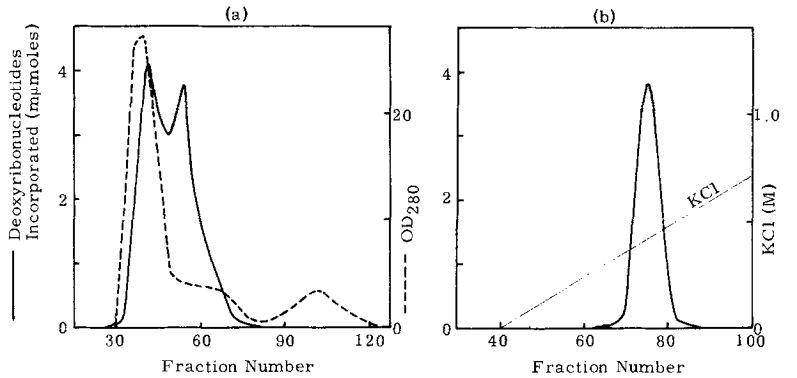

Figure 1. (a) Sephadex G-100 chromatography. (b) Phosphocellulose rechromatography.

0.15 M KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, and 1 M sucrose) and sonicated (Fraction I, 90 ml). The suspension was centrifuged at 20,000 x g for 90 min and the supernatant (Fraction II, 80 ml) was collected. Fraction II was adjusted to 20 mM $MgCl_2$ and centrifuged at 105,000 x g for 6 hours. The clear supernatant was recovered (Fraction III, 70 ml).

Sephadex G-100 chromatography. A Sephadex G-100 column (12.5 cm^2 x 65 cm) was equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). Fraction III (70 ml) was applied to the column and eluted with the same buffer. The polymerase activity was separated into two peaks (Fig. 1-a) and the first peak fractions were collected (Fraction IV ; 80 ml).

The DNA polymerase which was contained in the second peak fractions was separated into more than two active peaks by subsequent chromatography on DEAE-Sephadex column. This time, they were not investigated further.

DEAE-Sephadex chromatography. Fraction IV (75 ml) was applied to a column of DEAE-Sephadex (A-50) (3.14 cm^2 x 20 cm) equilibrated with 0.2 M potassium phosphate buffer (pH 6.5). The column was washed with 160 ml of the above buffer and then eluted with a linear gradient of KCl (a total volume of 300 ml) from 0 to 1 M dissolved in the above buffer. The fractions which were eluted out between 0.35 to 0.55 M KCl contained major polymerase activity.

These fractions were pooled (Fraction V, 80 ml) for further purification.

Phosphocellulose chromatography. A column of phosphocellulose (1.76 cm^2 x 15 cm) was equilibrated with 0.1 M potassium phosphate buffer (pH 6.5). Fraction V was diluted 1 : 5 (v/v) with a Dilution Buffer (0.02 M potassium phosphate buffer (pH 6.8)) and applied to the column. A linear gradient elution with KCl (a total volume of 200 ml) from 0 to 1 M dissolved in 0.1 M potassium phosphate buffer (pH 6.5) was carried out. DNA polymerase activity was eluted out at KCl concentration of 0.40 - 0.53 M. Active fractions were combined (Fraction VI, 32 ml).

Phosphocellulose rechromatography. A column of phosphocellulose (1.13 cm^2 x 20 cm) was equilibrated with 0.1 M potassium phosphate (pH 7.0). Fraction VI (30 ml) was diluted 1 : 5 (v/v) with the Dilution Buffer and applied to the column. After adsorption of the enzyme to phosphocellulose, a linear gradient elution with KCl (a total volume of 160 ml) from 0 to 0.7 M dissolved in the above buffer was performed. DNA polymerase activity was eluted at KCl concentration of 0.35 - 0.45 M as a symmetrical peak (Fig. 1-b). Active fractions were combined (Fraction VII, 28 ml), which can be stored in liquid nitrogen for 2 months without loss of activity.

Properties of Polymerase

Sucrose density gradient centrifugation of the enzyme. To test the homogeneity of the purified polymerase and the complex formation between the enzyme and DNA, the purified polymerase (Fraction VII) and activated DNA obtained from ascites cells were mixed and subjected to a neutral sucrose density gradient centrifugation. As is shown in Fig. 2, polymerase activity migrates as a single peak. Under these conditions, the complex formation of polymerase with ascites cells DNA can not be observed.

Optimal conditions of the enzymatic reaction. The reaction proceeded linearly for 90 min with 0.92 μg of enzyme protein, and was proportional upto 3 μg of enzyme under standard assay conditions. Maximal incorporation of ^3H-

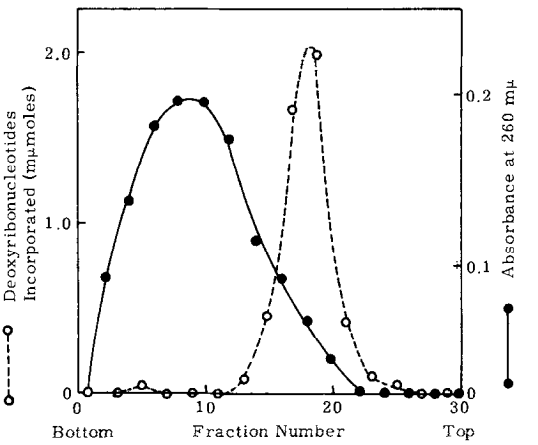

Figure 2. Sucrose density gradient sedimentation of enzyme. Fraction VII (160 units) was mixed with activated DNA (28 μg) obtained from ascites cells in the presence of 10 mM $MgCl_2$ and was layered over 5.0 ml of a 5 to 20 % linear sucrose gradient containing 0.15 M KCl, 10 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 1 mM dithiothreitol. Centrifugation was at 50,000 rpm for 6 hours at 2° in a Hitachi RPS 50 rotor. After collecting fractions from the bottom of the tube polymerase activity was measured as described in the text.

Table 2. Requirements for DNA Polymerase Activity

Condition	Relative Activity	Condition	Relative Activity
Control	100%	+Sodium pyrophosphate (2 mM)	73
-DNA	2	+p-Chloromercuribenzoate ($4x10^{-4}$M)	6
-2-mercaptoethanol	87	-dATP	20
$-Mg^{++}$, +EDTA (8 mM)	4	-dATP, -dCTP	13
$-Mg^{++}$, $+Mn^{++}$ (8 mM)	2	-dATP, -dCTP, -dGTP	9
$-Mg^{++}$, $+Ca^{++}$ (8 mM)	0	+Pronase (20 μg/ml)	1
+ATP (2 mM)	92	+DNase I (20 μg/ml)	0
+TTP (0.8 mM)	22	+RNase A (20 μg/ml)	100

Assay was performed by the standard method as described in the text with modifications indicated in the table. In control reaction, 4.5 mμmoles of total nucleotides were incorporated into acid-insoluble product.

TTP into acid-insoluble product was observed at pH 7.0 - 7.5, with 20 - 40 mM potassium phosphate, 3 - 8 mM magnesium chloride.

Requirements for polymerase reaction. As is shown in Table 2, purified polymerase (Fraction VII) requires template DNA, all four deoxyribonucleoside triphosphates, and magnesium ions for the reaction. Neither Mn^{++} nor Ca^{++}

Table 3. Relative Template Efficiency of Various DNAs

Template DNA	Relative Efficiency (%)
Calf thymus, DNase I treated	100
E. coli, native	2
E. coli, heat denatured	5
E. coli, DNase I treated	198
E. coli, micrococcal nuclease treated	5
Ascites hepatoma cells, DNase I treated	41
Poly d(A-T), native	14
Poly d(A-T), DNase I treated	20

Assay was performed by the standard method as described in the text except that the DNAs indicated were used. When 600 μM poly d(A-T) was used as template, 160 μM of dATP and TTP were added and dGTP and dCTP were omitted. All activities are expressed relative to that obtained with DNase I treated calf thymus DNA (2.0 mμmoles of total nucleotides were incorporated into acid-insoluble product in the reaction with DNase I treated calf thymus DNA).

can replace Mg^{++}. Addition of unlabeled TTP or sodium pyrophosphate depresses the incorporation of ^{3}H-TTP into acid-precipitable material. These results indicated that the triphosphates are the substrates for the polymerization reaction. The enzyme is strongly inhibited by p-chloromercuribenzoate, like E. coli DNA polymerase II (2, 3).

Template efficiency. As is shown in Table 3, the polymerase preferentially utilizes DNA treated with pancreatic DNase (activated DNA) as the template. Native DNA, heat-denatured DNA and micrococcal nuclease treated DNA are not effective templates. Poly d(A-T) is a better template than the native DNA, however, treatment of poly d(A-T) with pancreatic DNase showed no remarkable increase of template activity in contrast to the case of the similar treatment of the natural DNA.

DISCUSSION

Rat ascites hepatoma cells (AH 108 A) used in this experiment are rapidly growing tumor cells and have been maintained more than 350 generations without any variation of its character. These cells can be easily obtained in large amounts (i.e. 3-4 g packed cells from a rat), and are suitable material for the studies of enzymes involved in chromosomal replication.

When the soluble fraction of the above mentioned ascites cells was directly applied to a column of Sephadex G-100 without any prior fractionation, the polymerase activity was separated into two peaks (Fig. 1-a). From the fraction having higher molecular weight, a DNA polymerase was highly purified as described above. The DNA polymerase activity of rat liver and hepatomas has been reported to be separated into two peaks in Sephadex G-100 chromatography and the polymerase having higher molecular weight was found to increase in the rapidly growing tumor cells (12,13). Judging from the chromatographic pattern, the DNA polymerase purified this time can correspond to the enzyme which increases in tumor cells.

The distribution, the number of species of DNA polymerases in one kind of malignant cells and their metabolic roles are now under investigation.

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