CONVERSION OF DNA POLYMERASE EXTRACTED FROM RAT ASCITES HEPATOMA CELLS

Kazushi Tanabe and Taijo Takahashi

Laboratory of Biochemistry

Aichi Cancer Center Research Institute

Chikusa, Nagoya, Japan

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SUMMARY: DNA polymerase extracted fresh from rat ascites hepatoma cells possesses high molecular weight, maximal activity at neutral pH, and high sensitivity to N-ethylmaleimide (NEM). After physical and chemical treatment of the enzyme fraction, the appearance of low molecular weight DNA polymerase was detected by means of Sephadex gel filtration or sucrose density gradient centrifugation. This low molecular weight DNA polymerase possesses alkaline pH optimum, preference of native DNA as template/primer, and relative resistance to NEM.

Recently, there have been a number of reports which suggest that two or more molecular species of DNA polymerase occur in mammalian cells (1-8). The separation of the species was achieved by gel filtration, sucrose gradient centrifugation, DEAE- or phospho-cellulose column chromatography. The relationship between species of polymerase has, however, not been clear. In this paper, we report the evidence for a low molecular weight polymerase released after physical and chemical treatment of high molecular weight DNA polymerase extracted with hypotonic solution from rat ascites hepatoma AH130 cells.

MATERIALS AND METHODS

Extraction of DNA Polymerase from AH130 Cells. Rat ascites hepatoma AH130 cells were collected from ascites fluid after 7 days inoculation and washed once with cold isotonic NaCl and 4

times with cold 10 mM potassium phosphate buffer, pH 7.1, containing 1 mM ethylenediaminetetraacetate (EDTA) and 0.14 M KCl for removal of red cells. Washed cells were suspended in 10 volumes of hypotonic solution consisting of 10 mM 2-mercaptoethanol and 1 mM EDTA, and was stirred gently for 20 minutes with a magnetic stirrer (9). During the extraction, cells were swollen but not punctured, therefore neither DNA nor DNase was detected in the extract after centrifugation. Solid KCl was added to the supernatant after high speed centrifugation to 0.15 M final concentration. Supernatant fluid (Fraction I) after centrifuging once more at 12,000 rpm for 15 minutes was brought to pH 5.0 with 1 % acetic acid and centrifuged at 12,000 rpm for 15 min. The pellet was suspended in 10 mM potassium phosphate buffer, pH 7.1 containing 1 mM EDTA, 5 mM 2-mercaptoethanol and 0.15 M KCl (Buffer A) and was stirred gently for 4 hours at 0^{o}C. The undissolved material was removed by high speed centrifugation, and 3 ml of the supernatant fluid (Fraction II) was charged on to a Sephadex G-200 gel filtration column previously equilibrated with Buffer A and eluted with the same buffer.

DNA Polymerase Activity Assay. The reaction mixture contains the following in a total volume of 0.2 ml : 75 mM Tris-HCl buffer, pH 7.1 or 9.1, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.25 mM EDTA, 75 µM each of dATP, dGTP, dCTP, 10 µM of ^{3}H-dTTP (specific activity 250 µCi/µmole), 30 µg of activated (10) calf thymus DNA (heated or unheated), 0.1 mg of bovine serum albumin (crystalline) and enzyme fraction. After incubation at 37^{o}C for 30 minutes, the reaction was stopped by chilling and addition of 0.05 ml of 0.5 N NaOH. Carrier bovine albumin (0.5 mg) and 1.5 ml of cold 10 % CCl_3COOH containing 0.1 M sodium pyrophosphate were added and the mixture kept for 10 min. at 0^{o}C. After centri-

fugation at 3,500 rpm for 10 min. the pellet was dissolved in 0.1 ml of 0.5 N NaOH. Acid precipitation and alkaline dissolution was repeated twice. To the final pellet, 0.2 ml of 5 % CCl_3COOH was added and the suspension heated at 90°C for 20 min. The supernatant of the heated solution was transferred into a vial containing POPOP-PPO-dioxane scintillator and counted by Beckman Liquid Scintillation Counter.

RESULTS

When Fraction II from freshly harvested AH130 cells was charged on to a gel filtration column, DNA polymerase activity was recovered in a single peak near the elution front (Fig. 1a,

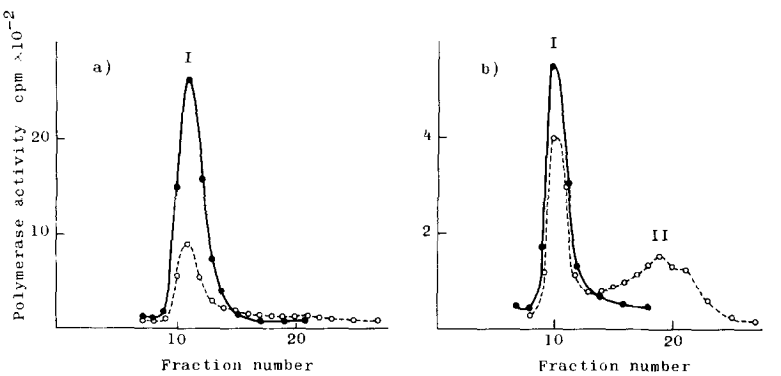

Fig. 1. Sephadex G-200 Gel Filtration of AH130 DNA Polymerase. 3 ml of Fraction II (see Materials and Methods) was loaded onto a Sephadex G-200 gel column (2.5 × 40 cm) equilibrated previously with Buffer A and eluted with same buffer by the upward method. a) Fraction II obtained fresh from AH130 cells. b) Fraction II after 10 days storage at 0°C. ●——● with heated template/primer at pH 7.1, o---o with unheated template/primer at pH 9.1.

peak I). From the experimental data using Sepharose 6B, the molecular weight of this activity was estimated as 600,000 or more. This DNA polymerase had a maximal activity at neutral pH, and was nearly equally active with both heated and unheated DNA

as template/primer. Furthermore, this activity was very sensitive to N-ethylmaleimide (NEM) or p-chloromercuribenzoic acid (pCMB).

When the gel filtration was repeated after seven to ten days storage of Fraction II at $0^{o}C$, a new peak of DNA polymerase activity was observed (Fig. 1b, peak II). This enzyme had a molecular weight of 70,000 to 80,000, by comparison with the elution profile of bovine serum albumin. In contrast to peak I enzyme, peak II polymerase had a maximal activity at alkaline pH (9 or higher), required only unheated template/primer, and was relatively resistant to NEM or pCMB inhibition.

This peak II polymerase was also detected in Fraction II prepared from AH130 cells which were frozen at $-80^{o}C$ (Fig. 2). Moreover, when 2-mercaptoethanol in hypotonic solution and elution buffer was replaced with dithiothreitol (DTT), peak II enzyme appeared without prolonged storage at $0^{o}C$ (Fig. 3).

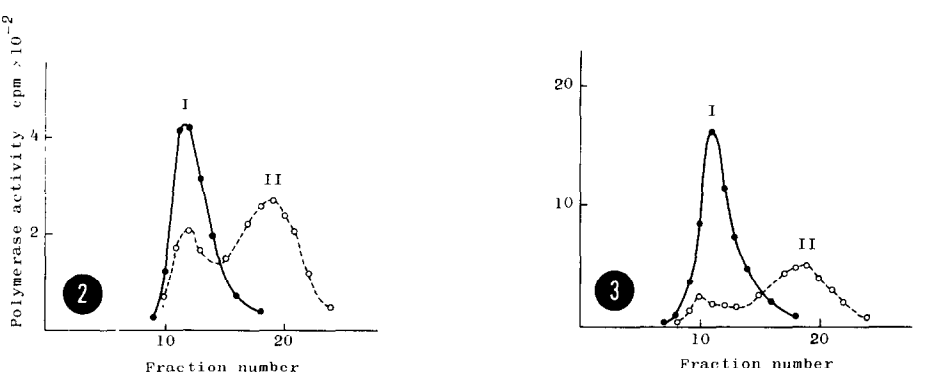

Fig. 2. Gel Filtration of DNA Polymerase from Frozen AH130 Cells. AH130 cells frozen at $-80^{o}C$ were thawed and used to prepare Fraction II. Gel filtration was performed as described in Fig. 1.

Fig. 3. Gel Filtration of DNA Polymerase from AH130 Prepared in the Presence of DTT. 2-Mercaptoethanol in hypotonic solution and Buffer A for the preparation of Fraction II was replaced with 0.5 mM DTT. Gel filtration was performed with Buffer A containing DTT after overnight storage of Fraction II at $0^{o}C$.

Appearance of the new, low molecular weight DNA polymerase activity was also observed by sucrose density gradient centrifugation. The profile of sucrose gradient centrifugation of Fraction I showed that DNA polymerase activity was associated with some particles smaller than ribosomal monomer (Fig. 4a).

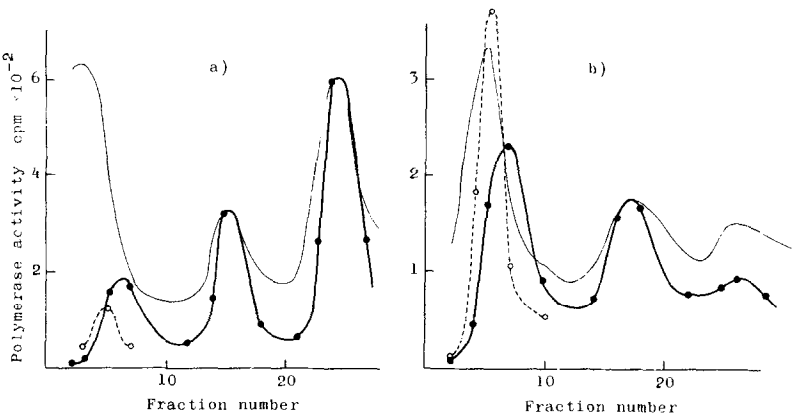

Fig. 4. Sucrose Density Gradient Centrifugation of AH130 DNA Polymerase. a). One ml of Fraction I was layered on 35 ml of 10-30 % (w/v) linear sucrose density gradient containing 10 mM potassium phosphate buffer, pH 7.1, 5 mM 2-mercaptoethanol, 1 mM EDTA. Sedimentation was carried out for 19 hours at 20,000 rpm at 2°C, using Beckman SW 27 rotor. Fractions of 1 ml were collected by ISCO fractionator. Direction of sedimentation was from left to right. —— O.D. at 260 nm. b). The supernatant of 105,000 ×g, one hour centrifugation of Fraction I was recentrifuged at 105,000 ×g for 15 hours and the pellet was dissolved in Buffer A. One ml of this sample was layered on a sucrose gradient and sedimented as a).

Similar result was reported by Baril et al. (11). Although these particles could not be spun down in one hour centrifugation at 105,000 ×g, most of polymerase activity could be found in the pellet after the additional 15 hour centrifugation at 105,000 ×g. The pellet of 15 hour centrifugation was dissolved in small volume of Buffer A and the sucrose density gradient centrifugation was repeated (Fig. 4b). Enzyme activity of larger parti-

cles was reduced and at the top of the gradient was found a new DNA polymerase activity which preferred double stranded template/primer and had pH optimum at 9.0.

DISCUSSION

Two (or more) DNA polymerases have been separated from many species of mammalian cells by means of gel filtration, DEAE- or phospho-cellulose chromatography or sucrose density gradient centrifugation (1-8). However, there has been no evidence concerning the relationship between these polymerases.

We have reported here the fact that the low molecular weight DNA polymerase was released by physical and chemical treatment from the high molecular weight (or complex) DNA polymerase and is distinct from the latter with respect to pH optimum, template/primer preference and NEM sensitivity. From this evidence, we suggest that the high molecular weight DNA polymerase is a complex form containing the low molecular weight enzyme.

Ove et al. (2) reported the appearance in rat hepatoma cells of a high molecular weight DNA polymerase different from the low molecular weight enzyme observed in normal and regenerating rat liver cells. Our data on normal rat liver DNA polymerase suggested that peak I as well as peak II activity on gel filtration existed in the fresh extract from normal cells (12). Thus the finding by Ove et al. (2) could be explained if the conversion of DNA polymerase (peak I → peak II) proceeds so rapidly in normal liver cells that peak I activity is lost during the extraction and purification process. On the other hand, the conversion proceeds slowly in hepatoma cells, therefore we can observe peak I as well as peak II after the extraction.

The agents responsible for this conversion, which appear

to be high in normal liver cells but very low in ascites hepatoma cells, has not yet been identified. We are now investigating whether the factor(s) is i) a SH compound in the cells, or ii) a proteolytic enzyme.

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REFERENCES

1. Iwamura, Y., Ono, T., and Morris, H.P., Cancer Res., 28, 2466 (1968).
2. Ove, P., Brown, O.E., and Laszlo, J., Cancer Res., 29, 1562 (1969).
3. Wallace, P.G., Hewish, D.R., Venning, M.M., and Burgoyne, L.A., Biochem. J., 125, 47 (1971).
4. Chang, L.M.S., and Bollum, F.J., J. Biol. Chem., 246, 5835 (1971).
5. Weissbach, A., Schlabach, A., Fridelender, B., and Bolden, A., Nature New Biology, 231, 167 (1971).
6. Chiu, J.F., and Sung, S.C., Nature New Biology, 239, 176 (1972).
7. Smith, R.G., and Gallo, R.C., Proc. Nat. Acad. Sci., 69, 2879 (1972).
8. Tsuruo, T., Tomita, Y., Satoh, H., and Ukita, T., Biochem. Biophys. Res. Commun., 48, 776 (1972).
9. Roychoudhury, R., and Bloch, D.P., J. Biol. Chem., 244, 3359 (1969).
10. Aposhian, H.V., and Kornberg, A., J. Biol. Chem., 237, 519 (1962).
11. Baril, E.F., Brown, O.E., Jenkins, M.D., and Laszlo, J., Biochemistry, 10, 1981 (1971).
12. Tanabe, K., and Takahashi, T., unpublished results.