A CONVERSION FACTOR FOR CYTOPLASMIC DNA POLYMERASE OF RAT LIVER

Kiyokazu Morioka and Hiroshi Terayama

Zoological Institute, Faculty of Science, University of Tokyo
Tokyo, Japan

Received October 8,1974

SUMMARY: DNA polymerase of 6-8S prepared from rat liver cytosol was found to be dissociated into active 3.3S DNA polymerase by column chromatography on phosphocellulose. This 3.3S enzyme was converted into a 5S form in the presence of a factor derived from cytoplasm in both high and low ionic strength media, with an accompanied change in the template specificity. The converting activity seems to be associated with a thermolabile protein of about 4-5S.

Two molecular species (3.3S and 6-8S) of DNA-dependent DNA polymerase distinct from the mitochondrial enzyme (1-3) have been purified from mammalian sources by Bollum and co-workers (4-7). Immunological relationship between these two species was shown by Chang and Bollum (8) and the interconversion between 3.3S and 6-8S forms of DNA polymerase was observed by Hecht (9). Later, however, the results not necessarily supporting the above findings have also been obtained by Holmes et al (10) and Spadari et al (11), showing that the interconversion may not be achieved so easily as suggested by Hecht, and furthermore the two species of DNA polymerase are immunologically unrelated at least in HeLa cells (11). On the other hand dissociation of the high-molecular-weight cytoplasmic DNA polymerase into a 5S or 7-10 x 10^4 molecular weight component has been reported by many investigators (7, 12-14), and Yoshida et al (15) have recently shown that the high-molecular-weight (6.7S-8.5S) DNA polymerases are dissociated into active smaller molecules (5.4S and 3.8S) upon freezing and thawing, 2M urea treatment or gel-filtration.

In the present study we have observed that the 6-8S DNA polymerase is converted to the 3.3S active form by column chromatography on phosphocellulose and that the 3.3S active molecule is further converted to the 5S form by the addition of a cytoplasmic factor. Preliminary characterization of this factor has been attempted.

Materials and Methods

Assay of DNA polymerase: The standard reaction mixture (0.25 ml) contained 50 mM Tris-Cl (pH 7.6), 6 mM $MgCl_2$, 2 mM β-mercaptoethanol, 80 μM each of dATP, dGTP and dCTP, 20 μM [^{3}H] dTTP (Radiochemical Center, Amersham, U.K. 70 or 140 mCi/mmole), 20 μg activated (16) calf thymus DNA, 10 μg crystalline bovine serum albumin and the enzyme solution (50-200 μl) which was added last. One unit of the enzyme activity was tentatively defined as the activity to incorporate 1 nmole of labelled dTTP into DNA during the incubation at 37° for 60 min.. After incubation a 100-μl aliquot was removed and processed according to the procedure of Bollum (17). Incorporation of [^{3}H] dTTP into DNA was almost proportional to the reaction time and the enzyme dose under the present experimental conditions.

Procedures for purification of DNA polymerase: All the following operations were carried out at 4-5° except otherwise specified. One series of experiments including sedimentation analyses was completed within 3 weeks and meanwhile the enzyme solutions were stored at 5° without a serious loss of the activity. Livers of male Wistar rats weighing about 150 g were homogenized (x 10 strokes) in 2.5 vols of extraction buffer (0.25 M sucrose, 50 mM Tris-Cl at pH 8.3, 25 mM KCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol), followed by centrifugation for 90 min. at 27,000 rpm in a Spinco RP 30 rotor. The supernatant was dialyzed against buffer A (50 mM Tris-Cl at pH 8.3, 0.1 M NaCl, 2 mM β-mercaptoethanol) and referred to as Frac. I. Proteins in Frac. I were precipitated differentially at 0.3-0.5 and 0.5-0.8 saturation of $(NH_4)_2SO_4$, dissolved in buffer A, dialyzed against buffer A and referred to as Frac. II* and Frac. II, respectively. Frac. II was then subjected to column chromatography on phosphocellulose. Linear gradient (0.1-1.0 M) NaCl in buffer B (50 mM Tris-Cl at pH 8.3, 2 mM β-mercaptoethanol) was run to eluate the components. The preliminary eluate (Frac. III-a) showing no (or little) DNA polymerase activity and a fraction corresponding to the highest DNA polymerase activity (Frac. III) were separated. The results of preliminary experiments omitting dATP, dGTP and dCTP from the standard reaction mixture containing Frac. II or Frac. III have revealed that either Frac. II or Frac. III may essentially be free from the terminal deoxynucleotidyl transferase (18).

Sucrose density gradient centrifugation analysis: Linear 10% to 25% (w/v) sucrose gradients prepared in 5 ml of buffer B were used. The centrifugation was performed at 4° in a SW 50L rotor in a Beckman L2-65B ultracentrifuge. In each run, a parallel gradient contained bovine serum albumin (4.4S) and bovine liver catalase (11.3S) as references. Sedimentation coefficients were determined according to the procedure of Martin and Ames (19).

Other procedures: Catalase was assayed by a conventional method (20) and proteins were assayed by measuring the absorbance at 280 nm or by the method of Lowry et al. (21) with bovine serum albumin as standard.

Results and Discussion

Aliquots of Frac. I (0.6 unit/mg protein) prepared from the

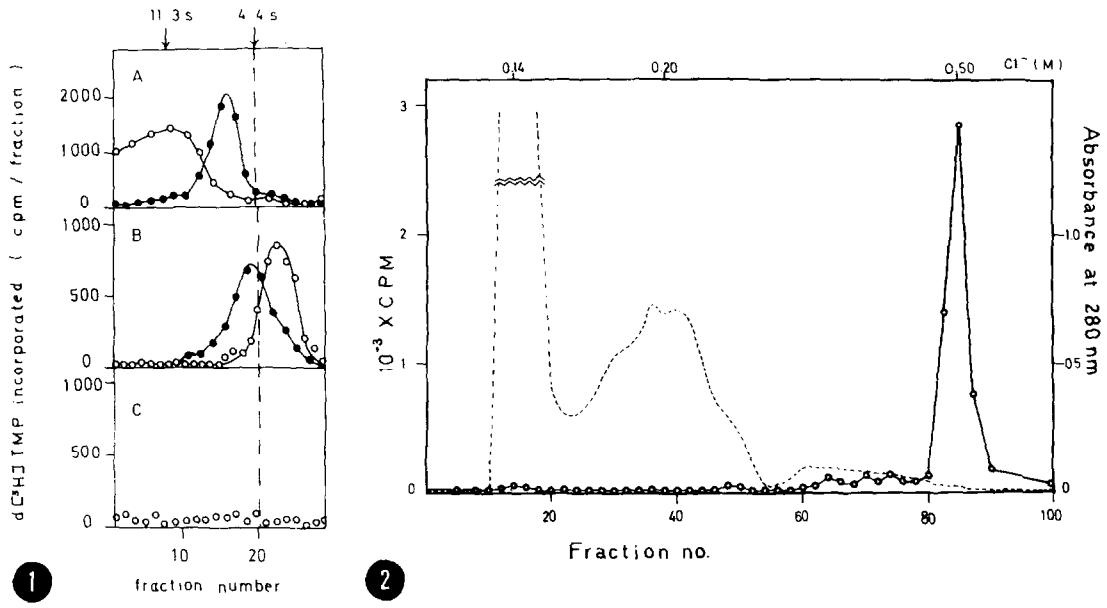

Figure 1. Sedimentation analysis of cytoplasmic DNA polymerase at different preparation steps.

A: Frac. II (●—●) and Frac. II* (○—○). An aliquot (0.2 ml) of Frac. II (3 mg protein/0.2 ml) or Frac. II* (8 mg protein/0.2 ml) was loaded onto the gradient prepared as described in the text. The centrifugation was carried out at 45,000 rpm for 13-h. A 0.2 ml aliquot of each fraction collected after centrifugation was assayed for DNA polymerase as described in the text.

B: Frac. III (○—○) and Frac. III + Frac. III-a (●—●). Aliquots (0.3 ml) of equal volume mixture of Frac. III plus buffer A (5 μg protein/0.3 ml) and Frac. III plus Frac. III-a (1 mg protein/0.3 ml) were analyzed in the same way as described above.

C: Frac. III-a alone (○—○). 0.3 ml of Frac. III-a (2 mg protein) was analyzed in the same way as described above. [^{3}H]dTTP (70 mCi/mmole) was used in the assay.

Fig. 2. Elution profiles of protein and DNA polymerase in Frac. II in chromatography on phosphocellulose.

A 10-ml aliquot of Frac. II (in buffer A, 1.5 mg protein/ml) was applied to a column of phosphocellulose (2 cm x 20 cm) which had been equilibrated with buffer A. Elution was carried out by running buffer B with a continuous gradient of NaCl (0.1-1.0 M NaCl) at a flow rate of 20 ml/h. Fractions were collected every 5 ml. A 50-μl aliquot of each fraction was assayed for DNA polymerase. ○—○ : DNA polymerase (cpm per reaction tube). -----: protein (absorbancy at 280 nm of each fraction). [^{3}H]dTTP of the same specific activity as described in Fig. 1 was used in the assay.

rat liver cytosol were subjected to gel-filtration on Sephadex G-150 and to sucrose density gradient centrifugation analysis, respectively. In the following sedimentation analyses including this one, sucrose gradients were always prepared in buffer B, while loaded DNA polymerase samples contained 0.1 M or 0.27 M of NaCl. The gel-filtration gave the elution diagram suggesting the very large molecular nature of the cytoplasmic DNA polymerase (being eluted at or near the void volume), while the sucrose density gradient centrifugation gave the typical sedimentation profile showing a somewhat broad band of the activity at 6-8S in accordance with the observation of other investigators (22, 23). In Fig. 1-A we show the sedimentation profiles of Frac. II (1.5 unit/mg protein) and Frac. II* (1.2 unit/mg protein) prepared as described in Materials and Methods. Frac. II exhibited a rather sharp activity peak arround 6.5S, while Frac. II* exhibited a very broad band in the region of larger S value. When Frac. II was subjected to column chromatography on phosphocellulose, almost all the enzyme activity was recovered in a very sharp band eluted at the NaCl concentration near 0.45 M as shown in Fig. 2. Frac. III (Fraction No. 84 in Fig. 2) thus separated had a specific activity of 1,000-3,000 units/mg protein. Little or no enzyme activity was found in Frac. III-a (Fraction No.10-20 in Fig. 2).

Frac. III and Frac. III plus Frac. III-a (each contains 0.27 M NaCl) were subjected to the sedimentation analysis, respectively. As shown in Fig. 1-B, Frac. III exhibited an activity peak at 3.3S in accordance with the results obtained by Berger et al. (24) who have isolated 3.5S DNA polymerase from the rat liver cytosol (they could not detect other species of DNA polymerase), while Frac. III plus Frac. III-a exhibited a new peak band at 5S. Frac. III-a, either in the presence of 0.1 M NaCl (Fig. 1-C) or of higher concentrations of NaCl (not indicated), exhibited little or no DNA polymerase activity in any of the subfractions in the centrifugation analysis.

In a separate experiment Frac. III-a was further fractionated by sucrose density gradient centrifugation into the following 7 subfractions ; larger than 12.7S, 12.7-10.5S, 10.5-8.3S, 8.3-6.2S, 6.2-4.0S, 4.0-1.8S, and smaller than 1.8S. Each of the above subfractions of Frac. III-a was added to Frac. III (final NaCl concentration of 0.27 M) and subjected to the sedimentation analysis. Fig. 3-A through G indicate that the cytoplasmic factor in Frac.

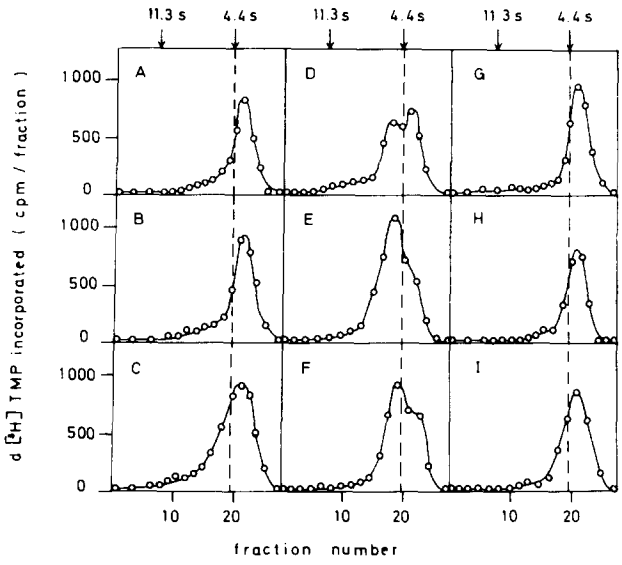

Figure 3. Effects of subfractions of Frac. III-a on the sedimentation profile of DNA polymerase in Frac. III.

0.3 ml of Frac. III-a was loaded onto the gradient prepared as described in the text. The centrifugation was carried out at 40,000 rpm for 18-h. After the centrifugation subfractions (0.75 ml each) were collected and dialyzed against buffer A. Thus Frac. III-a was divided into the following 7 subfractions, i.e. A (>12.7S), B (12.7-10.5S), C (10.5-8.3S), D (8.3-6.2S), E (6.2-4.0S), F (4.0-1.8S) and G (<1.8S). Each of the subfractions were dialyzed against buffer A and mixed with an equal volume of Frac. III and an aliquot (0.3 ml) of each mixture was loaded onto the gradient prepared as described in the text, centrifuged at 40,000 rpm for 18-h and analyzed as described in Figure 1. Effects of the subfractions A through G are shown in Figure A through G, respectively. Figure H and I indicate the sedimentation profile of Frac. III mixed with an equal volume of heat-treated subfraction E (at 65° for 30 min.) and of buffer A, respectively. [^{3}H]dTTP (140 mCi/mmole) was used for DNA polymerase assay.

III-a converting 3.3S DNA polymerase to the 5S form is most rich in the 6.2-4.0S subfraction. Fig. 3-H shows that the factor is thermolabile (65°, 30 min).

In order to examine the possibility that the conversion described above could be caused by simultaneous activation of an activity in the 5S region and inhibition of an activity in the 3.3S region, a 0.1-ml aliquot of each of the 20 subfractions of Frac. III-a separated by sucrose density gradient centrifugation was added to 0.1 ml Frac. III and DNA polymerase activity was assayed. The results showed that the DNA polymerase activity of

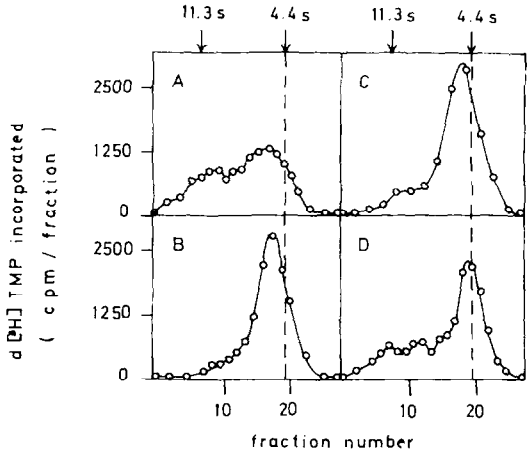

Figure 4. Effects of subfractions of Frac. III-a on the sedimentation profile of DNA polymerase in Frac. III under the conditions of low ionic Strength.

Frac. III (originally containing about 0.45 M NaCl) was dialyzed against buffer A and the Frac. III dialyzate was mixed with an equal volume of each of the dialyzed (against buffer A) Frac. III-a subfractions corresponding to D, E and F in Fig. 3 and the mixtures were subjected to the sedimentation analysis. Sedimentation profiles in Fig. 4-A, B and C correspond to the effect of Frac. III-a subfraction D, E and F, respectively. In Fig. 4-D, Frac. III dialyzate against buffer A was mixed with an equal volume of buffer A. Sedimentation analyses and DNA polymerase assay were carried out in the same way as described in Fig. 1, except for the specific activity of [^{3}H] dTTP (140 mCi/mmole) used.

Frac. III was not affected to any significant extent by the addition of any of these subfractions, suggesting that the conversion is more likely due to the shift in a molecular species from one sedimentation coefficient to another.

The cytoplasmic conversion factor seems not only to convert the purified 3.3S DNA polymerase to the 5S form in the presence of 0.27 M NaCl but also to convert the oligomeric DNA polymerase known to occur under lower ionic strength conditions (25) to the 5S form. As evidenced in Fig. 4, the purified DNA polymerase (Frac. III dialyzed against buffer A) exhibits the oligomeric sedimentation profile (Fig. 4-D) in the presence of 0.1 M NaCl, while addition of Frac. III-a subfractions seemingly containing the conversion factor (corresponding to D, E and F in Fig. 3) appears to convert the oligomeric forms of enzyme to the 5S form depending on the content of the converting factor (Fig. 4-A, B and C)

Table 1. Effect of Frac. III-a on the template specificity of DNA polymerase

Enzyme	Addition	Template DNA	pH	Activity(cpm)
Frac. II	buffer A	activated	7.4	2021
			7.8	3576
		denatured	7.4	124
			7.8	114
		native	7.4	478
			7.8	554
Frac. III	buffer A	activated	7.4	2017
			7.8	3842
		denatured	7.4	56
			7.8	49
		native	7.4	104
			7.8	62
Frac. III	Frac. III-a	activated	7.4	1638
			7.8	2499
		denatured	7.4	311
			7.8	534
		native	7.4	669
			7.8	1054

100-μl each of the enzymes and additives were added in the standard reaction mixture. Frac. II, Frac. III and Frac. III-a contained 1.5 mg, 3 μg and 0.6 mg protein, respectively. Denatured DNA was prepared by heating native calf-thymus DNA at 100° for 10 min, followed by rapid cooling on ice. [^{3}H]dTTP (140 mCi/mmole) was used.

The conversion of the enzyme seems to be accompanied by a change in the template specificity as evidenced in Table 1. It is interesting to see that the 5S enzyme (Frac. III plus Frac. III-a) can utilize denatured DNA as the template more efficiently than the 6.5S (Frac. II) or the 3.3S (Frac. III) enzyme.

These results presented above lead us to infer that the cytoplasmic factor in Frac. III-a may interact with the 3.3S DNA polymerase unit in singular or polymeric forms, converting it to the 5S form with accompanied changes in the template specificity, and thus might be responsible for the regulation of DNA replication.

References

1. Kalf,G.F. and Ch'ih,J.J., J.Biol.Chem. 243, 4904-4916 (1968)
2. Meyer,R.R. and Simpson,M.V., J.Biol.Chem. 245, 3426-3435 (1970)
3. Probst,G.S., and Meyer,R.R., Biochem.Biophys.Res.Commun. 50, 111-117 (1973)
4. Yoneda,M. and Bollum,F.J., J.Biol.Chem. 240, 3385-3391 (1965)
5. Chang,L.M.S. and Bollum,F.J., J.Biol.Chem. 246, 909-916 (1971)
6. Chang,L.M.S. and Bollum,F.J., Biochem. 11, 1264-1272 (1972)
7. Bollum,F.J., Chang,L.M.S., Tsiapalis,C.M. and Dorson,J.W., Methods Enzymol. 29, 70-80 (1974)
8. Chang,L.M.S. and Bollum,F.J., Science 175, 1116-1117 (1972)
9. Hecht,N.B., Biochim.Biophys.Acta. 312, 471-483 (1973)
10. Holmes,A.M., Hesslewood,I.P. and Johnston,I.R., Eur.J. Biochem. 43, 487-499 (1974)
11. Spadari,S., Muller,R. and Weissbach,A., J.Biol.Chem. 249, 2991-2992 (1974)
12. Chang,L.M.S., Brown,M. and Bollum,F.J., J.Mol.Biol. 74, 1-8 (1973)
13. Crerar,M., and Pearlman,R.E., FEBS Letters 18, 231-237 (1971)
14. Tanabe,K. and Takahashi,T., Biochem.Biophys.Res.Commun. 53, 295-301 (1973)
15. Yoshida,S., Kondo,T. and Ando,T., Biochim.Biophys.Acta. 353, 463-474 (1974)
16. Wicha,M. and Stockdale,F.E., Biochem.Biophys.Res.Commun. 48, 1079-1087 (1972)
17. Bollum,F.J., J.Biol.Chem. 234, 2733-2734 (1959)
18. Chang,L.M.S., Biochem.Biophys.Res.Commun. 44, 124-131 (1971)
19. Martin,R.G. and Ames,B.N., J.Biol.Chem. 236, 1372-1379 (1961)
20. Chance,B. and Maehly,A.C., Methods Enzymol. 2, 764-768 (1955)
21. Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J., J.Biol.Chem. 193, 265-275 (1951)
22. Hecht,N.B. and Davidson,D., Biochem.Biophys.Res.Commun. 51, 451-459 (1973)
23. Chang,L.M.S. and Bollum,F.J., J.Biol.Chem. 247, 7948-7950 (1972)
24. Berger,Jr.H., Huang,R.C.C. and Irvin,J.L., J.Biol.Chem. 246, 7275-7283 (1971)
25. Lazarus,L.H. and Kitron,N., J.Mol.Biol. 81, 529-534 (1973)