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DNA POLYMERASE-γ IS LOCALIZED IN MITOCHONDRIA

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SUMMARY: DNA polymerase-γ and DNA polymerase-mt prepared from rat liver showed the same properties [Tanaka,S. & Koike,K.(1977) Biochim.Biophys.Acta, 479, 290-299]. When the tissue was homogenized and fractionated using 0.3 M sucrose/4 mM $CaCl_2$, DNA polymerase-γ was exclusively detected in the mitochondrial fraction. Sucrose gradient centrifugation of postnuclear fraction also indicated that DNA polymerase-γ was solely sedimented with mitochondria. DNA polymerase-γ is thus a mitochondrial enzyme.

INTRODUCTION

As previously reported, DNA polymerase-mt (e.g. DNA polymerase extracted from purified mitochondria) is a high-molecular weight enzyme (9.2 S) and uses polyA.oligo(dT) as a template-primer with high efficiency (1). Because of these properties, DNA polymerase-mt may be classified as a DNA polymerase-γ (2). As DNA polymerase-γ activity was found in nuclei or cytoplasm from a variety of cells including tumor cells (3,4), two types of the enzyme might be expected to exist. However, there is no evidence that two types of the enzyme exist in the same cell, except a report of Spadari and Weissbach on HeLa cells (5). Their data strongly suggest that DNA polymerase-mt and DNA polymerase-γ are the same enzyme, but, the exact location of DNA polymerase-γ in the cell was obscure. We, therefore, prepared DNA polymerase-γ from rat liver to compare with DNA polymerase-mt, and investigated the intracellular distribution of DNA polymerase-γ with careful fractionation of organelles. The results presented in this paper show that DNA polymerase-γ localizes in mitochondria.

MATERIALS AND METHODS

1. Cell fractionation —— Nuclear, mitochondrial and postmitochondrial fractions were prepared according to the method of Lynch et al.(6) with our modifications. Two g of rat liver were homogenized in 20 ml of 0.3 M sucrose/4 mM $CaCl_2$ with a loosely-fitting teflon-glass homogenizer. The homogenate was passed through 4 layers of cheesecloth. The filtrate (cell homogenate) was

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centrifuged at 10,000 g for 15 min. The supernatant (postmitochondrial fraction) was removed and the pellet was washed once with 0.3 M sucrose/4 mM $CaCl_2$. The final pellet was suspended in 17 ml of 2 M sucrose/1 mM $CaCl_2$, layered over 13 ml of 2.2 M sucrose/1 mM $CaCl_2$ and centrifuged at 24,000 rpm for 20 min in a Beckman SW25.1 rotor. Nuclei was found at the bottom and mitochondria were in the top layer of 2 M sucrose/1 mM $CaCl_2$. The mitochondrial fraction was, then suspended in 30 ml of buffer A (1) and centrifuged at 10,000g for 15 min.

2. Extraction of DNA polymerase —— The precipitate of nuclei or mitochondria was suspended in buffer B [50 mM Tris-HCl, pH 8.0/50 mM $Mg(CH_3COO)_2$/1 mM EDTA/2 mM DTT] and 1 M NaCl, and sonicated for 10 sec twice by Artek Sonic 300 Dismembrator. Crude extract was obtained by centrifugation of the sonicated sample at 180,000g for 30 min. To cell homogenate or postmitochondrial fraction one-tenth volume of 10X buffer B and solid NaCl, at a final concentration of 1 M, were added and crude extract was obtained by the same procedure, as described above.

3. Sucrose density gradient analysis of postnuclear fraction —— Rat liver, homogenized in 8 volumes of 0.3 M sucrose with a teflon-glass homogenizer, was centrifuged at 1,000g for 10 min. The postnuclear fraction was carefully removed and centrifuged twice at 1,000g for 10 min. The final fraction (3 ml) was layered over 27 ml of a 0.5-0.8 M linear sucrose gradient containing 1 mM EDTA and centrifuged at 8,000 rpm for 20 min in a Beckman SW25.1 rotor. After centrifugation, 1.5 ml of fractions were collected from the gradient.

4. Assay of DNA polymerase —— The reaction mixture contained 60 µl of 113 µM dATP/dGTP/dCTP/6.6 µM [^{3}H]dTTP (2.7 Ci/mmole)/120µg/ml activated DNA (1)/12.5 mM Tris-HCl, pH8.5/6 mM $MgCl_2$/1 mM DTT/0.5 mg/ml BSA/0.17 M KCl/enzyme solution. After incubation at 35°C for 30 min, a 50 µl portion was pipetted onto a DE81 filter disc(Whatman) and washed with 0.3 M Na_2HPO_4 twice, and with H_2O and ethanol. The radioactivity of the disk was counted in a Beckman LS-250 scintillation counter. PolyA·oligo(dT)-directed activity was measured in the reaction mixture containing 25 mM Tris-HCl,pH7.5/0.5 mM $MnCl_2$/100 µg/ml polyA/12.5 µg/ml $oligo(dT)_{10}$/6.6 µM [^{3}H]dTTP (1.35 Ci/mmole)/1 mM DTT/0.5 mg/ml BSA/0.17 M KCl.

5. Assay of marker enzymes —— The assay of cytochrome c oxidase was performed according to the procedure of Orii and Okunuki (7). Monoamine oxidase was measured by the method of Tabor et al.(8). Glucose-6-phosphatase was determined by measuring the release of inorganic phosphate from glucose-6-phosphate. Acid phosphatase was measured by p-nitrophenyl phosphate as a substrate.

6. Chemicals —— [^{3}H]dTTP was purchased from New England Nuclear. Deoxynucleotides and synthetic polynucleotides were from Boehringer Manheim GmbH and PL Biochemicals. Other materials were described in the previous paper (1).

RESULTS

1. Comparison of DNA polymerase-γ and DNA polymerase-mt —— DNA polymerase-γ was purified from the liver of 4 weeks rats, as described in the legend of Table I. The properties of the enzyme after glycerol gradient centrifugation were compared with those of purified DNA polymerase-mt (1). As shown in Table I, DNA polymerase-γ and DNA polymerse-mt were sensitive to ethidium bromide and N-ethylmaleimide and distinct from DNA polymerase-α and DNA polymerase-β. Both enzymes were eluted at 0.35 M NaCl from the phosphocellulose column, and

Table I. Effects of inhibitors on DNA polymerases.

DNA polymerase-γ was prepared as follows. Frozen liver was homogenized in buffer A containing 0.5 M NaCl and 2.5 mM 2-mercaptoethanol. After 40 h of stirring at 4°C, the homogenate was centrifuged at 30,000 rpm for 30 min. To the supernatant, ammonium sulfate was added at 25% saturation. After centrifugation at 10,000 rpm for 20 min, ammonium sulfate was added to the supernatant at 65% saturation. The precipitate was dissolved in buffer C(50 mM Tris-HCl, pH8.0/1 mM EDTA/1 mM DTT) and layered on a DEAE-cellulose column. DNA polymerase-γ was eluted at 0.08 M NaCl by linear gradient of NaCl in buffer C. The enzyme fraction was then applied to a phosphocellulose column and eluted with a linear gradient of NaCl in buffer C. DNA polymerase-γ, which was eluted at 0.35 M NaCl, was centrifuged in the 15-30% glycerol gradient containing 0.5 M KCl in buffer C. DNA polymerase-α and DNA polymerase-β were prepared, as described previously (10).

		DNA pol-mt		-γ		-β	-α
None		100%	(100%)*	100%	(100%)*	100%	100%
EtdBr	2.5 μM	34	(30)	26	(69)	96	80
	10	10	(9)	4	(29)	94	59
	25	2	(1)	8	(15)	81	44
MalNEt	0.2 mM	47	(99)	9	(105)	129	36
	1.0	11	(23)	1	(82)	-	22
	10	0	(3)	0	(27)	114	19

* PolyA.oligo(dT) was used as a template-primer. EtdBr:Ethidium bromide. MalNEt: N-ethylmaleimide.

sedimented at 9 S in the glycerol gradient at high ionic strength. No significant difference in properties between DNA polymerase-γ and DNA polymerase-mt was detected.

2. Distribution of DNA polymerase-γ —— Nuclear, mitochondrial and postmitochondrial fractions were prepared from the liver of newborn rats. DNA polymerase was extracted from each fraction by sonication in the presence of 1 M NaCl. The extraction efficiency was more than 85 %. As shown in Fig. 1, two discrete classes, large and small, of DNA polymerases were observed in the cell homogenate by means of glycerol gradient centrifugation. The large enzyme, which copied polyA.oligo(dT) in high efficiency, corresponds to DNA polymerase-γ or DNA polymerase-mt (1). The sedimentation coefficient of the enzyme was slightly reduced, to 8 S, in the presence of 1 M KCl. The reaction catalyzed by the large enzyme continued linearly up to 30 min, and the ratio of activated DNA-directed activity to polyA.oligo(dT)-directed one was almost constant in each fraction. On account of its resistance to N-ethylmaleimide, the small enzyme

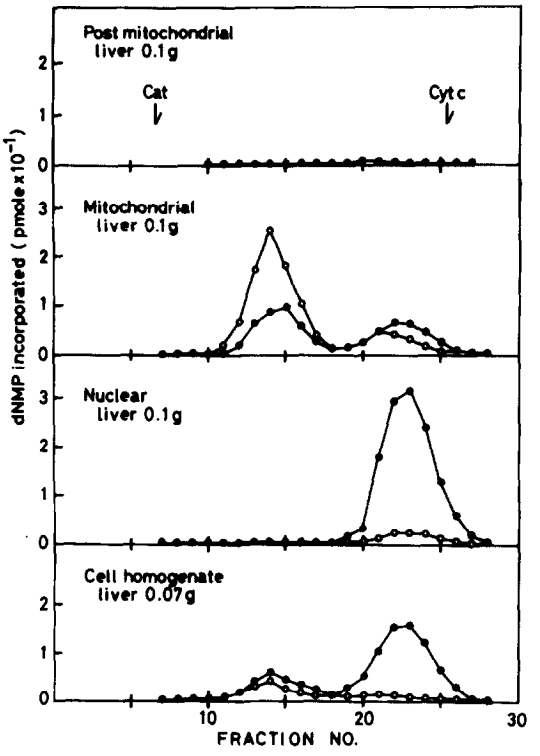

Fig. 1. Glycerol gradient centrifugation of DNA polymerases from subcellular fractions.

DNA polymerases were extracted from nuclear, mitochondrial or postmitochondrial fraction of 0.1 g liver in each or cell homogenate of 0.07 g liver, and concentrated to 0.3 ml by Ficol, followed by dialysis against buffer C containing 1 M KCl. Each sample was layered on 4.6 ml of 15-35 % glycerol gradient containing 1 M KCl in buffer C. After centrifugation for 20 h at 45,000 rpm at 2°C in a Beckman SW50.1 rotor, the gradient was fractionated and DNA polymerase activity was assayed. Activated DNA-directed activity(●—●). PolyA·oligo(dT)-directed activity(o—o).

(3.5 S) is DNA polymerase-β. This enzyme had only slight polyA.oligo(dT) activity. On the other hand, DNA polymerase-α activity was inhibited by the presence of 0.17 M KCl in the assay mixture (9).

By the present procedure, DNA polymerase-γ was found exclusively in the mitochondrial fraction, whereas DNA polymerase-β was mostly ratained in the nuclear fraction. The recovery of DNA polymerase-γ activity from the mitochondrial fraction was almost 100 % using activated DNA as a template-primer. When polyA·oligo(dT) was used, the enzyme activity recovered was over 400 % of that of the cell homogenate, suggesting the presence of inhibitory material(s)

in the extract. Calcium ion did not affect the recovery of DNA polymerase-γ or -mt in contrast to the case of DNA polymerase-α (10). The small enzyme observed in mitochondrial fraction was also resistant to N-ethylmaleimide. This could be explained as a contamination of DNA polymerase-β from unbroken cells. In fact, further purification of mitochondria by sucrose gradient centrifugation eliminated the small enzyme. In the postmitochondrial fraction, neither class of DNA polymerase was found. In the case of adult rat liver, DNA polymerase-γ represented a small part of the total DNA polymerase activity of the cell homogenate. However, this activity was recovered after phosphocellulose chromatography. In nuclear and postmitochondrial fractions, only a trace amount of the activity was detected even after phosphocellulose chromatography.

3. Sucrose gradient centrifugation of postnuclear fraction —— To further investigate the localization of DNA polymerase-γ in mitochondria without interference by the DNA polymerase-β of nuclei, the postnuclear fraction was centrifuged in the sucrose gradient. The distribution of the organelles was monitored by the activity of marker enzymes in the gradient. As shown in Fig. 2, mitochondria (cytochrome c oxidase and monoamine oxidase), lysosome (acid phosphatase), microsome (glucose-6-phosphatase) were clearly separated, under the condition used. DNA polymerase was extracted from each tube, as described in the legend of Fig. 2. Most of the DNA polymerase activity was found in the mitochondrial fraction with polyA·oligo(dT), as well as activated DNA, as a template-primer. Then, the crude extract was centrifuged in the glycerol gradient, but significant activity of DNA polymerase-γ was not detected in any other fraction (data not shown). These data clearly indicate that the DNA polymerase which sedimented with the mitochondria is DNA polymerase-γ (i.e. DNA polymerase-mt). When the sucrose gradient was centrifuged for 5 h at 22,000 rpm, the mitochondria were banded at a density of 1.19 g/ml together with DNA polymerase-γ. However, the separation of other organelles was not clear in this case, because of the similarity of their density.

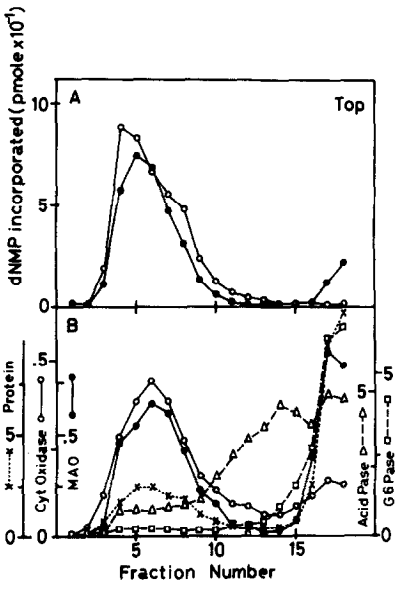

Fig. 2. Sucrose gradient centrifugation of postnuclear fraction.
(A) After centrifugation the gradient was fractionated and DNA polymerase was extracted from each tube, as described in MATERIALS AND METHODS. To the crude extract ammonium sulfate was added at 60% saturation, and the precipitate was dissolved in a small volume of buffer C before activity assay of DNA polymerase. (B) As to marker enzymes, one unit of the actitity is defined as follows. Initial decrease of absorbance of 0.1/min at A_{550nm} for cytochrome c oxidase(o—o). Production of 1 nmole benzaldehyde/min for monoamine oxidase (●—●). Formation of 1 μmole p-nitrophenol/30 min for acid phosphatase(△---△). Release of 1 μmole inorganic phosphate/30 min for glucose-6-phosphatase(□---□). Protein concentration is expressed in mg/ml(x···x).

DISCUSSION

Previous data have indicated that a species of DNA polymerase purified from mitochondria was indeed a mitochondrial origin, and had the ability to utilize polyA·oligo(dT) in high efficiency (1). On the other hand, only one class of DNA polymerase-γ has been reported so far (2), but its physiological function is not known. In this paper we compare the properties and distribution of DNA polymerase-γ with DNA polymerase-mt.

For the investigation of the two DNA polymerases, the following points were carefully considered. The quantitative analysis of each DNA polymerase activity was required. Depression of the enzyme activity by some inhibitor in the crude extract and inactivation of the enzyme through purification steps had to be avoided. Glycerol gradient centrifugation at high ionic strength had the

advantage to measure the activity of DNA polymerase-γ or -mt without contamination of DNA polymerase-β. Differential centrifugation was useful to obtain pure mitochondria (9), but a considerable amount of mitochondria contaminated other fractions. By the present method, nuclear and postmitochondrial fractions were free of mitochondria. DNA polymerase-γ activity in nuclear and postmitochondrial fractions detected by other investigators (3,4,11) might be contaminating mitochondria. As another method to investigate the localization of DNA polymerase-γ, we used sucrose gradient centrifugation to fractionate the postnuclear fraction. The activity of DNA polymerase-γ was exclusively found in the mitochondrial fraction.

Recently, Bolden et al.(11) have reported that in the HeLa cells, DNA polymerase-γ and DNA polymerase-mt were the same enzyme. Their conclusion is that as only a small part of DNA polymerase-γ is located in the mitochondria, DNA polymerase-mt should be included in DNA polymerase-γ. However, our present data clearly indicate that most (if not all) of DNA polymerase-γ activity associated with mitochondria.

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