FURTHER CHARACTERIZATION OF A MITOCHONDRIAL DNA POLYMERASE FROM HELA CELLS

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SUMMARY: DNA polymerase was extracted from HeLa cell mitochondria with high salt concentrations (1M) and Nonidet-P 40 (0.2%). Subsequently the enzyme was purified stepwise by DEAE-cellulose-, phosphocellulose-, hydroxyapatite-Ultrogel-, DNA-cellulose chromatography and preparative polyacrylamide gel electrophoresis. The purified enzyme exhibited a molecular weight between 100 000 - 110 000 and was devoid of endonuclease activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of this enzyme preparation revealed two protein bands suggesting that the mitochondrial DNA polymerase might consist of two subunits with the molecular weights of 45 000 and 60 000.

INTRODUCTION: In addition to the nuclear and cytoplasmic DNA polymerases α, β and γ, HeLa cells possess another DNA polymerase which is found in purified mitochondria (1,2). Because of its localization, the mitochondrial DNA polymerase (mt-polymerase) has not been well purified and previous studies have been unable to resolve certain questions about this enzyme. The partially purified enzyme has been previously characterized with respect to its template utilization and interaction, requirements for maximal activity and temperature sensitivity (2,3). Moreover, these available partially purified mt-polymerase preparations have been consistently shown to exhibit low levels of endonuclease activity (2,3), a pro-

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perty not shown by the other eukaryotic DNA polymerases. If true, this finding would have important implications in studying mitochondrial DNA synthesis.

The present report presents data on the further purification and characterization of the mt-polymerase. Following several additional chromatographic steps and polyacrylamide gel electrophoresis, the purified mt-polymerase was found to have a molecular weight of about 100 000. Analysis of the purified enzyme revealed two subunits with molecular weights of about 45 000 and 60 000. Endonuclease activity against circular mitochondrial DNA could not be observed in these purified preparations.

MATERIALS AND METHODS: HeLa S-3 cells, grown in F-13 medium (GIBCO) supplemented with 5 % calf serum, were harvested at a cell density of 3 - 4 x 10^5/ml. A total of 32 g (wet weight) of cells was fractionated as described previously (4) and mitochondria isolated from the cytoplasmic fraction by differential centrifugation (4). The washed mitochondrial pellet was resuspended in 1 M KPO_4, ph 7.5, 0.2% Nonidet P40 (N-P40), 0.5 mM dithiothreitol (DTT), at a concentration of approximately 1 mg mitochondrial protein/ml, and homogenized with a tight-fitting Dounce-type glass homogenizer. Following centrifugation of the homogenate at 150 000 x g for 30 minutes, 4°C, the supernatant, containing the solubilized mt-polymerase, was dialyzed overnight against 0.02 M KPO_4, ph 7.5, 0.5 mM DTT at 4°C. The dialyzed enzyme preparation was made 20% with respect to glycerol and passed through DEAE-cellulose (2). The protein which did not adsorb to the DEAE-cellulose (Whatman DE-52) was adsorbed onto a phosphocellulose column (Whatman P-11) and the DNA polymerase activity eluted with a linear KPO_4 gradient (0.02 - 0.5 M, ph 8.0) as described in detail by Fry and Weissbach (2). The pooled peak fractions of DNA polymerase activity obtained from phosphocellulose chromatography were diluted 5-fold with 0.02 M KPO_4, ph 7.5, and adsorbed to a combined hydroxylapatite-Ultrogel AcA 34 column (HT-AcA 34), (0.3 ml of hydroxylapatite layered onto 10 ml of AcA 34). Following a 5 ml wash with 0.02 M KPO_4, ph 7.5, the enzyme was eluted with a 50 ml linear KPO_4 gradient (0.02 - 0.5 M KPO_4, ph 7.5) (5). The pooled peak fractions of DNA polymerase activity from HT-AcA 34 were diluted 5-fold with 0.02 M KP04, ph 7.5 and adsorbed to a 3 ml double-stranded DNA cellulose column. After a 5 ml wash with 0.02 M KPO_4, ph 7.5, the enzyme was eluted with 30 ml linear KPO_4 gradient (0.02 - 0.5 M, ph 7.5). The peak fractions of DNA polymerase activity from DNA cellulose were subjected to polyacrylamide del electrophoresis and subsequently eluted from the gels with 0.2 M KPO_4, ph 7.5, containing 20% glycerol, 0.2% N-P40 and 0.5 mM DTT as described previously for DNA polymerase γ (5). Mt-polymerase activity was determined according to Fry and Weissbach (2).

Endonuclease activity was assayed under conditions identical to the DNA polymerase assay except that 0.6 μg of purified (^{3}H)-labelled covalently-closed supercoiled circular mitochondrial DNA (6 250 cpm/μg) was used as the substrate. After incubation, the amount of radioactivity in the covalently-closed circular DNA and in the nicked, circular DNA was determined with CsCl-ethidium bromide velocity sedimentation gradients (6(. Movement of radioactivity from the covalently-closed circular DNA to nicked circular DNA would be indicative of endonuclease activity

The methods of velocity sedimentation in linear sucrose gradients and of sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis for estimation of molecular weights have been described previously (7,5). The SDS-gels were scanned in a Gilford spectrophotometer Model 2400 at 550 nm after staining of the gels with Commassie blue R-250.

RESULTS: Fig.1 and Table 1 present the data on the purification of the mt-polymerase by the new procedure described in the Mate rials and Methods section. The additional chromatographic steps yield an enzyme preparation at least 6-fold more purified than that described before (2) [2]. Further purification of the enzyme was achieved by polyacrylamide gel electrophoresis after the DNA-cellulose chromatography step (Fig.1,Table 1). The final degree of purification could not be determined accurately because protein levels were too low for reliable estimation, even by the sensitive fluorometric method of Bohlen et al. (8). However, we estimate the specific activity of the final gel purified enzyme fraction to be at least 1000 units/mg.

The purified enzyme preparation exhibited a sedimentation value of 6 s in sucrose gradients (not shown). This would be consistent with the previously reported molecular weight of 106 000 (2). Analysis of the proteins in the mt-polymerase peak obtained from the HT-AcA chromatography step (Fig. 1A) or polyacrylamide gel electrophoresis (Fig. 1C) was also carried out in SDS-polyacrylamide gel electrophoresis (Fig. 2).

[2] It should be noted that the specific activity values for the mt-polymerase as listed in Reference (2) are 20-fold too high due to a computational error by the authors.

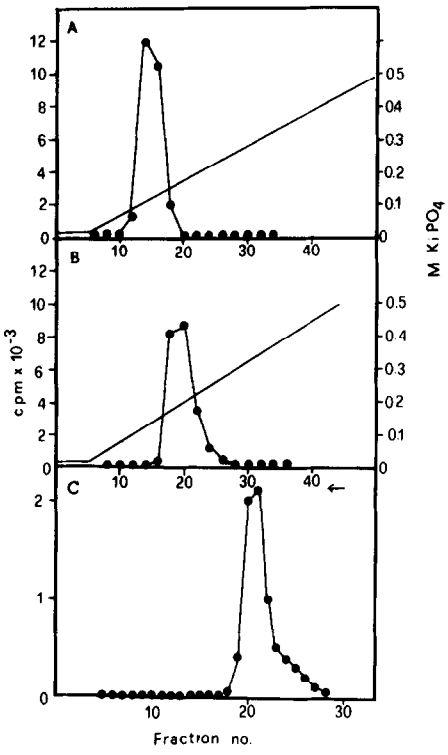

Fig. 1: Hydroxylapatite-Ultrogel AcA 34 chromatography, DNA cellulose chromatography, and polyacrylamide gel electrophoresis of HeLa cell mitochondrial DNA polymerase. (A) HT-AcA 34 chromatogram of the pooled peak fractions of DNA polymerase activity obtained from phosphocellulose chromatography. Preparation of the phosphocellulose peak, adsorption and elution on AcA 34 were carried out as described in Materials and Methods (2). A total of 50 fractions were collected. DNA polymerase activity was determined with 30 µl aliquots using an activated DNA template and deoxyribonucleoside triphosphates as substrates (the specific activity of (^{3}H)-thymidine triphosphate (^{3}H)TTP) was 90 cpm/pmole). (B) DNA-cellulose chromatogram of the pooled peak fractions of DNA polymerase activity from the HT-AcA 34 chromatography. Adsorption and elution were carried out as described in Materials and Methods. A total of 44 fractions were collected. 30 µl aliquots were used for the determination of DNA polymerase activity (specific activity of (^{3}H)TTP was 90 cpm/pmole). (C) Polyacrylamide gel electrophoresis of DNA Polymerase activity of the pooled peak fractions from DNA cellulose Chromatography. The gels were fractionated into 30 fractions, DNA polymerase activity eluted (5) and determined in 20 µl aliquots using (^{3}H)TTP with a specific activity of 390 cpm/pmole. The arrow indicates the direction of migration.

TABLE 1

Purification of mitochondrial DNA polymerase

Purification step	Total units	Total protein (mg)	Specific activity
Cytoplasm		1 122.00	
Washed mitochondria		23.92	
Dialyzed mitochondrial extract	144	15.90	9.05
DEAE-cellulose	88	12.85	6.84
Phosphocellulose	21	0.44	47.7
HT-AcA 34	24	0.13	184.6
DNA-cellulose	24	0.024	1000.0

The purification procedure is described in Materials and Methods. One unit of DNA polymerase is defined as the activity that catalyzes the incorporation of 1 nmole deoxyribonucleoside triphosphates into an acid precipitable form in 30 minutes. Specific activity refers to units/mg protein.

In both cases, two major polypeptide components at about 60 000 and 45 000 daltons were found. We assume from these results that the functional mt-polymerase is probably composed of two subunits of the above-mentioned molecular weights.

Previous studies of the mt-polymerase (2,3) raised the possibility that a nuclease was associated with this enzyme. To test this possibility, we incubated our mitochondrial DNA polymerase at two different stages in the purification procedure, with (^{3}H)-labelled mitochondrial DNA. As shown in Table 2, in neither case could we detect any nuclease activity in our mt-polymerase preparation. Our limit of sensitivity could be calculated to detect 1 phosphodiester bond breakage/100 000 phosphodiester bonds formation in these enzyme preparations.

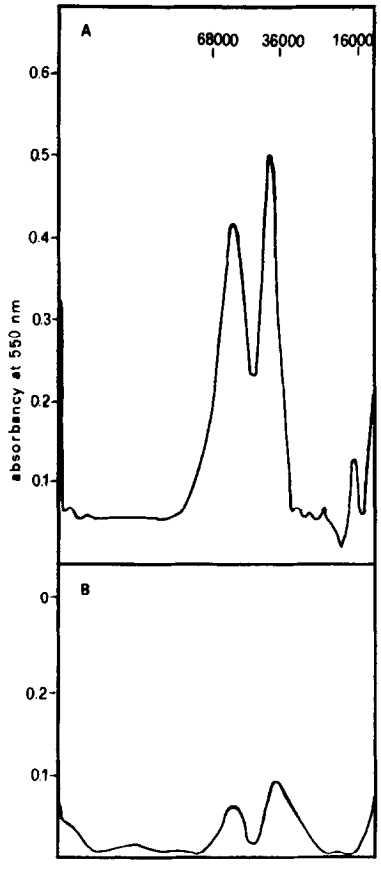

Fig.2: SDS-polyacrylamide gel electrophoresis of the mitochondrial DNA polymerase. (A) SDS-gel electropherogram of 3 µg of the mt-polymerase preparation obtained by HT-AcA 34 chromatography. (B) SDS-gel electropherogram of the mt-polymerase obtained after polyacrylamide gel electrophoresos. An estimated 500 ng of protein was used.
The molecular weight markers in panel (A) are 68 000 (bovine serum albumin), 36 000 (lactate dehydrogenase) and 16 000 (myoglobin)

DISCUSSION: Our results with a more highly purified mt-polymerase from HeLa cells verify the molecular weight determination of the enzyme by Fry and Weissbach (2),who reported the enzyme to be 106 000 daltons. Furthermore, we have presented evidence obtained from SDS-polyacrylamide gel electrophoresis that the enzyme may consist of two subunits of 60 000 and 45 000 daltons ($\pm$ 10%).

TABLE 2

Absence of endonucleolytic activity in mitochondrial DNA polymerase

Incubation	% Radioactivity in Closed circular DNA	Nicked circular DNA
A) No enzyme	43.8	56.2
B) With mt-polymerase		
1) Phosphocellulose fraction	43.3	56.7
2) Polyacrylamide gel fraction	42.8	57.2

The conditions of the endonuclease assay using purified mouse cell (^{3}H)-labelled mitochondrial DNA (3750 cpm/assay) as the substrate are given in Materials and Methods.

It has been suggested that the replication of circular mitochondrial DNA requires a transient breakage of the circular double-stranded molecule (9) and that an endonuclease activity capable of nicking the DNA might be associated with the mt-polymerase (2,3). In this context, it is of interest that our purified enzyme preparation is devoid of endonuclease activity towards mitochondrial DNA within the limits of detection by the test applied. In view of the suggested need of a nicking step (9) for mitochondrial DNA replication, the question thus arises whether mitochondria contain a separate and specific endonuclease, which is required for mitochondrial DNA synthesis and is different from other known cellular nucleases (10). The resolution of this question has to await further investigations.

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