ISOLATION OF TWO DNA POLYMERASES FROM A MITOCHONDRIAL MEMBRANE-DNA COMPLEX OF MOUSE CELL CULTURES

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SUMMARY: A membrane-DNA complex isolated from the mitochondria of thymidine kinase deficient mouse cells could be shown to contain in addition to mitochondrial DNA two different DNA polymerases: (i) Mitochondrial DNA polymerase 1 exhibiting characteristics of the DNA polymerase described for HeLa cell mitochondria and (ii) mitochondrial DNA polymerase 2 showing properties comparable to those of the DNA polymerase isolated from mouse liver mitochondria.

INTRODUCTION: DNA polymerases have been isolated from a variety of mammalian cell mitochondria. Common features of the enzymes from liver mitochondria are a molecular weight of about 150 000 daltons, stimulation by salt and sensitivity to ethidium bromide (1,2,3). On the other hand, the DNA polymerase from HeLa cell mitochondria appears to exhibit different properties showing a molecular weight of 106 000 daltons and no comparable salt stimulation (4,5,6). In context with this discrepancy the possibility was recently discussed that mammalian mitochondria may contain more than one DNA polymerase (3). Furthermore, the view was forwarded that new isolation procedures may help to cast more light on this problem (3).

Following this suggestion, the usual procedure to solubilize the DNA polymerase directly from isolated mitochondria was modified and the preparation of a membrane-DNA complex from isolated mitochondria included as an intermediate step of isolation of the mitochondrial DNA polymerase(s). Subsequently the DNA polymerase

activity was solubilized from this complex and partially characterized.

This new approach led to the isolation of two DNA polymerases from the mitochondrial membrane-DNA complex of the mouse cell cultures used in these experiments. One major activity corresponded to the enzyme that has been described for mouse liver mitochondria (3). A minor activity closely resembled the enzyme isolated from HeLa cell mitochondria (4,5,6,).

MATERIALS AND METHODS: Thymidine kinase deficient mouse cells (Cl-1D) grown as monolayer cultures in F-16 medium (GIBCO) supplemented with 5 percent calf serum, 200 IU penicillin/ml and 10 µg bromodeoxyuridine (BUDR)/ml were used in all experiments. This cell line incorporates exogenous thymidine exclusively into mitochondrial DNA and cannot replicate in medium containing hypoxanthine (15 µg/ml), aminopterin (20 µg/ml) and thymidine (4 µg/ml)(7,8). Cell cultures to be used for in vivo labelling of mitochondrial DNA with ^{14}C-thymidine were cultivated in medium without BUDR for two passages prior to the beginning of the experiment. Routine examinations of this cell line for mycoplasma were found to be negative.

The methods of cell fractionation and isolation of mitochondria by differential centrifugation (8,9), digitonin treatment of the organelles (10) the effectiveness of which was monitored by determinations of the mitochondrial outer membrane marker enzyme monoamino-oxidase (10,11) and the preparation of the mitochondrial membrane-DNA complex (12,13) have been described previously. The DNA polymerase activity of the membrane-DNA complex was examined in an incubation mixture reported by Shearman and Kalf (13) with the minor modification that 1 mM ß-mercaptoethanol was included. Mitochondrial DNA was extracted from the membrane-DNA complex by a slightly modified 'Hirt-procedure' using CsCl instead of NaCl for precipitation of high molecular weight DNA in the lysate (14,15). Isopycnic centrifugation of mitochondrial DNA in CsCl-ethidium bromide and the assay for radioactivity were performed as described elsewhere (8,9,15,16).

The DNA polymerase was solubilized from the isolated membrane-DNA complex by incubation for 30 minutes at 4°C in 0.5 M potassium phosphate ($K.PO_4$), ph 7.5, 0.5 mM dithiothreitol (DTT) and 0.2 percent Triton X-100. Insoluble material was subsequently removed by centrifugation at 150 000 x g for 45 minutes at 4°C. The supernatant was dialyzed overnight against 0.02 M $K.PO_4$, ph 7.5, 0.5 mM DTT, 0.2 percent Triton X-100 and 20 percent glycerol. The dialysate was loaded onto a DEAE cellulose (DE-52) column and the adsorbed DNA polymerase activity eluted with a linear gradient (0.02 - 0.5 M $K.PO_4$, ph 7.5) containing 0.5 mM DTT, 0.2 percent Triton X-100 and 20 percent glycerol (4). The protein which did not adsorb to DE-52 (mitochondrial DNA polymerase 1) as well as the pooled peak fractions of DNA polymerase activity eluted from DE-52 by the ion gradient (mitochondrial DNA polymerase 2) were loaded onto phosphocellulose (P-11) columns. Mitochondrial DNA polymerase 1 was eluted from P-11 as

described in detail before (4). Mitochondrial DNA polymerase 2 was eluted from P-11 with a linear 0.02 - 0.8 M $K.PO_4$ gradient. The method of velocity sedimentation in linear sucrose gradients containing high salt concentrations for estimation of melecular weights has been described previously (4,17).
DNA polymerase activity was determined according to Fry and Weissbach (4) except for the inclusion of 150 mM KCl into the incubation assay in the case of mitochondrial DNA polymerase 2 for recovery of optimal activity. The specific activity of (^{3}H)thymidine triphosphate used in all DNA polymerase assays was 300 cpm/pmole. Protein contents was estimated by the method of Lowry(18).

RESULTS: Preliminary experiments had to be carried out to demonstrate that the isolated mitochondrial membrane-DNA complex met the crucial criteria to contain DNA polymerase activity as well as an endogenous mitochondrial DNA template. For this purpose a mitochondrial membrane-DNA complex preparation was isolated from 1 g Cl-1D cells (wet weight, approximately 3 x 10^8 cells)

TABLE 1

Incorporation of (^{3}H)dTTP by a mitochondrial membrane-DNA complex under various conditions

System	pmoles of (^{3}H)dTMP incorporated
Complete system [a]	0.816
+ 50/ug activated DNA	2.216
+ DNA polymerase α [b]	1.768
+ pancreatic DNase 1 (40/ug)	0.048

a) The complete system consisted of 100 mM Tris-HCl, ph 8.4(25°); 30 mM $MgCl_2$; 1 mM ß-mercaptoethanol; 0.1 mM dATP, dCTP, dGTP; 1/uM (^{3}H)dTTP (specific activity 2 500 cpm/pmole), and membrane-DNA complex (100/ug of protein) in a total volume of 200/ul. Incubations were carried out in duplicate or triplicate for 60 minutes in a 37°C shaking water bath. The reaction was stopped by addition of 1 ml 10 percent cold trichloroacetic acid. Radioactivity of acid insoluble material was assayed as described elsewhere (9).

b) A 15/ul aliquot of a DE-52 fraction was used that catalyzed the incorporation of 58.27 pmoles (^{3}H)dTMP under appropriate conditions (4) on 50/ug of an activated calf thymus DNA template (19) in 30 minutes.

and the DNA polymerase activity examined under appropriate conditions in vitro (see Materials and Methods and TABLE 1) by determination of the activity to catalyze the incorporation of deoxyribonucleoside triphosphates into an acid insoluble form using (^{3}H)thymidine triphosphate as the labelled substrate. As shown in TABLE 1 the complete incubation mixture exhibited a significant intrinsic DNA polymerase activity which was still better demonstrated by the inclusion of activated calf thymus DNA (19). Addition of DNA polymerase α (DE-52 fraction),on the

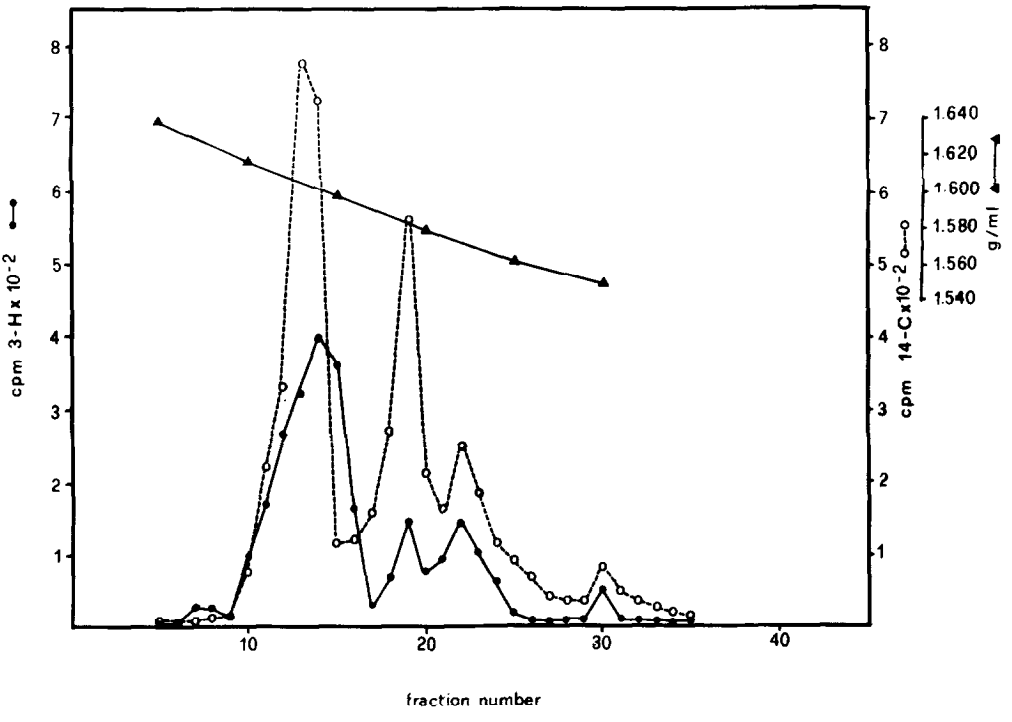

FIG. 1: Isopycnic centrifugation of doubly labelled mitochondrial DNA in CsCl-ethidium bromide solution. Mitochondrial membrane-DNA complex was isolated from 3 x 10^{8} Cl-1D cells labelled for 24 hours with (^{14}C)thymidine. Subsequently the complex was labelled in vitro with (^{3}H)TTP (see TABLE 1) and the DNA extracted from the complex as described in Materials and Methods. Centrifugation conditions: CsCl-ethidium bromide (250 µg/ml),mean density 1.57 g/ml, 65 fixed angle rotor,38 000 rpm, 40 hours, 17°C.

other hand,also enhanced the yield of acid insoluble (^{3}H)labelled material whereas addition of DNase 1 almost abolished the incorporation. These latter results thus indicated the presence of an endogenous DNA template. In order to show that this DNA was indeed mitochondrial DNA the mitochondrial membrane-DNA complex was isolated from 1 g (^{14}C)thymidine-(specific activity 59 mCi/mmole) labelled Cl-1D cells (0.1 uCi/ml culture medium for 24 hours) and subsequently labelled in vitro with (^{3}H)thymidine triphosphate as described above. FIG. 1 demonstrates that the radioactive material extracted from the complex after this experimental procedure was (^{14}C)- as well as (^{3}H)labelled and banded with the characteristics of replicating mitochondrial DNA (20) when subjected to isopycnic centrifugation in CsCl-ethidium bromide.

Subsequently experiments were carried out to isolate and partially characterize the intrinsic DNA polymerase activity of the mitochondrial membrane-DNA complex. For this purpose the mitochondrial membrane-DNA complex was prepared from 6 g Cl-1D cells (wet weight, approximately 2 x 10^{9} cells) and the DNA polymerase activity solubilized from the complex as described in Materials and Methods. Following dialysis the preparation was subjected to column chromatography on DEAE cellulose (Whatman DE-52) and subsequently on phosphocellulose (Whatman P-11).

Approximately 1 - 2 percent of the DNA polymerase activity of the dialysate did not adsorb to DE-52 (mitochondrial DNA polymerase 1) and was eluted at 0.1 M salt from P-11 columns that had been loaded with the DE-52 flow through thus resembling the DNA polymerase that has been described for HeLa cell mitochondria (4,6).

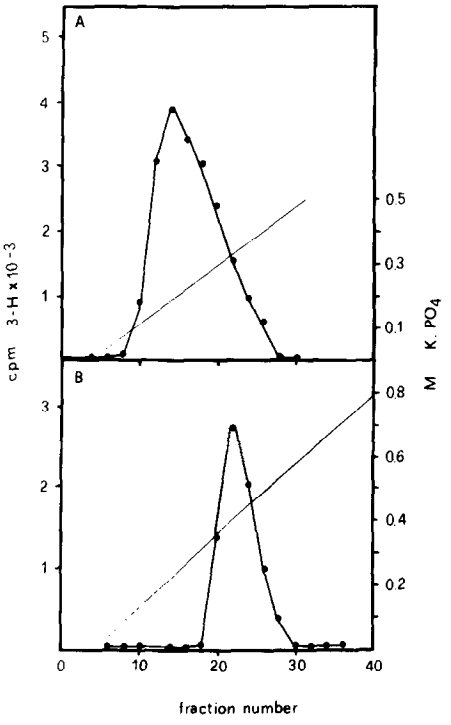

FIG. 2: DE-52- and P-11 chromatography of mitochondrial DNA polymerase 2 following solubilization from the membrane-DNA complex. (A) DE-52 chromatography of mitochondrial DNA polymerase 2. Following solubilization the dialyzed preparation was loaded on a 3 ml DE-52 column and eluted with a 30 ml $K.PO_4$ gradient (0.02-0.5 M, ph 7.5) as described in Materials and Methods. A total of 35 fractions were collected. DNA polymerase activity was determined with 50 µl aliquots as described previously (4) except for the inclusion of 150 mM KCl in the incubation mixture for recovery of optimal activity. (B) P-11 chromatography (3 ml column) of the pooled peak fractions of DNA polymerase activity from the DE-52 chromatography. After adsorption DNA polymerase activity was eluted with a 30 ml $K.PO_4$ gradient (0.02 - 0.8 M, ph 8.0). A total of 40 fractions were collected and DNA polymerase activity determined with 50 µl aliquots as mentioned above.

The major part of the DNA polymerase activity of the dialysate did adsorb to the DE-52 resin (mitochondrial DNA polymerase 2) from which it was eluted at 0.2 M $K.PO_4$. Elution from P-11 columns which had been loaded with the pooled peak fractions of DNA polymerase activity from DE-52 chromatography occurred

TABLE 2

Purification of mitochondrial DNA polymerase 2

Purification step	Total protein (mg)	Total units	Specific activity
Cell homogenate	298.21	-	-
Cytoplasm	180.36	-	-
Isolated mitochondria	48.50	-	-
Digitonin particles	21.86	31.2	1.43
Dialysate	1.42	5.04	3.55
DEAE cellulose	0.205	1.76	8.58
Phosphocellulose	0.068	1.08	15.88

One unit of DNA polymerase is defined as the activity that catalyzes the incorporation of 1 nmole (^{3}H)dTTP into an acid insoluble form in 30 minutes. Specific activity refers to units/mg protein.

TABLE 3

Stimulation by KCl of mitochondrial DNA polymerase 2 at different stages of purification

KCl concentration	Incorporation of pmoles (^{3}H)dTMP catalyzed by a preparation of mitochondrial DNA polymerase 2			DNA polymerase α (DE-52 fraction)
	dialysate	DE-52 fraction	P-11 fraction	
0	4.3	-	-	174.8
50 mM	15.8	1.8	-	90.9
100 mM	22.0	5.7	5.6	39.4
150 mM	24.8	7.5	9.0	15.5
200 mM	24.3	6.5	7.9	7.7
250 mM	14.6	4.5	7.1	3.2

Incubations were carried out in duplicate for 30 minutes at 37°C (see also Materials and Methods) with 50/ul of the DNA polymerase preparations indicated.

at 0.4 M $K.PO_4$ (FIG. 2). TABLE 2 gives additional data on the purification procedure of this enzyme.

Prominent features of the main DNA polymerase activity (mitochondrial DNA polymerase 2) were its significant stimulation by KCl at different stages of purification (TABLE 3) (1,2,3) and sensitivity to ethidium bromide (TABLE 4) (2,3) when compared to DNA polymerase α of the same cells.

TABLE 4

Comparison of the sensitivity to ethidium bromide of mitochondrial DNA polymerase 2 and DNA polymerase α

Ethidium bromide concentration (ug/assay)	Incorporation of pmoles (^{3}H)dTMP catalyzed by	
	Mitochondrial DNA polymerase 2 (P-11 fraction)	DNA polymerase α (DE-52 fraction)
0	8.1	180.6
0.5	5.0	254.3
2.0	2.7	196.6
5.0	0.8	134.0
10.0	0.0	110.0

Incubations were carried out in duplicate for 30 minutes at 37°C with 50 ul aliquots of the DNA polymerase preparations indicated under the respective optimal conditions(see TABLE 3)

The molecular weight determinations of the salt stimulated mitochondrial DNA polymerase 2 by velocity sedimentation in sucrose gradients containing 0.4 M KCl (not shown) were in agreement with a molecular weight of about 150 000 (8.8 s) whereas the minor activity representing mitochondrial DNA polymerase 1 exhibited a sedimentation coefficient of 6 s as reported before (6).

DISCUSSION: To the knowledge of the authors this report describes for the first time the successful isolation of two DNA polymerases from mammalian cell mitochondria. This was achieved by a new isolation procedure which essentially consisted of the preparation of an active mitochondrial membrane-DNA complex as an intermediate step of the isolation of the mitochondrial DNA polymerases. Both enzymes,the minor activity of mitochondrial DNA polymerase 1 with a molecular weight of 106 000 (4,6) as well as the main activity of mitochondrial DNA polymerase 2 with a molecular weight of about 150 000 (3), appeared to be integral part of our membrane-DNA complex preparation.

According to our calculation the total DNA polymerase activity associated with the mitochondrial membrane-DNA complex (predominantly mitochondrial DNA polymerase 2) amounts to approximately 1 percent of the DNA polymerase activity of the cytoplasmic fraction as tested under our conditions using an activated DNA template (19). This percentage represents most probably a minimum estimate since it can be assumed that a considerable amount of mitochondrial DNA polymerase activity was lost during the lengthy isolation procedure.

The presence of two different DNA polymerases in the mitochondria apparently parallels the situation in the nucleus of mammalian cells (21,22,23). It is tempting to also assume different functions for the mitochondrial enzymes as it has been suggested for the DNA polymerases α and β in the nucleus (22,23,24,25). The determination of the activities of these two mitochondrial DNA polymerases during the cell cycle may help to clarify this point.

In this context the observation has to be pointed out,however, that chloramphenicol treatment of the cell cultures prior to isolation significantly decreases the activity of mitochondrial DNA

polymerase 1 (26). A plausible explanation for this phenomenon may be that this enzyme is of procaryotic origin. However, the activity of mitochondrial DNA polymerase 1 can also be drastically reduced by a cycloheximide pretreatment of the cells (26), an observation which argues against a contamination of the cultures used, e.g. by mycoplasma not detectable in routine examinations. Nevertheless, further thorough investigations are needed for a final elucidation of this problem.

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