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# MITOCHONDRIAL DNA POLYMERASE FROM XENOPUS LAEVIS OOCYTES

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SUMMARY

The DNA polymerase of Xenopus laevis oocytes, with characteristics similar to those of the γ polymerases of other systems, can be extracted from the mitochondrial pellet and from the mitochondria purified on a sucrose gradient. It elutes from phosphocellulose at about 0.4 M KCl and sediments faster than 4 S in high salt glycerol gradients. At low KCl concentrations it uses Poly(A) better than activated DNA as a template; the reverse is true at KCl concentrations higher than 150 mM. Various experiments are presented that indicate that it is the mitochondrial DNA polymerase.

In a previous report we have described the major DNA polymerase activity able to use Poly(A)·oligo(dT) as a template in Xenopus laevis oocytes (1). This enzyme has characteristics similar to those of the γ polymerases of other systems, in that it efficiently uses Poly(A)·oligo(dT) as a template, has a sedimentation coefficient higher than 4S and is sensitive to N'ethyl-maleimide (2). The enzyme has a strict cytoplasmic location in these cells and is present in a particulate structure so that detergent is needed for its solubilization (1, 3). When detergent was omitted during the homogenization of the oocytes, virtually all the enzyme could be extracted from a 12,000 xg pellet; since mitochondria are the major component of this pellet, we decided to investigate the possibility that the enzyme is the mitochondrial DNA polymerase. In this paper we present experiments that support this hypothesis.

## MATERIALS AND METHODS

Materials. Deoxynucleotide-5'-triphosphates were obtained from Schwartz Mann, Orangeburg, N.Y. [$^3$H]methyl-deoxy-thymidine-5'-triphosphate (40-50

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Ci/mmol) was purchased from Amersham. Poly(A)·oligo(dT) was from P.L. Biochemicals. Calf thymus DNA (Type V, from Sigma) was 'activated' according to Loeb (4). Cytochrome c was from Sigma.

Cell fractionation. Stage six oocytes were prepared as previously described (1). Cells were thawed and homogenized, without detergent, in the presence of 5 vols. of B buffer (sucrose 0.24 M, Tris-HCl pH 7.5 50 mM, EDTA 1 mM, dithiothreitol 5 mM) with 15 strokes in a hand homogenizer fitted with a Teflon piston. The homogenate was centrifuged at 800 xg for 10 min.; this supernatant was centrifuged at 10,000 xg for 20 min., and the pellet resuspended in the original volume of B buffer. The cycle of centrifugations was repeated twice to avoid large contamination of the 'mitochondrial pellet' by α polymerase. Mitochondria were further purified by layering 1 ml of resuspended mitochondrial pellet on a 20-65% linear sucrose gradient containing TEMG buffer (Tris-HCl pH 7.5 50 mM, EDTA 1 mM, 2-mercaptoethanol 1.4 mM, glycerol 20% w/v). Gradients were centrifuged at 37,000 rpm for 16 hrs in an SW41 Spinco rotor, fractionated and the fractions with density between 1.17 and 1.18 gms/ml containing the peak of cytochrome c oxidase activity pooled and stored at 4°C in high sucrose. Bacterial contamination was effectively contained by using only autoclaved solutions and routinely checked by standard microbiological techniques.

Enzyme purification. The procedure to solubilize the enzyme was the same for all starting materials: mitochondrial pellet, fractions from sucrose gradients etc. The enzyme was solubilized by making the fraction 0.75 M in KCl and 1% Triton X-100. After standing at 4°C for 30 min., the extract was centrifuged at 38,000 rpm for 45 min. in a 50 Ti fixed angle rotor; the supernatant was absorbed batchwise to DEAE cellulose equilibrated with TEMG buffer containing 0.6 M KCl to remove most nucleic acids. The unabsorbed material, containing all the DNA polymerase activity was diluted with TEMG buffer to 0.25 M KCl and absorbed to a phosphocellulose column equilibrated with the same buffer at the same KCl concentration. After extensive washing with TEMG 0.28 M KCl, the activity was eluted with a 0.28-0.6 M KCl gradient. In the experiments of Figure 3, in which several fractions had to be examined for presence and amount of Poly(A) dependent DNA polymerase, solubilization and purification were according to the same procedure except that the phosphocellulose step was performed batchwise: the enzyme was absorbed to the resin inthe presence of 0.28 M KCl and eluted with 0.6 M KCl.

DNA polymerase assay. Assays were run for 30 min. at 37°C. Each assay contained, in 50 µl final volume, 5 or 10 µl of the enzyme fraction, Tris-HCl pH 8.5 50 mM, KCl 50 mM, MgAc 10 mM, dithiothreitol 2.5 mM, dATP, dCTP and dGTP 0.1 mM each, [$^3$H]TTP 5 µM with a specific activity of 2200 cpm/pmol; templates were present at 10 µg/ml; bovine serum albumin was present at 250 µg/ml.

Endogenous DNA synthesis. Assays were run for 30 to 180 min. at 30°C. Each assay contained, in a final volume of 100 µl, 25 µl of the fraction, Tris-HCl pH 8.5 50 mM, KCl 50 mM, MgAc 10 mM, dithiothreitol 2.5 mM, dATP, dCTP and dGTP 0.1 mM each, and [$^3$H]TTP 10 µM with a specific activity of 4400 cpm/pmol.

Cytochrome c oxidase assay. Each assay contained, in a final volume of 1.2 ml, 30 µg of reduced cytochrome c; the buffer was 50 mM $KPO_4$ pH 7.5, sucrose 5% w/v, bovine serum albumin 50 µg/ml, Triton X-100 0.4%. 5 µl of the fraction or of a dilution of the fraction were added and the decrease in absorbancy at 550 nm monitored.

Protein concentration was determined by the method of Lowry et al. (5).

RESULTS

Figure 1 shows the chromatography on a phosphocellulose column of DNA polymerase activities present in the mitochondrial pellet prepared, as described in Materials and Methods, from Xenopus laevis stage 6 oocytes. The chromatogram shows two peaks of activity able to use Poly(A)·oligo(dT) as template, eluting respectively at 0.34 and 0.43 M KCl. The two peaks were not always separated and sometimes the elution pattern would show, as previously reported (1), a single peak of activity eluting around 0.4 M KCl. The chromatogram of Figure 1 also shows, in the flow-through fractions and at 0.28 M KCl, two peaks of activity that are detectable with activated DNA but not with Poly(A)·oligo(dT). These are a contamination of the mitochondrial pellet by a small amount (less than 5% of the total present in these cells) of α polymerase, an enzyme very abundant in Xenopus oocytes (3). This contamination is reduced or absent when the mitochondria used to prepare the extract are first purified on a sucrose gradient.

The two peaks of DNA polymerase activity at 0.34 and 0.43 M KCl are, as previously reported (1), sensitive to N'ehtylmaleimide. Figure 2 shows, for each of them, the velocity sedimentation in high salt glycerol gradients, and the utilization of activated DNA and Poly(A)·oligo(dT) as templates at various salt concentrations. The fact that the unusual stimulation by high salt when activated DNA is used as template is identical for the two peaks, the fact that they have an identical sedimentation velocity, and the fact that sometimes the activity elutes as a single peak from phosphocellulose have led us to the conclusion that the two peaks are two forms of the same enzyme, and therefore in this paper they will be referred to as one enzyme.

The fact that the enzyme eluting around 0.4 M KCl and able to use Poly(A)·oligo(dT) as template is present in the mitochondrial pellet or in mitochondria purified on a sucrose gradient does not prove by itself that it is a mitochondrial enzyme. Unfortunately, one or more inhibitors of the Poly(A) dependent Poly(dT) synthesizing activity (probably nucleases) are

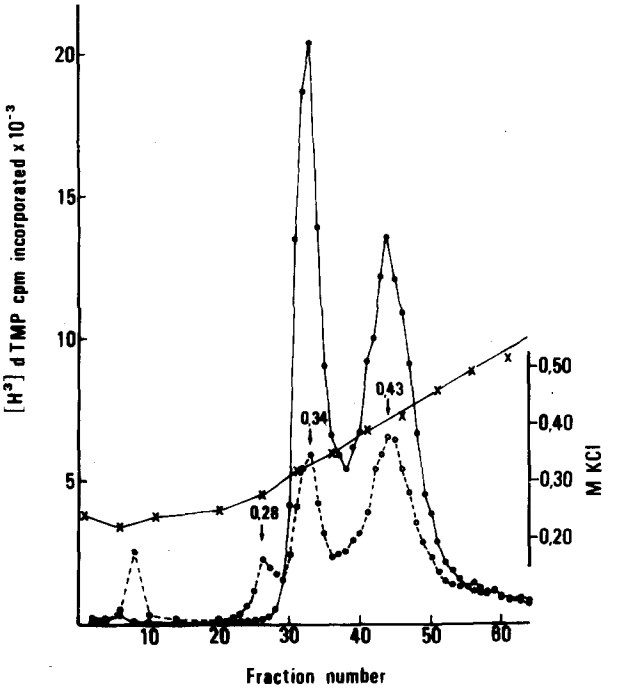


Figure 1. Phosphocellulose chromatogram of DNA polymerases present in mitochondrial pellet. 5 µl of the fraction (0.5 ml) were assayed as described in Materials and Methods. Poly(A)·oligo(dT)⟷, activated DNA○--○were used as templates.

present in the crude extract but not in the mitochondrial pellet; these inhibitors elute from phosphocellulose with the DNA polymerase. For this reason, experiments to compare the recovery of the DNA polymerase and of cytochrome c oxidase (a known mitochondrial enzyme) from different subcellular fractions were difficult to interpret; nonetheless the results obtained (not shown) were consistent with the notion that the two enzymes are located in the same structure in the cell.

If a mitochondrial pellet is centrifuged to equilibrium on a sucrose gradient and the fractions are assayed for cytochrome c oxidase activity (Fig. 3, panel B) and for endogenous DNA synthesis (Fig. 3, panel A), the two activities peak at the same fractions; this DNA synthesis is sensitive to the presence of 10 µg/ml of ethidium bromide, a known inhibitor of mitochondrial DNA synthesis (not shown). When DNA synthesized endogenously by fractions

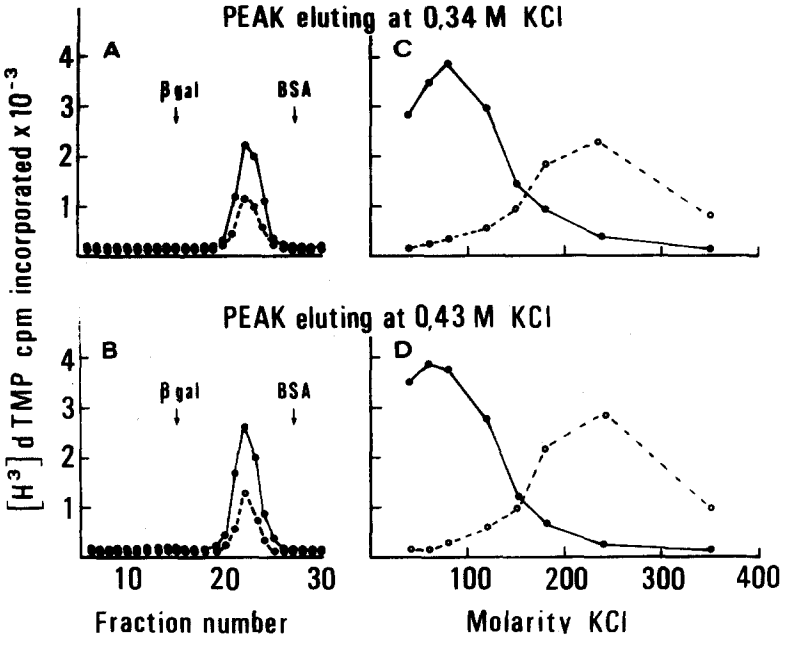


Figure 2. A and B. Sedimentation in glycerol gradient. Fractions from phosphocellulose were adjusted to 300 mM KCl and lyaered on a linear 10-30% glycerol gradient containing Tris-HCl pH 7.5 50 mM, KCl 300 mM, Dithiothreitol 10 mM; the gradient was centrifuged in a Spinco rotor SW41 at 39,000 rpm for 18 hrs at 4°C. BSA and β-galactosidase were used as markers in the same gradient. 10 µl of each fraction were assayed as in Materials and Methods. Poly(A)·oligo(dT)⟷ or activated DNA○---○. C and D. Salt dependence of polymerase activity. 5 µl of dyalized phosphocellulose fraction were assayed as in Materials and Methods. Poly(A)·oligo(dT)⟷; activated DNA○---○.

15, 16, 17 of Figure 3 is banded to equilibrium in a CsCl gradient containing ethidium bromide, the pattern obtained reproduces exactly the pattern expected for the product of mitochondrial DNA synthesis, i.e., with approximately one-third of the labeled material in a supercoiled form (Fig. 4). The characteristics of the reaction (sensitivity to ethidium bromide), and of its product (one-third supercoiled), indicate that the endogenous DNA synthesis, which peaks in these gradients with the cytochrome c oxidase, is due to the mitochondrial DNA synthesizing machinery.

If the DNA polymerase we were studying was of mitochondrial origin, the fractions of the sucrose gradient in Figure 3 showing the highest activity for cytochrome c oxidase and mitochondrial DNA synthesis should also contain

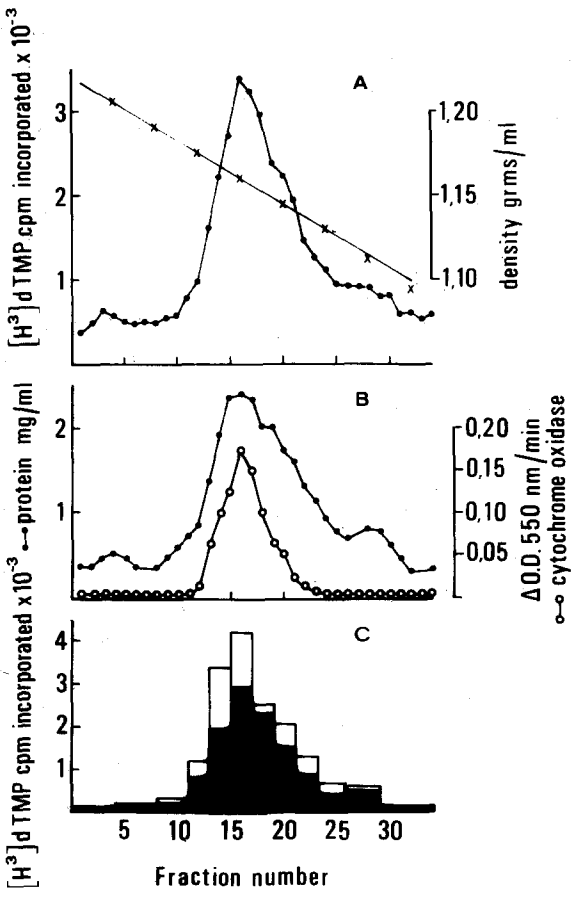


Figure 3. The mitochondrial pellet was centrifuged to equilibrium in a sucrose gradient. Panel A: Fractions were assayed for endogenous DNA synthesis ⟷; Panel B: protein concentration ⟷ and cytochrome c oxidase activity ○–○; Panel C: fractions were pooled in groups of three; from each pool the DNA polymerase was solubilized, purified and assayed (see Materials and Methods). Activated DNA ■ and Poly(A)·oligo(dT) □ were used as templates.

the highest amount of the DNA polymerase. That this is in fact the case can be seen in Figure 3, panel C which shows the amount of DNA polymerase present in the various regions of the gradient.

It could still be that the DNA polymerase is part of some structure other than mitochondria, that by coincidence has the same density as mitochondria and sediments with them. In a solubilization experiment (not shown) we treated a mitochondrial suspension with various amounts of detergent, centrifuged it at low speed and measured in the pellet and in the supernatant

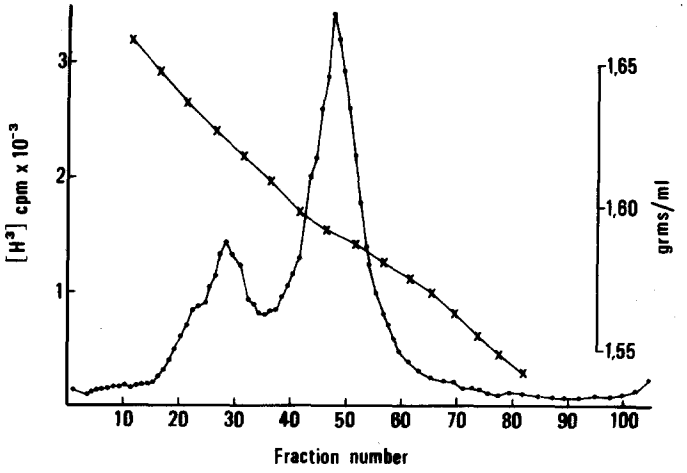


Figure 4. CsCl-EtBr density gradient centrifugation of the product of the endogenous DNA synthesis of pooled fractions 15, 16, 17 of Figure 3. The endogenous DNA synthesis was run as in Materials and Methods, stopped by adding an equal volume of EDTA 20 mM and Sarcosyl to 1% final concentration; after phenol extraction the aqueous phase was brought to 5 ml containing EtBr 300 µg/ml and CsCl to give a density of 1.6 g/ml. Centrifugation was for 48 hrs at 30,000 rpm in an SW50.1 Spinco rotor. TCA precipitable material from each fraction was colleted on GFC filters and counted.

the amount of each of the three activities we could assay, namely cytochrome c oxidase, mitochondrial DNA synthesis (endogenous DNA synthesis), and Poly(A) dependent DNA polymerase. At any given detergent concentration these three activities are released in the same percentage into the supernatant, indicating that they are contained in the same subcellular structure, i.e., mitochondria.

## DISCUSSION

Bolden et al. (6) have reported that the $\gamma$ polymerase in both HeLa cells and rat liver is the mitochondrial DNA polymerase. In this paper, we present several experiments that indicate that in Xenopus laevis oocytes, too, the enzyme that most resembles the $\gamma$ polymerase of other systems is the mitochondrial DNA polymerase. Unlike the situation in HeLa cells and rat liver, the enzyme in Xenopus laevis oocytes seems to be present only in mitochondria and seems to be the only polymerase present in them. The close association of this enzyme with the mitochondrial DNA synthesizing machinery

supports the possibility that it is responsible for mitochondrial DNA synthesis. If this is the case, it would suggest that it is not the presence of the polymerase that controls mitochondrial DNA synthesis, because this synthesis is not active in stage 6 oocytes and will only resume much later after fertilization. We have preliminary evidence that indicates that in fact it is the availability of deoxy-NTPs inside the mitochondria that plays the major role in the regulation of mitochondrial DNA replication in these cells.

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