DNA Polymerase of Mitochondria Is a γ-Polymerase

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Mitochondria isolated from rat liver cells or mycoplasma-free HeLa cells contain a single DNA polymerase activity which is closely related to, or identical to, the DNA polymerase γ activity found in the homologous cell. In rat liver cells, about 16% of the total cytoplasmic γ-polymerase activity is found associated with mitochondria and in HeLa cells about 20% of the total cellular γ-polymerase is mitochondria associated.

Since mitochondria possess no unique DNA polymerase activity, the number of DNA polymerases now known in mammalian cells is reduced, from the previously proposed four enzymes, to three — DNA polymerases α, β, and γ.

The first studies of Kalf and Ch'ih and Meyer and Simpson (1, 2), which revealed that mitochondria isolated from rat liver contained a DNA polymerase activity, also concluded that the mitochondrial-associated DNA polymerase differed from the other cellular DNA polymerases. Since those studies it has become clear that animal cells contain three major and distinct DNA polymerases called α, β, and γ (3, 4) and it was assumed that the mitochondrial DNA polymerase (mt-polymerase) represented the 4th, albeit minor, cellular polymerase. Although the enzyme has not been highly purified, the properties of the mt-polymerase from murine, rat liver, and HeLa cells show it to have a molecular weight of about 150,000 and to be stimulated by 0.05 to 0.10 M salt (5-7).

Several other reports which dealt with cultured human cells, either HeLa (8, 9) or KB cells (10), found that isolated mitochondria contained two DNA polymerases. One of these had chromatographic properties, and a template specificity similar to DNA polymerase γ and the other, a new DNA polymerase of 105,000 daltons which differed in its properties from the cellular DNA polymerases α, β, and γ (8, 11, 12).

However, we have recently found that the HeLa cells used in some of these experiments (8, 11, 12) were contaminated with a mycoplasma strain which was clearly detectable only under anaerobic assay conditions (13). We have, therefore, reinvestigated mitochondria isolated from either mycoplasma-free HeLa cells or rat liver for DNA polymerase activity and have found, in both cases, a single DNA polymerase in the purified mitochondria. This mitochondrial associated enzyme closely resembles the γ-polymerase found in the corresponding cytoplasm of these cells.

**RESULTS**

The DNA polymerase associated with mitochondria, isolated from either rat liver or HeLa cells, can be extracted with high salt containing solutions which solubilize 75 to 90% of the total DNA polymerase activity. Further purification of the mitochondrial associated DNA polymerase (DNA polymerase mt) (3) was achieved by DEAE-cellulose and phosphocellulose chromatography. These mitochondrial DNA polymerases were then compared to the γ-polymerase prepared from the homologous cytoplasm. The following section deals first with the rat liver mitochondrial associated DNA polymerase and cytoplasmic γ-polymerase and a later section will deal with the corresponding human (HeLa cell) enzymes.

Purification of Rat Liver Cytoplasmic γ-Polymerase and Mitochondrial-associated DNA Polymerases — An extract of purified rat liver mitochondria was chromatographed successively on DEAE-cellulose and phosphocellulose as shown in Fig. 1. In these chromatographic studies the DEAE-cellulose and phosphocellulose columns were assayed with both an "activated" DNA template and the oligomer ribohomopolymer complex (dT)12-18·poly(A). The former is a versatile and ubiquitous template for the α-, β- and γ-DNA polymerases while the latter template is utilized only by the β- and γ-polymerases (4). As can be seen, the mitochondrial DNA polymerase, which elutes as a single enzymatic peak on DEAE-cellulose (Fig. 1A) at 0.10 to 0.15 M KPO₄, copies both an activated DNA and a poly(A) template and the peak of DNA polymerase activity with these two templates almost coincides. Since the β-polymerase is never found in the cytoplasm in our experience (4, 14), and since it is known not to be retained under these conditions on DEAE-cellulose (14, 15), the chromatographic behavior and template characteristics of the mitochondrial-associated DNA polymerase on DEAE-cellulose shows it to be that expected of the cytoplasmic DNA polymerase γ. Furthermore, when the mitochondrial-associated DNA polymerase activity, as isolated from DEAE-cellulose, is further chromatographed on phosphocellulose (Fig. 1B), a single enzymatic activity eluting at 0.25 to 0.30 M KPO₄ is obtained which again copies both activated DNA and (dT)12-18·poly(A).

Rat liver cytoplasm contains a substantial amount of DNA polymerase γ activity which is distinct from other known cellular DNA polymerases (16, 17). To isolate this activity, the cytosol, prepared as described under "Methods" was chromatographed immediately following this paper. (Figs. 1S through 4S and Tables I through III are found on pp. 3355-3356.) For the convenience of those who prefer to obtain the supplementary material in the form of 13 pages of full size photocopies, it is available as JBC Document Number 76M-1564. Orders should specify the title, authors, and reference to this paper, the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.95 per set of photocopies.

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The enzyme peak, containing a total of 108 mg of protein (bovine to 26, A) was pooled and diluted with an equal volume (70 ml) of 0.02 under "Methods" (see miniprint supplement). B, phosphocellulose chromatography of the rat liver mitochondrial DNA polymerase. The DNA polymerase peak obtained from DEAE-cellulose (Fractions 18 to 21, A) was pooled and diluted with an equal volume (70 ml) of 0.02 M potassium phosphate, pH 7.5, containing 0.5 mM dithiothreitol. The enzyme peak, containing a total of 108 mg of protein (bovine serum albumin included), was applied to a 7-ml column of phosphocellulose. Elution was performed as described under "Methods" and aliquots of the indicated fractions were assayed for DNA polymerase activity as in A. ●, (dT)$_{12-18}$ poly(A) template; ○, activated salmon sperm DNA template.

tographed on DEAE-cellulose (Fig. 2A). Two separable DNA polymerases are resolved in this step. One of these enzymatic peaks, eluting at 0.15 to 0.20 M KPO$_4$, copies activated DNA but not (dT)$_{12-18}$ poly(A) and is presumably the α-polymerase which is known to be found in the cytoplasm when cells are broken by conventional dounce homogenization in many aqueous solutions. The other DNA polymerase activity which elutes at about 0.10 M KPO$_4$ is clearly the γ-polymerase as it copies both (dT)$_{12-18}$ poly(A) and activated DNA. Further chromatography of this γ-polymerase peak, obtained by DEAE-cellulose chromatography, on phosphocellulose is shown in Fig. 2B. A single peak of DNA polymerase γ eluting at about 0.30 M KPO$_4$ is obtained and this enzyme copies, as expected, activated DNA and a poly(A) template.

Both the mitochondrial-associated DNA polymerase and the cytoplasmic γ-polymerase when further chromatographed on DNA-cellulose also show one peak of DNA polymerase activity which elutes at 0.25 M KPO$_4$ (data not shown). It is thus apparent that in rat liver, the mitochondrial-associated DNA polymerase seems very similar to the cytoplasmic γ-polymerase in its behavior on DEAE-cellulose, phosphocellulose, and DNA-cellulose column chromatography and in its ability to copy (dT)$_{12-18}$ poly(A), a template which cannot be used by DNA polymerase α (18).

Because of the low levels of these polymerases in rat liver, it has been difficult to obtain an extensive purification. Table IIS shows that after the phosphocellulose chromatography step, these enzymes were purified about 100-fold (DNA polymerase mt) or 800-fold (DNA polymerase γ) from either the purified mitochondria or the cytosol. Nevertheless, as shown below, other data obtained with these enzyme preparations indicate quite clearly that the mitochondrial associated DNA polymerase is, in fact either identical to or a closely related form of the γ-polymerase found in the mitochondria-free cytoplasm.

The pH dependence and divalent metal requirement were the same for both rat liver DNA polymerases γ and mt. Optimum activity was observed at pH 8 to 9 in the presence of 0.5 mM Mn$^{2+}$ or 5 to 10 mM MgCl$_2$. Both enzyme activities are stimulated optimally in 0.25 to 0.3 M KCl as shown in Fig. 1A and 1B but show a differential effect when KPO$_4$ is added in the incubation (Fig. 2A and 2B). In the presence of 100 mM KPO$_4$, the mt-polymerase was unaffected whereas the γ-polymerase was inhibited about 80%. This represents one of the few differences between the cytoplasmic and mitochondrial γ-polymerases and as will be shown later, this difference in response to KPO$_4$ is found with the HeLa cell γ-polymerases.

A further study of the requirements and inhibitors of the rat liver γ-polymerase and mitochondrial associated enzyme is presented in Table IIS. Dithiothreitol is not an absolute requirement for activity with either of the polymerases. Both activities are strongly inhibited in the presence of 10 mM N-ethylmaleimide or 25 μM ethidium bromide and are relatively insensitive to inhibition by Ara ATP and Ara CTP. Template studies with the rat liver mitochondrial-associated DNA polymerase and cytoplasmic γ-polymerase showed that both copy a poly(A) template strand much more efficiently than a poly(dA) template (Table IIS) This phenomena is characteristic of the γ-polymerase class of enzymes (18, 19) and confirms the poly(A) template copying ability of both enzymes noted in Figs. 1 and 2.

Another useful tool for identifying a DNA polymerase is a molecular weight determination. Since eukaryotic DNA polymerases are known to aggregate readily in solutions of low ionic strength (4), velocity sedimentation analysis of the par-
tially purified polymerases was performed in high salt- and low salt-containing linear glycerol gradients as described under "Methods." The partially purified rat liver DNA polymerases mt and γ were first dialyzed at low ionic strength (0.02 M KPO₄). When the enzyme preparations were brought to 0.25 M KCl and then applied onto high salt containing 10 to 30% glycerol gradients, the polymerases were found to sediment with the same s value (Fig. 3A). Both enzymes exhibited one major peak of DNA polymerase activity sedimenting at about 4 S, and slightly faster than myoglobin. Assuming a globular shape of the proteins and a partial specific volume of 0.736, a molecular weight of about 60,000 was estimated for the rat liver mitochondrial and the rat liver γ-polymerases. When the dialyzed, partially purified, polymerases in 0.02 M KPO₄ were applied to low salt glycerol gradients, containing no KCl, both enzymes appeared as multiple heterogenous forms, although DNA polymerase mt under these conditions appeared to form larger aggregates than DNA polymerase γ (Fig. 3D). The mitochondrial polymerase activity separated into a major peak (11.3 S), two minor peaks (greater than 11.3 S), and another minor peak of activity (6.5 S). No DNA polymerase activity was detected at 4 S under these sedimentation conditions, so that the enzymatic species of 60,000 daltons present in high salt has been completely converted to aggregated, higher molecular weight species ranging from about 120,000 (6.5 S) to about 240,000 (11.3 S) or greater. DNA polymerase γ activity on the other hand separates into two peaks in low salt solutions, the original peak, which sediments at approximately 4 S and a new peak sedimenting at about 6.5 S. Since the mt-polymerase seems to be a form of the γ polymerase, the aggregation of the rat liver γ-polymerase in low salt to form dimers or multimers as shown here, and reported elsewhere for the enzyme obtained from other sources (19), should also be expected to be observed with the mt-polymerase. Probst and Meyer (5) have reported a molecular weight of 150,000 for the rat liver mitochondrial DNA polymerase. Their determinations were done by sucrose velocity gradients centrifugation in 0.02 Tris/HCl and these low salt conditions would be expected to favor aggregation of the mt-polymerase as shown here.

Comparison of HeLa Cell Mitochondrial DNA Polymerase and DNA Polymerase γ—The HeLa cell DNA polymerase γ obtained from the cytoplasm has been highly purified and its characteristics carefully examined by Knopf et al. (20). This work serves as a basis for the studies done with mitochondrial DNA polymerase isolated from mycoplasma-free HeLa cells. When these mitochondria are extracted with 1 M KCl and the resulting extract, after dialysis to remove the salt, is chromatographed on DEAE-cellulose, a single peak of DNA polymerase activity, which copies both activated DNA and (dT)₃₀₋₅₀poly(A), is observed (Fig. 3E). This enzyme, when further chromatographed on phosphocellulose, again shows a single peak of enzymatic activity which copies both templates (Fig. 3F). The elution position of the mitochondrial-associated DNA polymerase from DEAE-cellulose (about 0.15 M KPO₄) and from phosphocellulose (0.2 to 0.3 M KPO₄) are the same as those described for the HeLa cytoplasmic DNA polymerase γ by Knopf et al. (20). The mitochondrial-associated DNA polymerase was purified about 300-fold from mitochondria after the phosphocellulose step (Table II).

The HeLa mitochondrial associated DNA polymerase was further compared to the HeLa γ-polymerase by several criteria. As shown in Table II, a divalent cation such as Mn²⁺ is required for the activity of both DNA polymerase γ and mt from HeLa cells. Ethidium bromide and N-ethylmaleimide are effective inhibitors of both enzymes though the mitochondrial polymerase seems more sensitive to the latter reagent. Neither Ara CTP or Ara ATP will inhibit the γ or mt-polymerases from HeLa cells.

Both enzymes show maximal activity in 0.12 to 0.16 M KCl as shown in Fig. 1A and 1B where enzyme activity is measured as a function of KCl concentration. The effect of KPO₄, which is known to stimulate the HeLa γ-polymerase under proper conditions, was also tested (Fig. 2C and 2D). DNA polymerase γ was stimulated in the presence of 20 mM KPO₄ and was inhibited by concentrations above 50 mM KPO₄. Knopf et al. (20) have reported a stimulation of DNA polymerase γ by 50 mM KPO₄ in the presence of 130 mM KCl. In our experiments, the mitochondrial DNA polymerase was essentially unaffected by KPO₄, up to 0.1 M. This difference in response to KPO₄ was also noted with the rat liver DNA polymerase γ and mt (Fig. 2A and 2B).

Velocity sedimentation of the HeLa cell mitochondrial DNA polymerase in linear glycerol gradients containing 0.25 M KCl revealed a single enzymatic peak sedimenting at 8.1 S (data not shown). DNA polymerase γ is reported to have an s value of 7.8 under these conditions (20). The rate of heat inactivation of both enzymes at 45° was identical (Fig. 4B) with 80% of the activity being lost by 10 min. The template studies shown in Table II also show the HeLa mitochondrial associated polymerase and the γ-polymerase to copy templates in a very similar manner. A synthetic ribonucleopolymer such as (dT)₃₀₋₅₀poly(A) is copied 3 to 10 times faster than either (dT)₃₀₋₅₀poly(dA) or an activated DNA template. As shown here, (dG)₃₀₋₅₀poly(dC) is an exceptional template for the γ and mt-polymerases from HeLa cells.

In summary, the template characteristics of HeLa cell DNA

![Glycerol gradient sedimentation analysis of the rat liver γ-polymerase in high salt.](https://example.com/glycerol-gradient-sedimentation-analysis-of-rat-liver-g-polymerase-in-high-salt)
Polymerase mt strongly resemble those of the HeLa cell DNA polymerase γ and the general characteristics of both enzymes would indicate they are, in fact, the same, or closely related, species.

**DISCUSSION**

A previous report from this laboratory (8) had indicated that HeLa cell mitochondria contained two DNA polymerases. One of these resembled DNA polymerase γ and the other was a unique enzyme unrelated to the other cellular DNA polymerases. Subsequent testing for mycoplasma under anaerobic conditions revealed that the HeLa cells used in those experiments were contaminated with this organism. Hence, mitochondria from rat liver or from mycoplasma-free HeLa cells were reexamined and found to contain a single DNA polymerase activity which is very similar to the γ-polymerase isolated from the cytoplasm of the same cells.

The important observations are that the mitochondrial-associated DNA polymerase in rat liver or HeLa cells has a molecular weight identical to the homologous γ-polymerase when measured in solutions of high ionic strength, and can utilize various templates in a manner similar to the corresponding cellular γ-polymerase. In addition, both the mt- and γ-polymerases show the same response to KCl, to a varied extent which is very similar to the γ-polymerase isolated from the cytoplasm of the same cells.

We, therefore, conclude that the mitochondrial DNA polymerase is either DNA polymerase γ or a closely related form of this enzyme. The γ-polymerase was first isolated from both nuclei and cytoplasm prepared by conventional aqueous techniques and it is worth noting that, of the total DNA polymerase γ activity found in the rat liver cell cytoplasm, about 16% is associated with the purified mitochondria isolated from the cytoplasm. A similar calculation of the DNA polymerase γ activity in HeLa cell cytoplasm showed that 29% of the total enzyme activity was associated with the mitochondria and the rest was found in the postmitochondrial supernatant. Since the Dounce homogenization technique used to break HeLa cells in these studies leads to the appearance of about 80% of the DNA polymerase γ activity in the cytoplasmic fraction (21), HeLa cell mitochondria would appear to contain 20 to 25% of the total cellular γ-polymerase activity. In both rat liver and HeLa cells there are minor differences between the γ-polymerase which is found in the cytoplasm and that isolated from the mitochondria. These include a difference in response to high KPO₄ concentrations and a difference in the size of the aggregated forms of the rat liver polymerases under low ionic strength conditions (Fig. 3). It is not clear whether these differences reflect alternate forms of the γ-polymerase such as reported by Spadari and Weissbach (21), or whether γ-polymerases at various stages of purification show small variations in their characteristics because of the presence or absence of accessory proteins or components which affect the enzyme activity or structure. A comment on the molecular weight of the rat liver mitochondrial DNA polymerase is pertinent here. We find that the rat liver mt- and γ-polymerases in the presence of high salt (0.25 M KCl) have an s value of about 4 and a calculated molecular weight of about 60,000. However, under conditions of low ionic strength, the mitochondrial enzyme formed larger aggregates of 11 S and higher with a smaller component at 6.5 S. Probst and Mayer (5) have reported the rat liver mitochondrial DNA polymerase to have an s value of 9.2 in sucrose gradient of low ionic strength (0.025 M Tris/HCl).

We also find that the rat liver cytoplasmic γ-polymerase can form an aggregate at low ionic strength which sediments at 6.5 S though larger aggregates in the 11 S region were not observed (Fig. 3). Since the basis and mechanism of aggregation of these enzymes are unclear, we cannot explain the differences between these two forms of the γ-polymerase as found in these experiments.

With the knowledge that the mitochondrial associated DNA polymerase is a γ-polymerase, the number of major DNA polymerase classes known to be common to higher eukaryotic cells is now reduced to 3: the α-, β-, and γ-polymerases (3, 4). It is, of course, unclear if the γ-polymerase is the enzyme utilized in the synthesis of mitochondrial DNA since its association with the mitochondria in a broken cell preparation may be artifactual. It is possible that one or more of the other cellular DNA polymerases are used for mitochondrial DNA synthesis but are removed from the organelle upon cell disruption. If, on the other hand, the γ-polymerase or a closely related form proves to be the key polymerizing enzyme in the replication of mitochondrial DNA, one would then ask if this enzyme has any other role in the cell, or whether its function is related only to mitochondrial DNA synthesis. The finding that about 20% of the γ-polymerase is apparently found in the nucleus of HeLa cells might suggest a wider role for the γ-polymerase and the enzyme has also been implicated in the synthesis of adenovirus DNA (22). Investigations with mutants which are defective in mitochondrial function might be a useful approach to the relationship of DNA polymerase γ and mitochondrial DNA synthesis (23).

**REFERENCES**

Mitochondrial Associated DNA Polymerase 

Supplemental Material

The DNA Polymerase of Mitrionitronia in a 1.25-hL polymerization buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM MgCl₂, 0.1 mM dNTPs, 1 mM DTT, 10% glycerol) at 37°C for 30 min. The DNA polymerase was purified to homogeneity by preparative SDS-PAGE and then eluted from the gel. The purified enzyme was assayed for DNA polymerase activity using the method of Kunkel et al. (1985). The purified enzyme was used for further experiments.

**Previous Experiments**

Supplemental Figure 1: Supplementation with 1.25-hL polymerase buffer. (A) The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min. (B) The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min. (C) The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min.

**Materials and Methods**

Assay Mixture

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<th>Component</th>
<th>Concentration</th>
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<tbody>
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<td>T4 DNA Polymerase</td>
<td>0.25 U/μL</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.25 mM each</td>
</tr>
<tr>
<td>DNA template</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>DNA polymerase activity buffer</td>
<td>10X</td>
</tr>
</tbody>
</table>

**Results**

Supplemental Figure 2: Effects of temperature on DNA polymerase activity. The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min. (A) The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min. (B) The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min.

**Discussion**

The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min. The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min. The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min.

**Tables**

Table 1: Purification of DNA polymerase from mitochondrial extracts. The DNA polymerase activity was determined using a DNA polymerase assay kit (Roche). The activity was measured at 37°C for 30 min. | Value |
<table>
<thead>
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<th></th>
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<tr>
<td>Total activity</td>
<td>0.25 U</td>
</tr>
<tr>
<td>Specific activity</td>
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</table>

**Acknowledgments**

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**References**


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It is apparent that these two enzymes are very similar in their kinetic and their effect on 
DNA replication. Figure 4 shows the effect of two different inhibitors on the activity of 
DNA polymerase associated with mitochondria. The results indicate that both inhibitors 
have a similar effect on the activity of the enzyme. The DNA polymerase associated with 
mitochondria is inhibited by both inhibitors, while the DNA polymerase associated with 
the nucleus is not inhibited by either of the inhibitors.

**Fig. 4.** Test inhibition of the mitochondrial DNA polymerase. The DNA polymerase activity of the nuclei and mitochondria was determined in the presence of various concentrations of the inhibitors. The DNA polymerase activity was measured by the incorporation of tritiated thymidine into DNA.

**REFERENCES**

Note: References 1-7 will be found in the parent paper.

DNA polymerase of mitochondria is a gamma-polymerase.
A Bolden, G P Noy and A Weissbach


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