DNA Polymerase of Mitochondria Is a y-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) resembled 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 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 columns and 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 chroma-
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 miniprint supplement. B, phosphocellulose chromatogram of the rat liver mitochondrial DNA polymerase. The preparation of mitochondrial extract, chromatography, elution, and determination of DNA polymerase activity was performed as described under "Methods." (see miniprint supplement). The preparation of mitochondrial DNA polymerase activity was performed as described under "Methods." (see miniprint supplement). The preparation of mitochondrial DNA polymerase activity was performed as described under "Methods." (see miniprint supplement). DEAE-cellulose chromatogram of rat liver mitochondrial DNA polymerase. The DNA polymerase peak obtained from DEAE-cellulose (Fractions 18 to 26, 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. This 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.

Mitochondrial Associated DNA Polymerase

Fig. 1. DEAE-cellulose and phosphocellulose chromatography of the rat liver mitochondrial polymerase. A, DEAE-cellulose chromatogram of the rat liver mitochondrial DNA polymerase. The preparation of mitochondrial extract, chromatography, elution, and determination of DNA polymerase activity was performed as described under "Methods." (see miniprint supplement). B, phosphocellulose chromatogram of the rat liver mitochondrial DNA polymerase. The DNA polymerase peak obtained from DEAE-cellulose (Fractions 18 to 26, 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.

Mitochondrial Associated DNA Polymerase

Fig. 2. Chromatography of rat liver DNA polymerase γ. A, DEAE-cellulose chromatogram of rat liver mitochondrial DNA polymerase γ. Elution and assays of DNA polymerase activity was carried out as described under "Methods." B, phosphocellulose chromatogram of rat liver mitochondrial DNA polymerase γ. Pooled fractions, 16 to 21, from DEAE-cellulose column A, containing 8.5 mg of protein were diluted and applied to a 36-ml column of phosphocellulose. DNA polymerase activity was eluted and assayed as described under "Methods." (dT)_{12-18}, poly(A) template; ○, activated salmon sperm DNA template.

Mitochondrial Associated DNA Polymerase

Because of the low levels of these polymerases in rat liver, it has been difficult to obtain an extensive purification. Table I shows that after the phosphocellulose chromatographic step, these enzymes were purified about 100-fold (DNA polymerase mt) or 800-fold (DNA polymerase γ) from either the purified mitochondrial 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 Mn²⁺ or 5 to 10 mM MgCl₂. 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 KPO₄ is added in the incubation (Fig. 2A and 2B). In the presence of 100 mM KPO₄, 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 KPO₄ 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 II. 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 II). 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-
ially 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 to 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 190,000 (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 0.20 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)ₙ,-poly(A), is observed (Fig. 3SA). This enzyme, when further chromatographed on phosphocellulose, again shows a single peak of enzymatic activity which copies both templates (Fig. 3SB). The elution position of the mitochondrial-associated DNA polymerase from DEAE-cellulose (about 0.15 M KPO₄), and from phosphocellulose (0.25 to 0.30 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 III).

The HeLa mitochondrial associated DNA polymerase was further compared to the HeLa γ-polymerase by several criteria. As shown in Table IIIA, a divalent cation such as Mg²⁺ 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. 1SC and 1SD 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. 2SC and 2SD). 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. 2SA and 2SB).

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. 4S) with 80% of the activity being lost by 10 min. The template studies shown in Table IIIA also show the HeLa mitochondrial associated polymerase and the γ-polymerase to copy templates in a very similar manner. A synthetic ribopolymer such as (dT)ₙ,-poly(A) is copied 3 to 10 times faster than either (dT)ₙ,-poly(dA) or an activated DNA template. As shown here, (dG)ₙ,-poly(dC) is an exceptional template for the γ and mt-polymerases from HeLa cells.

In summary, the template characteristics of HeLa cell DNA polymerase...
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 series of inhibitors, and heat inactivation studies showed the two enzymes to act in a similar manner. The behavior of these enzymes on DEAE-cellulose, phosphocellulose, and DNA-cellulose column chromatography is also the same.

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 given 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 mitochondrial DNA polymerase to have an s value of 9.2 in a sucrose gradient of low ionic strength (0.025 M Tris/HC1). 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**

Supplemental Material

The role of mitochondrial DNA in cell motility

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EXPERIMENTAL PROCEDURE

Mitochondria-associated DNA polymerase was purified from mouse liver by sequential centrifugation, followed by heparin-Sepharose and DEAE-Sepharose chromatography. The enzyme was then applied to a CM-Sepharose column and eluted with a linear gradient of NaCl. The purified enzyme was then analyzed by SDS-PAGE and Western blotting using antibodies specific for the enzyme.

The purified enzyme was then used to study its role in cell motility. Mitochondria were isolated from mouse liver and incubated with the purified enzyme. The motility of the mitochondria was then measured using a video-based motility assay. The results showed that the purified enzyme significantly increased the motility of the mitochondria, suggesting a role for mitochondrial DNA polymerase in cell motility.

TABLE 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Value (mean ± SEM)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.2 ± 0.03</td>
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The results showed a significant increase in motility upon treatment with the purified enzyme. Further studies are needed to understand the mechanism by which mitochondrial DNA polymerase regulates cell motility.

**REFERENCES**


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Mitochondrial Associated DNA Polymerase

It is apparent that these two enzymes are very similar in their activities and that they both require the presence of metal ions, as well as being sensitive to the presence of actin and ATP. The presence of actin and ATP is essential for the activity of both enzymes, with actin being particularly important in the case of the mitochondrial enzyme. The presence of ATP also appears to be necessary for the activation of the mitochondrial enzyme, as it is not active in the absence of ATP. The presence of metal ions, particularly zinc, is also required for the activity of both enzymes. The presence of zinc is essential for the activity of the mitochondrial enzyme, while it is not necessary for the activity of the actin-associated enzyme.

References:


Fig. 4: Time course of the mitochondrial DNA polymerase activity. The assay was performed as described in the Methods section. The enzyme was incubated with the template/primer at 37°C for 30 min, and the incorporation of dNTPs was determined. The incorporation was linear up to 30 min. The data are representative of at least three experiments. The error bars represent the standard deviation from the mean.
DNA polymerase of mitochondria is a gamma-polymerase.
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