*DNA BIOSYNTHESIS IN MITOCHONDRIA: PARTIAL PURIFICATION OF A DISTINCT DNA POLYMERASE FROM ISOLATED RAT LIVER MITOCHONDRIA**

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It is becoming increasingly evident that mitochondria possess a considerable degree of autonomy in their process of biogenesis.[1] That mitochondria can also synthesize their own DNA has been suggested by *in vivo* autoradiographic[2–6] and biochemical studies,[7–9] and has been proved conclusively by biochemical studies on isolated mitochondria.[10–12] In this report we now present evidence that the mitochondrion contains its own DNA polymerase, distinct from that in the nucleus.

Experimental.—Preparation of mitochondrial DNA polymerase: Rat liver mitochondria were isolated from 175–200-gm rats according to the method of Schneider and Hogeboom[13] as described previously.[10] A typical preparation (100 gm of liver) yielded about 1 gm of mitochondrial protein. The packed pellet was frozen in a dry ice-acetone bath and ground with levigated alumina (2 parts alumina to 1 part wet weight of mitochondria). All further operations were carried out at 0–4°. The paste was extracted with 20–25 ml of 0.05 *M* Tris buffer, pH 8.0 (25°) containing 0.02 *M* Mg acetate, 0.005 *M* β-mercaptoethanol, 0.001 *M* EDTA, and 1.0 *M* NaCl, and was centrifuged 60 min at 165,000 × *g* (Spinco 50 Ti rotor). The supernatant fluid was passed through a Sephadex G-25 column (3 × 20 cm) equilibrated with Tris-ME (0.025 *M* Tris pH 8.0 [25°] and 0.005 *M* β-mercaptoethanol). The desalted extract (fraction Mt-I) was stored at −125°C. Fraction Mt-I was thawed and slowly brought to 25% saturation with saturated $(NH_4)_2SO_4$ adjusted to pH 7.0 with NH_3. After centrifugation, the supernatant solution was brought to 43% saturation. The precipitate was collected, dissolved in Tris-ME buffer, and dialyzed in a rapid dialyzer[14] against 3–4 changes of the Tris-ME for 3–4 hr (fraction Mt-II).

A DEAE-cellulose column (0.9 × 7 cm) was washed, and fraction Mt-II (7–20 mg of protein per run) was applied and fraction collecting (2.4 ml) begun immediately. The column was then washed with about 35 ml of Tris-ME and eluted with 200 ml of a linear NaCl gradient (0–0.35 *M* NaCl in Tris-ME) at a flow rate of about 0.8 ml/min. The active fractions were pooled and concentrated by precipitation at 50% saturated $(NH_4)_2SO_4$. The dialyzed fraction (Mt-III) was stored at 4° and was used for periods up to 2 weeks.

Preparation of nuclear DNA polymerase: Rat liver nuclei were prepared by the method of Blobel and Potter[15] modified as follows: (1) their TMK buffer was changed to 0.05 *M* Tris pH 8.0 (25°), 0.005 *M* Mg acetate, 0.025 *M* KCl, 0.005 *M* β-mercaptoethanol, and 0.001 *M* EDTA; (2) the larger-volume Spinco SW25.2 rotor replaced the SW39 and the centrifugation time was increased to 1 hr at 25,000 rpm; (3) the sucrose was then decanted and the upper 75% of the tube was cut off. The nuclear pellets were combined and resuspended in 75 ml TMK. A typical preparation (180 gm of liver) run in 6 batches yielded about 850 mg of protein.

The nuclear suspension was sonicated 1–2 min until all nuclei were lysed. NaCl (4 *M*) was slowly added to a concentration of 1 *M*. The extract was dialyzed 4–6 hr against three changes of TMK containing 0.15 *M* NaCl. (The complexes which occur between DNA and polymerase or histone are dissociated by 1.0 *M* NaCl. Subsequent reduction of the NaCl concentration to 0.15 *M* results in the precipitation of DNA-histone complex while avoiding reassociation of the DNA with the polymerase.) The precipitate was removed by centrifugation and the supernatant solution constitutes fraction Nc-I.

Fraction Nc-I was immediately subjected to $(NH_4)_2SO_4$ fractionation, and the protein precipitating between 43 and 75% saturation was collected. The rapidly dialyzed material constitutes fraction Nc-II. Fraction Nc-II was chromatographed on DEAE-cellulose as described for the mitochondrial enzyme. However, the nuclear enzyme is not retained by the column, the bulk of the polymerase activity appearing at the front. The active fractions were pooled and concentrated with $(NH_4)_2SO_4$ at 75% saturation. The dialyzed material (fraction Nc-III) was fairly stable when stored at 4°.

Enzyme assays: The composition of the reaction mixtures is given in the legends. In some experiments the levels of TTP were below enzyme saturation. However, properties of the enzyme such as response to salt, Mg^{+2} optimum, and primer specificity, were not affected by changes in substrate concentration or by the extent of loss of enzyme activity through decay. TTP incorporation of 100-μl samples was determined by the filter paper method.[20]

Protein determination: Protein was measured by the biuret method[16] in the crude fractions and by ultraviolet absorption[17] in the partially purified fractions.

Results.—In order to make a valid comparison between the mitochondrial and nuclear DNA polymerases, it was first necessary to isolate and purify these enzymes to a reasonable degree. The use of isolated and purified mitochondria and nuclei facilitated the purification, particularly with respect to contamination of one enzyme by the other.

Purification of mitochondrial DNA polymerase: The increase in the specific activity of the enzyme starting with intact mitochondria is about 11,000-fold, and starting with the crude lysate is about 1,100-fold (Table 1). To what extent this increase represents the removal of inert protein is not clear. For example, the tenfold increase observed when mitochondria are lysed can largely be accounted for by the removal of permeability barriers. Further, the sedimentation and gel filtration step leading to fraction Mt-I results in a large increase in specific activity with less than a threefold purification on a protein basis. This may occur primarily from the removal of endogenous TTP. In addition, optimal assay conditions change during the purification. Although each fraction was assayed at near-optimal conditions, the changes in optima indicate the presence of interfering enzymes which would be likely to result in falsely low specific ac-

TABLE 1. *Purification of rat liver mitochondrial and nuclear DNA polymerases.*

	Mitochondrial Enzyme (Mt)		Nuclear Enzyme (Nc)	
Enzyme fraction	Specific activity (units/mg protein)	Total activity (units)	Specific activity (units/mg protein)	Total activity (units)
Intact organelle	0.014	13	6.0	5720
Disrupted organelle	0.14	132	6.7	6650
Salt extract (fraction I)	8.1	2470	24	5430
$(NH_4)_2SO_4$ (fraction II)	28	2370	91	2240
DEAE-cellulose (fraction III)	155	1330	465	780

The enzyme activity for each fraction was measured at near-optimal conditions. The activity of intact and disrupted mitochondria was determined under incubation conditions described by Parsons and Simpson.[10] The reaction mixtures for assaying the other fractions consisted of 25 mM Tris pH 8.0 (25°), 100 μg/ml calf thymus DNA (Worthington Biochemical Corp.; native DNA for Nc and denatured[20] for Mt fractions), 0.015 mM each of dATP, dCTP, dGTP, and H^3-TTP (specific activity, 300 mc/mmole), Mg acetate (7.5 mM for Mt-III and all Nc fractions; 20 mM for Mt-I and Mt-II), 0.5 mM EDTA, 5 mM β-mercaptoethanol, 2 mM ATP, and either 0.15 *M* NaCl (Mt fractions) or 0.025 *M* KCl (Nc fractions). The reactions were run in a final volume of 125 μl which contained 25 μl (0.1–1.0 enzyme unit, 4–100 μg protein) of the fraction tested, and the tubes were incubated at 37° for 1 hr. An enzyme unit is defined as the incorporation of 0.1 mμmole of TTP per hour at 37°.[24]

tivities, particularly of less purified fractions. Experiments in progress indicate that no change in the optimal assay conditions for fraction Mt-III occurs when this preparation is subjected to hydroxylapatite chromatography.

On the other hand, the somewhat unstable nature of the enzyme preparations results in a continued loss of activity throughout purification (from 30%/day in fraction Mt-I to 6%/day in fraction Mt-III). This loss, if corrected for, would raise the specific activity of fraction Mt-III. Considering all these factors, we estimate the purification of the enzyme to be not less than 60-fold, and probably higher. Since mitochondria account for about 25 per cent of total liver protein,[18] this would represent at least a 240-fold purification based on whole liver as the starting substance.

Properties of the mitochondrial DNA polymerase: Like other DNA polymerases,[19] the enzyme requires Mg^{+2}, DNA primer, and the four dXTP's. Table 2 shows that the enzyme possesses an absolute requirement for DNA and that, with fraction Mt-III, this can be met by either denatured[20] or native calf thymus DNA. In contrast, crude fraction Mt-I shows a fivefold preference for the denatured form. This change may be caused by the presence in fraction Mt-I of a native DNA-specific nuclease which might give rise to inhibitory 3′-phosphate ends.[21] The requirement for ATP in fraction Mt-I probably results from the enzymatic degradation of a DNA precursor(s), especially dGTP[22] and its regeneration by ATP.

The enzyme exhibits one interesting property: it is stimulated by salt to an unusual extent, about six- to sevenfold (Fig. 1). This is observed with either native or denatured DNA primer and at all stages of purification. It is noteworthy that the optimal NaCl concentration is rather high, about 0.15 *M*. Salt effects are not uncommon among mammalian DNA polymerases but the effect is relatively moderate, about twofold, and the optimal salt concentration is much lower.[19] The effect appears to be largely nonspecific since K^+ and NH_4^+ can be

TABLE 2. *Properties of rat liver mitochondrial and nuclear DNA polymerases.*

Experimental conditions	Incorporation (%) Enzyme Fraction: Mt-I	Mt-III	Nc-III
Complete system	100	100	100
− dATP, dCTP, dGTP	9	7	43
− Mg^{+2}	<1	<1	<1
− DNA	<1	<1	<1
− denatured DNA + native DNA	22	124	
− native DNA + denatured DNA			16
− ATP	29	104	107
− 0.15 *M* NaCl	18	15	
− 0.15 *M* NaCl, − 0.02 *M* Mg Acetate, + 0.02 *M* $MgCl_2$	18		
− 0.15 *M* NaCl + 0.15 *M* KCl	131		
− 0.15 *M* NaCl + 0.15 *M* NH_4Cl	98		

The reaction mixtures and conditions of incubation were as described in Table 1 except that C^{14}-TTP (0.005 mM, specific activity 30.3 mc/mmole) was used for fraction Mt-I and H^3-TTP (0.0005 mM, specific activity 9 c/mmole) used for fractions Mt-III and Nc-III. These concentrations are below saturation levels. This fact and the loss of enzyme activity with storage result in the enzyme activities reported in this table and in Figs. 1–3, being lower than in Table 1, where saturation levels of substrate along with freshly prepared enzyme preparations were used. The data given were taken from several experiments. 100% incorporation for Mt-I is equivalent to 35–70 $\mu\mu$moles/mg protein, for Mt-III 550–840 $\mu\mu$moles/mg, and for Nc-III 1.0–2.3 mμmoles/mg.

substituted for Na^+, and acetate for Cl^- (Table 2). The extent of purification of the polymerase plus the linearity of the reaction kinetics over periods as long as 24 hours argue against the interpretation that the salt effect merely results from inhibition of a nuclease rather than being a property of the enzyme.

The optimal Mg^{+2} concentration of fraction Mt-III is about 7.5 mM, much less than that of the crude preparation (Fig. 2). The rate of incorporation of substrate over a 24-hour incubation period is fairly linear, indicating enzyme stability under conditions of incubation. There is, however, a decrease in labeling after one hour with fraction Mt-I, again suggesting the presence of nuclease activity in this fraction.

Purification of the nuclear DNA polymerase: The data shown for the purification of the nuclear polymerase (Table 1) are more valid than those given for the mitochondrial enzyme; most of the problems encountered with the latter did not occur with the nuclear enzyme. However, similar problems of instability exist in the early steps, so that a calculated figure for over-all purification is probably again a minimal estimate. Thus, fraction Nc-III has been purified at least 75-fold from intact nuclei, and 900-fold based on the content of nuclear protein in whole liver.[18]

Properties of the nuclear enzyme: The rather high reaction rate in the absence of the three unlabeled dXTP's, even in our most purified nuclear preparation, Nc-III (Table 2), indicates contamination by a terminal addition enzyme.[38] Its presence probably has no great effect on the polymerase assay since unlabeled dXTP's are known to inhibit the incorporation of the labeled dXTP by the terminal

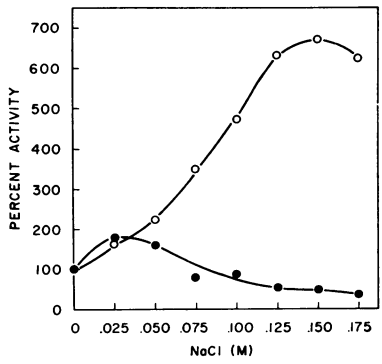

FIG. 1.—Effect of NaCl on mitochondrial and nuclear DNA polymerases. The reaction mixtures and conditions of incubation were as described in Table 2, except that the salt concentration was as given in the curve. The amounts of protein used per tube were 4.4 μg for Nc-III and 8.8 μg for Mt-III. For comparison, data are expressed as per cent activity; for the mitochondrial enzyme (○), 100% is equivalent to 0.6 $\mu\mu$mole for the nuclear enzyme (●), 5.4 $\mu\mu$moles.

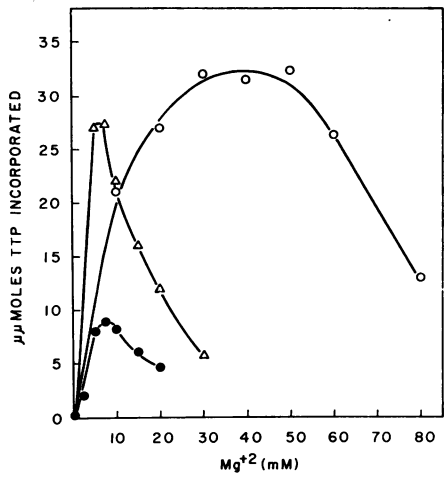

FIG. 2.—Effect of Mg^{+2} on mitochondrial and nuclear DNA polymerases. The reaction mixture and condition of incubation were as described in Table 2, except that the Mg^{+2} concentration was as given in the curve, and the amounts of protein used per tube were 460 μg for fraction Mt-I (○), 26 μg for Mt-III (●), and 15 μg for Nc-III (△).

addition enzyme.[19, 38] The polymerase shows an absolute requirement for DNA primer, with a five- to sixfold preference for native DNA.

Like the mitochondrial enzyme, early fractions show an ATP dependence which is no longer apparent with Nc-III. The requirement for Mg^{+2} is absolute (Table 2), with an optimal concentration of about 6 mM (Fig. 2). Like many other DNA polymerases,[19] the enzyme is moderately stimulated (twofold) by 0.025–0.05 *M* salt (Fig. 1) and, like the mitochondrial enzyme, no ion specificity is observed. Fraction Nc-III is capable of maintaining the reaction rate almost constant for at least 24 hours, indicating the lack of appreciable nuclease activity.

Is the mitochondrial enzyme distinct? The DNA polymerase isolated from mitochondria and the DNA polymerase isolated from nuclei appear to be two distinct enzymes; they differ in their properties as proteins and they differ in their properties as enzymes.

The contrast in their properties as proteins became evident during the purification. More than 90 per cent of the recovered activity of the mitochondrial enzyme precipitated at 25–43 per cent saturated $(NH_4)_2SO_4$, whereas only 10 per cent of the nuclear enzyme appeared in this fraction. On the other hand, roughly 85 per cent of the nuclear enzyme precipitated at 43–75 per cent $(NH_4)_2SO_4$ saturation, with only 6 per cent of the mitochondrial enzyme appearing here. On DEAE-cellulose chromatography (Fig. 3), each enzyme elutes at a characteristic and widely different NaCl concentration. The mitochondrial enzyme peak elutes at 0.11–0.12 *M* NaCl (Fig. 3*A*). On the other hand, the nuclear enzyme is not retained on the column, with virtually all the activity eluting with the Tris-ME wash (Fig. 3*B*).

Inasmuch as the two enzymes are derived from different organelles, contaminants in the two preparations might be different and could differentially alter the behavior of possibly identical enzymes on the DEAE column. To eliminate this possibility, fractions Mt-II and Nc-II were first mixed and then co-chromatographed. The two major peaks (I and II, Fig. 3*C*) retain positions identical with those obtained when chromatographed separately (cf. Fig. 3*A* and *B*). The peaks were identified as the mitochondrial and nuclear enzymes, respectively, by their primer specificity and response to salt.

In the nuclear elution profile, two small enzyme peaks (III and IV, Fig. 3*B*) representing 1–10 per cent of the total recovered activity are always observed. The nature of these activities is not clear. However, it does not appear that either of these peaks corresponds to mitochondrial DNA polymerase from their lack of identical behavior on chromatography and co-chromatography (Fig. 3). Preliminary experiments further indicate that their enzymatic properties correspond closely to those of the major nuclear peak.

The mitochondrial and nuclear enzymes can also be distinguished by enzymatic properties. The two enzymes show striking differences in their response to salt and to DNA primer. The mitochondrial enzyme is stimulated six- to sevenfold by an optimal salt concentration of about 0.15 *M* (Fig. 1). In contrast, the nuclear enzyme is stimulated only moderately by salt (about twofold at 0.025 *M*) and is inhibited by the optimal mitochondrial salt concentration of 0.15 *M*. The difference in primer specificity using commercial calf thymus DNA can be seen by

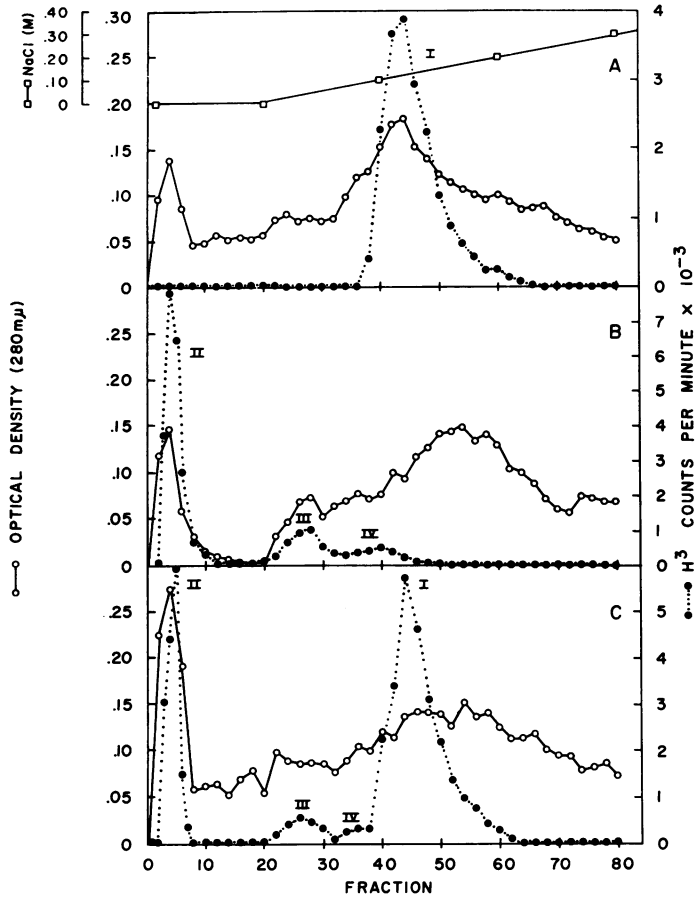

FIG. 3.—DEAE-cellulose elution profile. (*A*) Mitochondrial enzyme; (*B*) nuclear enzyme; (*C*) mixture of mitochondrial and nuclear enzymes.

The assay conditions used to locate the peaks for runs *A* and *B* were, respectively, as described in Table 2. For co-chromatography run *C*, equal amounts of each enzyme activity were used. Inasmuch as both nuclear and mitochondrial polymerases were present in this run, assay conditions intermediate between those used for runs *A* and *B* were employed, namely, the reaction mixture contained only native DNA and the salt concentration was 0.10 *M* NaCl, the KCl being omitted. These conditions lower the activity of each enzyme about 20%.

comparing fractions Mt-III and Nc-III in Table 2. While the mitochondrial enzyme can use either native or denatured DNA, the nuclear polymerase shows a fivefold preference for the native form. Similar results have been obtained when rat liver nuclear DNA was substituted for calf thymus DNA, and when the primer specificity was tested in the absence of added salt.

Discussion.—DNA polymerases have been isolated from highly purified preparations of rat liver mitochondria and nuclei and have been purified at least 60–75-fold from the disrupted organelles, and 240-fold and 900-fold, respectively, based on whole liver. In terms of specific activity, the purity of our nuclear enzyme preparations exceeds any isolated from rat liver thus far[23–25] and is

about equal to the specific activity of a preparation from ascites tumor cells.[26] The highly purified calf thymus enzyme[27] possesses a 5-fold greater activity than our nuclear enzyme and a 15-fold greater activity than our mitochondrial enzyme. No comparisons are possible of specific activities of mitochondrial DNA polymerase preparations obtained in other laboratories, since only preliminary results with crude extracts have been reported.[28, 29]

In light of these results, we feel that the evidence at this point is sufficient to justify the tentative conclusion that the mitochondrial and nuclear DNA polymerases are distinct from each other. It is, of course, hazardous to draw definitive conclusions about the nature and properties of enzymes which have not been completely purified, particularly ones requiring multiple substrates and a complex primer. For example, it is well known that in DNA polymerase preparations, nucleases and polymerase-DNA complexes can change the behavior and properties of the polymerase. The drastic change seen in Mg^{+2} optimum on purification of the mitochondrial enzyme could be caused by such factors. Likewise, nuclease action could well explain the marked preference for denatured DNA found in crude mitochondrial extracts,[29, 30] in contrast to the lack of such preference in our more highly purified preparation. However, the lack of degradation of labeled DNA during long incubation periods argues against significant nuclease activity in fraction Mt-III or Nc-III, while the absolute requirement for DNA primer and the use of DEAE-cellulose would argue against the presence of enzyme-bound DNA. The A_{280}/A_{260} ratios were about 1.27 for the Nc-III and 1.54 for the Mt-III preparations. Further purification of these enzymes, now in progress, should settle these and similar questions.

The occurrence of a distinct DNA polymerase in mitochondria raises a number of questions. The problem of whether the mitochondrial enzyme is simply a contaminant from the nucleus appears to be settled; we have found no evidence for the presence of mitochondrial enzyme in the nucleus or vice versa. Another question raised is whether this enzyme acts as a true replicative enzyme, or a repair enzyme, or possibly as both. Preliminary experiments on isolated *intact mitochondria*, using density labeling techniques, indicate that the DNA synthesis which occurs is in fact a replicative rather than a repair process.[31]

Recent evidence indicates that the components of the protein biosynthesis system of mitochondria appear to be distinct from their counterparts in the cytoplasm.[30, 32–37] We now add the enzyme DNA polymerase to the growing list of distinct components in the mitochondrion, presumably involved in the biogenesis of this organelle.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; TTP, thymidine-5′-triphosphate; dATP, deoxyadenosine-5′-triphosphate; dCTP, deoxycytidine-5′-triphosphate; dGTP, deoxyguanosine-5′-triphosphate; dXTP, any deoxynucleoside-5′-triphosphate (TTP, dATP, dCTP, or dGTP); ATP, adenosine-5′-triphosphate; DEAE-cellulose, *O*-(diethylaminoethyl) cellulose.

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