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Partial Purification of Mitochondrial RNA Polymerase from Rat Liver

(rifampicin/α-amanitin/$(NH_4)_2SO_4$/DEAE-Sephadex/divalent cations)

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ABSTRACT **Mitochondrial RNA polymerase activity from rat liver has previously been demonstrated in intact organelles. This activity has now been solubilized, partially purified, and shown to be a true polymerase, free of nuclease. The enzyme is derived from mitochondria and is not from contaminating bacteria or nuclear components. The enzyme is distinguished from its nuclear counterparts by its behavior on ammonium sulfate fractionation and lack of inhibition by α-amanitin. Rifamycin inhibits the crude enzyme, but only inconsistently inhibits the more purified preparation.**

Intact mitochondria perform both nucleic acid and protein biosynthesis (1–7). Analysis of the components of these biosynthetic systems has demonstrated that several of them are different from their cytoplasmic or nuclear counterparts (8–14). The DNA differs from the nuclear genome in several of its physical characteristics (8, 9), the ribosomes differ from those of the cytoplasm in size and the molecular weights of their RNA constituents (10), several tRNAs and tRNA synthetases are distinct from their cytoplasmic counterparts (11–13), and the mitochondrial DNA polymerase differs from the nuclear DNA polymerase (14). On the basis of hybridization studies, some RNA species present in mitochondria are believed to be coded by the mitochondrial genome (15–18), as are some mitochondrial proteins (19–21). Such findings lend support to the concept that the mitochondrion is, in part, autonomous, and that its genome countributes to its biogenesis. The concept of mitochondrial autonomy would be strenthened if similar studies concerning RNA polymerases revealed that the mitochondrial enzyme differs in its properties from the corresponding nuclear enzymes. Shmerling (22) reported that RNA polymerase from intact rat liver mitochondria was sensitive to rifamycin, whereas nuclear RNA polymerase activity was not. Wintersberger and Wintersberger (23), on the other hand, were not able to demonstrate sensitivity to this drug in either intact rat liver or yeast mitochondria.

Wintersberger and Wintersberger (23) were unable to solubilize the yeast mitochondrial enzyme without loss of its activity. However, Tsai *et al.* (24) have solubilized and purified this enzyme and report that it differs from the corresponding nuclear enzyme in its response to α-amanitin. The mitochondrial enzyme was found to be insensitive to rifamycin. Gadaleta *et al.* (25) have solubilized the mitochondrial enzyme from rat liver with detergents and report that it is inhibited by rifamycin.

We wish to report the solubilization and partial purification of mitochondrial RNA polymerase from rat liver. The partially purified enzyme, as well as the enzyme of the intact organelle, is insensitive to inhibition by α-amanitin. The enzyme is inhibited by 0.2 M ammonium sulfate, both in the intact organelle and in the partially purified form. The most highly purified enzyme fraction is not as sensitive to rifamycin inhibition as is the intact organelle or a less purified, soluble enzyme fraction, both of which are extensively inhibited by this compound.

The observed enzyme activity is not due to contaminating bacteria or nuclear constituents. Present evidence indicates that the mitochondrial enzyme differs from two nuclear RNA polymerases that have been partially purified from intact nuclei (26, 27, and manuscript in preparation) and thus further supports the concept that mitochondria are, in part, autonomous.

MATERIALS AND METHODS

Rifampicin was the rifamycin derivative used for inhibition studies. α-amanitin was kindly supplied by Prof. Th. Wieland. All other biochemicals were obtained commercially.

Polymerase Assay. All assay mixtures contained 50 mM Tris, (pH 7.5), 60 mM KCl, 1.6 mM spermidine, 1.0 mM $MgCl_2$, 0.45 mM DL-malate, 7.5 mM pyruvate, 1 mM each of ATP, CTP, and GTP, 0.1 mM [^{3}H]UTP (40 Ci/mol), and 1–3 mg/ml of intact mitochondrial protein or 0.02–1.0 mg/ml of solubilized enzyme. All incubations were at 37°C. Fractions tested for inhibition by rifamycin were preincubated with the inhibitor for 30 min at 0°C. In experiments where nucleoside diphosphates replaced the triphosphates, they were present in the same concentrations as the triphosphates, with [^{3}H]UDP of 40 Ci/mol. Calf-thymus DNA, when it was included, was present at 40 μg/ml. Labeled, acid-insoluble RNA was counted after it was washed on filter discs (28) by liquid scintillation in 0.4% 2,5-bis-[2-(5-*tert*-butylbenzoxazolyl)]thiophene in toluene. A unit of enzyme is defined as the pmoles of [^{3}H]UTP incorporated into RNA per mg of mitochondrial protein per 20 min.

Nuclease Assays. Mitochondrial DNase and RNase activities were monitored by an increase in absorbance at 260 nm at room temperature. The DNase assay mixture contained 0.14 M Tris (pH 8.0), 20 μg of calf-thymus DNA, 0.07 M $MgSO_4$, and 0.25 ml of DEAE-Sephadex fraction (about 0.2 mg of protein) in Buffer C (see below), in a final volume of 3.0 ml. The RNase assay was identical, except that 200 μg of yeast RNA was used as substrate. Buffer C consists of 0.05 M Tris (pH 7.9)–0.1 mM EDTA–0.1 mM dithiothreitol–5% glycerol (29).

Solubilization and Purification of RNA Polymerase. Mitochondria were prepared from rat liver with 0.25 M sucrose–

1 mM EDTA (1), except that a 4% homogenate was used to improve the yield of organelles. After four washes in isolation medium, the organelles were suspended in 0.05 M Tris (pH 7.5), 0.2 M KCl, 0.01 M $MgCl_2$, 5% glycerol, 0.1 mM EDTA (Buffer G, ref. 29) at 50–60 mg of mitochondrial protein per ml. Fine glass beads were added in a 3:1 (w/v) ratio and the suspension was homogenized in a Virtis blender at a setting of "70" for 10 min at 0°C. The homogenate was filtered through glass wool and washed with sufficient Buffer G to give a protein concentration of about 15 mg/ml. After centrifugation for 30 min at 160,000 × *g*, the high-speed supernatant fraction was brought to 33% saturation with solid ammonium sulfate (23.1 g/100 ml). After centrifugation for 15 min at 27,000 × *g*, the supernatant was brought to 60% saturation with solid ammonium sulfate (17.1 g/100 ml) to precipitate the enzyme. The centrifugation was repeated and the pellet was suspended in 0.01 M Tris (pH 7.9)–0.01 M $MgCl_2$–0.1 mM EDTA–0.1 mM dithiothreitol–5% glycerol (Buffer A) (29) containing 55% ammonium sulfate (38.5 g/100 ml). After it was stirred at 0°C for 15 min, the suspension was centrifuged as above and the pellet, containing the enzyme, was dissolved in Buffer A at a concentration of about 20 mg/ml. After dialysis of the solution against Buffer A to remove residual ammonium sulfate, DNase was added, to a final concentration of 10 μg/ml, and the suspension was incubated 1 hr at 37°C. After extensive dialysis against Buffer C, and clarification by centrifugation at 27,000 × *g* for 15 min, the enzyme was applied to a DEAE-Sephadex column (2.5 × 18 cm), which was washed with Buffer C and eluted with Buffer C containing 0.3 M KCl to separate it from DNA fragments that remained on the column. After dialysis against Buffer C to remove the KCl, the eluted enzyme was applied to a second DEAE-Sephadex column and eluted batch-wise with Buffer C containing 0.13 M KCl, followed by Buffer C containing 0.23 M KCl, which elute fractions VI and VII, respectively.

Other Methods. Protein was assayed by the method of Lowry *et al.* (30), with bovine-serum albumin as a standard. Bacterial contamination was monitored by plating fractions on nutrient-agar plates and counting colonies after 24 and 48 hr of incubation at 30°C. Mitochondria were counted by an unpublished procedure (Parsons, P., and M. V. Simpson, in preparation).

RESULTS

Intact mitochondria

Intact mitochondria incorporated [^{3}H]UTP for as long as 60 min into a cold-acid-insoluble product. The incorporation is inhibited 55–60% by 100 μg/ml of actinomycin D incubated at 0°C for 45 min with the enzyme before assay. Also, incubation before assay with 10 μg/ml of rifamycin, a potent inhibitor of bacterial RNA polymerases (31–33), prevents mitochondrial RNA synthesis after 10 min of incubation, and results in a 45 and 70% inhibition after 30 and 60 min, respectively. Our results agree with those of Shmerling (22), who also found inhibition of RNA polymerase from intact rat liver mitochondria by rifamycin. Wintersberger and Wintersberger (23) however, were not able to demonstrate inhibition of either yeast or rat-liver mitochondria. Gadaleta *et al.* found inhibition of solubilized mitochondrial RNA polymerase by rifamycin (25). That the labeled product synthesized is RNA is indicated by the fact that it is labile to hot Cl_3CCOOH and, after extraction, is 76% solubilized by RNase I. In addition, the product synthesized by the solubilized enzyme is completely stable to DNase and is alkali labile.

TABLE 1. *Properties of mitochondrial RNA polymerase in intact organelles*

Experimental conditions	Enzyme activity (pmol per mg of protein per 20 min)	Inhibition (%)
Complete System	175	—
+ 1 mM Mn^{++}	175	0
+ 2 mM Mn^{++}	127.8	27
+ 5 mM Mn^{++}	42	76
− Mg^{++}, + 1 mM Mn^{++}	152.3	13.1
− Mg^{++}, + 5 mM Mn^{++}	75.3	57.3
− Mg^{++}, + 1 mM Mn^{++}, + 0.08 M $(NH_4)_2SO_4$	100.5	34
− Mg^{++}, + 1 mM Mn^{++}, + 0.24 M $(NH_4)_2SO_4$	54.8	64
+ 0.2 M $(NH_4)_2SO_4$	49	72.1
+ 40 μg/ml of α-amanitin	175	0

Intact mitochondria can incorporate appreciable amounts of label in the absence of added Mg^{++}, presumably because there is endogenous Mg^{++} present. This incorporation can be stimulated by addition of Mg^{++}, and reaches an optimum at 1 mM added ion. Table 1 indicates that 1 mM Mg^{++} is somewhat more stimulatory than 1 mM Mn^{++}. In addition, 1 mM Mn^{++} does not seem to inhibit the Mg^{++} activity. Higher concentrations of Mn^{++} in the presence of 1 mM Mg^{++}, however, do inhibit the polymerase activity. The qunatitative significance of the data is somewhat obscure because the endogenous concentrations of Mg^{++} and Mn^{++} are not known. This table also demonstrates that both the Mg^{++}- and Mn^{++}-catalyzed RNA polymerase activities of intact mitochondria are extensively inhibited by the presence of ammonium sulfate. Finally, the enzyme is completely insensitive to α-amanitin, a compound that inhibits nuclear RNA polymerase activity from rat liver (34, 35).

Bacterial contamination

Considerable research has been done to evaluate the possible contributions made by contaminating bacteria to mitochondrial protein and DNA synthesis (1, 36–39). Since rifamycin is a potent inhibitor of bacterial RNA polymerases (31–33), it was imperative in the present studies on intact mitochondria to show that the inhibition was due to its effect on the mitochondrial enzyme and not on any possible contaminating bacterial enzyme. Bacteria present in the mitochondrial RNA polymerase assay, during incubation with intact organelles, were allowed to multiply on nutrient agar, and various quantities were added to *subsequent* preparations. The results are shown in Table 2. No contribution to the assay results is made by contaminating bacteria until some 2.4 × 10^7 organisms are present. This is 1000 times more bacteria than are present in routine preparations (2.0 × 10^4). In routine preparations, there are some 2.7 × 10^5 times as many mitochondria present as bacteria.

TABLE 2. *Mitochondrial RNA polymerase activity in the presence of added bacteria*

Bacteria added	Bacteria/ml of incubation mix ($\times 10^{-5}$)	Enzyme activity (pmol per mg of protein per 20 min)
Expt. 1*		
Control	0.20	100
+ 2.5×10^5	2.6	100
+ 1.0×10^6	10	100
Expt. 2†		
Control	0.20	116
+ 2×10^6	22	63
+ 5×10^7	240	257
+ 5×10^8	9500	430

* 5.3×10^9 mitochondria/ml were present in this experiment.
† 9.9×10^9 mitochondria/ml were present in this experiment.

Purified enzyme

The purification of the mitochondrial enzyme is shown in Table 3. Subsequent experiments have shown that the same extent of purification can be obtained by stepwise elution of a single DEAE-Sephadex column, as described in *Methods* for the second DEAE-Sephadex column. Fraction IV enzyme shows the same response to α-amanitin and to Mg^{++} and/or Mn^{++} as does the intact organellar enzyme. However, the solubilized fraction is only inhibited half as much by 0.2 M ammonium sulfate as is the intact enzyme, with either Mg^{++} or Mn^{++} as the added divalent cation, and is 37 and 34% dependent on the presence of nucleoside-triphosphate precursors and DNA, respectively. The observed activity of this fraction is not due to polynucleotide phosphorylase (EC 2.7.7.8), since nucleoside diphosphates could not effectively replace the nucleoside triphosphates. Concentrations of phosphate as high as 4.0 mM caused no inhibition of the observed incorporation, whereas polynucleotide phosphorylase is inhibited by 0.4 mM phosphate (29). As expected, the solubilized enzyme is extensively inhibited by rifamycin, as shown in Fig. 1. 100 μg/ml of actinomycin D inhibited this fraction 60–65% after a 30 min incubation at 0°C before assay (not shown). Assay for the presence of mitochondrial nucleases in this fraction reveals the presence of both DNase and RNase. The RNase probably obscures the true purification of the enzyme at this step, since the apparent incorporation is probably the difference between RNA synthesis and degradation.

The enzyme has been further purified by DEAE-Sephadex chromatography. Two enzyme fractions have been characterized after stepwise elution from this column. Both fractions are devoid of nuclease activity. However, some endogenous DNA elutes with these fractions. We feel this DNA is complexed with the enzyme, and is therefore protected from the DNase digestion earlier in the purification. The second fraction eluted from this column (Fraction VII) is characterized in Table 4. The behavior of this fraction with respect to its requirement for Mg^{++} and Mn^{++} is similar to that obtained with Fraction IV enzyme. Also, the Mg^{++}-stimulated activity is inhibited by ammonium sulfate. The residual activity in the absence of added nucleoside triphosphates is largely inhibited by nucleoside diphosphates. The mechanism of the residual activity is not understood, however, and must await further studies. Surprisingly, whereas intact mitochondrial and Fraction IV enzyme were extensively inhibited by rifamycin, Fraction VII enzyme seems to be less sensitive to this inhibitor; it is not consistently inhibited. In addition, the inhibition of this fraction by actinomycin D is lower than that of Fraction IV enzyme. The enzyme continues to be insensitive to α-amanitin inhibition, however.

The characteristics of the first fraction eluted from DEAE-Sephadex (Fraction VI) are very similar to those of Fraction VII.

DISCUSSION

The data presented clearly indicate that the incorporation seen is due to the presence in mitochondria of at least one RNA polymerase. No significant amount of polynucleotide posphorylase activity could be detected in the purified fractions, nor is there any contribution by contaminating bacteria to the RNA synthesis obtained under our conditions of assay. The relatively low inhibition by actinomycin D in intact organelles is probably due to a partial permeability barrier to this inhibitor (1, 40). For example, to obtain 90% inhibition of mitochondrial DNA polymerase activity (ref. 1 and Parsons and Simpson, in preparation), concentrations of actinomycin D as high as 200 μg/ml had to be used, even when the reactions were run in the presence of phosphate, which allows the mitochondria to swell and thus become more permeable. While we obtain appreciable inhibition of the solubilized RNA polymerase enzyme (Fraction IV) with this inhibitor (60–65%) at high concentrations, one expects to see more inhibition. We suggest that the manner in which this enzyme is associated with endogenous template under our conditions of purification makes it inaccessible to this inhibitor. This also seems indicated by the inhibition data with actinomycin D on Fraction VII enzyme. The extent of inhibition correlates well with the extent of DNA dependence, suggesting that only the activity obtained with added DNA template is sensitive to this inhibitor. The triphosphate-dependent incorporation of this fraction is at least 30% above what can be accounted for by the actinomycin D-inhibited

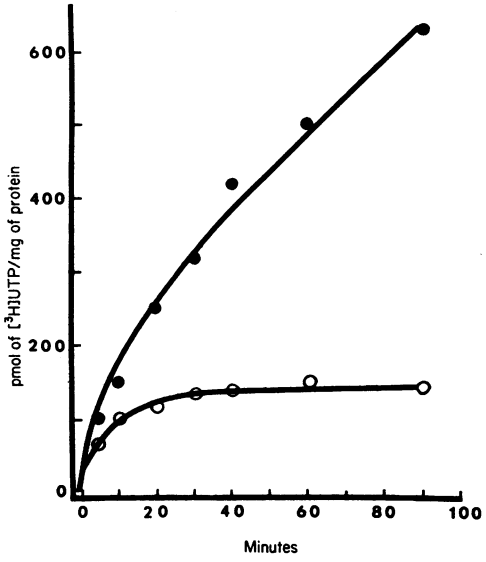

FIG. 1. RNA polymerase activity of fraction IV, and its inhibition by rifamycin. ●, control; ○, rifampicin.

activity plus a possible residual polynucleotide phosphorylase activity. This suggests that unless some novel polymerization reaction is occurring, this 30% of the observed incorporation activity is not accessible to actinomycin D. Additional studies are necessary to clarify this point.

We have sought means to demonstrate that the mitochondrial RNA polymerase differs from those RNA polymerases residing in the nucleus. Rifamycin effectively inhibits the RNA polymerase activity of microorganisms (31–33), but has no effect on nuclear RNA polymerases from higher cells (32, 41, 42), including rat liver (22). Although Wintersberger and Wintersberger (23) could not demonstrate rifamycin inhibition of rat-liver mitochondria, Shmerling (22) and Gadaleta *et al.* (25), in studies on intact and detergent-solubilized mitochondria from rat liver, respectively, did obtain inhibition. In our studies, we have also obtained extensive inhibition of intact mitochondria and Fraction IV enzyme with this compound. We do not understand why we can't demonstrate consistent, extensive inhibition in the most highly purified fractions. These fractions are quite unstable, however, and lose their RNA polymerase activity after only a few days when stored at −20°C. Perhaps the inconsistent sensitivity to rifamycin is related to this instability. The inhibition obtained indicates that the mitochondrial enzyme from rat liver is different from the corresponding enzyme from yeast (24).

Another criterion used to distinguish between the mitochondrial and nuclear RNA polymerases is their respective responses to the specific RNA polymerase inhibitor, α-amanitin. In contrast to rifamycin, α-amanitin is an effective inhibitor of nuclear RNA polymerase activity from rat liver (34, 35), but not of the bacterial enzymes (34). At least two enzymes from nuclei have been described (26, 34). One of these is clearly inhibited by α-amanitin, while the other is not. Our results show that the mitochondrial enzyme is not inhibited by this compound, thus distinguishing it from the nuclear enzyme which is inhibited. In addition, we have recently extracted and partially purified two RNA polymerases from rat-liver nuclei (manuscript in preparation), and find that one enzyme is inhibited by α-amanitin while the other is not. Moreover, the enzyme that is not inhibited remains in the supernatant fraction after 55% ammonium sulfate fractionation, while the mitochondrial enzyme is precipitated under this condition, indicating that the two enzymes are different. Thus, it seems clear that the mitochondrial enzyme activity is different from either of two nuclear enzyme activities in rat liver. These findings further support the concept that mitochondria are, at least in part, autonomous. We realize that there are certain hazards involved in studying enzymes that are only partially purified, and are further purifying these enzymes in order to test the present conclusions more extensively.

TABLE 3. *Purification of mitochondrial RNA polymerase*

Enzyme fraction	Total mg of protein	Specific activity (pmol per mg per 20 min)	Total units ($\times 10^{-3}$)	Purification, fold
I Intact organelle	810	43.3	35.1	1
II Homogenate	675	42.3	28.6	0.98
III High-speed supernatant	270	45.2	12.2	1.04
IV 55% $(NH_4)_2SO_4$ pellet	180	62.7	11.3	1.45
V 1st DEAE-Sephadex	57.5	341	19.6	7.90
VI 2nd DEAE-Sephadex	16.3	468	7.6	10.80
VII 2nd DEAE-Sephadex	25.7	517	13.3	11.94

TABLE 4. *Properties of partially purified mitochondrial RNA polymerase (Fraction VII)*

Experimental conditions	Enzyme activity (pmol per mg of protein per 20 min)	Inhibition (%)
Expt. 1		
Complete	313	—
+ 0.2 M $(NH_4)SO_4$	156	50
+ 5 mM Mn^{++}	97	69
− Mg^{++}, + 1 mM Mn^{++}	232	26
− Mg^{++}, + 5 mM Mn^{++}	150	52
− DNA	225	28
− triphosphates	102	67
− triphosphates, + diphosphates	29.7	90
+ Actinomycin D, (100 μg/ml)	238	24
+ Rifamycin (10 μg/ml)	313	0
+ α-amanitin (40 μg/ml)	313	0
Expt. 2		
Complete	193	—
+ Rifamycin (10 μg/ml)	126	35
+ α-amanitin (40 μg/ml)	386	0

NOTE ADDED IN PROOF

The mitochondrial RNA polymerase has now been purified 140-fold, is 85% inhibited by rifamycin and has an estimated molecular weight of 64–68,000. H. Kuntzel and K. P. Schafer have purified mitochondrial RNA polymerase from *Neurospora crassa*, which is sensitive to rifamycin and has an estimated molecular weight of 64,000. (*Nature* **231**, 265 1971).

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