LOW MOLECULAR WEIGHT SUBUNIT OF A RIFAMPICIN-RESISTANT MITOCHONDRIAL RNA POLYMERASE FROM YEAST

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1. Introduction

Studies on mitochondrial DNA-dependent RNA polymerases from a variety of sources have shown that these enzymes exhibit properties clearly distinguishing them from the respective nuclear RNA polymerases (for review see [1]). Most strikingly these enzymes have in some cases been reported to consist only of subunits of low molecular weight. Thus Küntzel and Schäfer [2] have shown that the enzyme from *Neurospora crassa* is a polypeptide with a mol. wt. of 64 000. Similar values were reported for the mitochondrial RNA polymerases from liver [3,4] while the enzyme from *Xenopus laevis* is composed of even smaller subunits (mol. wt. 46 000–50 000 [5]). Purified RNA polymerases from *Neurospora crassa* and liver are found rifampicin-sensitive, the *Xenopus* enzyme, in contrast, is resistant to the drug.

Mitochondrial RNA polymerases from yeast have been solubilized, purified and characterized by several groups [6–11], however, while some authors find the enzyme to be rifampicin-resistant [6–9], others describe the isolation of a rifampicin-sensitive enzyme [10,11]. Scragg has recently reported that the rifampicin-sensitive mitochondrial RNA polymerase from yeast consists of a subunit with a mol.wt. around 60 000 [11], Eccleshall and Criddle [9], in contrast, found the rifampicin-resistant enzyme to contain high molecular weight subunits similar to those of the nuclear RNA polymerases. This latter observation raises the question as to whether these 'complex mitochondrial RNA polymerases' are in fact nuclear contaminants. We show in this report that the rifampicin-resistant mitochondrial RNA polymerase can be obtained in pure form from wild type and respiration deficient petite cells. This enzyme consists of a polypeptide chain with a mol. wt. of 67 000.

2. Materials and methods

2.1. *Preparation of mitochondria*

Cells of the haploid wild type strains D 273-10 B (α_1 ρ^+) or the petite mutant D 273-10 B-1 (α_1 ρ^-) were grown to late log phase in a medium containing 0.3% yeast extract, 0.5% peptone and 1% galactose. Cells were harvested and converted into spheroplasts essentially as described by Cabib [12]. Spheroplasts were washed twice with 1 M sorbitol and suspended in 5 vol of 0.7 M sorbitol containing 0.01 M Tris–HCl (pH 7.5) and 0.08% bovine serum albumin and were homogenized for 20 sec at top speed in a Waring Blendor. Remaining intact cells and nuclei were removed by 10 min centrifugation at 1000 *g* (Sorvall rotor GSA) and the mitochondria were isolated from the supernatant by a 20 min centrifugation at 22 000 *g*. The crude mitochondrial pellet was resuspended in the homogenization medium and the two-step centrifugation procedure was repeated twice. Mitochondria were then suspended in 30 ml of 0.7 M sorbitol containing 0.01 M Tris–HCl (pH 7.5) and 0.01 $MgCl_2$ and pancreatic deoxyribonuclease added to a final concentration of 100 μg/ml suspension. After incubation for 30 min at 0°C mitochondria were re-isolated by centrifugation and washed twice in homogenisation medium containing 10 mM EDTA pH 7.5.

2.2. *Solubilization and purification of mitochondrial RNA polymerase*

Solubilization of mitochondrial RNA polymerase

was achieved by one of the following two methods: either mitochondria were disrupted mechanically as described previously (7,8) except that the protease inhibitor phenylmethylsulfonylfluorid was added to the buffer to a final concentration of 1 mM. The homogenate was then treated with deoxyribonuclease and salt as described earlier (7,8). Alternatively, mitochondria from 60 g of cells were suspended in 17 ml of 0.01 M Tris–HCl (pH 8.0) containing 20% sucrose, 0.1 M KCl, 5 mM dithiothreitol and 1 mM phenylmethylsulfonylfluorid to give a final vol of 20 ml. Then 20 ml of a lysis mixture consisting of 1% Nonidet P 40 (Shell), 1 M KCl, and 0.01 M EDTA (pH 8) was added at 0°C and the mixture was gently stirred for 15 min. Centrifugation for 90 min at 30 000 rpm (Spinco centrifuge rotor 30) resulted in a clear supernatant which contained the RNA polymerase. This extract was dialysed against 0.05 M Tris–HCl (pH 7.9) containing 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, 25% glycerol (TGMED) and 50 mM ammonium sulfate and applied to a column (1.7 × 15) of DEAE-cellulose (Whatman DE 52) which had previously been equilibrated with the same buffer. After non-absorbed protein was washed off the column, RNA polymerase was eluted by a gradient of 0.05 M–0.5 M ammonium sulfate in TGMED. Fractions of 2.5 ml each were collected and tested for RNA polymerase activity. Active fractions were combined and dialysed against TGMED buffer with 10% glycerol. Five ml each of the dialysed solution were then layered on top of 30 ml gradients of 15–30% glycerol in a buffer containing 0.05 M Tris–HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA and 5 mM dithiothreitol. Centrifugation was carried out for 18 hr at 25 000 rpm and −2°C in the SW 27-rotor of the Spinco centrifuge. Gradients were then divided into approximately 30 fractions and tested for RNA polymerase activity. Active fractions were combined and dialysed against TGMED buffer containing 50% glycerol. Specific activities of the final enzyme preparation were between 0.8 and 1 nmole UMP incorporated/mg protein in 15 min at 35°C.

2.3. *Other methods*

RNA polymerase activity was determined as previously described [8].

Polyacrylamid gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the method of Weber and Osborn [13]. Approximately 50 μg of protein were dissociated in 0.01 M phosphat buffer (pH 7.5) containing 0.5% sodium dodecyl sulfate and 0.1% β–mercapto-ethanol by heating at 100°C for 3 min. Electrophoresis was carried out in 7.5% polyacrylamid gels in the presence of 0.1% sodium dodecyl sulfate. Protein bands were stained with Coomassie Blue.

3. Results

Two alternative procedures were applied to solubilize the RNA polymerase from purified yeast mitochondria. In one of these mitochondria were disrupted mechanically using methods which have been described earlier [7,8]. In the second procedure disruption was achieved using the nonionic detergent Nonidet P 40. Although both procedures yield identical enzymes we have preferred the second method throughout most of this work because not only does Nonidet P 40 (at a final concentration of 0.5%) effectively solubilize mitochondrial RNA polymerase but in addition its presence in the soluble extract which is applied to the DEAE cellulose column allows a much more efficient removal of contaminating protein from the column. Thus enzyme eluted from the DEAE cellulose column with a salt gradient can be purified to homogeneity by a single centrifugation step in a glycerol gradient. Furthermore this method gives reproducible results either with wild type yeast mitochondria or with mitochondria from a respiratory deficient petite mutant.

Some characteristics of the enzyme purified by the above procedure are summarized in table 1. Mitochondrial RNA polymerase is almost completely dependent on the addition of DNA template. In agreement with results obtained with the enzyme from *Neurospora crassa* [2] or *Xenopus laevis* [5] yeast mitochondrial RNA polymerase is most active with poly d(A-T) as template and also shows a strong preference for native mitochondrial DNA as compared to calf thymus DNA. Actinomycin D inhibits the reaction as expected for a DNA dependent polymerase while neither α-amanitin nor rifampicin show any significant inhibition. This latter property is independent of the template used.

Electrophoretic analysis of the purified enzyme in polyacrylamid gels containing sodium dodecyl sulfate results in a single protein band (fig. 1). No other protein components can be detected in our final preparat-

Tabel 1
Properties of homogeneous yeast mitochondrial RNA polymerase

	pMol UMP incorporated/ 15 min at 35°C	%
Standard assay	1.4	100
plus native calf thymus DNA	0.8	57
plus poly d(A–T)	12.6	900
plus native yeast nuclear DNA	1.5	106
plus native yeast mitoch. DNA	5.7	408
no template	0.1	7
plus α-amanitin (20 μg/ml)	1.3	94
plus rifampicin (25 μg/ml)	1.3	94
plus actinomycin D (5 μg)	0.9	64
plus actinomycin D (10 μg)	0.5	36
plus actinomycin D (25 μg)	0.2	14

Standard assays were those described previously [8] with denatured calf thymus DNA as template. Assays contained 1.5 μg of enzyme. To test for the effect of the various drugs these were added to standard assays to give the final concentrations indicated. In tests for template specifity denatured calf thymus DNA was replaced by the template shown. All templates were used at a final concentration of 100 μg/ml. Percent are calculated with respect to the activity measured in the standard assay.

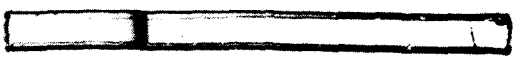

Fig. 1. Polyacrylamid-gel-electrophoresis of yeast mitochondrial RNA polymerase in the presence of sodium dodecyl sulfate. Conditions for dissociation of the enzyme and for the electrophoresis are described in Materials and methods.

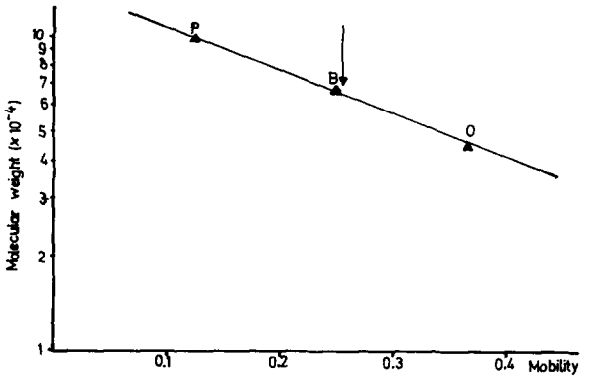

Fig. 2. Estimation of the molecular weight of the polypeptide subunit of mitochondrial RNA polymerase. Reference proteins were phosphorylase B (P), bovine serum albumin (B) and ovalbumin (O). The arrow indicates the mobility of the mitochondrial RNA polymerase subunit.

ion of yeast mitochondrial RNA polymerase. In particular no polypeptides with an electrophoretic mobility similar to that of the heavy subunits from nuclear RNA polymerases [14] can be seen. Phosphorylase b (mol. wt. 100 000), bovine serum albumine (mol. wt. 68 000) and egg albumine (mol. wt. 45 000) were used to assess the molecular weight of yeast mitochondrial RNA polymerase which is found to be 66 000–67 000 (fig. 2). The mitochondrial RNA polymerase isolated from a petite mutant (D 273-10 B-1ρ^-) is composed of subunits which are electrophoretically indistinguishable from those of the enzyme from wild type cells.

4. Discussion

Many of the properties of the homogeneous mitochondrial RNA polymerase from yeast described in this paper are similar to those found earlier with less pure preparations [8]. A notable difference is the strong preference with which poly d(A-T) or mitochondrial DNA are used as template by the pure enzyme as compared to less pure preparations. In this respect the enzyme closely corresponds to those isolated from *Neurospora crassa* [2] and from *Xenopus laevis* [5]. Our pure yeast enzyme is again fully resistant to rifampicin, a property shared by pure mitochondrial RNA from *Xenopus laevis* but not by the enzymes isolated

from *Neurospora crassa* or liver mitochondria or by the enzyme preparation isolated from yeast mitochondria by Scragg [10]. We do not know the reason for this difference which is especially surprising if one considers that the pure mitochondrial RNA polymerases obtained so far otherwise have many properties in common [1], including now the low molecular weight of the polypeptide chains. Scragg [11] has reported that his rifampicin-sensitive mitochondrial RNA polymerase from yeast consists of subunits with a mol. wt. around 60 000 being only slightly lower than the 66 000–67 000 found by ourselves. Whether this difference is significant we do not know. On the other hand, the rifampicin-resistant mitochondrial RNA polymerase from *Xenopus laevis* is even smaller than the rifampicin-sensitive enzymes from *Neurospora*, yeast or liver. The fact that the mitochondrial RNA polymerase from a petite mutant consist of a subunit electrophoretically indistinguishable from that of wild type cells confirms the earlier observation [6] that an enzyme with properties similar to those of the mitochondrial RNA polymerase from wild type cells is present in petite mutants. We want to emphasize that despite the low molecular weight of the polypeptide chain of yeast mitochondrial RNA polymerase this enzyme readily forms aggregates with molecular weights up to about 500 000 in buffers of low ionic strength. This property is responsible for the effective enzyme purification which is achieved by glycerol gradient centrifugation (see also for instance [2]). Complete dissociation of the aggregates into the low molecular weight subunit requires fairly drastic treatment with dissociating agents such as sodium dodecyl sulfate. We found that carrying out this treatment at 37°C as described by Weber and Osborn [13], leads to incomplete dissociation of the polymerase and protein bands corresponding to dimers and oligomers of the subunit are found in acrylamide gels in addition to the low molecular weight polypeptide. On the other hand if care is taken to completely dissociate the enzyme, polyacrylamid gel electrophoresis reveals only a single component. We finally want to stress that the specific activity of the purified enzyme is very low, a property again shared by other mitochondrial RNA polymerases. Thus, similar specific activities were found for the enzyme from liver mitochondria [3,4], while the polymerase from *Xenopus laevis* mitochondria [5] is about 4 times more active than our enzyme. The low specific activity of mitochondrial RNA polymerase compared with that of the nuclear enzyme B from yeast or the RNA polymerase from *E. coli* has also been discussed by Scragg [11]. These findings indicate that the enzymes inside the mitochondria must exist in a form which allows the faster RNA synthesis occurring in the organelle in vivo.

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