Mitochondrial Transcription Complex from *Saccharomyces cerevisiae*

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A DNA protein complex has been isolated from the mitochondria of *Saccharomyces cerevisiae*. The complex transcribes RNA complementary to mtDNA in a nonrandom manner. The RNA polymerase activity contained in the transcription complex is not dependent on the addition of exogenous template. The activity is rendered template-dependent by autolysis and can be further purified by heparin-Sepharose 4B chromatography. The activity is inhibited by heparin, Mn**+,** and increasing ionic strength. The activity requires Mg**+** and ribonucleotides. The preferred template for the template dependent activity is poly[d(dAT)]. The majority of the RNA synthesized by the transcription complex from endogenous DNA is complementary to the DNA strands directing the synthesis of the large and small ribosomal RNA. In yeast the 21 S and 14 S rRNA genes are widely separated, therefore the transcription of these two regions but not of the intervening regions by the transcription complex suggests the existence of at least two transcriptional promoters on the yeast mitochondrial genome.

Yeast mitochondrial biogenesis is a complex process that involves the assembly of components specified by both the nuclear and mitochondrial genomes. Whereas most mitochondrial proteins are translated on cytoplasmic ribosomes from mRNA transcribed in the nucleus, a small number of peptides essential for oxidative phosphorylation are coded for by mtDNA. These peptides are transcribed by a protein-synthetic apparatus that is itself in part mitochondrially coded. MtDNA contains the genes for a large (21 S) and a small (14 S) rRNA as well as approximately 25 tRNA genes, including at least one for each amino acid. In addition, a peptide (Var-1) associated with the small mitochondrial ribosomal subunit is specified by mtDNA (1-3).

The capacity for performing genetic analysis of both the nuclear and mitochondrial genomes as well as the ease of large scale biochemical manipulations makes yeast particularly attractive for the study of mitochondrial biogenesis. Since yeast is a facultative anaerobe, it can survive mutations eliminating oxidative phosphorylation if grown on a fermentable carbon source. The mitochondrially coded peptides required for electron transport and ATP synthesis have been genetically defined. Three of seven subunits of cytochrome oxidase, two of the subunits of oligomycin-sensitive ATPase, and a peptide associated with the cytochrome bc** complex have been genetically mapped. The genes associated with these peptides are, respectively, OX1-1, 2, and 3, OLI-1 and 2, and COB-BOX. The COB-BOX gene contains four intervening sequences, and preliminary evidence indicates that the OXI-3 gene is split as well. In addition the 21 S rRNA gene contains a 1200-base pair intervening sequence (1-3).

Yeast nuclear RNA polymerases have been well characterized (4-10) and yeast nuclear transcription is presently the subject of investigation (11), but the regulatory elements and transcriptional controls in yeast mitochondria have yet to be elucidated. Studies on the interrupted genes and long transcriptional units on mtDNA may define transcriptional requirements for the splicing and processing of RNA in addition to contributing to our knowledge of organelar biogenesis. The involvement of RNA polymerase in the processing of primary transcripts has been postulated (12).

One approach to the study of mitochondrial transcription is the reconstitution of the RNA-synthetic machinery from purified components. Although our studies are concerned primarily with mitochondrial RNA polymerase and its template, mtDNA, additional factors may be required to mimic *in vivo* transcription *in vitro*, as has been reported for nuclear transcription systems (13-17). Whereas mtDNA has been extensively characterized both physically and genetically (1, 2, 18), there is no consensus on the properties of the mitochondrial RNA polymerase despite several reports on its purification (19-22). The specific activities are sufficiently low to suggest that alternative approaches to mitochondrial transcription must be employed.

We have isolated a protein-DNA complex from yeast mitochondria that transcribes mtDNA faithfully, partially obviating the need for reconstitution. Similar complexes have been isolated from *Euglena* chloroplasts (23) and from poliovirus-infected cells (24). We have also developed a purification scheme for the soluble mitochondrial RNA polymerase, which is described in the following paper.

The organization and termini of the yeast mitochondrial transcription units are not known. In HeLa mitochondria, there appears to be complete symmetric transcription of the mitochondrial genome, followed by degradation of the noncoding RNA complementary to the L strand (25, 26). We now present evidence that complete symmetric transcription of the yeast mitochondrial genome does not occur, and that transcription is initiated from at least two promoters.

**MATERIALS AND METHODS**

*Strains and Growth of Cells—*We used the grande strain of *Saccharomyces cerevisiae* MH41-7B grown to midlog phase on 2% glucose, 1% yeast extract, and 1% Bacto-peptone unless otherwise noted.

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Occasionally, galactose was substituted from glucose with no observable change in results. Cells were harvested by centrifugation either with a Sharples continuous flow centrifuge or by centrifugation at 2000 × g for 5 min in a Sorvall GSA rotor.

Preparation of Mitochondria—Cells were washed with cold water and centrifuged at 2000 × g for 5 min in a Sorvall SS-34 rotor. After the supernatant had been discarded, the pellet was resuspended in 10 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 0.1% bovine serum albumin, 5% glycerol, and 0.5 mM MgCl₂. The column was washed with 0.1 M sucrose, 10 mM MgCl₂, and 200 g in a Sorvall GSA rotor, and the broken cells and nuclei were removed as described in procedure b. The unbroken cells were resuspended in 1.5 volumes of BB, and breakage was repeated; cells were passed through the Manton-Gaulon press a total of three times. This method is suitable for 500 to 1500 g of cells. All three procedures result in suitable disruption and centrifugation were then repeated. This method allows rapid breakage of 10 to 200 g of cells. (c) The cells were washed with 1.2 M sorbitol as described in procedure b and then suspended in 1.5 volumes of BB and passed through the Manton-Gaulon press at 6000 p.s.i. The homogenate was centrifuged for 10 min at 2000 × g in a Sorvall GSA rotor, and the supernatant had been discarded, and the unbroken cells were suspended in an equal volume of BB. Cell breakage and centrifugation were then repeated. This method allows rapid breakage of 10 to 200 g of cells. (c) The cells were washed with 1.2 M sorbitol as described in procedure b and then suspended in 1.5 volumes of BB and passed through the Manton-Gaulon press at 6000 p.s.i. The homogenate was centrifuged for 10 min at 2000 × g in a Sorvall GSA rotor, and the broken cells and nuclei were removed as described in procedure b. The unbroken cells were resuspended in 1.5 volumes of BB, and breakage was repeated; cells were passed through the Manton-Gaulon press a total of three times. This method is suitable for 500 to 1500 g of cells. All three procedures result in breakage of 60 to 90% of the cells.

The cell homogenates from the three procedures were treated identically for the isolation and washing of mitochondria. The homogenates were centrifuged for 10 min at 2000 × g in a Sorvall SS-34 rotor, the supernatant was discarded, and the mitochondrial pellets were washed twice by resuspension in BB and centrifugation for 10 min at 40,000 × g. The mitochondrial pellets were resuspended in 500 ml of yeast mito-
Preparation of Heparin-Sepharose 4B—Heparin-Sepharose 4B was prepared by coupling of heparin to cyanogen bromide-activated Sepharose 4B according to Sternbach et al. (34).

Hybridizations—Hybridizations to DNA immobilized on nitrocellulose filters either directly (35) or after agarose gel electrophoresis (36) were performed as described. RNA-RNA hybridizations were performed by incubation of [3H]RNA or 32P-transcription complex-synthesized RNA to a vast excess of unlabeled mitochondrial RNA in a hybridization mixture containing 0.23 M sodium chloride and 0.04 M sodium phosphate (pH 6.8), unlabeled mitochondrial RNA, and radioactive RNA transcribed in vitro. The mixtures were sealed into glass capillaries and completely denatured by heating to 110°C for 5 min, followed by quick cooling in an ice-salt bath. Samples to be hybridized were then incubated at 60°C for 16 h to the specified Rot values. At the specified time, the contents of the capillaries (total volume less than 20 μl) were emptied into 2.0 ml of 50 mM Tris-Cl, pH 7.4. One-half of this sample was precipitated with 5% trichloroacetic acid, and the other half was incubated for 1 h at 37°C with 10 μg/ml each of pancreatic RNase and T1 RNase. Precipitates were collected and counted in a toluene-based scintillation fluid. We compared ribonuclease-treated samples with nonribonuclease-treated samples to determine the per cent hybridization. In order to determine the amount of self-annealing occurring with the transcription complex-synthesized RNA, we performed hybridizations as described above, except that no cold mitochondrial RNA was included in the hybridization mixture.

Determination of DNA Content in the Transcription Complex—Aliquots (50 μl) of the transcription complex in PEG in either 20% or 50% glycerol and 0.5 mM MgCl2 were diluted to 1 ml with 10 mM Tris-Cl, pH 7.5, and 10 mM MgCl2, and the fluorescence in the presence of 1 μg/ml of ethidium bromide was determined (excitation at 548 nm, emission at 590 nm). An identical sample was treated for 0.5 h at 37°C with 280 μg/ml of RNase-free DNase I prior to the addition of ethidium bromide and fluorescence determination. The difference in fluorescence between the two samples was compared to a standard curve generated by identical treatment of known quantities of mtDNA. In all cases, digestion with DNase reduced the fluorescence to background levels.

DNase Assays—Aliquots (25 μl) of Sepharose 4B fractions were incubated in 100 μl of 100 mM Tris-Cl, pH 7.9, 10 mM MgCl2, 2 mM dithiothreitol, and 1 μg of [3H]E. coli DNA (10,000 cpm/μg). Samples were incubated for 15 min at 37°C, precipitated with 5% trichloroacetic acid, and spun for 5 min in an Eppendorf microcentrifuge. The supernatants were counted in Instagel (Packard).

RESULTS

Due to the low specific activities and instability of most reported preparations of yeast mitochondrial RNA polymerase (19-22), we purified an RNA polymerase-mtDNA complex. The buffer used to lyse the mitochondria contains a number of components which stabilize the RNA polymerase...
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activity and its interaction with the endogenous template. In particular, Mg$^{2+}$ and the nucleotides ATP and GTP, which are known to stabilize the interaction between E. coli RNA polymerase and promoters, were included in the buffer (37).

Mitochondria lysed with 0.25 M KCl and 0.5% NP-40 appear to lose 90% of their RNA polymerase activity. This loss is recovered following Sepharose 4B chromatography, indicating that the RNA polymerase is either inhibited or masked by nucleases in the crude extract. The RNA polymerase activity elutes from the Sepharose 4B column in two peaks (Fig. 1a). One peak of activity is found in the fractions excluded from the column and contains less than 1% of the mitochondrial protein. The other peak elutes with the bulk of the protein in the included fractions. Synthesis of RNA in the absence of exogenously added template is supported by the fractions from the excluded volume, whereas the second peak is totally dependent on added template. The distribution of activity between the complexed and soluble peaks has been found to be a function of the stage of growth at which the cells used for preparation of the polymerase are harvested. As shown in Fig. 2, the amount of activity in the transcription complex decreases as the cells progress toward the stationary phase. It is difficult to assess the amount of RNA polymerase actually present in the second peak, since soluble nucleases also elute in the same fraction. It is possible that the decrease in activity in the transcription complex reflects a physiological regulatory event. The transcription complex has been isolated from a $\rho^-$ strain, CEO$_n$, but, as predicted, is not found in a DNA-less ($\rho^+$) strain (Fig. 1, b and c).

The RNA polymerase activity in the transcription complex is very tightly associated with DNA. Attempts to liberate the activity by use of phase partition (38), streptomycin or Polymin P precipitations (39) followed by high salt washes, treatment with micrococcal or pancreatic nucleases (40), and chromatography on hydroxylapatite followed by high salt elutions (41) were all unsuccessful. In all of the above procedures, the activity either remained associated with the endogenous template or was lost. Upon storage of the complex for 2 or 3 days at 4°C in PED with 20% glycerol and 0.5 mM MgCl$_2$, the RNA polymerase activity became increasingly template dependent, with a concomitant decrease in the DNA content of the complex (Fig. 3a). The time necessary to render the activity 90% template dependent could be hastened if the leading edge of the peak of soluble proteins from the Sepharose 4B column is pooled with the transcriptionally active fractions. The leading edge of the peak of soluble protein contains a DNase activity which may be responsible for the degradation of the endogenous DNA and the release of RNA polymerase activity from the template (Fig. 3b). The rate at which the complex becomes template dependent is decreased if the first peak of activity is stored in 50% glycerol with 0.5 mM MgCl$_2$ at -20°C.

Once released from the DNA, the RNA polymerase activity was further purified by heparin-Sepharose and DEAE-cellulose chromatography. Stepwise elution of the activity from the columns was utilized, since gradient elution resulted in dilution of the RNA polymerase activity and subsequent loss of activity. Only small amounts (less than 50 pg, optimally) of

**Fig. 4.** Properties of the RNA polymerase activity in the transcription complex. Transcription complex and RNA polymerase fractions were prepared and assayed as described under “Materials and Methods” in the presence of the indicated additions. a. Divalent cation dependence, $\bigcirc$--$\bigcirc$, MnCl$_2$; $\bullet$--$\bullet$, MgCl$_2$. b. Inhibition of RNA polymerase activity by MnCl$_2$ and KCl; $\bigcirc$--$\bigcirc$, MnCl$_2$; $\bullet$--$\bullet$, KCl. c. Inhibition of the RNA polymerase activity by heparin. $\bigcirc$--$\bigcirc$, template-dependent activity, upper scale; $\bigcirc$--$\bigcirc$, template-independent activity, lower scale.
the yeast mitochondrial RNA polymerase have been purified by the procedure described above; quantities of the enzyme sufficient for structural and transcriptional studies are better purified by the procedure described in the accompanying paper (42). Although both preparations have similar specific activities (300 nmol of UMP incorporated/mg of protein/10 min when poly[d(AT)] is used as template), the activity isolated from the transcription complex is more labile and variable.

The RNA polymerase purified from the transcription complex possesses enzymatic properties and requirements similar to those of the highly purified mitochondrial RNA polymerase. The activity is inhibited by salt. Concentrations as low as 30 mM KCl caused significant inhibition (Fig. 4b). The polymerase activity requires Mg++; Mn++ will not substitute and is, in fact, inhibitory even in the presence of a large excess of Mg++. (Fig. 4, a and b). The activity prefers poly[d(AT)] 15-fold to natural DNAs. The activity is not sensitive to α-amantin and is inhibited by rifampicin only at extremely high concentrations.

Seventy per cent of the activity of the transcription complex is inhibited by heparin (Fig. 4c), whereas 30% remained resistant even to large amounts of heparin. We decided to determine whether the heparin-sensitive activity represented that portion of transcription resulting from in vitro initiation. RNA was synthesized by the complex in the presence of [γ-32P]ATP or [γ-32P]GTP. No incorporation of radioactivity was detected by acid precipitation of the RNA. Furthermore, analysis of an alkaline hydrolysate of newly synthesized RNA revealed a lack of the expected adenosine tetraphosphate and guanosine tetraphosphate. We conclude that the complex does not initiate with ATP or GTP. The absence of initiation in the complex must be considered tentative, however. The presence of very large mitochondrial RNAs (29, 43, 44) suggests that the mitochondrial transcription units are long, and that initiation may be a rare event. Furthermore, the possibility that initiation does not occur with a purine deserves special consideration in view of the recent discovery of a transcription unit initiating with CTP in E. coli (45). If the complex does not initiate, then the heparin inhibition may reflect a complex mode of action such as that occurring with bacterial RNA polymerases (46, 47). Once it becomes template dependent, the RNA polymerase activity is 100% inhibited by heparin (Fig. 4c).

The net incorporation of UMP by the transcription complex continues for only 20 min. This is consistent with the lack of

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**Fig. 5. Time course of RNA synthesis by transcription complex.** Transcription complex was prepared and allowed to synthesize RNA for the stated time in the presence of [3H]UMP. The RNA was acid-precipitated and counted for radioactivity as described under "Materials and Methods."

**Table I**

Hybridization of RNA transcribed from RNA polymerase-DNA complex

| Nucleic acid in excess | RNA transcribed from transcription complex (% RNA hybridized) | cRNA transcribed from mtDNA by E. coli RNA polymerase (% RNA hybridized) |
|-----------------------|-------------------------------------------------------------|------------------------------------------------------------------------|
| Yeast mtDNA           | 48                                                          | 60                                                                     |
| Yeast nuclear DNA     | 0.4                                                         | 0.4                                                                    |
| E. coli DNA           | 0.2                                                         | 0.4                                                                    |
| Salmon sperm DNA      | 0.3                                                         | ND*                                                                    |
| Yeast mitochondrial RNA | 0.5                                                         | 29                                                                     |

* Not determined.
detectable initiation (Fig. 5). The size of the in vitro product isolated after 1 min of incubation is about 11 S and decreases with time despite an increase in acid-precipitable RNA (data not shown). This is probably due to the presence of contaminating ribonucleases. Thus far, it has not been possible to inhibit the RNases without simultaneously inhibiting the RNA polymerase activity.

The RNA synthesized by the transcription complex hybridizes with mtDNA, but not with heterologous DNAs, thus attesting to the mitochondrial origin of the transcription complex (Table I). This result is consistent with the lack of detectable nuclear DNA contaminating the mitochondria as analyzed by analytical buoyant density ultracentrifugation (Fig. 6). The RNA synthesized in vitro neither self-anneals nor anneals with a large excess of cold mitochondrial RNA, showing that the transcription supported by the transcription complex is asymmetric and is transcribed from the same DNA strand that specifies the RNA synthesized in vivo. The RNA transcribed in vitro hybridizes principally to those regions that code for the 14 S and 21 S rRNAs. RNAs from other regions of the genome are present in reduced amounts (Figs. 7 and 8a and b). The pattern of hybridization is the same for RNAs synthesized for 1, 5, or 20 min in vitro (Fig. 8a). In contrast, when E. coli RNA polymerase is used to transcribe mtDNA, all regions of the genome are represented equally (Fig. 8c). In addition, at least 30% of the RNA synthesized by the bacterial enzyme anneals with excess mitochondrial RNA, indicating that transcription by that enzyme is nonphysiologic (Table I).

**DISCUSSION**

The purification of RNA polymerase from the mitochondria of yeast (19–22), rat liver (48, 49), Xenopus laevis (50), and Neurospora crassa (61) has thus far failed to yield an enzyme that has allowed faithful transcription in vitro of mitochondrial RNA. For yeast, there is still no consensus on the properties or characteristics of the mitochondrial RNA polymerase. We have isolated a transcription complex that promotes
the transcription in vitro of coding regions of the yeast mitochondrial genome, and we have partially purified the RNA polymerase from this complex. Transcription complexes similar to that which we described have been useful in the examination of transcription in *Euglena gracilis* chloroplasts (23) and in polyoma virus (24). In addition, agarose gel filtration of protein DNA complexes has been used for analysis of DNA-binding proteins from T- infected *E. coli* cells (52).

Several lines of evidence show that the transcription complex and its associated RNA polymerase activity are mitochondrial in origin. 1) DNA isolated from the same mitochondria that are used in the preparation of the transcription complex bands as a single peak by both preparative and analytical CsCl ultracentrifugation and has the buoyant density of mtDNA. Furthermore, the DNA exhibits a restriction enzyme fragment pattern characteristic of mtDNA (data not shown). Hence, we conclude that the DNA recovered in the excluded volume of the Sepharose 4B column is mitochondrial, and this strongly suggests that the associated RNA polymerase activity is also mitochondrial. 2) The RNA transcribed by the complex hybridizes to mtDNA with the correct strand specificity and primarily to regions known to code for the mitochondrial rRNAs. The possibility that yeast nuclear RNA polymerase fortuitously transcribes mtDNA with appropriate biologic specificity would seem to be remote. 3) The RNA polymerase-DNA complex is not found in the mitochondria of *p* petite strains; this suggests an association between the transcribing activity and mtDNA. 4) The properties of the RNA polymerase activity isolated from the transcription complex are very different from the properties of any known yeast nuclear RNA polymerase. The RNA polymerase found in the transcription complex is fully resistant to a-amanitin and is inhibited by Mn2+ and by ionic strengths not inhibitory to the nuclear enzymes. The mitochondrial RNA polymerase uses poly[d(A-T)] as template with much greater efficiency relative to other templates than do the nuclear RNA polymerases (4-10). 5) Finally, the RNA polymerase activity isolated from the complex is inhibited by antibodies raised to the purified soluble mitochondrial RNA polymerase; this is distinct from the nuclear enzymes (see accompanying paper).

The presence of the transcription complex in the mitochondria of petite strains of yeast indicates that the RNA polymerase is the product of a nuclear gene. Since the RNA polymerase is specified by the nucleus, it is reasonable to expect that the regulation and synthesis of this enzyme play a central role in the depression of the mitochondrial genome. In fact, South and Mahler (53) found that the RNA polymerase activity present in whole mitochondria rises prior to the expression of induced enzymes such as malate dehydrogenase and cytochrome oxidase and falls as the cells progress toward stationary phase. We have observed that the transcription complex from logarithmically growing yeast possesses more RNA polymerase activity than does transcription complex from stationary yeast, indicating that the transcription we observe may reflect the physiologic state of the mitochondria.

The RNA synthesized by the transcription complex has properties similar to those of mitochondrial RNA synthesized in vivo. The RNA synthesized in vivo neither self-aneals nor anneals with excess cold mitochondrial RNA; it is, therefore, likely that this material is transcribed asymmetrically. The alternative possibility that the complex exclusively transcribes regions of the mitochondrial genome which are not transcribed in vivo is unlikely, since the material synthesized in *vitro* and radiiodinated mitochondrial RNA hybridizes similarly to nitrocellulose blots of restriction fragments of mtDNA. In both cases, hybridization occurs predominantly to the fragments that code for the 21 S and 14 S rRNAs. Analysis of total mitochondrial RNA on agarose-urea or methyl mercury hydroxide-agarose gels reveals that the 21 S and 14 S rRNAs are the most abundant mitochondrial species (29, 43). We conclude that the bulk of the RNA synthesized in vitro is specified by the same strand and the same regions that serve as template for RNA synthesis in *vitro*.

In *S. cerevisiae*, the large and small rRNA genes are separated by one-third of the mitochondrial genome (25 kb) (53-61). Since we observed extensive transcription of the two rRNA regions relative to the transcription of the sequences between these genes, we conclude that the two rRNAs are probably transcribed from separate promoters. The lack of detectable initiation in *vitro* indicates that the synthesis which we observed reflects the in vivo situation. If both the 21 S and 14 S rRNAs were processed from the same primary transcript, the DNA-protein complex would have to degrade specifically the RNA coded for by the nonribosomal regions of the genome. One would expect absent or minimal hybridization of the RNA to the 3' distal sequences with short incubations and increasing hybridization to these sequences as the time of transcription in *vitro* is increased. In fact, acid-precipitable RNA continues to accumulate for 15 min, and the same hybridization patterns are observed with RNAs isolated after incubations of 1, 5, or 20 min. If transcription from a single promoter had occurred, the sequences between the rRNAs would have been degraded instantaneously by an activity in the protein-DNA complex. We believe this possibility to be much less likely than the existence of separate promoters for the 14 S and 21 S rRNA genes. This view is supported by the observation that pettes containing only one or the other rRNA gene transcribe and process that rRNA normally. The possibility of nonspecific transcription followed by specific processing also appears unlikely considering the specific nature of the hybridization of the RNA transcribed by the complex.

The existence of separate promoters for each of the RNA genes makes it likely that additional transcriptional regulation occurs. Our conclusion that the rRNA genes are over-transcribed relative to the other sequences in the genome implies that the non-rRNA genes are under a different transcriptional control. One possibility is that regulation occurs at termination. Thus, if there were read-through transcription at the terminators for the rRNA genes, the sequences downstream would be underrepresented relative to the rRNA sequences. Alternatively, the non-rRNA genes may have completely independent transcriptional controls, at both termination and initiation sites. Once again, the existence of apparently normal transcription in pettes suggests that the controls for each gene are separate. Characterization of RNA synthesis from transcription complexes isolated from various petites might prove useful for the analysis of the transcriptional organization of the mitochondrial genome. The transcription complex provides a reference point for the examination of transcriptional activity obtained by reconstitution in *vitro* of the RNA synthetic apparatus from its components.

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