Synthesis and processing of ribosomal RNA in isolated yeast mitochondria

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ABSTRACT

The synthesis and processing of the 15S and 21S rRNAs have been studied in isolated yeast mitochondria. When mitochondrial transcripts were labeled with [α -³²P]UTP in an incubation mixture containing 50 μ M ATP, the transcripts from the genes for the large and small ribosomal RNAs accumulated in the form of putative precursor molecules. The labeled pre-21S rRNA was converted to mature 21S rRNA during a chase period in the presence of 1 mM ATP. Thus, the maturation of 21S rRNA, a process which includes trimming at the 3' end and, in omega⁺ strains, the excision of a 1.1 kb intervening sequence, can occur in isolated mitochondria and appears to be dependent on ATP. In contrast, the maturation of 15S rRNA by the removal of approximately 80 nucleotides from the 5' end of a 15.5S transcript is severely restricted in isolated mitochondria, even in the presence of 2.5 mM ATP.

INTRODUCTION

Recent studies of the mitochondrial genetic system in yeast have revealed several rather surprising characteristics of the mitochondrial genome and have stimulated interest in the transcription and processing of mitochondrial RNAs. Of great importance has been the demonstration that the genes for cytochrome *b* (1,2), subunit I of cytochrome *c* oxidase (3), and the 21S rRNA (4,5) in yeast mitochondrial DNA have a mosaic organization. Complex splicing and processing pathways have been proposed for the cytochrome *b* mRNA on the basis of transcripts that accumulate in various cells with mutations in the COB gene (6,7). Furthermore, DNA sequence information combined with a detailed examination of transcripts in cells carrying intron mutations in the COB gene have raised the possibility that one of the COB introns encodes a polypeptide required for the splicing of COB mRNA (2). Extensive unidentified open reading frames within intervening sequences have also been found in the COX I (3) and 21S rRNA genes (5).

Although the analysis of transcripts in mutant and wild-type cells has provided a rough outline of the pathways for RNA splicing and maturation,

the difficulty in performing kinetic experiments in whole cells appears to be a barrier to a refinement of these studies. It is reasonable to expect that mitochondrial RNA synthesis and processing might be more readily studied at the subcellular level.

Accordingly, we have examined the RNAs transcribed in vitro by intact mitochondria isolated from wild-type cells. In this report we describe incubation conditions in which labeled high molecular weight forms of the two rRNAs accumulate in isolated mitochondria. We show that the splicing of the pre-21S rRNA, but not the processing of a putative precursor for the 15S rRNA, can occur normally in isolated mitochondria.

MATERIALS AND METHODS

Cell Growth and Isolation of Mitochondria. The Saccharomyces cerevisiae strains were D273-10B ($\alpha \rho^+$, ATCC 24657) and a diploid from the cross A6 (a his7 trp5 $\rho^+ \omega^+$) X D273-10B ($\alpha \rho^0$). The Saccharomyces carlsbergensis strain was NCYC-74. Cells were grown at 28°C in a semisynthetic medium (8) containing 1% galactose and harvested in the midlogarithmic phase of growth. The preparation of isolated mitochondria was essentially similar to the conditions described by Woodrow and Schatz (9) except that spheroplasts were not regrown in osmotically stabilized growth medium prior to cell breakage.

RNA Synthesis by Isolated Mitochondria. In a typical assay, isolated mitochondria (0.15-0.4 mg/ml; final volume 0.5 ml) were incubated in a reaction mixture containing 50 mM bicine, pH 7.4, 10 mM $MgCl_2$, 50 mM KCl, 10 mM KH_2PO_4 , 0.1 mM of all amino acids, 0.05 mM ATP, GTP and CTP, 1-5 μ M UTP, 5 mM α -keto glutarate, 5 mM phosphoenol pyruvate, 6 units/ml of pyruvate kinase, 2 mg/ml of bovine serum albumin, and 0.6 M mannitol. The reaction was carried out at 28°C with gentle shaking in 20 ml glass scintillation vials. Labeling was initiated with the addition of either $[5-^3H]UTP$ (5 μ Ci/ml; approximately 1-5 Ci/mmol) or $[\alpha-^{32}P]UTP$ (5-30 μ Ci/ml; 1-40 Ci/mmol). When mtRNA was to be extracted for electrophoretic analysis, the incorporation was stopped with 2 mM UTP. For the determination of total radioactivity incorporated into RNA, the reaction was stopped with 5% trichloroacetic acid. The TCA-precipitable material was trapped on glass-fiber filters and washed with 5% TCA, ethanol-ether (2:1), and ether. The filters were dried and counted in a toluene-based scintillation fluid.

Extraction of RNA. Mitochondria were isolated by centrifugation and suspended at up to 10 mg/ml in 0.05 M Tris-Cl, pH 7.4, 10 mM EDTA, 1% SDS, 100 μ g/ml proteinase K. The proteins were digested for 1 hour at room temperature,

NaCl was added to 0.15 M and the suspension was extracted 2 times with an equal volume of phenol: chloroform: isoamyl alcohol (50:50:1). RNA was precipitated from the aqueous phase with 2 volumes of ethanol at -20°C. The precipitate was obtained by centrifugation at 10,000 rpm for 10 minutes in the Sorvall SS-34 rotor. The pellet was washed with 95% ethanol and dissolved in 1.5 mM Na citrate, pH 6.35, 15 mM NaCl, 0.1% SDS.

Electrophoresis of RNA and Isolation from Gels. Gels contained a mixture of 2.2% polyacrylamide and 0.5% agarose in a Tris-borate buffer (10). RNA was applied in a sample buffer containing 2.3 mM Tris-Cl, pH 7.5, 0.23 mM EDTA, 0.25% SDS, 7 M urea, 20% sucrose, 0.05% each bromphenol blue and xylene cyanol. Samples were heated to 90°-100°C just prior to electrophoresis. Where methyl mercuric hydroxide was used as a denaturant (11), it was added to the gels (7.5 mM) and to the samples after heating (7.5 mM). Where glyoxal was used as a denaturant, the RNA was glyoxalated as described (12) prior to electrophoresis. RNAs to be isolated from gels were localized either by staining in 1 µg/ml ethidium bromide or by autoradiography, cut from the gels and extracted as described (13).

Hybridization Conditions. Restriction fragments of yeast mtDNA (strain D273-10B) were separated by agarose gel electrophoresis and blotted to nitrocellulose strips as described (14,15). Hybridizations were carried out in a volume of 2.5 ml of 6X SSCP (14) plus 1% SDS at 65°C for 48 hrs, washed, and subjected to autoradiography. Where indicated, 5-10 µg of unlabeled mitochondrial 21S or 15S ribosomal RNA, purified from total mtRNA by gel electrophoresis were added to the hybridization reactions as competitor.

RESULTS

RNA synthesis in isolated mitochondria. The conditions for the *in vitro* incorporation of radiolabeled UTP into mitochondrial RNA were basically similar to those described in the accompanying paper by Groot *et al.* (16) with the major exception that we have used significantly lower concentrations of nucleotide triphosphates. Our routine assay mixture contained 50 µM ATP and 1-5 µM UTP as compared to 2.5 mM ATP and 100 µM UTP used by Groot *et al.* (16). Low UTP concentrations were employed in this study in order to obtain high levels of total radioactivity incorporated and high specific radioactivity of the labeled RNA. The reaction, however, was linear with time for up to 75 min and was proportional to the amount of mitochondrial protein added in the range of 0.1 to 1.0 mg/ml. The specific activity for UTP incorporation into RNA was 0.5 pmoles UTP incorporated/min/mg protein at 1 µM UTP. The rate

was 50% higher in the presence of 2 mM ATP and 30-fold greater at 2 mM ATP, 100 μ M UTP.

The properties of the reaction are consistent with those expected for RNA synthesis in isolated mitochondria. The incorporation of UTP was more than 98% inhibited at 40 μ g/ml ethidium bromide, and 80% inhibited at 40 μ g/ml actinomycin D, but was insensitive to α -amanitin. Over 80% of the labeled RNA was protected from attack by exogenous pancreatic RNase in the reaction mixture. The labeled products, therefore, appear to be mitochondrial transcripts located predominantly within intact mitochondria.

Electrophoretic analysis of RNA labeled in isolated mitochondria is shown in Figure 1. The electrophoretic profile of the RNA labeled in vitro is characteristically different from the uniformly labeled mtRNA derived from cells grown in the presence of 32 P-phosphate. A large fraction of the

Figure 1: Electrophoretic analysis of RNA labeled in isolated mitochondria. A. Mitochondria isolated from wild-type yeast strain A6/D273-10B were labeled for 15 min in 5 μ M UTP (10 Ci/mmol) and the assay mixture described in Methods. At the end of the labeling period, 1 mM nonradioactive UTP was added and the incubation was continued for the times indicated. RNA was extracted and subjected to electrophoresis in gels containing 2.2% polyacrylamide and 0.5% agarose. The gel was dried and autoradiographed. B. The labeling conditions were as in A above. The chase was for 60 min in either 1 mM UTP (lane 1) or 1 mM UTP, 2 mM AMP. The bands designated by numbers are referred to in the text. The standard is uniformly 32 P-labeled mtRNA from cells grown in 32 P-phosphate.

total radioactivity migrated more slowly than the 21S rRNA and a heavily labeled band migrated slightly behind the 15S rRNA while little or no radioactivity was associated with 15S rRNA. There was also relatively little labeled RNA in the 4S region of the gel (not shown). We therefore routinely ran the 4S RNA off the bottom of the gel in order to increase the resolution of the larger RNA species.

A chase period of up to 60 min in 2 mM UTP had little effect on the general distribution of radioactivity (Fig. 1A). Those bands which were present at the end of the 15-min labeling period became more intense and the diffuse radioactivity in the background was reduced as incomplete chains were elongated and terminated during the chase period. Thus, even after a prolonged chase period in excess UTP, most of the radioactivity was associated with distinct RNA species which did not correspond in electrophoretic mobility to the major RNAs found in wild-type mitochondria.

While the electrophoretic behavior of the labeled RNA did not change significantly during a chase with UTP alone, the addition of either 2 mM AMP, ADP, or ATP to the chase medium yielded surprising results (Fig. 1B). There was a substantial decrease in the radioactivity in Band 1 (see Fig. 1B) and a corresponding increase of ^{32}P -RNA in the 21S band. At the same time, there was little change in the relative amounts of radioactivity in either band 3 or the 15S rRNA. Although only the effects of 2 mM AMP are shown in Figure 1B, identical changes were obtained with ADP and ATP.

These results suggest the possibility that the labeled RNA in band 1 represents a precursor to 21S rRNA which accumulates because the splicing and processing events involved in its conversion to mature 21S rRNA require higher levels of ATP than are normally available in the incubation mixture. In the presence of an ATP regenerating system, the addition of any adenine nucleotide should relieve this deficiency. If such an interpretation is correct, RNA synthesis in isolated mitochondria would have potential for studies on the pathways of RNA splicing and processing.

As a first step in exploring these possibilities, it was necessary to clearly demonstrate that the slowly migrating species were not simply stable aggregates of the newly synthesized RNA. Electrophoresis of the labeled RNA after denaturation with either glyoxal or methyl mercuric hydroxide yielded a profile of radioactivity similar to that described above (Fig. 2). At least five distinct major radioactive bands were resolved in both gel systems and only a single band at 21S corresponded to a major RNA from mitochondria labeled in vivo. Several minor bands were resolved in the methyl mercury

Figure 2: Electrophoresis of ^{32}P -labeled mt transcripts under denaturing conditions. Labeled mtRNA was either (A) glyoxylated (12) or (B) treated with methyl mercuric hydroxide (11) and subjected to electrophoresis as described in Methods. Lanes A-1 and B-1 contain RNA labeled in isolated mitochondria exactly as described in the legend to Figure 1, sample B-1. Lanes A-2 and B-2 contain uniformly ^{32}P -labeled mtRNA from cells grown in ^{32}P -phosphate.

gels. These gels also clearly separated two bands in the region of the 15S rRNA (Fig. 2B). In the RNA labeled in isolated mitochondria, the major radioactive band at approximately 15.5S and the minor band at 15S corresponded, respectively, to a minor 15.5S species and to the 15S rRNA in RNA labeled *in vivo*.

Others have previously observed a minor 15.5S RNA in mtRNA from wild-type yeast which corresponded in electrophoretic mobility to a major species of mtRNA in petite mutants retaining the 15S rRNA gene (17-19). Recently, this 15.5S RNA has been identified as a putative precursor to 15S rRNA with an extension of 70-90 nucleotides at the 5'-end (19). It appears likely, therefore, that the 15.5S RNA and the other RNAs transcribed in isolated mitochondria are normal intermediates in the metabolism of mtRNA rather than products of aberrant transcription or aggregates of newly synthesized RNA.

The identification of putative precursors to 21S and 15S rRNA by hybridization to the corresponding rRNA genes. If any of the major transcripts made during RNA synthesis in isolated mitochondria are precursor forms of RNA, then these transcripts should hybridize to those restriction fragments known to code for the mature molecules. Furthermore, their hybridization should be specifically competed by the addition of the bona fide mature RNAs. Since the genes coding for 15S and 21S rRNA have been identified and localized on the restriction map of mtDNA and the 15S and 21S rRNAs can be readily obtained, we have used this protocol to screen the transcripts made in isolated mitochondria for the presence of precursors to rRNA.

Radioactively labeled putative precursors (corresponding to bands 1, 2 and 3 in Fig. 1B) were purified from total mtRNA by gel electrophoresis and extracted from the gels. These purified ^{32}P -labeled RNAs were then hybridized to nitrocellulose strips to which HindII fragments of total mtDNA had been fixed (Fig. 3). In agreement with the notion that the *in vitro* labeled species in band 1 is a precursor to the 21S rRNA, this RNA hybridized predominantly to a 2900 base pair HindII fragment (indicated on the left) known (20,21) to consist almost entirely of sequences coding the 21S RNA (Fig. 3A). The addition of excess unlabeled mature 21S rRNA effectively competed the hybridization of the labeled "precursor" (Fig. 3B) while the addition of excess unlabeled 15S rRNA did not (Fig. 3C). Similarly, the 15.5S putative precursor to the 15S rRNA (band 3, Fig. 1B) hybridized predominantly with a 7200 base pair fragment known (20,21) to contain most of the 15S rRNA gene (indicated on the right) (Fig. 3D). This hybridization was competed by the addition of excess unlabeled 15S rRNA (track E), but not the addition of 21S rRNA (track F). The sequence identity of the putative 15S precursor to the mature 15S rRNA was further confirmed by hybridization-competition experiments using HpaII fragments of mtDNA (not shown). RNA in band 2 hybridized to 3 HindII fragments, but in this case neither the 21S nor the 15S rRNA were effective competitors of the hybridization (not shown).

These results demonstrate that two RNA species labeled in isolated mitochondria, band 1 and band 3, are transcribed from those regions of mtDNA which code for the 21S and 15S rRNAs respectively. Furthermore, the hybridization-competition experiments show that these putative precursors contain largely the same sequences as the mature molecules. However, since the "precursor" species do contain sequences not present in the mature rRNA, the addition of mature rRNA would not be expected to completely compete the hybridization of the "precursors" (the 2900 bp HindII fragment does contain a short

Figure 3. Hybridization of putative ribosomal RNA precursors to HindII fragments of mtDNA and competition by bona fide rRNAs. The putative 21S rRNA precursor (band 1) and the putative 15S rRNA precursor (band 3) were purified by gel electrophoresis from mtRNA labeled in isolated mitochondria. These labeled RNAs (30,000 cpm per reaction) were hybridized to nitrocellulose strips to which HindII fragments of mtDNA had been fixed (approximately 1 μ g per strip), after electrophoresis on a 0.8% agarose gel. The ethidium bromide stained pattern of DNA fragments is shown next to each panel. The arrow on the left indicates the DNA fragment (2900 base pairs) known to carry a large portion of the 21S rRNA gene, while the arrow on the right indicates the fragment (7200 base pairs) known to carry most of the 15S rRNA gene (7,8). The reaction contained (a) 32 P-21S "precursor" (band 1, Fig. 1B), (b) 32 P-21S "precursor" plus unlabeled 21S rRNA, (c) 32 P-21S "precursor" plus unlabeled 15S rRNA, (d) 32 P-15S "precursor" (band 3, Fig. 1B), (e) 32 P-15S "precursor" plus unlabeled 15S rRNA, (f) 32 P-15S "precursor" plus unlabeled 21S rRNA. Following hybridization and washing, the strips were autoradiographed.

portion of 21S rRNA intervening sequence). In fact we did not observe complete competition (Fig. 3), although we cannot exclude the possibility that the incomplete competition is simply due to insufficient competitor. With respect to this point, the RNA in band 2 also hybridized to the same HindII fragments as did the RNA species in band 1, but in this case the 21S rRNA was not an effective competitor of the hybridization (not shown). Since the labeled RNA in band 2 is likely to be highly contaminated with unlabeled 21S rRNA, the hybridization we have detected with band 2 may be mostly due to complementarity in the region of the intervening sequence and therefore not subject to further competition by mature 21S rRNA. The results of these hybridization experiments are, however, clearly consistent with the idea that

precursor forms of ribosomal RNA accumulate during transcription in isolated mitochondria.

The effect of incubation conditions on RNA transcripts labeled in mitochondria from *S. cerevisiae* and *S. carlsbergensis*. The putative precursor to the 21S rRNA we have observed (band 1, Fig. 1B) did not appear as a prominent labeled RNA when mitochondria from *S. carlsbergensis* were labeled in the presence of 2.5 mM ATP (16). The preceding experiments would suggest that the critical variable in the two experimental systems is the level of ATP available to drive (or regulate) processing reactions. Alternatively, the 21S rRNA gene in *S. carlsbergensis* lacks an intervening sequence and thus the processing of transcripts from this gene might follow a pathway which is not subject to the same rate-limiting factors as in *S. cerevisiae*. In the experiment depicted in Figure 4, mitochondria from *S. cerevisiae* and *S.*

Figure 4: Comparison of transcripts labeled in mitochondria isolated from *S. cerevisiae* and *S. carlsbergensis*. Mitochondria obtained from the two different yeast strains were labeled with [α - 32 P]UTP under two different conditions. *S. cerevisiae* (lane 2) and *S. carlsbergensis* (lane 1) were labeled for 45 min in the incubation mixture described in Methods with 50 μ M ATP, 1 μ M UTP (40 Ci 32 P-UTP/mmol). *S. cerevisiae* (lane 3) and *S. carlsbergensis* (lane 2) were labeled in the reaction mixture described by Groot *et al.* (16) with 2.5 mM ATP, 100 μ M UTP (1 Ci 32 P-UTP/mmol). The mitochondria were all labeled for 45 min and then chased for 30 min after adding 5 mM UTP. 32 P-labeled RNA was extracted and subjected to electrophoresis in the presence of 7.5 mM methyl mercuric hydroxide. *S. cerevisiae* (lane 1) contained uniformly labeled mtRNA from strain D273-TOB.

carlsbergensis were labeled with [α - 32 P]UTP for 45 min under our conditions and those of Groot *et al.* (16). In each case the labeling was followed by a 30 min chase in 5 mM UTP. The results show clearly that the accumulation of transcripts larger than the 21S rRNA is determined by the incubation conditions and not by differences between the two yeast strains. Furthermore, the conversion of the putative pre 15S rRNA precursor to its mature size appears to be as severely restricted in mitochondria from *S. carlsbergensis* as in those from *S. cerevisiae*.

DISCUSSION

The results presented in this preliminary study and in the companion paper (16) demonstrate the utility of isolated organelles for studies on the biosynthesis and processing of mtRNA. It is evident from our data that the adenine nucleotide concentrations can be manipulated to allow the accumulation of a putative precursor to the 21S rRNA in one stage of the incubation and then to observe the processing reactions as subsequent events. A putative precursor to the 15S rRNA also accumulated in isolated mitochondria but its conversion to the mature 15S rRNA was not significantly enhanced by increasing the concentration of adenine nucleotides or by any other conditions tested so far. Although they have not been the subject of this investigation, it is reasonable to assume that the synthesis and processing of other mitochondrial transcripts could also be studied in this system.

The present data do not answer the question of whether either of the putative precursors we have identified are primary transcription products or processing intermediates. Nor is it certain that these RNAs are physiologically important *in vivo*. Results from other laboratories do, however, support the concept that the large RNAs transcribed in isolated mitochondria correspond to species detected in whole cells.

Recently, Levens *et al.* (22) have used vaccinia guanylyl-transferase to label 5'-ends of yeast mtRNAs produced by transcriptional initiation. The 5'-end of 15S rRNA was not labeled suggesting that the 5'-end of a pre-15S rRNA was removed during maturation. However, there was a "cappable" transcript slightly larger than mature 15S rRNA. A similar 15.5S RNA was a prominent transcript in mitochondria from petite mutants which retain the 15S rRNA gene and a minor species in wild-type mitochondria (17-19). This species contains the entire sequence of the 15S rRNA plus an extension of approximately 80 nucleotides at the 5'-end (19). Thus, the available evidence strongly supports the idea that the 15.5S RNA is the primary transcript

of the 15S rRNA gene and is the physiological precursor to 15S rRNA.

Precursors to the 21S rRNA have been detected in the mtRNA from wild-type and petite mitochondria (24,25). The largest is an approximately 5 kb transcript which is processed to mature 21S rRNA by removal of a 3'-extension and excision of the intron sequence (25). The 5'-end is not processed (22). Bands 1 and 2 in Figure 1B most likely have counterparts among the precursors to the 21S rRNA detected by others (22,24,25).

The experiments presented here suggest a significant role for adenine nucleotides, presumably in the form of ATP, in the processing of precursors to the 21S rRNA. ATP could participate directly in processing reactions as a substrate or co-factor. For example, the splicing of yeast tRNA precursors involves an ATP-dependent ligation reaction (26). When this splicing reaction is studied in vitro, an ATP-independent endolytic reaction produces half-tRNA molecules which accumulate when the ATP concentration is below 100 μ M. While it is possible a similar reaction occurs in the splicing of 21S rRNA precursors in S. cerevisiae, it would not apply to S. carlsbergensis which has an uninterrupted 21S rRNA gene.

Alternatively, ATP might influence RNA processing as a regulatory factor. It is interesting to note that guanine nucleotides (probably GDP or GTP) greatly stimulate protein synthesis in isolated mitochondria even though these exogenously added nucleotides do not seem to participate directly in the translation process (27).

Finally, it is worth considering the question of why the processing of pre-21S rRNA is able to proceed in isolated mitochondria at the same time there is almost complete blockage of the apparently less complex removal of an 80 nucleotide 5'-extension from the precursor to 15S rRNA. We are attracted to the possibility that the processing of 15S rRNA requires the RNA to be in a ribonucleoprotein complex containing small subunit proteins. Thus, the processing could be extremely sensitive to a deficiency in one or more ribosomal proteins. All of the small subunit proteins except one are synthesized in the cytoplasm and imported into the mitochondria, a process which is disrupted in isolated mitochondria. Furthermore, since all petite mutants lack var 1, the one small subunit protein translated on mitochondrial ribosomes, the impaired processing of 15S rRNA in petite mutants (17-19) could also be due to defective assembly of the small ribosomal subunit. In fact, even with extremely sensitive immunological procedures, we have not detected stable ribonucleoprotein particles containing small subunit proteins in petite mutants that transcribe the gene for 15S rRNA (28).

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