

RNase P Activity in the Mitochondria of *Saccharomyces cerevisiae* Depends on Both Mitochondrion and Nucleus-Encoded Components

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A requisite step in the biosynthesis of tRNA is the removal of 5' leader sequences from tRNA precursors. We have detected an RNase P activity in yeast mitochondrial extracts that can carry out this reaction on a homologous precursor tRNA. This mitochondrial RNase P was sensitive to both micrococcal nuclease and protease, demonstrating that it requires both a nucleic acid and protein for activity. The presence of RNase P activity in vitro directly correlated with the presence of a locus on yeast mitochondrial DNA previously shown by genetic and biochemical studies to be required for tRNA maturation. The product of the locus, the 9S RNA, and this newly described mitochondrial RNase P activity cofractionated, providing further evidence that the 9S RNA is the RNA component of yeast mitochondrial RNase P.

tRNA genes in yeast mitochondria are transcribed into large RNA precursors, either alone or contiguous with mRNA, rRNA, or additional tRNA transcripts (14, 16, 20, 24). Maturation steps include endonucleolytic cleavage of 5' leading and 3' trailing sequences, addition of CCA at the 3' terminus, and modification of some of the bases. Mitochondrial tRNAs can be correctly transcribed and matured in yeast strains which cannot carry out mitochondrial protein synthesis (12, 17). Since mitochondrial protein synthesis is not required for mitochondrial tRNA synthesis, all of the proteins involved in mitochondrial tRNA maturation must be encoded in the nucleus, translated in the cytoplasm, and imported into the mitochondria. Even though the nuclear genome provides all of the proteins necessary for tRNA biosynthesis, previous genetic studies have shown that the mitochondrial genome also contributes to this process (13).

There is one mitochondrial gene besides the tRNA genes themselves that is necessary for the production of mature mitochondrial tRNA. Called the tRNA synthesis locus, this gene is located between the tRNA^{Met} and tRNA^{Pro} genes on the yeast mitochondrial genome (23). The tRNA synthesis locus has been characterized by DNA sequence analysis. It is a particularly unusual sequence in that it is composed of predominantly A+T-rich DNA with short interspersed G+C-rich elements (15). Despite the unusual structure of the locus, it is transcribed to yield an RNA molecule called the 9S RNA. S1 mapping and primer extension experiments indicate that the 9S RNA is about 450 bases long. To better understand the function of the 9S RNA, we have characterized mitochondrial tRNA gene transcripts in mutants with and without the locus.

Mitochondrial DNA deletion mutants that lack this locus accumulate tRNA precursors (6, 11, 23, 24). Characterization of tRNA gene transcripts in such mutants shows that they are correctly processed on the 3' end but retain 5' tRNA precursor leader sequences (13). tRNA precursors with 5' leaders arise in two different ways. In some cases, the 5' leader carries a di- or triphosphate, indicating that the 5' first nucleotide was the initiating nucleotide during transcription (3, 11, 18, 19). Transcript mapping shows that other 5' tRNA leaders result from cleavage events that separate precursors

from polycistronic RNA molecules (6, 20, 24). tRNA precursors of both origins require the tRNA synthesis locus for 5' leader processing (23).

Cleavage of the 5' leader from tRNA precursors is carried out by an enzyme known as RNase P (21). *Escherichia coli* (21), *Bacillus subtilis* (7), veal heart (1), *Schizosaccharomyces pombe* (9), and HeLa mitochondrial (5) RNase P activities have been described. A common feature in all of these RNase P's is that the enzyme is composed of both a protein and an RNA moiety (1, 5, 7, 9, 10). Remarkably, the RNA component of *E. coli* and *B. subtilis* RNase P's can, under certain conditions, catalyze the removal of 5' precursor tRNA leaders by itself, in the absence of any protein component (8).

The previous genetic biochemical results obtained in yeast mitochondria coupled with the knowledge of RNase P from other organisms strongly suggest a hypothesis that mitochondrial DNA deletion mutants unable to remove 5' leaders from mitochondrial tRNA precursors are deficient in mitochondrial RNase P activity because they are missing the tRNA synthesis locus and thus the 9S RNA. To test the hypothesis suggested by the in vivo studies, in vitro assays were developed to detect yeast mitochondrial RNase P from both wild-type and petite deletion mutants. Results of experiments described here demonstrate the presence of RNase P in mitochondria. An examination of this activity in petite deletion mutants implicates the 9S RNA as the mitochondrially encoded component of mitochondrial RNase P. Further, using this in vitro system, we have shown for the first time that there is a nucleus-coded component(s) in mitochondrial RNase P.

MATERIALS AND METHODS

Purification of precursor RNA. Mitochondria were isolated as described previously (13). RNA was extracted from frozen ND40 mitochondrial pellets by phenol extraction in the presence of 50 mM sodium acetate (pH 5.5)-1% sodium dodecyl sulfate. The RNA was concentrated by ethanol precipitation and separated from other nucleic acids on a 10% polyacrylamide-6 M urea gel. RNA was stained with ethidium bromide and visualized with UV light, and the tRNA^{Met} precursor (pMet) was excised in a small fragment

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of polyacrylamide. The gel fragment was crushed and the pMet_f was eluted into 10 mM Tris (pH 7.5)–0.1 mM EDTA by shaking overnight at 4°C. The eluate was concentrated by evaporation and filtered/desalted through a column of Bio-Rad P-30.

Radiolabeling of pMet. To label the 5' end of the pMet_f, 0.5 µg of the pMet_f was incubated with 0.5 U of alkaline phosphatase (New England Nuclear Corp.) in 10 mM Tris (pH 8) at 55°C for 20 min. After phenol extraction, the pMet_f was labeled with T-4 polynucleotide kinase (Boehringer Mannheim Biochemicals or New England Nuclear) in the presence of [γ -³²P]-ATP (New England Nuclear) as directed by the manufacturer. The 3' end of the pMet_f was radiolabeled through the addition of [³²P]pCp by RNA ligase (P-L Biochemicals, Inc.) as described by Romaniuk and Uhlenbeck (22).

Partial purification and assay, mitochondrial RNase P. Fresh or frozen mitochondrial pellets (13) were suspended in 2 ml of 1 M ammonium sulfate–20 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–0.2 mM dithiothreitol–1% Tween 20. The mitochondria were disrupted by sonication (three 20-s pulses) with the microtip of a Heat Systems-Ultrasonics sonicator. The sonicate was centrifuged at 48,000 × *g* for 10 min. Supernatant from that centrifugation was dialyzed against 50 mM NH₄Cl–10 mM MgCl₂–50 mM Tris (pH 7.5) for 1 h with four changes of buffer. The dialysate was applied to a DEAE-Sephadex A25-120 column at a ratio of 4 mg of protein per g of dry resin and washed with 10 column volumes of the 50 mM NH₄Cl buffer. The column was then washed with a total of 10 column volumes of 200 mM NH₄Cl–50 mM Tris (pH 7.5)–10 mM MgCl₂. The first 4 column volumes from the 200 mM NH₄Cl wash were collected for assay. The rest were discarded. The RNase P activity was eluted with 4 column volumes of 500 mM NH₄Cl–50 mM Tris (pH 7.5)–10 mM MgCl₂. Each column volume was collected as one fraction. Aliquots from each step in the procedure were assayed in the presence of 50 mM Tris (pH 8)–6 mM MgCl₂–50 mM NH₄Cl. Equal amounts of 5' and 3' radiolabeled pMet_f were added as substrates and the reaction was allowed to proceed at 37°C for 15 min. *E. coli* RNase P (a generous gift from S. Altman) was assayed under identical conditions as a positive control. Products were separated by electrophoresis on a 10% acrylamide–6 M urea gel and visualized by autoradiography. RNase P-containing fractions were stable at 4°C for at least 4 months.

Micrococcal nuclease incubation. Mitochondrial extracts containing RNase P activity were incubated in the presence of 25 mM Tris (pH 7.5)–10 mM CaCl₂–0.1 µg of micrococcal nuclease (Sigma Chemical Co.) per µl with or without 100 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGAA). After 15 min at 37°C, aliquots from each incubation were assayed. The products were analyzed as described above.

Protease incubation. RNase P extract was incubated in 20 mM Tris (pH 7.5) with or without 0.5 µg of pronase E (Sigma) per µl. After 30 min at 37°C, aliquots from each reaction were assayed for RNase P activity as described above.

Isolation, transfer, and hybridization of RNA. RNA was purified by phenol-chloroform extraction from whole mitochondria or from the fractions from the RNase P preparation. The RNAs were applied to Zeta-probe paper (Bio-Rad Laboratories) in the presence of 40 mM Tris–20 mM sodium acetate–1 mM EDTA (pH 7.4). Each slot contained the RNA from an equivalent amount of starting material. Hybridization conditions were 50% formamide, 50 mM

NaPO₄ (pH 7), 0.1% sodium dodecyl sulfate, 0.2% Denhardt solution (4), and 200 µg of herring sperm DNA per ml at 50°C.

Preparation of 9S probe. The *Hpa*II fragment containing the 3' end of the 9S gene was cloned into the *Cla*I site of pBR322, resulting in plasmid pHP321 (15). The *Hind*III-*Eco*RI fragment from pHP321 was then cloned into pSP65 (Promega Biotec), resulting in plasmid pMH66. After complete digestion of pMH66 with *Hind*III, transcripts complementary to the 9S RNA were produced, using SP6 polymerase as recommended by the manufacturer (Promega, Boehringer Mannheim, New England Nuclear).

RESULTS

Preparation of mitochondrial precursor tRNA. Before the purification of mitochondrial RNase P could be accomplished, it was necessary to find a substrate with which to assay the activity. The yeast mitochondrial mutant ND40 retains only the tRNA^{Met} gene within its mitochondrial genome (13). The tRNA synthesis locus is not present, but the tRNA^{Met} gene is transcribed. The result is the production of the pMet_f transcript which is correctly processed except for the presence of a 26-base 5' leader sequence (11). RNA extraction of ND40 mitochondria enables us to isolate several micrograms of the precursor tRNA^{Met}. This could then be radiolabeled at the 5' or 3' end or both. An equal amount of radioactivity from 5' and 3' labeled species is mixed to allow the fate of both products of the RNase P reaction to be followed by electrophoresis.

Detection of RNase P activity in wild-type cells. An RNase P activity can be detected in extracts of highly purified mitochondria, using the pMet_f as a substrate (Fig. 1). Various concentrations of different cations and salts at several pH conditions were tested in preliminary experiments to arrive at the assay conditions used here. Rigorous optimization of reaction conditions can only be accomplished on more purified enzyme. The products of this reaction are the same as those produced by *E. coli* RNase P. The tRNA-sized product comigrates with authentic mitochondrial tRNA^{Met} (11) and the 5' product is the expected 26 bases long. To detect the mitochondrial RNase P, it is necessary to dilute crude fractions such as the sonicate, 48,000 × *g* supernatant, pellet, and dialysate prior to assay. This dilutes other nucleases in the extract sufficiently so that the substrate and products are not completely destroyed during the incubation. The amount of RNase P activity remaining in the pellet fraction varies from experiment to experiment. In the experiment shown in Fig. 1, the pellet fraction assayed was also fourfold more concentrated than the supernatant. Therefore, the approximate partitioning of activity was 80% in the supernatant and 20% in the pellet.

There are at least two nuclease activities that remove 5' leader sequences from the pMet_f. One is the RNase P activity. The other is a nuclease which produces a product retaining the 3' end label that migrates on gels slightly slower than authentic tRNA^{Met} (data not shown). The latter activity is present in the flowthrough fraction (F, Fig. 1). The RNase P activity can be largely separated from the other nuclease(s) by DEAE chromatography. The RNase P activity is retained by DEAE and is only eluted by high salt. Visual inspection of the autoradiogram of products produced by these high-salt fractions of wild-type extracts shows that they are substantially free of interfering nucleases because the label in the 5' and 3' products appears to be equally represented. In contrast, there appears to be more 3' product than 5' product in samples produced by the unfractionated extracts. This is

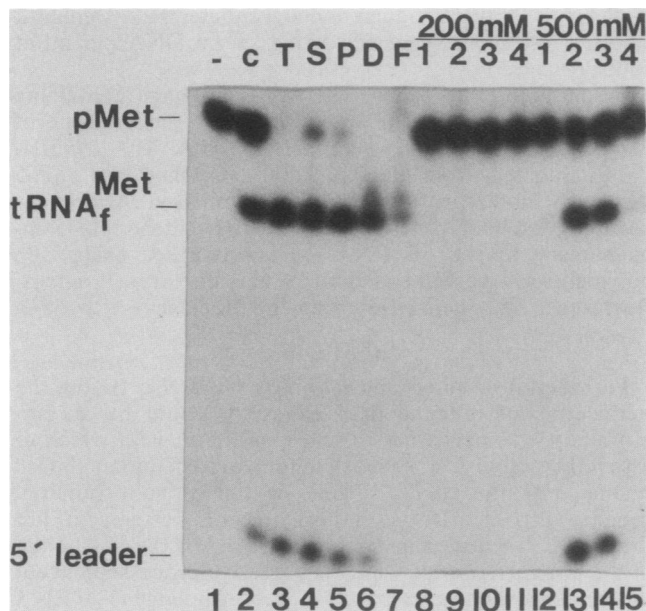


FIG. 1. Detection of *S. cerevisiae* mitochondrial RNase P. Aliquots from each step in the partial purification were assayed for RNase P activity. Purification and assay conditions are given in Materials and Methods. Lanes: 1, incubation of pMet_f without enzymes; 2, incubation of pMet_f with *E. coli* RNase P; 3, an aliquot from the initial sonicate; 4, supernatant from the 48,000 × *g* spin; 5, pellet from the 48,000 × *g* spin, taken up into one-fourth the original volume of breakage buffer; 6, dialysate; 7, flowthrough from DEAE column; 8 to 11, fractions from the 200 mM NH₄Cl wash of the DEAE column; 12 to 15, fractions from the 500 mM NH₄Cl elution of the DEAE column. The sonicate, supernatant, and pellet aliquots were diluted 1:10 in reaction buffer before assaying. The dialysate was diluted 1:5, while the column fractions were assayed without dilution.

due to the contaminating nucleases mentioned above. In this experiment, these nucleases are present in the flowthrough of the DEAE column, but not in the low- or high-salt wash. The most important criterion for following the mitochondrial RNase P was the appearance of both products, the tRNA_f molecule and the 5' leader.

RNase P activity in petite deletion mutants. The presence of the tRNA synthesis locus has been positively correlated with fully processed tRNAs *in vivo* (23). In its absence, tRNAs are correctly processed except for the presence of a 5' leader. Experiments were performed to investigate whether the newly discovered *in vitro* RNase P activity could be correlated with the action of the tRNA synthesis locus *in vivo*. Two petite mitochondrial deletion mutants were used in these experiments. One, ND157, contains only the synthesis locus and the tRNA^{ASP} gene. The tRNA^{ASP} is correctly transcribed and processed *in vivo*. The other, ND40, is the strain from which the substrate for these experiments is isolated. It contains a tRNA_f^{Met} gene, but no synthesis locus. The tRNA_f^{Met} transcript produced by ND40 mitochondria is a precursor with a 26-base 5' extension (11). Mitochondrial pellets from ND157 and ND40 were lysed and assayed for RNase P activity. RNase P activity is very difficult to detect in crude extracts of petite mitochondria but can easily be measured after the extracts are fractionated by DEAE chromatography. Figure 2 shows the results of an analysis of RNase P activity in petite deletion mutant fractions. Three parallel columns were run. One was loaded with ND157

extract, one with ND40 extract, and one with a mixture of both. Each column received 0.6 mg of protein (2) from each extract. The columns were eluted, 0.5-ml fractions were collected, and 0.001 ml of each fraction was assayed (Fig. 2). Both ND40 and ND157 have substantial amounts of nucleases that produce fragments of the pMet_f. The appearance of these activities in high-salt fractions from columns loaded with extracts from petites is variable. In this experiment, the washing of the DEAE columns was not complete enough to elute these nucleases prior to the application of high salt. In other experiments (data not shown), a more thorough 200 mM NH₄Cl elution washes these nucleases from the column. A 3' labeled product appears in the flowthrough as well as in both low- and high-salt fractions of ND40 and ND157. The ND157 high-salt fractions, but not the ND40 fractions, had an activity that produced a 5' product. Therefore, ND157, the mutant with the synthesis locus, does have an RNase P activity.

There are several possible explanations for the lack of production of a 5' product by ND40 fractions. One is that ND40 mitochondria have an inhibitor that actively prevents the production of a 5' product. To determine whether the lack of a 5' product in the RNase P assay of ND40 fractions was due to such an inhibitor, mixing experiments were performed. Fractions were assayed from a column that had equal protein from both ND40 and ND157 mitochondrial extracts applied. If the ND40 extracts contain a substance that interferes with RNase P activity, then it should inhibit that activity in ND157 extracts and no 5' product should be produced in the mixture. A 5' product is produced by the eluate from the ND40 plus ND157 column, demonstrating that the lack of such a product in the ND40 fractions is not due to the presence of an inhibitor. Another explanation is that ND40 extracts preferentially degrade the 5' product, so although it is produced it is rapidly destroyed. The mixing experiment argues against this possibility as well, because the 5' product produced by the mixed fractions is as stable as the 5' product produced by ND157 fractions alone. The most straightforward explanation consistent with all the previous *in vivo* and the current *in vitro* results is not that ND40 contains an inhibitor or preferentially destroys the 5' product but rather that it has no RNase P. Therefore, the presence of an RNase P activity *in vitro* can be correlated with the presence of the synthesis locus *in vivo*.

The second important result from this comparison is the confirmation that we are actually measuring a mitochondrial enzyme activity. If the activity was due to nuclear RNase P contaminating mitochondrial preparations, both petite mitochondrial extracts would contain the contaminant. Since the ND40 extracts do not have RNase P activity, this demonstrates that our mitochondrial preparations are not contaminated with the nuclear RNase P.

Dependence on yeast mitochondrial RNase P upon a nucleic acid. RNase P activities from every source investigated to date have been dependent upon both a protein and an RNA moiety for activity (1, 5, 7, 9, 10). To investigate whether the yeast mitochondrial RNase P is also dependent upon a nucleic acid, the activity was assayed in the presence of micrococcal nuclease, with and without EGTA. Micrococcal nuclease is a nonspecific nuclease which has an absolute dependence upon calcium for activity. Chelation of the calcium by EGTA renders the nuclease inactive, allowing the RNase P assay to proceed without destruction of substrate by the micrococcal nuclease. Results of one such experiment can be seen in Fig. 3. Treatment of mitochondrial extracts with micrococcal nuclease abolishes the

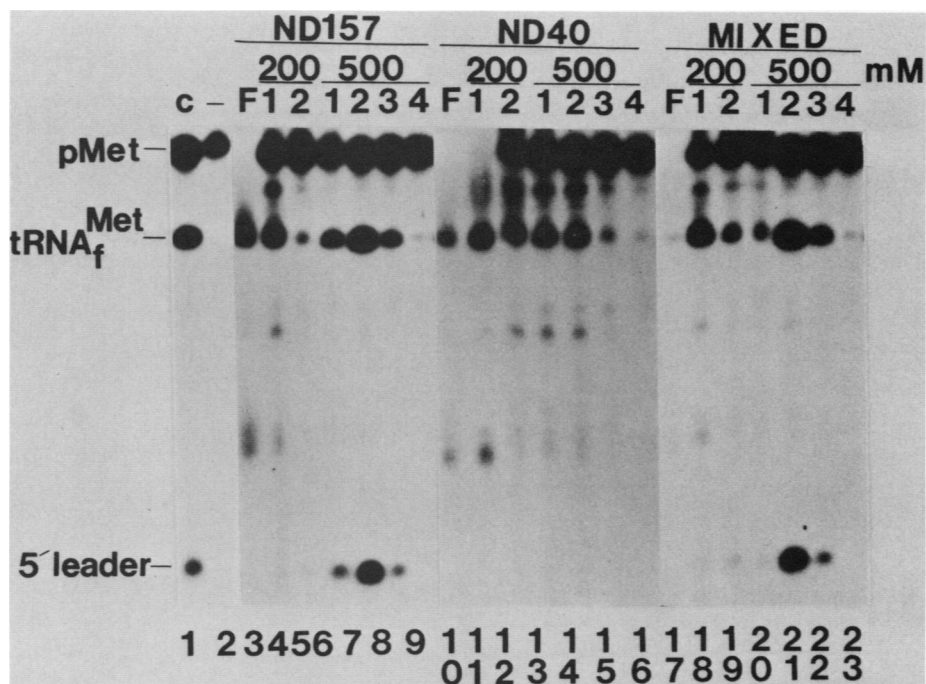


FIG. 2. RNase P activity positively correlated with the tRNA synthesis locus in yeast mitochondria. Fractions from DEAE columns were assayed for RNase P activity. Lanes: 1, *E. coli* RNase P; 2, incubation under assay conditions, but with no enzyme; 3, flowthrough, ND157; 4 and 5, 200 mM wash, ND157; 6 to 9, 500 mM elution, ND157; 10, flowthrough, ND40; 11 and 12, 200 mM wash, ND40; 13 to 16, 500 mM elution, ND40; 17, flowthrough, ND40 plus ND157; 18 and 19, 200 mM wash, ND40 plus ND157; 20 to 23, 500 mM elution, ND40 plus ND157.

RNase P activity. Incubation of the RNase P under the same conditions but in the presence of EGTA leaves the activity slightly diminished, but present. Therefore, *Saccharomyces cerevisiae* mitochondrial RNase P is dependent upon a nucleic acid for activity. Mitochondrial RNase P is not sensitive to DNase I (data not shown). The results from these nuclease experiments imply that the nucleic acid moiety in this enzyme is an RNA molecule.

Dependence of the yeast mitochondrial RNase P upon a protein. The RNA moieties from *E. coli* and *B. subtilis* RNase P's can catalyze tRNA processing under certain salt conditions in the absence of protein (8). No yeast mitochondrial deletion mutants carry out mitochondrial protein synthesis, yet some of them have a mitochondrial RNase P activity. If there is a protein component of mitochondrial RNase P, then it must be coded by nuclear DNA. The availability of an *in vitro* assay enabled a determination as to whether the mitochondrial RNase P has a protein-dependent activity. RNase P was assayed with or without prior treatment by protease, as outlined in Materials and Methods. Results from the protease experiments can be seen in Fig. 4. Treatment of the mitochondrial RNase P with protease abolishes its activity. In a control incubation without protease, the RNase P activity is retained. From these experiments, it is possible to conclude that *S. cerevisiae* mitochondrial RNase P, under these assay conditions, is dependent upon both protein and nucleic acid moieties for activity.

Correlation between the mitochondrial RNase P and the tRNA synthesis locus gene product. As shown above, there is a positive correlation between RNase P activity *in vitro* and the presence of the tRNA synthesis locus *in vivo*. Since tRNA processing occurs even in the absence of mitochondrial protein synthesis, and the activity requires a mitochondrial gene, the tRNA synthesis locus must provide the nucleic acid upon which the RNase P depends, and not the

protein. Only one transcript, the 9S RNA, has been identified as being a product of the synthesis locus (15). Hybridization studies were used to discover whether 9S RNA sequences cofractionate with the RNase P activity. Aliquots from each step in an RNase P preparation were extracted for RNA. The RNAs were transferred to nylon membrane and hybridized with a probe specific for the 9S RNA. ND40 RNA was used as a negative control and wild-type MH41 RNA was used for the positive control. Results from this experiment can be seen in Fig. 5. Aliquots from fractions which were positive for RNase P activity (Fig. 1) also contain the 9S RNA as detected by hybridization. The relative activity of the 500 mM elution fractions (2>3>4>1) is also mirrored by the relative intensity of hybridization to RNAs from these fractions. This result further strengthens the positive correlation between the RNase P activity and the 9S RNA. RNAs isolated from the same fractions and separated by gel electrophoresis were transferred to paper and hybridized with the same probe. Most of the 9S RNA sequences in these fractions are smaller than those isolated from mitochondria by phenol extraction. Our current data do not allow us to determine if the activity we measure *in vitro* depends on residual full-length 9S or whether fragments of it are sufficient to support activity.

DISCUSSION

The description of an RNase P activity in highly purified *S. cerevisiae* mitochondria is reported here. The analysis of mitochondrial enzymes can be complicated by functionally analogous nuclear activities. In this case, the availability of mitochondrial mutants with and without RNase P activity enabled us to determine that our highly purified mitochondria were not substantially contaminated with nuclear RNase P. When purified yeast nuclear and mitochondrial

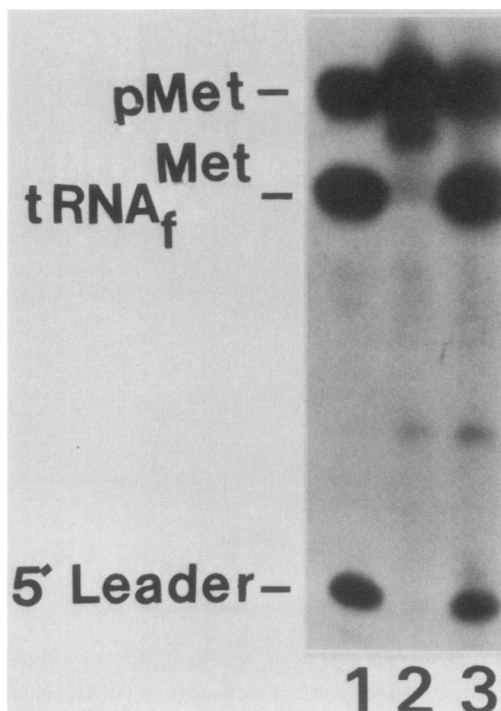


FIG. 3. Nuclease-sensitive *S. cerevisiae* mitochondrial RNase P. Mitochondrial RNase P was assayed in the presence of micrococcal nuclease with or without EGTA in the reaction mixture as outlined in Materials and Methods. Lanes: 1, assay for RNase P activity without prior incubation; 2, incubation with nuclease without EGTA prior to assay; 3, incubation with micrococcal nuclease in the presence of EGTA prior to assay.

RNase P are available, it will be of interest to compare their structures and mechanisms of action.

Detection of this RNase P in yeast mitochondrial extracts was made more difficult by the existence of other nucleolytic activities that produce a product which migrates only slightly slower than authentic $\text{tRNA}_f^{\text{Met}}$. These activities are present in petite mutants whether or not they accumulate precursor tRNAs in vivo. Relative amounts of nuclease varied from extract to extract but were consistently higher in petite mitochondria. The ratio of total nuclease activity to RNase P activity in petite mitochondria made it almost impossible to detect the latter in crude extracts. Although these nucleases produce 3' labeled products from the pMet_f , these contaminating activities do not yield an intact 5' product. It was necessary to follow the actual RNase P activity by the presence of this 5' product. Many procedures were used to attempt to separate RNase P from the nonspecific nucleases, including sucrose gradients, ammonium sulfate precipitation, sizing columns, and DEAE. Of these, DEAE gave the cleanest separation. We are currently optimizing the DEAE chromatography and exploring other chromatographic and electrophoretic steps for purification of the enzyme.

pMet_f is a uniquely appropriate substrate for mitochondrial RNase P assays. It is produced in ND40, a petite mutant without the synthesis locus. It has been shown to have the start site for $\text{tRNA}_f^{\text{Met}}$ gene transcription at the 5' end of its 26-base leader (3, 11). Because equal amounts of radioactivity from both 5' and 3' end-labeled samples were mixed, it was possible to visualize 5' and 3' products to an equivalent degree. This is in contrast to uniformly labeled

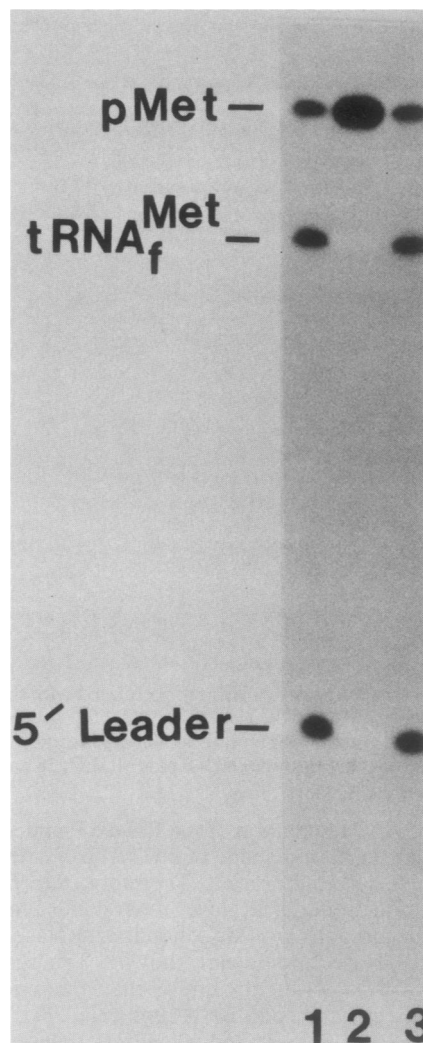


FIG. 4. Protease-sensitive *S. cerevisiae* mitochondrial RNase P activity. Yeast mitochondrial RNase P was assayed with or without prior incubation with pronase. Lanes: 1, incubation under protease conditions, but without the protease prior to assay; 2, incubation with protease prior to assay; 3, no incubation prior to assay.

precursor tRNAs, which yield a lighter autoradiographic signal for the shorter 5' product than the longer 3' product. The disadvantage is that the pMet_f used here is not uniformly labeled so we could not fingerprint the products. However, the 3' product produced by the most pure fractions comigrates with authentic mitochondrial $\text{tRNA}_f^{\text{Met}}$. Furthermore, the 3' and 5' products that are produced by mitochondrial RNase P appear identical by high-resolution gel electrophoresis to those produced by *E. coli* RNase P (11). Remarkably, yeast mitochondrial RNase P does not efficiently process a heterologous precursor tRNA from *E. coli* (M. J. Hollingsworth and C. Guerrier-Takada, unpublished data). Therefore, it is fortunate that we were able to obtain the homologous pMet_f from a petite deletion mutant for our assays.

The mitochondrial enzyme we describe here is both protein and nucleic acid dependent, as have been all of the other RNase P activities investigated to date (1, 5, 7, 10). The evidence that the 9S RNA is the nucleic acid component of the mitochondrial enzyme is persuasive. It is possible to

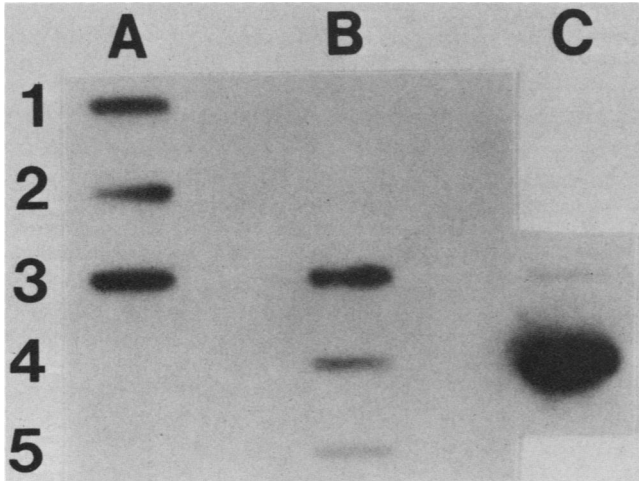


FIG. 5. Hybridization of 9S probe to RNA extracted from different steps in the RNase P preparation. (Column A) Rows: 1, supernatant, $48,000 \times g$; 2, pellet, $48,000 \times g$; 3, dialysate; 4, flowthrough, DEAE column; 5, first fraction, 200 mM NH_4Cl wash. (Column B) Rows: 1, second fraction, 200 mM NH_4Cl wash; 2, first fraction, 500 mM NH_4Cl elution; 3, second fraction, 500 mM NH_4Cl elution; 4, third fraction, 500 mM NH_4Cl elution; 5, fourth fraction, 500 mM NH_4Cl elution. (Column C) Rows: 3, 10 μg of ND40 mitochondrial RNA; 4, 10 μg of MH41 mitochondrial RNA. ND40 does not have the 9S gene. MH41 is the wild type from which the RNase P is actually isolated.

positively correlate the mitochondrial RNase P activity *in vitro* with the presence of the tRNA synthesis locus *in vivo*. The tRNA synthesis locus encodes only one abundant transcript, known as the 9S RNA (15). It was previously shown that the presence or absence of the synthesis locus dictates whether 5' leaders are processed from mitochondrial tRNA gene transcripts (13). Since the presence of the locus parallels RNase P activity, the relationship between RNase P activity and 9S RNA was also investigated. Hybridization of 9S probes to RNA prepared from mitochondrial extracts showed that the 9S RNA cofractionated with the RNase P activity. The M1 RNA of *E. coli* RNase P and P RNA of *B. subtilis* RNase P have been shown to be the catalytically active subunits of the enzymes. Under certain salt conditions, it is possible for the RNAs to carry out tRNA processing on their own without the accompanying protein (8). The best proof that the 9S RNA is the nucleic acid moiety of yeast mitochondrial RNase P would be to determine if the 9S RNA can also process tRNA in the absence of protein. Efforts are currently being made to obtain a large enough quantity of the 9S RNA to answer this question.

Normally one need not check an enzyme for protein dependence. In other organisms, however, the catalytic subunit of the enzyme is the RNA (8), and in yeast mitochondria RNase P is found under circumstances in which there can be no mitochondrial protein synthesis. It was possible, then, that the mitochondrial RNase P required only RNA for activity. This necessitated the protease experiment shown in Fig. 4. Proof that the activity is protein dependent permits the conclusion that the protein moiety of mitochondrial RNase P is encoded in the nuclear genome. Although a mitochondrial RNase P from HeLa cells has been described recently (5), no conclusions on the location of the genes which encode the components of the HeLa mitochondrial enzyme are available.

The yeast mitochondrial RNase P is unique in that its

nucleic acid is encoded within the mitochondria while its protein is encoded by the nuclear genome. Although it is not unusual for mitochondrial enzyme activities to be codependent upon mitochondrial and cytoplasmic translation products, this is an unusual case of an enzymatic activity whose mitochondrially produced subunit is not a polypeptide. It is not known whether the synthesis of the two subunits of this enzyme are coordinately regulated.

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