Characterization of yeast mitochondrial RNase P: an intact RNA subunit is not essential for activity *in vitro*

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Received June 19, 1989; Revised and Accepted July 25, 1989

ABSTRACT

We have previously described a mitochondrial activity that removes 5′ leaders from yeast mitochondrial precursor tRNAs and suggested that it is a mitochondrial RNase P. Here we demonstrate that the cleavage reaction results in a 5′ phosphate on the tRNA product and thus the activity is analogous to that of other RNase Ps. A mitochondrial gene called the tRNA synthesis locus encodes an A+U-rich RNA required for this activity *in vivo*. Two regions of this RNA display sequence similarity to conserved sequences in bacterial RNase P RNAs. This sequence similarity coupled with the analogous activities of the enzymes has led us to conclude that the RNAs are homologous and that the tRNA synthesis locus does code for the mitochondrial RNase P RNA subunit. The smallest and most abundant transcript of the tRNA synthesis locus is 490 nucleotides long. However, during purification of the holoenzyme, RNA is degraded and pieces of the original RNA are sufficient to support RNase P activity *in vitro*.

INTRODUCTION

Mitochondrial transfer RNAs are transcribed as precursors that must be processed to attain their mature form. The enzyme responsible for the cleavage of the 5′ leader from precursor tRNAs is Ribonuclease P (RNase P) and such an activity has been described in mitochondrial extracts from rats (1), humans (2) and yeast (3). None of these enzymes is as well characterized as those from *E. coli* and *B. subtilis* or those from the nucleus of eukaryotic cells.

RNase Ps from *E. coli* and *B. subtilis* consist of a small protein and an RNA subunit of 350−400 nucleotides (4,5,6,7). The RNA can catalyze the cleavage of 5′ leaders from tRNA precursors in the absence of the protein (8,9,10). Although the RNAs from *E. coli*, *B. subtilis*, and five other eubacteria have limited primary sequence similarity (7,11), phylogenetic comparisons of these RNAs indicate that they share common features of primary and secondary structure. Eukaryotic RNase Ps responsible for the processing of cytoplasmic tRNAs also consist of an RNA and protein component (12,13,14,15). While the eukaryotic RNAs can be arranged in secondary structure models that share features with the catalytic bacterial RNAs there has been no demonstration of RNA catalysis *in vitro*. Nonetheless, recovery of active, heterologous enzyme generated by mixing *E. coli* RNase P RNA with human protein or *E. coli* protein with human RNA (16) demonstrates that the subunits of these enzymes from widely divergent organisms are functionally analogous.

Mitochondria contain their own genome, separate from that in the nucleus. In *Saccharomyces cerevisiae*, this genome codes for a small number of polypeptides and the RNAs required for their translation (17). In addition to the RNase P cleavage, primary transcripts of yeast mitochondrial tRNAs are processed to their mature forms by endo-

nucleolytic removal of 3′ trailers (18), addition of the CCA at the 3′ end (19), and base modifications (20). Strains unable to make mitochondrial proteins due to mutations in their mitochondrial DNA can still process tRNA, so it is clear that the proteins needed for mitochondrial tRNA biosynthesis are coded for by nuclear genes (21,22). A subset of mitochondrial mutants that contain deletions of a 1400 bp region between the $tRNA_f^{Met}$ and $tRNA^{Pro}$ genes are unable to remove 5′ leaders from mitochondrial precursor tRNAs (23). This region, called the tRNA synthesis locus (21), contains a gene that codes for an RNA composed predominantly of adenosine and uridine with short interspersed guanosine- and cytidine-rich elements. The accumulation of tRNA precursors with 5′ leaders in strains lacking this RNA led us to propose that it is the RNA component of yeast mitochondrial RNase P (24).

The yeast mitochondrial 5′ endonuclease activity has been identified *in vitro* and is present only in mitochondrial extracts from yeast strains containing a tRNA synthesis locus. It is sensitive to both protease and nuclease, indicating the requirement of both a protein and an RNA component for activity (3).

We present here additional work on the characterization of the mitochondrial RNase P from *S. cerevisiae*. To confirm the mitochondrial activity as a true RNase P we have determined by fingerprint analysis that the tRNA product terminates with a 5′ phosphate. Biochemically, the mitochondrial enzyme is different from the bacterial RNase Ps with respect to ion selectivity and ionic strength optima. Nonetheless, the RNAs do share limited sequence similarities in two regions previously identified as highly conserved among bacteria (11). The most abundant product of the tRNA synthesis locus is an RNA 490 nucleotides long; however, none of this length RNA remains in our most purified enzyme fractions. Analysis of RNA recovered from these fractions shows that short pieces of the original RNA are able to support RNase P activity *in vitro*.

MATERIALS AND METHODS

Production of Precursor tRNAs.

A mitochondrial $tRNA_f^{Met}$ gene was constructed from the following oligonucleotides (5′ to 3′): GGTCATGACCCTAAAATGTT, TGACCTAATTATATACGTTCAAATCG, AATTCCTGGTAGCAATAATACGATTTGAACGTATATAATTA, AACCAATTACATCATATTGCATTTATATAATTAATACTTATATTATATTTGCA, AATATAATATAAGTATTAATTATATAAATG, TATTATTGCTACCAGG, and CATATGATGTAATTGGTTAACATTTTAGGGTCA. These were annealed and ligated with 0.2 pmoles PstI/EcoRI cleaved pSP64 (Promega Biotech) using 18 U T4 DNA ligase (New England Biolabs). Recombinant DNAs were transformed into *E. coli* strain HB101 and the resulting plasmid pMH101 was isolated, linearized with BstN1 and transcribed with Sp6 polymerase according to manufacturer's instructions (Promega Biotech) for use in fingerprinting experiments.

pMM6 was constructed by subcloning the EcoRI/HindIII fragment from pMH101 into EcoRI/HindIII cut pT3T7.19 (Bethesda Research Laboratory). pMM6 was linearized with BstN1 and transcribed using T7 polymerase according to manufacturer's instructions (Bethesda Research Laboratory) to produce precursor for processing studies.

Preparation of RNase P

Mitochondria were prepared from *Saccharomyces cerevisiae* as previously described (21) for enzyme used in the characterization studies. Mitochondria for other experiments were

prepared as described (18) except the yeast cell walls were digested with 2.5 mg/gram cells of Yeast Lytic Enzyme 70000 (ICN), and the cells were lysed by two cycles with a Parr Bomb. After preparation, mitochondria were frozen in liquid nitrogen and stored at −70°.

Frozen mitochondria were ground to a powder in a Waring blender equipped with a stainless steel chamber pre-cooled with liquid nitrogen. The powder was thawed in 100 mM NH_4Cl, 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 0.2 mM benzamidine, 1 μM leupeptin, 1 μM pepstatin, and 10% glycerol (Lysis buffer). The resulting protein concentration was 5 mg/ml. Sodium deoxycholate was added to a final concentration of 0.25% and the extract was stirred on ice for 5 minutes. After centrifugation at 30,000×g for 45 minutes, the supernatant was applied to a DEAE Sepharose (Pharmacia) column that contained 1 ml resin per 10 mg protein. The column was washed with one column volume of lysis buffer, followed by 25 column volumes of 150 mM NH_4Cl, 10 mM $MgCl_2$, 50 mM Tris-Cl pH 7.5, 10% glycerol (TMG + 150 mM NH_4Cl). The activity was eluted with the same buffer containing 350 mM NH_4Cl. The DEAE eluant was diluted 1.8-fold with TMG, and applied to an FPLC Mono Q HR 10/10 column at 4 ml/minute. The column was washed with 25 ml TMG, followed by 160 ml TMG + 250 mM NaCl. Activity was eluted with a linear gradient of 250 mM to 800 mM NaCl in TMG.

Active fractions from the Mono Q column were pooled, diluted 2-fold with 500 mM NaCl + TMG, and brought to 25 mM $MgCl_2$, 33% Cs_2SO_4 (BRL). The solution was centrifuged at 40,000 rpm for 40 hours at 10° in a Beckman 70Ti rotor. The gradient was fractionated with an ISCO Model 185 density gradient fractionator using a Fluorinert WP-40 chase solution. The buoyant density of the extract was determined with a Mettler/Parr Model DMA 55M calculating density meter. The active fractions were pooled and concentrated in a centriprep 30 concentrator (Amicon) followed by desalting on a NAP-25 column (Pharmacia). Protein concentrations were determined with the BCA protein assay (Pierce Chemical) according to manufacturer's directions.

Assay and Quantification of RNase P activity

Activity was assayed by incubating the enzyme with 4−10 nM radiolabeled pMet (transcribed from pMM6), 50 mM Tris-Cl, pH 8.0, 10 mM $MgCl_2$, 75 mM NH_4Cl for fifteen minutes at 37°C. For quantification of enzyme activity in crude extracts, aliquots of the reaction mix were removed at 1 minute intervals. All reactions were stopped by addition of 1/3 volume of 6 M urea, 50% sucrose, 10 mM aurintri-carboxylic acid, .01% xylene cyanol, and .01% bromphenyl blue. The reaction products were separated by electrophoresis through a 10% acrylamide/6 M urea. The products were visualized by autoradiography. The autoradiogram was used as a template for the excision of substrate and product RNAs from the gel. Radioactivity was quantified by liquid scintillation counting. Activity is expressed as femtomoles of substrate converted to products per minute.

RNA Fingerprinting

Precursors were uniformly radiolabeled with either α^{32}P-UTP or α^{32}P-CTP and purified by electrophoresis through polyacrylamide/urea gels. The RNA was mixed with 25 μg of carrier RNA and digested with 4 units of RNase T1 (Calbiochem) for 30 minutes at 37°C. The fragments were applied to a cellulose acetate paper strip and separated by electrophoresis followed by thin layer homochromatography on DEAE cellulose plates (35,36). Secondary digestions for identification of individual oligonucleotides were done using RNase T2 followed by separation on thin layer plates as in (35).

Isolation and Characterization of RNA in Mitochondrial Extracts
One volume of 25 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.2% SDS, and 0.25% bentonite was added to thawed mitochondria. The resulting solution was extracted with phenol/$CHCl_3$ and nucleic acids were precipitated with ethanol.

For northern analysis, the RNAs were separated by polyacrylamide gel electrophoresis and transferred to Zetaprobe according to manufacturer's instructions (Biorad). The membranes were pre-washed for 1 hour at 65° in 0.1×SSC, 0.5% SDS. Pre-hybridization was carried out in 5× SSC, 50 mM sodium phosphate buffer, pH 6.5, 1% SDS, 10× Denhardt's solution, 100 μg/ml *E. coli* tRNA (Sigma Chemical, Type XX) for 4 hours at 65°. The oligonucleotides used as probes were 5′-9S (TATTTATGACTTTCCTATTA), complementary to the region between positions 92−102 (Figure 6), 3′-9S (TTAAGCATATTTCTGTAT), complementary to positions 465−473, and GCIII (ACTCCTGCGGGGTCCGCCCG), complementary to the GC cluster between positions 371−391. Oligonucleotides labeled with T4 kinase and γ^{32}P-ATP were used in overnight hybridizations at 30°C, 37°C, and 62°C, respectively in 6×SSC, 50 mM sodium phosphate, pH 6.5, 10× Denhardt's solution, and 100 μg/ml *E. coli* tRNA. Washes were done three times in 6× SSC for 10 minutes at 30°.

S1 analysis was done by a modification of the procedure of Berk and Sharp (37). Probes were mixed with 60 μg of either mitochondrial RNA or *E. coli* tRNA in 400 mM NaCl, 40 mM Pipes pH 6.4, 1 mM EDTA to a total volume of 10 μl. After incubation overnight at 45°C each reaction was added to 100 μl ice-cold 250 mM NaCl, 30 mM NaOAc pH 4.5, 1 mM $ZnSO_4$ and 75 μg/ml herring sperm DNA, divided into 4 aliquots and treated with either 0, 600, 700, or 800 units S1 nuclease (BRL) at 30°C for 1 hour. Reactions were terminated by extraction with phenol/chloroform and the products recovered by ethanol precipitation. Products were separated on 8% polyacrylamide/8M urea gels and visualized by autoradiography. The probe for mapping the 5′ end was prepared from a plasmid, pS-9S-2, which contains the entire genome of the yeast strain ND157. This genome consists solely of the RNase P RNA and $tRNA^{Asp}$ genes (24). The mitochondrial DNA was linearized with Sau3A and cloned into the BamHI site of Bluescript M13(−) plasmid vector (Stratagene). One μg of single-stranded pS-9S-2 was annealed to 20 pmol of the oligonucleotide 5′ 9S and extended in the presence of 50 μM dGTP, dCTP, TTP, α-^{32}P-dATP plus 1 unit Klenow fragment of DNA polymerase I for 30 min. at 37°C. 2.5 μM cold dATP was then added and incubation continued another 30 min prior to digestion of the mixture with HinfI. The single-stranded 580-base fragment extending from the primer to an upstream HinfI site was isolated from a denaturing gel and used for 5′ mapping. To obtain the probe for 3′ end determination, double-stranded pS-9S-2 plasmid DNA was digested with HinfI and HindIII. The HinfI end was labeled by a fill-in reaction with α^{32}P-dCTP and Klenow enzyme (38). The labeled, single-stranded 592-base HinFI-HindIII fragment corresponding to the 3′ end of the tRNA synthesis locus was isolated from a denaturing gel.

The location of the RNA in the Cs_2SO_4 gradient was established by 'Slot-Blot' analysis. The individual fractions were diluted with 10 volumes DEPC-treated water and 500 ml filtered onto a microfiltration apparatus(Minifold II, Schleicher & Schuell) fitted with a Zetaprobe membrane. The membrane was pretreated as above. The probe was an *in vitro* transcribed full-length complement to the RNase P RNA, derived by cloning the NarI/SstI fragment from ND157 mitochondrial DNA into pT3/T7.19. Hybridization was carried out at 65° overnight in prehybridization buffer and washed in: 2× SSC, 0.1% SDS at

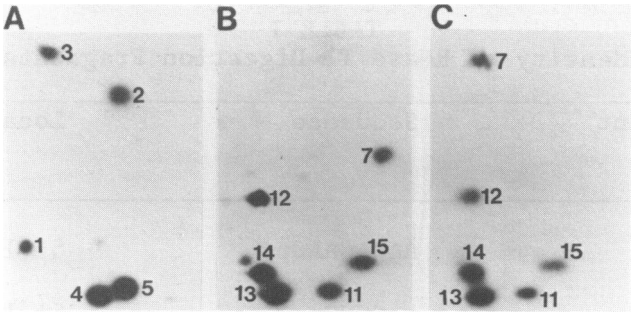

Figure 1: Fingerprint analysis of yeast mitochondrial RNase P reaction products. Products from the yeast mitochondrial RNase P are uniformly labeled at C (tRNAMet) or U (5′ leader). Analysis in the first dimension was paper electrophoresis; in the second, homochromatography on DEAE cellulose plates. Details of the method are given in Materials and Methods. A: The 5′ leader. B: tRNAMet, as isolated from a yeast mitochondrial RNase P reaction. C: Same substrate as in B, but treated with calf alkaline phosphatase prior to fingerprint analysis. Identities of each oligonucleotide are given in Table I.

room temperature, 0.1× SSC, 0.5% SDS at room temperature, and 0.1× SSC, 0.5% SDS at 65°; each for 15 minutes.

For hybrid selection experiments, single-stranded DNA complementary to the RNase P RNA was obtained by cloning the 1400 bp Nar I/Sau 3A fragment from ND157 mitochondrial DNA into the AccI and Bam HI sites of M13 mp8 (39). 80 μg DNA was applied to 11×11 mm nitrocellulose filters which were air dried and baked at 80°C *in vacuo*. The filters were boiled for one minute in H_2O before use. RNA from 120 μl of the Cs_2SO_4 pool (containing 8.5 μg protein) was labeled at the 3′ end (40) with 100 μCi ^{32}P-pCp (NEN) and T4 RNA ligase (New England Biolabs), extracted with phenol and recovered by ethanol precipitation. The labeled RNA was suspended in 10 mM Pipes pH 6.4, 0.4 M NaCl, dotted onto a filter, and hybridized in the Pipes/NaCl solution overnight at 45°C. After hybridization, the filters were washed ten times in 1× SSC, 0.5% SDS and five times in 2 mM EDTA, all at room temperature. The bound RNA was removed from the filter by boiling for 5 minutes in 1 ml H_2O. The RNA solution was quick-frozen in dry ice/EtOH and lyophilized to dryness. Hybrid-selected RNAs were separated from each other by electrophoresis.

The hybrid-selected RNA was treated with .0004 U RNase T1 (Calbiochem) in 10 mM Tris-HCl, pH 7.5 .1 mM EDTA, 5 μg cold carrier tRNA at 37° for 30 minutes in a total volume of 5 μl. RNA was digested with 1 unit T1 in the same conditions, but without carrier RNA. RNA size ladders were generated by treating each RNA with base from a BRL RNA sequencing kit according to manufacturer's instructions. Products were separated on a 15% acrylamide, 8 M urea gel. Digestion products were detected by autoradiography.

RESULTS

Characterization of Precursor and Products.

RNase Ps are endonucleases that leave 5′ phosphates and 3′ hydroxyls on their products (25, 6, 26). We have previously shown that the mitochondrial 5′ tRNA processing enzyme is an endonuclease (3); however, the exact structure of the tRNA product was not established. This information is necessary to confirm rigorously that the endonuclease

TABLE I
Identity of RNase T1 Digestion Fragments

Fragment	Sequence	Location
1	AAUACAAGp	5′ leader
2	CUUGp	5′ leader
3	CUGp	5′ leader
4	CAAAUAUAAUAUAAGp	5′ leader
5	UAUUAAUUAUAUAAA	5′ leader
7	(p)UGp	$tRNA_f^{Met}$
11	UUAACAUUUUAGp	$tRNA_f^{Met}$
12	UCAUGp	$tRNA_f^{Met}$
13	ACCUAAUUAUAUACGp	$tRNA_f^{Met}$
14	UUCAAAUCGp	$tRNA_f^{Met}$
15	UAUUAUUGp	$tRNA_f^{Met}$

activity is analogous to the RNase P from *E. coli* and *B. subtilis*. Therefore, we performed fingerprint analysis on the products of the *in vitro* RNase P processing reaction.

The radiolabeled products of the RNase P reaction were isolated from an acrylamide/urea gel, treated with RNase T1 and separated by cellulose acetate electrophoresis followed by thin layer chromatography. Results from the analysis are illustrated in Figure 1. Each oligonucleotide from the T1 digestion was eluted and digested with ribonuclease T2 to confirm its identity (Table 1). Figure 1A shows the results of a fingerprint of the 5′ leader, and 1B shows the pattern of oligonucleotides obtained in a fingerprint of the tRNA product.

To determine whether the 5′ end of the tRNA product of RNase P cleavage contains a 5′ phosphate, the tRNA was treated with alkaline phosphatase prior to digestion with T1 (Panel C). Any change in migration of a T1 digestion product after phosphatase treatment would be due to the loss of a 5′ phosphate. Oligonucleotide #7 shifted to a new position following phosphatase treatment. The rest of the oligonucleotides were not affected by phosphatase treatment. The oligonucleotide labeled #7 in figure 1 originates from the 5′ end of the tRNA. Therefore we conclude that, as expected for an RNase P, cleavage of

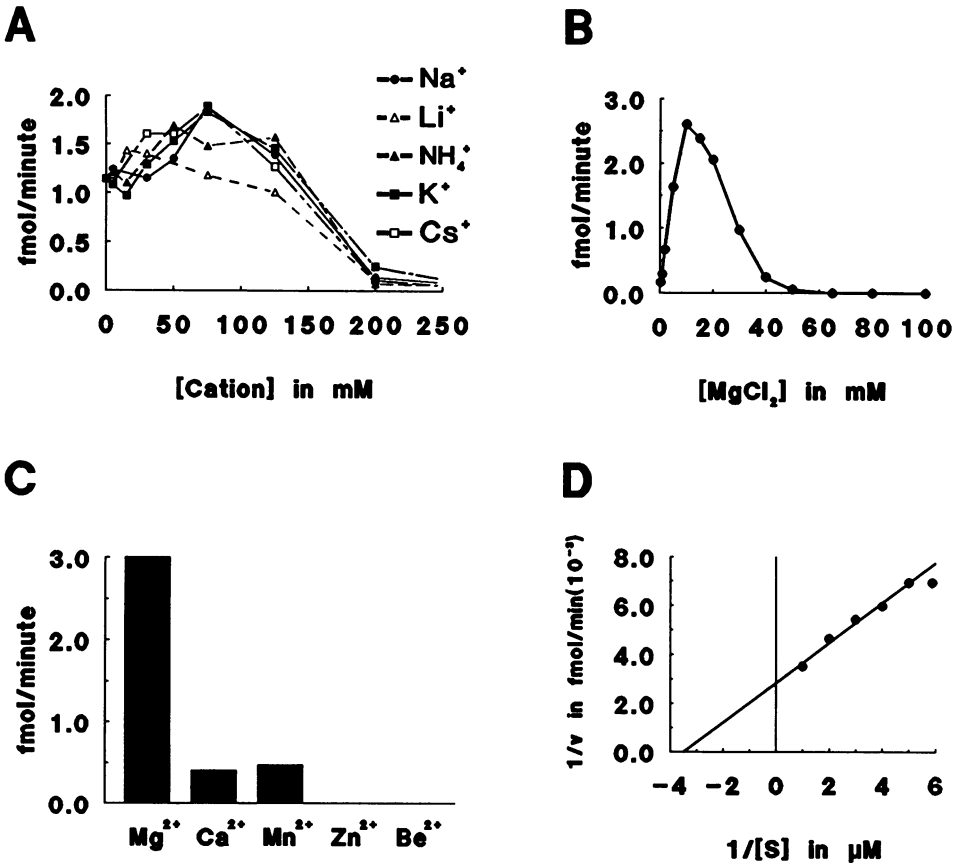

Figure 2: Ion dependence of the RNase P reaction. Assays performed as in methods with 4.0 nM pMet, 15 mM $MgCl_2$, and 0.5 μg protein from an active fraction from a DEAE column. A: Monovalent cation dependence; B: $MgCl_2$ dependence at 75 mM NH_4Cl. C: Divalent cation selectivity in 75 mM NH_4Cl; 15 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM $MnCl_2$. Zn^{2+} and Be^{2+} did not support the reaction at any concentration tested. D: Lineweaver-Burke plot of assay in 10 mM $MgCl_2$ and 75 nM NH_4Cl.

the tRNA precursor by this enzyme activity leaves a 5′ phosphate on the tRNA product.

Determination of Optimal Conditions for RNase P Activity.

We measured RNase P activity in a variety of conditions to determine ionic strength optima, divalent and monovalent cation selectivity, pH and temperature optima, and the kinetic parameters of the reaction. Figure 2 shows the results of these studies.

Yeast mitochondrial RNase P activity is enhanced by monovalent cations but does not require them (Figure 2A). The reaction proceeds two-fold faster in the presence of 75 mM monovalent cation than in its absence, and drops quickly at concentrations higher than 125 mM. The RNase P has little selectivity for monovalent cations; only LiCl inhibits catalysis slightly at concentrations above 30 mM. In contrast, the activity shows both specificity and a requirement for divalent cations. Figure 2B shows the enzyme requires 10 mM $MgCl_2$ for maximum activity. Other divalent cations were tested over a range of concentrations for their ability to substitute for $MgCl_2$. $MnCl_2$ and $CaCl_2$ can substitute

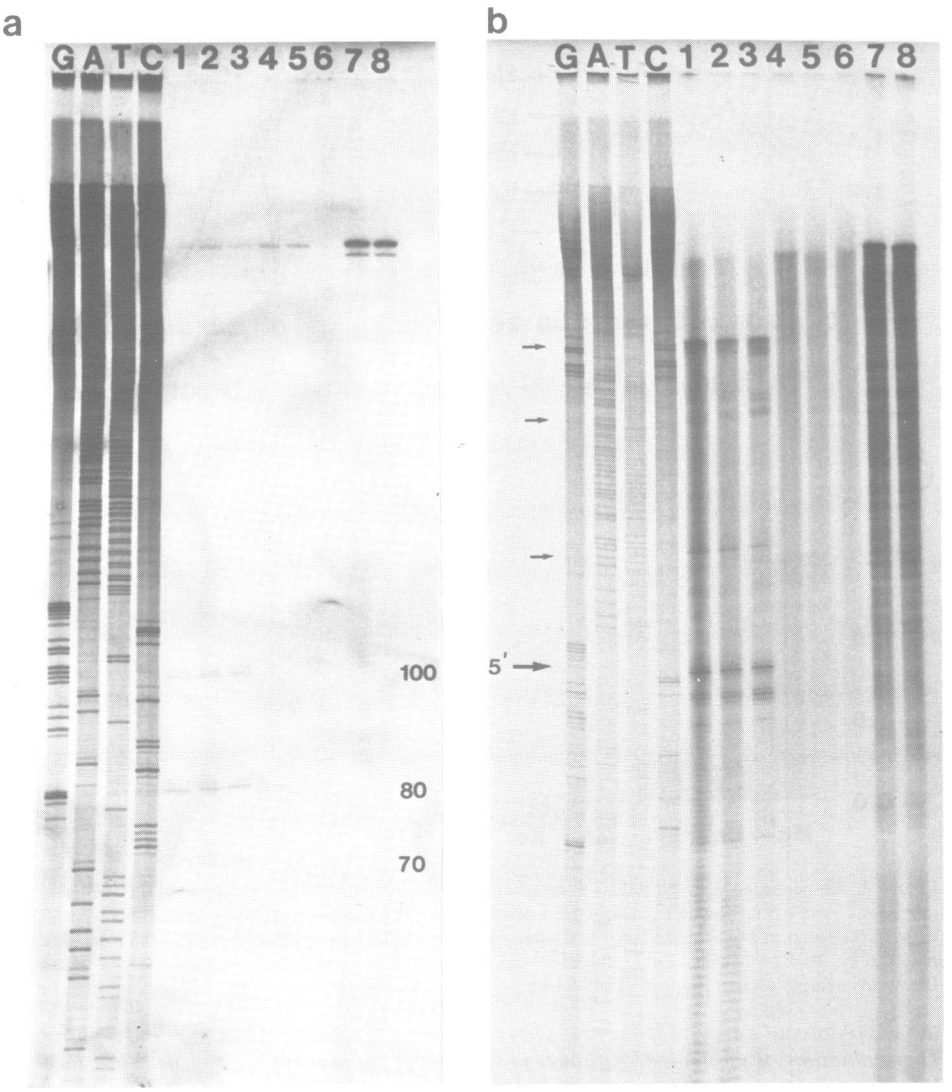

Figure 3: S1 mapping of RNase P RNA extracted directly from mitochondria. Panel A: To determine the 5′ end of the RNA, DNA fragments (see methods) protected from 600(Lane 1,4), 700(Lane 2,5), and 800(Lane 3,6) units of S1 nuclease in the presence of mitochondrial RNA (mito RNA; Lanes 1−3,7) or *E. coli* (Lanes 4−6,8) RNA were separated by electrophoresis next to a di-deoxy sequencing ladder. Lanes 7 and 8 were from a DNA fraction with no S1 nuclease added. G A T and C are the sequencing reactions from the same primer and DNA as were used to make the S1 probe. The small arrows indicate putative precursors to the RNase P RNA. Panel B. To determine the size of DNA fragments protected by the 3′ end of the RNase P RNA, an appropriate fragment (see methods) was annealed to mitochondrial RNA (Lanes 1−3,7), treated with 600(Lane 1,4), 700(Lane 2,5), and 800(Lane 3,6) units of S1 nuclease and separated by electrophoresis next to di-deoxy sequencing fragments which served as single stranded size markers. Controls included S1 reactions with *E. coli* RNA (lanes 4−6) and reactions with both RNAs but without S1 (lanes 7, 8).

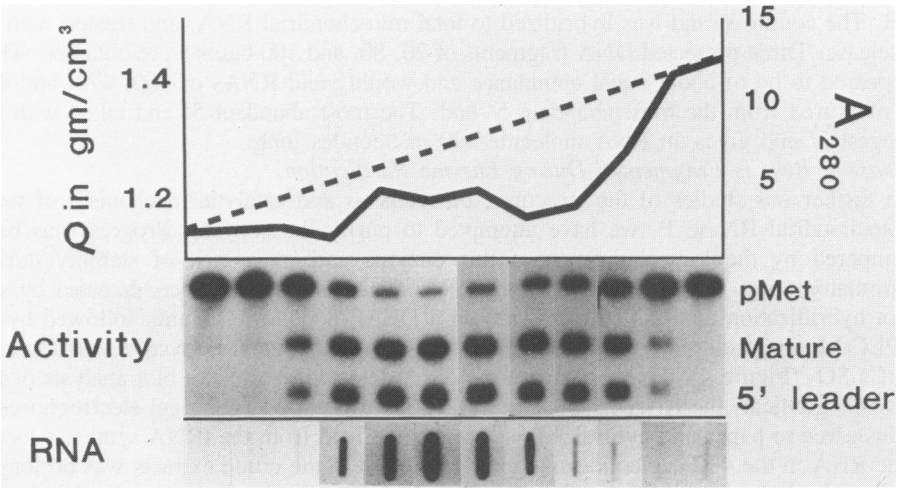

Figure 4: Isopycnic centrifugation in Cs_2SO_4. Active fractions from the Mono Q extract were brought to 33% Cs_2SO_4 as described in Methods, and were centrifuged at 165,000×g for 38 hours. The graph shows absorbance at 280 nm (solid line) and density of Cs_2SO_4 (dashed line). Yeast mitochondrial RNase P activity, and RNase P RNA as measured by hybridizatation of an *in vitro* transcribed probe are shown below. Hybridization is to RNA bound to a charged nylon membrane by filtration of fractions through a 'slot-blot' apparatus as described in methods.

for $MgCl_2$, but only support the activity about 15% as well as 10 mM $MgCl_2$ (Figure 2C). The enzyme has broad optima with respect to pH and temperature, with maximal values of 8.0, and 37°, respectively (data not shown). The Km(app) for pMet is 230 nM (Figure 2D).

Localization of the 5' and 3' Ends of the RNase P RNA.

The tRNA synthesis locus is located between the $tRNA_f^{Met}$ and $tRNA^{Pro}$ genes on yeast mitochondrial DNA. The most abundant RNA transcribed from this region, detected by Northern analysis using probes derived from the tRNA synthesis locus, is an RNA of about 450 nucleotides (24). We have examined the 5′ and 3′ ends of the RNA by S1 mapping (Figure 3). A uniformly labeled DNA probe made *in vitro* by extending an oligonucleotide primer hybridized to cloned mitochondrial sequences containing the tRNA synthesis locus was annealed to total mitochondrial RNA and the mixture digested with S1 nuclease. Several DNA fragments were protected by mitochondrial RNA, indicating that the RNA does not have distinct 5′ ends. The most abundant RNA 5′ end falls within a GC cluster 148 bases down-stream from the $tRNA_f^{Met}$. The highest molecular weight fragments (top arrow) correspond to the point at which the probe sequence diverges from mitochondrial sequences, indicating that some RNAs extend at least 288 bases from the site of hybridization of the oligonucleotide used to generate the probe. Additional protected fragments fall between the two discussed above. We propose that the RNAs that protect these longer DNAs are precursors to the most abundant and presumably active RNase P RNA. Their sizes are consistent with larger RNAs detected by Northern analysis (Figure 7B).

3′ end S1 mapping utilized a restriction fragment extending from the HinfI site at nucleotide 392 to a HindIII site beyond the mitochondrial sequences. This fragment was isolated, labeled at the 3′ end and the strands separated by electrophoresis in a denaturing

gel. The coding strand was hybridized to total mitochondrial RNA, and treated with S1 nuclease. Three protected DNA fragments of 70, 80, and 100 bases were obtained. They appeared to be of about equal abundance and would yield RNAs of 460, 470, and 490 if measured from the most abundant 5′ end. The most abundant 5′ end taken with the longest 3′ end gives an RNA molecule 490 nucleotides long.

RNase P RNA is Fragmented During Enzyme Purification.

To further our studies of the structure, biosynthesis and catalytic mechanism of yeast mitochondrial RNase P, we have attempted to purify the enzyme. Progress has been hampered by the low abundance of this enzyme and by a lack of stability during chromatography. RNase P activity and tRNA synthesis locus products detected by slot blot hybridization analysis cofractionate on a DEAE-Sepharose column followed by an FPLC-Mono Q column (Morales, unpublished) and a subsequent isopycnic centrifugation in Cs_2SO_4 (Figure 4). To characterize the RNA detected by the slot blot analysis of the Cs_2SO_4 gradient, the RNA in the active fractions was separated by gel electrophoresis, transferred to paper and hybridized with probes derived from the tRNA synthesis locus. The RNA in the 490 nucleotide size range observed in the crude extracts was no longer present in the active fractions after the first chromatographic step was completed. The RNAs derived from the synthesis locus that were recovered from the Cs_2SO_4 gradients were in the range of 100 nucleotides (Northern data not shown).

To characterize these RNAs further they were radiolabeled at their 3′ ends with ^{32}P-pCp and T4 RNA ligase both with and without prior phosphatase treatment, selected by hybridization to the tRNA synthesis locus DNA and separated by gel electrophoresis. No difference in the pattern of RNA was detected with these different labeling protocols. RNAs labeled with ^{32}P-pCp were characterized further. Figure 5A shows the profile of the RNAs from the active fractions of the Cs_2SO_4 gradient before and after isolation by hybrid selection. The patterns are very different and it is clear that a number of RNAs are greatly enriched by the hybrid selection protocol. The scarcity of guanosine residues in the synthesis locus enabled us to use partial T1 cleavage of most of the hybrid selected RNAs to determine their position within the synthesis locus product. The RNAs labeled A−F were excised from the gel and digested with RNase T1. Figure 5B shows the results of the RNase T1 digestion of these RNAs. [Figure 6 shows the sequence of the RNase P RNA].

The T1 digestion pattern of bands B and C is uniquely consistent with a fragment having a 5′ end at nucleotide 415 (Figure 6) and extending to nucleotide 490, the nucleotide position corresponding to the largest of the three 3′ ends detected by S1 nuclease analysis. Fragments D and E must come from one of two regions with more than 60 bases between G residues, 104−187 or 239 to 302(Figure 6). The close spacing of G's at the 5′ end of these fragments indicates they originate from the former region. The approximate boundaries of fragments A−D is shown in Figure 6. Fragment A from Figure 5 was resistant to T1 digestion(data not shown) and was less than 25 nucleotides long, so it could correspond to any of several regions. Fragment F was not enriched by the selection protocol. The digestion pattern of this fragment did not correspond to any region of the RNase P RNA and it was probably recovered because of its abundance.

We have repeated these experiments in a second preparation with RNAs labeled at their 5′ and 3′ ends(see Methods). Both labeling protocols suggest that fragments corresponding to the 3′ end of the RNA are most abundant followed by those corresponding to the 5′ end. 5′ labeling identified one additional RNA that spans from about nucleotides 275 to 350 which is less abundant than fragments origination from the ends. Therefore, RNAs

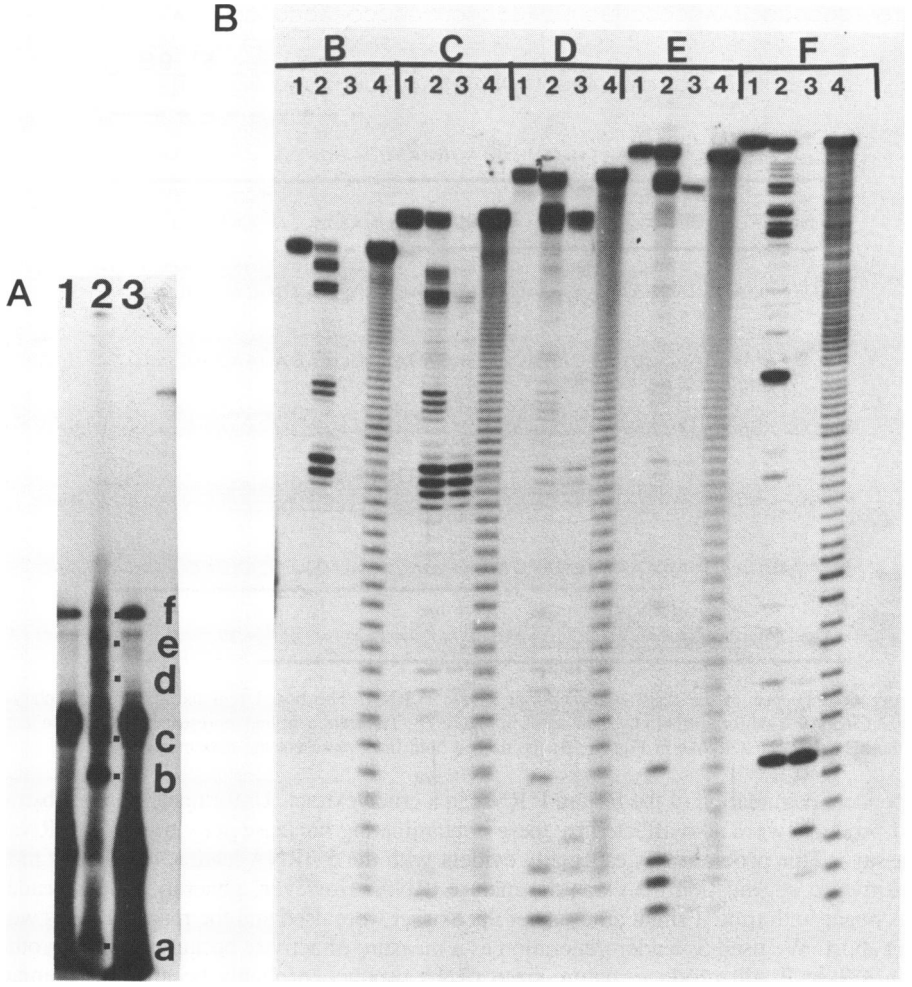

Figure 5: Characterization of RNAs present in fractions from a Cs_2SO_4 which contained RNase P activity. RNAs from the active fractions were radiolabeled at their 3′ termini and separated by electrophoresis. Lane 1: RNA prior to hybrid selection. Lane 2: RNA isolated by hybrid selection as described in Methods. Lane 3: RNA remaining in solution after hybridization. Small squares between lanes 2 and 3 mark RNAs A−F in lane 2. A−E were enriched by hybrid selection. F was not enriched but was purified and sequenced nonetheless. Figure 5B: RNase T1 digestion of hybrid selected RNA. Untreated RNAs B−F(Lanes 1) were digested partially with T1(Lanes 2), digested completely with T1(Lanes 3) or hydrolyzed with base to make a ladder(Lanes 4).

recovered from the active fractions are coded by the tRNA synthesis locus and are fragments of the the *in vivo* transcript.

Effect of RNase P RNA Fragmentation on RNase P Activity.

To determine the effect of RNase P RNA degradation on activity, we assayed the enzyme in an extract where the activity of an intact RNase P complex could be directly compared to that of an RNase P with degraded RNA. This was possible because there is a time-

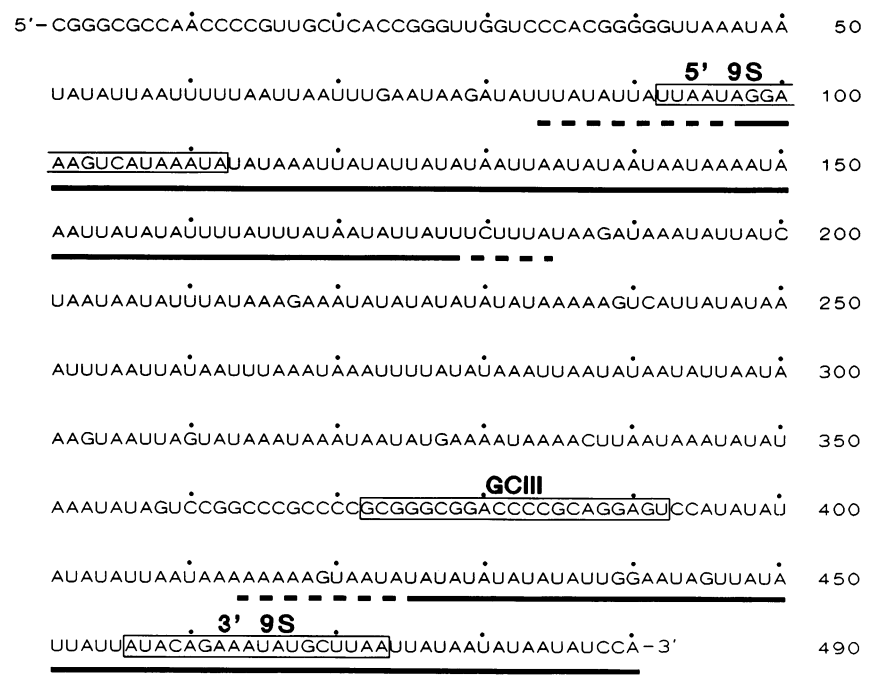

Figure 6: Sequence of the yeast mitochondrial RNase P RNA. The boxed regions are complimentary to oligonucleotides used for northern hybridizations in Figure 7B. The solid bold line represents the minimum length of the hybrid selected RNAs in Figure 5B; the dashed bold lines represent the maximum length.

dependent degradation of the RNase P RNA in a crude extract. Unfortunately, the substrate and products were also affected by these contaminating nuclease activities during RNase P assays. This problem was especially evident with the 5′ tRNA leader, which was more sensitive to degradation than was the mature tRNA. However, generation of 5′ leaders was linear with time if small amounts of the extract were used and the reaction times were kept short. We used 5′ leader generation as a measure of activity because nucleases other than RNase P will produce mature-sized tRNA product, probably because the compact nature of the tRNA provides some protection from degradation.

Figure 7 shows the effect of RNA fragmentation on RNase P activity in the extracts. Frozen mitochondria were lysed and the extract assayed for RNase P activity at 20°C. Samples were taken at 0, 30, 60, and 120 minutes after incubation. Nucleic acids were isolated, and RNA was subjected to northern analysis. During the 120 minutes of incubation in the crude extract, at most 30% of RNase P activity was lost (Figure 7A). When the RNA isolated at each time point was separated by electrophoresis and hybridized to oligonucleotides complementary to various regions of the tRNA synthesis locus, two RNA fragments were detected (Figure 7B). One contained sequences from the 5′ end of the RNA and the other from the 3′ end. This indicated that most of the activity can be maintained in the absence of full length RNA. An oligonucleotide directed to a central region of the RNase P RNA did not hybridize to any RNA at 120 minutes. This demonstrates that the sequences detected by this probe are not required to support activity *in vitro*.

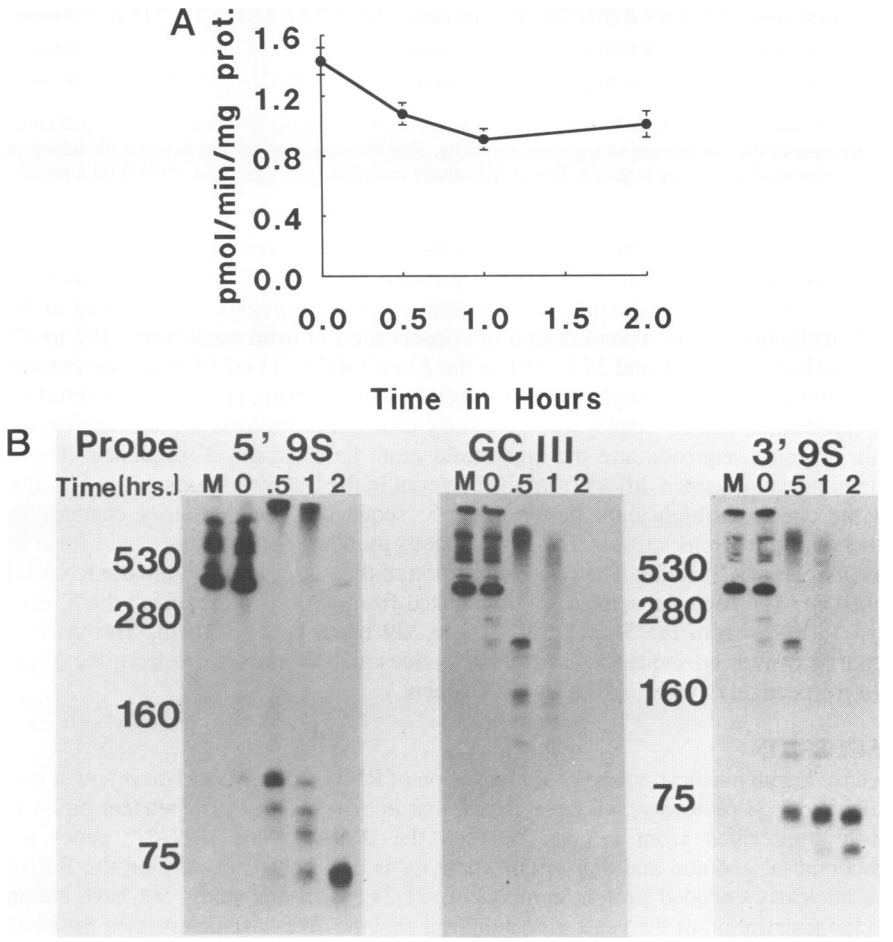

Figure 7: Enzyme activity and length of the RNase P RNA after incubation in a crude extract. Frozen mitochondria were lysed as in 'Methods' except at a concentration of 2.5 mg protein/ml. At the indicated times, an aliquot was removed and (A) 6.3 ng protein was assayed under standard conditions in 10 nM pMet and a total volume of 60 μl. At one minute intervals, 10 μl of the assay mixture was removed and analyzed as described to determine activity. RNA was isolated from the extract at the same times and analyzed by northern hybridization to the 5' oligonucleotide probe, oligonucleotide probe to the GCIII cluster and 3' oligonucleotide probe. Lane 'M' is RNA isolated from frozen mitochondria. See Figure 6 for location of regions complementary to the oligonucleotide probes.

Alignment of the Mitochondrial RNase P RNA with RNase P RNAs from Bacteria. Recently, a secondary structure model for the eubacterial RNase P RNA was derived from a phylogenetic comparison of 7 RNAs from bacteria (11). To identify regions of the mitochondrial RNase P RNA that might be important to RNase P function, we have tried to align their sequences. Except for two regions of striking primary sequence homology (Figure 8), we were unable to find similarity in more than 8 of 11 positions after scanning

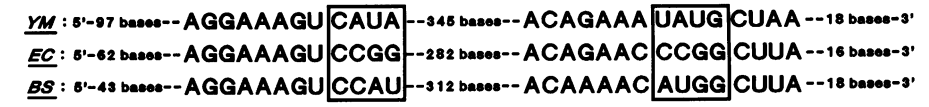

Figure 8: Comparison of RNase P RNAs from *S. cerevisiae* mitochondria (24) and this work) and eubacteria (11). Because of the low amount of sequence similarity, only the conserved regions between the mitochondrial and the eubacterial RNAs are included. Boxed regions are complimentary bases that could form a psuedo-knot structure.

overlapping windows (PC Gene Software). One of the conserved regions we have identified extends from position 97 to 108 in the mitochondrial RNase P RNA and positions 62 to 73 in *E. coli*. In the 7 bacterial RNAs examined (11) this region is conserved in 10 out of 12 nucleotides. The second region of conservation is from nucleotides 457 to 471 in the mitochondrial RNA and 347−361 in the *E. coli* RNA; 11 of 15 bases are conserved. These two regions are thought to form a pseudo-knot structure (11) in the bacterial RNA. The nucleotides which display compensatory changes in bacteria are the last two bases of the 5′-most sequence and the eighth and ninth bases in the 3′ sequence. The yeast mitochondrial sequence differs from the bacterial in the last three bases of the 5′ sequence and the eighth through tenth bases of the 3′ sequence. Compensatory changes in the mitochondrial regions suggest that an analogous pseudo-knot structure could form in the yeast mitochondrial RNA. The relative position of the two regions within the RNA is also conserved. The bacterial sequences are situated from 43 to 60 bases from the 5′ end and 16 to 18 bases from the 3′ end with 265 to 329 bases between them. The yeast mitochondrial conserved sequences are 96 nucleotides and 18 nucleotides from the 5′ and 3′ ends, respectively, with 347 bases in between.

DISCUSSION

We have begun purification and characterization of RNase P from *Saccharomyces cerevisiae* mitochondria. Previously, we have shown that *in vivo* activity is dependent on an RNA that is transcribed from a gene between the $tRNA_f^{Met}$ and $tRNA^{Pro}$ genes in the mitochondrial genome and that *in vitro* activity is dependent not only on the RNA, but on a nuclearly encoded protein component (21,24,3). In this study, we have continued the characterization of the yeast mitochondrial enzyme. We have determined the cleavage specificity and optimal reaction conditions for the mitochondrial RNase P. In addition, we have determined the size of the RNA *in vivo*, and shown that it need not be intact for activity *in vitro*.

We originally proposed that the tRNA synthesis locus coded for the RNA component of the mitochondrial RNase P based on genetic evidence. Mature tRNAs are only present in strains containing the tRNA synthesis locus (21), and only extracts from such strains contain the mitochondrial RNase P activity (3). There are other explanations for these results. For example, the locus could encode an RNA necessary for the synthesis of the RNase P, not required directly for its activity. Copurification of tRNA synthesis locus transcripts with RNase P activity through two chromatography steps and a Cs_2SO_4 gradient, plus the sequence conservation between the bacterial RNase P RNAs and this mitochondrial RNA provide compelling support for the veracity of our original hypothesis.

The optimal ionic conditions for mitochondrial RNase P differ from those optimal for *B. subtilis* RNase P (10). The latter enzyme prefers a higher ionic strength, 60−90 mM $MgCl_2$ and 100−200 mM NH_4Cl compared to 10mM $MgCl_2$ and 75mM monovalent

cation for the mitochondrial enzyme. Like the mitochondrial enzyme, the *B. subtilis* enzyme has a preference for $MgCl_2$, but in contrast to the mitochondrial enzyme, bulky ions such as NH_4^+, K^+, and Cs^+ support the reaction 2–3 fold better than Li^+ or Na^+. If, as proposed for the *B. subtilis* enzyme, salt aids in electrostatic shielding of phosphates between the substrate and the RNA, then the shielding conferred by the larger ions is either not a factor for the mitochondrial enzyme, or it is mitigated by some disadvantage these larger ions might present.

The size of the mitochondrial RNase P protein component may be a factor in its lower ionic strength preference. Although we have no direct measure of the size of the protein component(s) of this enzyme, a buoyant density of 1.28 gm/cm^3 in Cs_2SO_4 indicates that the complex is 90% protein (27). Thus, the protein component would be predicted to have a molecular weight of 380 to 500 kd if the RNA associated with the active enzyme is composed of 70 nucleotide fragments from both the 5′ and 3′ ends of the original RNA. If only one of the fragments was required for activity then the protein would still be predicted to have a molecular weight of 208 Kd. There are other factors that can affect buoyant density, such as lipid binding or RNA base composition. Regardless, it appears that this enzyme is much more proteinaceous than its *E. coli* or *B. subtilis* counterparts (28,29,30). The human HeLa RNase P RNA also has a buoyant density of 1.28 g/cm^3 indicating a larger proportion of protein as well. Perhaps the larger protein component in these eukaryotic enzymes provides better ion shielding than the bacterial protein and thus eliminates the need for high ionic strengths in the assay.

The eubacterial RNAs are well known for their ability to catalyze the cleavage reaction in the absence of protein (8,10). Experiments in which ionic strength was varied over a wide range with mitochondrial RNase P RNA made *in vitro* (Harris, Wise & Hollingsworth, unpublished results) have revealed no evidence of RNA alone catalysis by the yeast mitochondrial enzyme. This does not imply that the RNA is not the catalytic subunit in this RNase P, although we cannot rule out the possibility. The protein component may participate directly in catalysis, or play a non-catalytic but essential role such as maintaining the A/U rich RNA in the correct conformation.

We have determined the size of tRNA synthesis locus transcripts obtained by either direct phenol extraction of mitochondria or phenol extraction of active enzyme fractions after several purification steps. The size of the yeast mitochondrial RNase P RNA predicted from S1 mapping of RNA from crude extracts is 490 nucleotides long. RNAs similar to this in size must be responsible for RNase P activity *in vivo*. This size is longer than the 450 bases reported when the 5′ end was predicted based on primer extension of a DNA restriction fragment complementary to the RNA (24). The primer extension results are reproducible, as they were again obtained during the course of these studies, but presumably premature termination of elongation before the GC cluster led us to predict a false 5′ terminus downstream of the three ends determined by S1 nuclease protection. Since none of these three ends remains in the RNAs isolated from active fractions we do not know if RNA containing only one or all of these ends is actually part of the enzyme *in vivo*. S1 mapping also reveals several 3′ ends in tRNA synthesis locus RNAs from crude extracts. The fragmented RNAs found in partially purified extracts (Figure 6) only have the 3′ end that would give rise to the longest RNA. Whether the other ends are found on RNAs that are part of the enzyme *in vivo* or whether they arise from some artifact of the mapping technique is not certain.

The degradation of the RNA during purification shows that intact RNA is not required

for *in vitro* activity. Recovery of fragments from both the 5′ end and 3′ end that contain sequences conserved in the evolutionary separation of eubacteria and yeast mitochondria suggest the functional importance of these two regions to the assembly and/or activity of RNase P. Protection from endogenous nuclease digestion suggests these regions are likely to be binding sites for the protein component. However, despite its conservation, the 3′ homologous sequence is not absolutely required for *in vitro* activity in *E. coli*. Studies have shown that this RNase P RNA with large deletions in the 3′ region can still function, although with a much lower specific activity (31). We can not tell from our data what fragments are necessary to support *in vitro* activity.

If only a small portion of the RNA is required for activity, what is the purpose of the remainder? One function may be to allow correct folding of the RNA. Another may be to serve as a scaffold for the large protein component during assembly of the enzyme. Portions of the enzyme may have other undetected functions. There is precedent for multiple functions both with *E. coli* RNase P and with other fungal mitochondrial RNA processing enzymes. Yeast mitochondrial leucyl-tRNA synthetase and Neurospora mitochondrial tyrosyl-tRNA synthetase are both required for amino-acylation and mitochondrial mRNA splicing (32,33). *E. coli* RNase P has recently been found to possess a 3′ endonuclease activity for a precursor of a $tRNA^{Leu}$ amber suppressor (34).

The regions conserved between yeast mitochondrial and the eubacterial RNAs lie within the sequences nominally demonstrated in eubacteria to be involved in a pseudo-knot structure (11). Even though the differences between the yeast and bacterial regions can be interpreted as compensatory and would seem to preserve this interaction, there is not enough evidence to propose a pseudo-knot in the mitochondrial RNAs; eubacteria and fungal mitochondria are too evolutionarily distant. A phylogenetic study between the *S. cerevisiae* RNase P RNA and other fungal mitochondrial RNase P RNAs is in progress.

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