Yeast Mitochondrial RNase P

SEQUENCE OF THE RPM2 GENE AND DEMONSTRATION THAT ITS PRODUCT IS A PROTEIN SUBUNIT OF THE ENZYME*

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We report here the sequence of the RPM2 gene which codes for the 105-kDa protein previously purified from the mitochondria of Saccharomyces cerevisiae and shown by genetic techniques to be required for mitochondrial RNase P activity. The sequence predicts a primary translation product of 1202 residues with a molecular mass of 139 kDa and no obvious sequence similarity to any known protein in the data bases. There are 122 amino-terminal amino acids predicted by the gene that are not found in the purified protein, some of which may play a role in mitochondrial targeting of the protein. Antibodies raised against a trpE-105-kDa fusion protein recognize a 105-kDa protein in wild-type cells but not in cells carrying a disruption of the RPM2 gene. Immune, but not preimmune serum, immunoprecipitates the RNase P RNA and the mitochondrial RNase P activity. Thus, the 105-kDa protein forms a complex with RNase P RNA and is required for RNase P activity as predicted for a bona fide subunit of the enzyme.

RNase P is the enzyme responsible for removing the 5' leader sequence from precursor tRNAs to generate mature tRNAs. In bacteria, the RNase P holoenzyme is composed of both protein and RNA (1) but in vitro the bacterial RNAs can carry out the reaction alone, in the absence of protein (2). This observation has lead to extreme interest in the structure and mechanism of action of the RNA itself and a structural model of bacterial RNase P RNA has been derived from phylogenetic comparisons (3-5). The molecular mechanism by which bacterial RNase P RNA cleaves tRNA precursors remains unknown. Nuclear eukaryotic RNase P enzymes are less well understood but seem to require both protein and RNA (6-10). In no case have the eukaryotic RNA subunits been shown to be catalytic on their own. Mitochondrial RNase P activity in yeast is also dependent on a RNA subunit which is coded by mitochondrial DNA, and protein coded by nuclear DNA (11, 12).

In vivo in bacteria, both RNA and protein are required (1, 13). The primary structure of the protein subunits from Escherichia coli (14), Bacillus subtilis (15), Proteus mirabilis (16), Micrococcus luteus (17), Streptomyces bikiniosis (18), and Pseudomonas putida(1 have been determined through the cloning and sequencing of their genes. All are around 14 kDa in size and have an abundance of charged amino acids and some similarities in sequence. The role of the protein is unclear. The E. coli RNase P holoenzyme is more efficient than the RNA alone in vitro. One proposal for the role of the protein component is to provide ionic shielding between the negatively charged precursor tRNA and the RNase P RNA (20). Kinetic studies have shown that the $K_m$ for the RNA alone and the holoenzyme reactions are the same, but the catalytic efficiency ($k_{cat}$) of the holoenzyme is 20 times greater. Thus, the protein may not only contribute to substrate binding, but also may enhance the off-rate of mature tRNA, the product of the reaction. The protein may also be important for maintaining or changing RNA conformation during catalysis (21).

Very little is known about the subunit structure of eukaryotic RNase Ps and as none of the eukaryotic RNAs have been shown to be catalytic in the absence of protein, it is even possible that some functions of the bacterial RNAs may have been taken over by the protein subunit in eukaryotes. Buoyant densities of the human nuclear enzyme (8) and the yeast mitochondrial enzyme (22) predict they are more proteinaceous than most of their bacterial counterparts and the proteins may be larger. A 40-kDa protein associated with human RNase P RNA and activity has been identified by immunological techniques (23), but it is not known if it is actually required for activity.

Our interest in mitochondrial biogenesis has lead us to an examination of mitochondrial RNase P in yeast. In previous work we have reported that the RNA subunits of this enzyme in a variety of yeasts are unusually A-U-rich (95% A+U) and that they vary in size from about 150 to 500 nucleotides depending on the species of origin (11, 24-26). We have identified a 105-kDa protein from yeast mitochondria which fractionates with RNase P activity (27) and showed that it is required for RNase P activity in vivo by constructing a strain carrying a disruption of the RPM2 gene which codes for the protein. We present here the DNA sequence of the RPM2 gene and the deduced primary structure of the 105-kDa protein. We also provide evidence that the protein forms a complex with the RNase P RNA as expected for a bona fide subunit of the enzyme.

EXPERIMENTAL PROCEDURES

Subcloning and Sequencing—Restriction and modification enzymes were purchased from New England Biolabs. They were used as recommended by the supplier. A 4.5-kilobase BglII fragment from the original complementing plasmid (27) was subcloned into BamHI digested pBluescript (Stratagene), and a 3.4-kilobase EcoRI fragment was subcloned into EcoRI-digested pBluescript. These two fragments overlapped and contained the entire open reading frame plus flanking DNA.

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** The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) L08269.

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1. N. Ogasawara and H. Yoshikawa, unpublished results.
sequences. The two recombinant plasmids were used to obtain a series of overlapping clones by ExoIII/mung bean nuclease deletion according to the manufacturer's protocol (Stratagene). Sequencing was performed by the dye-deoxy chain termination method (28) using both M13-40 universal and reverse primers (United States Biochemical Corporation). Primers were synthesized by Marlene Steffen, Department of Biochemistry Core Facility, University of Louisville. Both strands of the DNA were sequenced. Genbank (release 73) and EMBL (release 32) data banks were searched for similar sequences with the FASTA program (29). Prosite, release 9.1, August 1992 and the transcription consensus motifs of Verdier (30) were also compared to the RPM2 gene sequence. We also used the bacterial RNase P sequences to derive and validate an RNase P protein profile (31).

Production of Fusion Protein—A 944-bp $f$glIII-HindIII fragment coding for amino acids 145-460 of the RNase P protein was cloned into the pATH2 vector (35) which had been cut with BamHI and HindIII. The construct was transformed into E. coli (JM101). The fusion protein was induced by depletion of tryptophan and purified as described previously (33).

Immunization and IgG Purification—Preimmune serum was harvested prior to injection of the antigen. The first immunization was with 137 μg of fusion protein in 1 ml of RIBI adjuvant (RIBI ImmunoChem Research Inc.) prepared according to the manufacturer's instructions. At monthly intervals, the rabbit was injected subcutaneously with 100 μg of fusion protein in 500 μl of incomplete Freund's adjuvant (Sigma). 10-15 ml of blood were collected 7-8 days after the last immunization. The polyclonal IgG was purified from serum by passage through a Protein G-Sepharose 4 column (MABTrap G, Pharmacia LKB Biotechnology Inc.) twice according to manufacturer's instructions. This purification was necessary to remove ribonucleases present in the serum.

Western Blotting—Mitochondria were isolated from the wild-type W303-1A and disrupted strain W303-1A RPM2::LEU2-6 (27). 100 μg of protein was loaded on 10% polyacrylamide SDS gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes (34), and immunoreactive proteins were detected by enhanced chemiluminescence (ECL) according to the manufacturer's protocol (Amersham Corp.).

Immunoprecipitation of RNase P RNA—300 μg of preimmune or immune IgG was mixed with 50 μl of Protein A-agarose (Pierce Chemical Co.) and rotated for 2 h at 4 °C. The agarose particles, with bound IgG, were washed with 1 ml of 50 mM NaPO$_4$, pH 7.0, three times, then suspended in 50 μl of DAE fraction (27) which contains RNase P activity and incubated with rotation for 2 h at 4 °C. After a 1-min centrifugation, the supernantant was extracted with an equal volume of phenol/chloroform (1:1), and the pellet was washed three times with 50 mM NaPO$_4$, pH 7.0, suspended in the same buffer and extracted with phenol/chloroform (1:1). The aqueous layers were recovered, and the nucleic acids were precipitated with cold ethanol, suspended, and separated by electrophoresis on a 10% polyacrylamide-urea gel. Northern blot analysis was performed as described (35) using a mitochondrial RNase P RNA-specific oligonucleotide probe.

Immunoprecipitation of RNase P Activity—150 μg of preimmune IgG and immune IgG were incubated with 50 μl of Protein A-agarose at 4 °C for 2 h. The IgG-Protein A-agarose complex was then washed with 50 mM NaPO$_4$, pH 7.0, three times. 10 μl of the beads were incubated with 10 μl of DEAE fraction at 4 °C for 2 h. After centrifugation, the supernatants and pellets were assayed for RNase P activity as described (35).

RESULTS

In previous studies of yeast mitochondrial RNase P, we purified the enzyme to near homogeneity and identified a 105-kDa protein which cofractionated with activity. Using a oligonucleotide designed from the NH$_2$-terminal sequence of this protein, we isolated the RPM2 gene by screening a yeast genomic DNA library and by gene disruption experiments demonstrated that its gene product is required for RNase P activity (27). To determine the sequence of the gene, 31 subclones and five oligonucleotide primers were used (Fig. 1). The nucleotide and deduced amino acid sequence of the RPM2 gene as well as 200-bp upstream from the translational start site and 181-bp downstream from the stop codon were determined and are shown in Fig. 2.

Examination of the flanking sequences reveals two putative TATA sequences (Fig. 2) but no obvious consensus sequences that might dictate the start site of transcription. The last 221 nucleotides of the sequence shown in Fig. 2 matches the beginning of a sequence reported by Emori et al. (36) to code for the Y7 subunit of the yeast proteasome. The start site of translation of the Y7 open reading frame is only 380 nucleotides from the termination site of translation of RPM2. Although the 3’-flanking sequence is very AT-rich, there are no obvious sequences which match sequences known to function in transcription termination in yeast (37, 38). Some of the 33 TAA repeats of the 3’ region may be sufficient for efficient transcription termination of this gene. The putative translation start site context is gAAAUAUGCU and thus differs in two positions from the consensus (A/Y)(A/U)AAUGUCU derived by Cigan and Donahue (39). Codon usage bias, calculated as described by Sharp and Cowe (40) indicates that the RPM2 gene falls into the class of yeast genes which are expressed at a low level.

The single open reading frame of 3606-bp predicts a protein of 1202 amino acids with a calculated molecular mass of 139,366 daltons. The amino-terminal amino acids obtained by direct protein sequencing (27) fall between amino acids 123-143 of the deduced protein sequence. The 122-amino-acid NH$_2$-terminal extension may represent the signal sequence for targeting the protein to the mitochondria. The amino-terminal portion shares sequence features common for mitochondrial targeting signals such as the absence of acidic residues, presence of basic residues, and hydroxylated amino acids (41). The amino acid sequence was used to search the data bank for similar sequences, but no such sequence was found. The amino acid sequence was also compared with the known prokaryotic RNase P proteins but no matches which were statistically significant were found.

Previously we showed that the RPM2 gene is required for mitochondrial RNase P activity since mitochondrial tRNA precursors accumulate in strains carrying a disrupted RPM2 gene (27). We had also demonstrated that the RPM2 gene product and the RNase P RNA, a product of the RPM1 gene, cofractionate during purification but a direct association between the two had not been established. To determine if the RPM2 gene product forms a complex with the RPM1 gene product, we initiated an immunoprecipitation approach. Part of the RPM2 gene coding for amino acids 145-460 was cloned into an expression vector (pATH) to produce antigen. The resulting antibody was used first in Western blot analysis (Fig. 3). Preimmune serum does not recognize a 105-kDa protein in wild-type cells but the immune serum does. Only the 105-kDa protein disappears from cells carrying a disrupted copy of the RPM2 gene demonstrating that the only difference detected between the yeast proteins recognized by preimmune
Yeast Mitochondrial RNase P Protein

FIG. 2. DNA sequence and predicted protein sequence of RP52. Numbering of the DNA sequence shown in the right margin begins with the initiating AUG codon. In the 5'-flanking region, the putative "TATA" boxes are underlined.

![DNA Sequence](image)

![Predicted Protein](image)

and immune and is the presence of the 105-kDa protein.

RNase P activity was assayed in the presence of a constant amount of IgG by varying the ratio of preimmune to immune IgG. To determine if RNase P activity could be immunoprecipitated with immune IgG-protein A-agarose complex, but the activity in the supernatant of the preimmune sample remains. We were able to recover a small amount of activity in the pellet of the immune but not preimmune serum, an immunodepletion experiment begins with the initiating AUG codon. In the 5'-flanking region, the putative "TATA" boxes are underlined.

![Image](image)

![Diagram](image)
recovery has to do with difficulty in recovering activity from immunoprecipitated material rather than to some nonspecific inhibitor present in immune but not preimmune serum. Thus, mitochondrial RNase P activity is dependent on the 105-kDa protein in vitro as predicted for a subunit of the enzyme.

A second prediction of the hypothesis that the RPM2 gene is a protein subunit of mitochondrial RNase P is that the antibody to the RPM2 gene product should immunoprecipitate the RNase P RNA and the product of the RPM1 gene. To determine whether the RPM1 gene product is immunoprecipitated with the antibody, RNA was isolated from the supernatants and pellets of the immunodepletion experiment described above. Fig. 6 shows the Northern analysis of these RNAs using an RNase P RNA-specific probe. The RNase P RNA was present in the supernatant of the sample treated with preimmune IgG but in the pellet of the sample treated with immune IgG. It was quantitatively immunoprecipitated by the anti-RPM2 antibodies, demonstrating that the RNase P RNA forms a complex with the 105-kDa protein.

DISCUSSION

We report here the DNA sequence of a nuclear gene required for the activity of mitochondrial RNase P in Saccharomyces cerevisiae. The amino acid sequence has no apparent similarity to any protein in current data bases from Genbank or EMBL. The nucleotide sequence and the amino acid sequence have been deposited in the Genbank, EMBL and the DNA Data Bank of Japan. The RPM2 gene is upstream of the gene coding for the Y7 subunit of the yeast proteasome (36). The DNA sequence of the gene predicts that the protein is synthesized as a larger protein with a NH₂-terminal extension of 122 amino acids. One likely explanation for this difference is that proteins imported into the mitochondrial matrix have NH₂-terminal leader sequences which serve as targeting signals and are cleaved upon import into the organelle (42). These signal sequences share a consensus amino acid composition rich in both positively charged and hydroxylated residues. A 122-amino-acid leader sequence is unusually long for a targeting sequence and only the NH₂-terminal portion of it is highly enriched in both positively charged and hydroxylated amino acids. An alternate possibility is that part of the extension is removed upon import into the mitochondria and the rest removed during assembly of the enzyme. Further, some proteolysis may have occurred during purifi-
Yeast Mitochondrial RNase P Protein

19795

cation resulting in a shorter protein than is actually present in vivo. Finally, the presence of a putative branch point (nucleotides 243-250) and several 3' splice site consensus sequences raises the possibility that splicing may play a role in RPM2 expression. Further work will be necessary to clarify these issues.

Is the 105-kDa protein a subunit of yeast mitochondrial RNase P? Polyclonal antiserum raised against a portion of the RPM2 protein did not appear to inhibit RNase P activity but inhibition of activity might not be expected as it is likely that the RNA subunit carries out the actual catalysis. On the other hand, if binding of the antibody had changed the conformation of the protein sufficiently to affect the structure of the RNA, inhibition could have resulted. Despite this lack of inhibition, the antibody quantitatively precipitates the RNase P RNA demonstrating a physical connection between the 105-kDa protein and the mitochondrial coded RNase P RNA as would be expected if the two are subunits of the same enzyme. The most straightforward interpretation of this data, in conjunction with our biochemical purification of the enzyme and the gene disruption experiments demonstrating that the 105-kDa protein is required for RNase P activity (27), is that the S. cerevisiae mitochondrial RNase P consists of a mitochondrial-coded RNA and a nuclear-coded 105-kDa protein. Nonetheless, the question is not yet completely settled.

For example, there is no data to eliminate the possibility that a nuclear coded RNA plays a role in yeast mitochondrial RNA P. In addition, as the preparation of Morales et al. (27) was not completely homogeneous, it remains a formal possibility that some minor protein in our purified preparations could also be important to activity. Using the cloned RPM1 and RPM2 genes, it should be possible to produce both products in amounts sufficient for reconstitution experiments. Recovery of activity from two independently produced subunits would provide direct proof that the enzyme in mitochondria, like in prokaryotes, consists of one RNA and one protein.

Although the RNAs from prokaryotes and from yeast mitochondria differ greatly in size and in primary sequence, they do share two short regions of sequence similarity which form part of a pseudoknot thought to be necessary for arranging the catalytic core of the RNA (24-26). A comparison of the protein we isolated with the proteins of bacterial RNase P enzymes demonstrates great differences in size and in primary sequence, and we were unable to recognize even a short region with significant sequence similarity between them. This is in contrast to the obvious sequence similarity between other bacterial and yeast mitochondrial enzymes involved in protein synthesis such as aminoseryl-tRNA synthetases (42), translation factors (43), and release factor (44). Unlike these enzymes, the basic catalytic mechanism of RNase P resides in the RNA and thus there may be less pressure to maintain similarity in its associated protein. Like the RNase P protein, most yeast mitochondrial ribosomal proteins sequenced to date bear no similarity to the eubacterial ribosomal proteins (45).

The RPM2 gene product is clearly the largest RNase P-associated protein identified to date. The lack of sequence similarity between it and the prokaryotic proteins raises the issue as to whether it is functionally analogous to its bacterial counterparts. If it is, why might it be so much bigger? One suggested role of prokaryotic RNase P proteins is to stabilize the active conformation of the RNA in vivo. It may be that the larger size and A-U-rich character of the mitochondrial RNA in S. cerevisiae requires a larger protein to fulfill this role. We should point out that we do not know the size of the analogous protein in other yeasts and as mitochondrial RNase P RNAs vary so much in size it is possible the proteins do as well. Unlike the prokaryotic RNAs, the eukaryotic RNAs do not function without their protein subunits in vitro. Whether this is because they cannot take on a catalytically active form in the absence of the protein or whether the protein plays some direct role in enzyme activity is not known. If the former is correct, a larger protein may be required, not only to stabilize a catalytic core structure, but to play an active role in its formation. Alternatively, a larger size might be required if the eukaryotic proteins play some direct role in catalysis.

Another possible explanation for the large size of this protein may be that only part of the RPM2 gene product corresponds in function to the smaller prokaryotic proteins and that there are additional domains which contribute to the larger size. Such domains might dictate the organization of the enzyme into a tRNA processing complex and/or be required to interact with proteins during assembly of the enzyme. Some yeast mitochondrial ribosomal protein homologs of E. coli ribosomal proteins have a functionally analogous core with additional domains of unknown function (45-50).

Finally, it is possible that the large size of the RPM2 protein is necessary to accommodate a second function that is not yet known. There are interesting precedents for multifunctional mitochondrial RNA processing enzymes. The mitochondrial tyrosyl-tRNA synthetases from Neurospora crassa (51) and Podospora anserina (52), and the leucyl-tRNA synthetase of yeast (53, 54) are required for splicing of group I introns in mitochondrial large tRNA and mRNAs. The P. anserina and N. crassa proteins share three regions of amino acid similarity, including the NH2-terminal domain, not found in other tyrosyl-tRNA synthetases (55), which was shown previously to be necessary for splicing activity but not catalysis.

There are other examples of gene products involved in splicing which also have a second known mitochondrial function. The second function of PET54 is in translation of mitochondrial COX3 mRNA (56). The second function of NAM1 (57) as a transcription factor (58) is controversial. Finally, the MSS5 (59, 60), and the MRS2 (19) gene products have a second but as yet unknown function in addition to their role in splicing introns from mitochondrial mRNA precursors.

Regardless of the final explanation for the lack of apparent similarity between the yeast mitochondrial protein we have isolated and bacterial RNase P protein subunits, it does raise interesting questions about the evolutionary origins of these proteins, and of the relation of structure to function in RNase P enzymes. Characterization of the gene for one eukaryotic RNase P protein subunit is a step toward understanding such structure-function relationships as well as providing a foundation for further studies on the biosynthesis of the enzyme and its role in mitochondrial biogenesis.

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Yeast Mitochondrial RNase P Protein

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