An RNase P RNA subunit mutation affects ribosomal RNA processing

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ABSTRACT

RNase P is a ribonucleoprotein endoribonuclease responsible for the 5′ maturation of precursor tRNAs in all organisms. While analyzing mutations in conserved positions of the yeast nuclear RNase P RNA subunit, significant accumulation of an aberrant RNA of ~193 nucleotides was observed. This abundant RNA was identified as a 3′ extended form of the 5.8S rRNA. This strain also displays a slightly elevated level of other rRNA processing intermediates with 5′-ends at processing site A2 in the internal transcribed spacer 1 (ITS1) region of the rRNA primary transcript. To test whether pre-rRNA in the region of ITS1/5.8S/ITS2 is a substrate for RNase P *in vitro*, nuclear RNase P was partially purified to remove contaminating nucleases. Cleavage assays were performed using an rRNA substrate transcribed *in vitro* which includes the 5.8S region and its surrounding processing sites in ITS1 and ITS2. Discrete cleavages of this rRNA substrate were coincident with the peak fractions of nuclear RNase P, but not with fractions corresponding to mitochondrial RNase P or ribonuclease MRP RNA. The cleavage activity is sensitive to treatment with micrococcal nuclease, also consistent with an activity attributable to RNase P. The strong RNase P cleavage sites were mapped and their possible relationships to steps in the rRNA processing pathway are considered. These observations suggest an intimate relationship between the processes of tRNA and rRNA maturation in the eukaryotic nucleus.

INTRODUCTION

RNase P is one of a growing list of ribonucleoproteins (RNPs) found to be involved in a wide variety of biosynthetic pathways in eukaryotes. Essential RNPs and their functions include the small nuclear RNPs (snRNPs) in mRNA splicing (1,2), ribosomes in translation (3–5), telomerase in chromosome end formation (6,7), small nucleolar RNPs (snoRNPs) in rRNA processing (8,9), and RNase P in precursor tRNA (pre-tRNA) 5′-end maturation (10). Of these, only RNase P is considered a ribozyme, following demonstration that the *E.coli* RNA subunit is catalytic *in vitro* in the absence of protein (11). RNase P from Bacteria, Archaea and Eucarya contains a similar sized RNA (~300–400 nt), but, unlike the bacterial enzymes, the RNA subunits from Archaea and Eucarya are not active without protein (12,13). The RNA subunits from different phyla show only small patches of sequence conservation, but retain similar structural features (14,15). Only two protein subunits have been purified to homogeneity from the eukaryotes, the *S.cerevisiae* mitochondrial (105 kDa) (16) and the *S.pombe* nuclear (100 kDa) (17) proteins. The eukaryotic proteins are of a significantly greater molecular weight compared to the 14 kDa *E.coli* C5 protein (18). Although the 369 nt RNase P RNA from *S.cerevisiae* nuclei has been extensively characterized, the protein content of the holoenzyme remains to be determined (15,19–21).

E.coli RNase P is able to recognize a number of different RNA substrates in addition to pre-tRNAs. Examination of temperature sensitive mutants revealed accumulation of precursor RNAs including 4.5S RNA (26,27), 10Sa (28,29) and the polycistronic mRNA from the histidine operon (30). In all instances the substrate resembles a pre-tRNA and is consistent with the proposed minimal structure requirement for an RNase P substrate (31). A recent study (32) suggests that bacterial RNase P holoenzyme has a greater substrate range than the RNA subunit alone, implicating the subunit in recognizing substrates other than pre-tRNAs.

The eukaryotic RNase P that processes cytoplasmic pre-tRNAs is thought to function in the nucleus and 5′ and 3′ processing precede splicing (22,23). Since yeast mutants that fail to splice tRNAs accumulate end-matured tRNAs in the nucleus (24,25), RNase P cleavage (an earlier event) is probably occurring in the nucleus.

Eukaryotic nuclear RNase P is closely related to ribonuclease MRP ribonucleoprotein, an endonuclease known to be involved in rRNA processing (33–37). In humans, RNase P appears to share Th antigen association with RNase MRP (38–40). A 100.5 kDa polypeptide has been shown to associate with both enzymes in yeast and may be analogous to the Th antigen. A temperature sensitive mutation in this yeast protein, Pop1, results in defects in rRNA processing and tRNA processing, as might be anticipated for a protein playing a functional role in both enzymes (41). In addition to the common protein association, the RNA components of RNase P and RNase MRP possess some sequence and structural similarity (14,42,43), leading to suggestion that the two
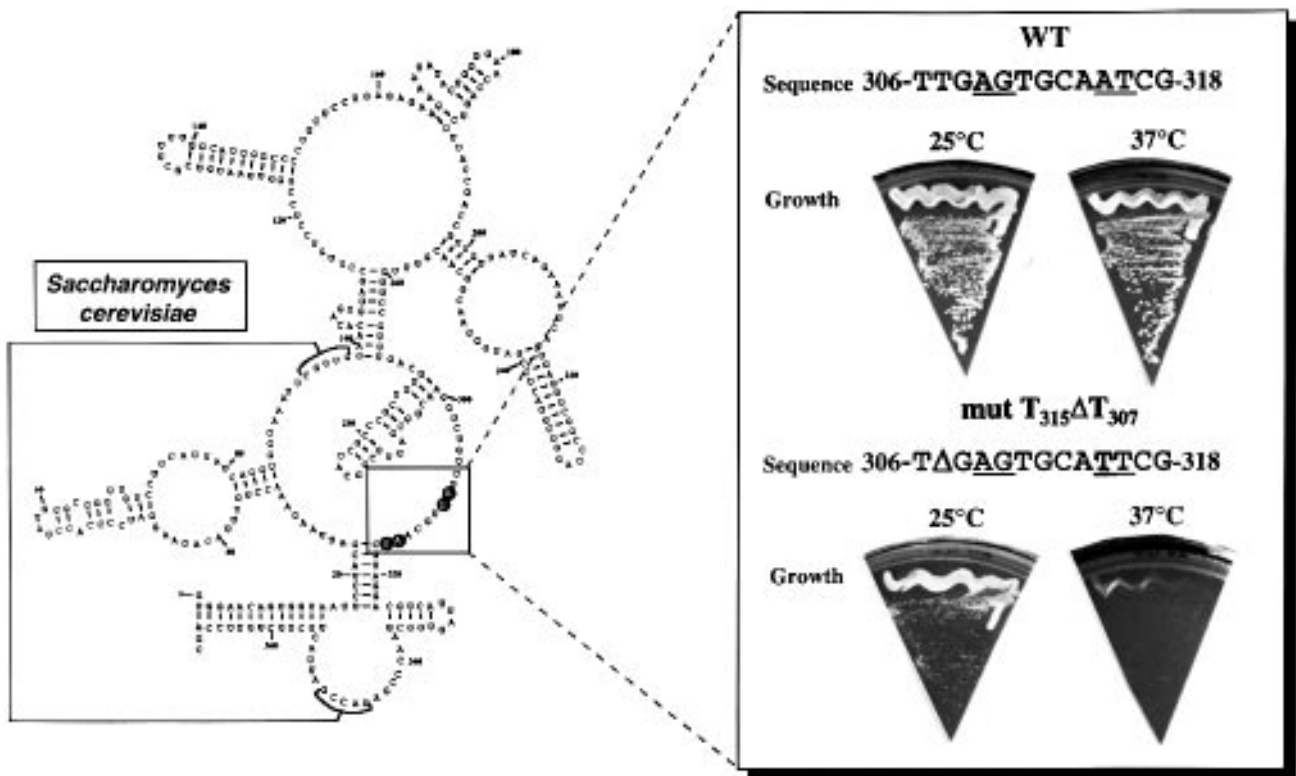
Figure 1. Growth phenotypes of an *RPR1* mutant. RNase P RNA secondary structure shown was proposed on the basis of phylogenetic analysis and solution structure probing (15). Blackened circles with nucleotides indicated in white represent strictly conserved positions that were randomized for generation of a gene library from which the mutant originated. The growth phenotype of this *RPR1* mutant ($T_{315}\Delta T_{307}$) is shown at 25°C and 37°C relative to an otherwise isogenic strain carrying a wild-type copy of *RPR1*.

eukaryotic enzymes are derived from a common progenitor. These observations, in addition to *in situ* localization of at least some RNase P RNA to the perinucleolar compartment with RNase MRP (44,45), has led to further speculation that there might be undiscovered similarities in the substrates recognized by the two enzymes (43,46,47).

In the process of characterizing mutants with defects in the *S.cerevisiae* nuclear RNase P RNA subunit, the appearance of an anomalous RNA prompted us to investigate whether RNase P was involved in the maturation of rRNA, as well as tRNA.

MATERIALS AND METHODS

Yeast strains and growth conditions

For construction of the temperature sensitive mutant used in these studies, *S.cerevisiae* strain JLY-1 (19) containing a *rpr1*::HIS3 disruption in strain W3031A (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) and plasmid YCp50-*RPR1* with wild-type copy of the *RPR1* gene were used. JLY-1 was transformed with plasmid pRS315 (*LEU* marker) containing either the *RPR1* mature coding sequence or the mutated *RPR1* coding sequence inserted between wild-type *RPR1* 5′ and 3′ flanking gene sequences; the wild-type *RPR1* containing plasmid (*URA3* marker) was eliminated by growth on 5-fluorooritic acid (48). Protease-deficient strain PP1002 (*MATα ade2 leu2-3,112 pep4-3 rna3 rna82*) (gift of P. Piper & Kerstin Stråby) was used as the source of RNase P holoenzyme (20). Media were prepared as described (19). Growth of the temperature sensitive (*ts*) mutant used in these studies and its isogenic wild-type strain was maintained in selective medium lacking histidine and leucine at 25°C for isolation of RNA.

RPR1 mutation $T_{315}\Delta T_{307}$ was derived by sequence randomization at highly conserved positions in the *RPR1* RNA (Fig. 1). Detailed characterization is described elsewhere (49). Nucleotide T_{315} represents an A to T transversion and a single nucleotide deletion is found at position 306 or 307. DNA sequencing revealed no other mutations in the plasmid-born *RPR1* gene (50).

Pulse-labeling of RNA

RNA was labeled by addition of 500 μCi [^{32}P]orthophosphate to 1 ml of cells at OD$_{600}$ of 1.0 for 5 min. RNA was prepared as previously described (51). Samples were subjected to electrophoresis on an 8% denaturing acrylamide gel and exposed on a Molecular Dynamics PhosphorImager 445 SI.

Primers and probes

Oligonucleotides used for PCR, primer extension and northern hybridization are designated P1–P5. P1 and P5 were used as PCR primers in construction of the rDNA template (see RNase P reactions). P1 included a T7 promoter. Oligonucleotides P2, P3 and P4 were end-labeled with T4 polynucleotide kinase (Gibco-BRL) and [γ-^{32}P]ATP (New England Nuclear) and purified by 15% denaturing polyacrylamide gel electrophoresis. Oligonucleotide sequences are as follows:

P1, 5′-TAATACGACTCACTATAGGCCAAACGGTGAGAGA-TTTCTGTGC-3′;
P2, 5′-TCCAGTTACGAAAATTCTTGTTTT-3′;

P3, 5′-CGCATTTCGCTGCGTTCTTCATCG-3′;
P4, 5′-ACAGAATGTTTGAGAAGGAAATG-3′; and
P5, 5′-ACTTTAAGAACATTGTTCGCCT-3′.

A primer which anneals to positions +24 through +40 of the yeast alcohol dehydrogenase 1 (*ADH1*) RNA [52] was added to control for primer extension reactions. An oligonucleotide which anneals to positions +62 through +88 of the U6 snRNA was used for total RNA blot quantitation [53,54].

The *RPR1* probe was the complement of the coding region cloned into plasmid pGEM-3Z [20,55] and transcribed using [α-^{32}P]UTP. The ribonuclease MRP RNA (RNase MRP) coding region antisense probe was synthesized from the RNase MRP gene (*NME1* or *PPR2*) in pBluescript II KS$^+$ (gift of D. Tollervey).

Northern blot analysis

Total RNA was prepared from yeast carrying wild-type or T$_{315}$ΔT$_{307}$ *RPR1* genes. Two μg of each RNA was subjected to electrophoresis on 8% denaturing polyacrylamide gels and transferred to Maximum Strength Nytran (Schleicher & Schuell) using a Genie Electrophoretic Blotter (Idea Scientific). For analysis of both large and small RNAs, ~10 μg of WT and mutant RNAs was added to denaturing RNA loading buffer (95% formamide, 0.1% SDS, 5 mM EDTA, 0.1% bromophenol blue and xylene cyanol), incubated for 10 min at 65°C and separated on a 1.2% native agarose gel. A denaturing agarose gel was also run with these samples and confirmed the results obtained from northern analysis of the non-denaturing gel for the large rRNAs. For these analyses, RNA was capillary blotted to Nytran [56]. Hybridizations with P3 and P4 were done at 55°C and 50°C, respectively, for blots of denaturing polyacrylamide gels. For quantitation of total RNA loaded, the radioactive U6 probe was used in a separate hybridization at 40°C. The northern blot of the 1.2% agarose gel was probed with P4 and the ADH1 probe at 40°C. Following hybridization, membranes were exposed for PhosphorImager analysis.

For detecting specific RNAs in RNase P purification chromatography, 10 μl from Mono Q fractionation was treated with stop mix (0.25 mg/ml proteinase K, 0.5% SDS, 25 mM EDTA) extracted, and precipitated. Samples were separated on 6% denaturing acrylamide gels, electroblotted, and hybridized at 55°C to ^{32}P-labeled antisense *RPR1* or *NME1* RNA.

Primer extensions

Primer annealing and extension used SuperScript II (Gibco) at 45°C as recommended by the manufacturer. Products were separated on 8% denaturing polyacrylamide gels. Gels were dried and exposed to film or phosphor screen. Positions of primer extension stops were determined by comparison with adjacent chain termination sequencing reactions performed using identical primers on the rDNA template used for synthesis of the rRNA substrate. Primer extension of the aberrant rRNA (Fig. 4C) was done following elution of this RNA from 8% denaturing poly-acrylamide gel.

Isolation of RNase P

Extracts were prepared from *Saccharomyces cerevisiae* strain PP1002 as described [57]. RNase P activity was purified from the extracts using SP Sepharose (Pharmacia), DEAE cellulose (Whatman DE52), and FPLC Mono Q H/R 5/5 (Pharmacia). The yeast extract was ammonium sulfate precipitated and resuspended in buffer HGMDN (20 mM HEPES pH 7.9, 10% glycerol, 10 mM MgCl$_2$, 1 mM DTT, 0.5% Nonidet P-40), adjusted to 0.15 M KCl, and applied in a final volume of 300 ml (with addition of HGMDN + 0.15 M KCl) to 120 ml SP Sepharose. Extensive washing was followed with single-step elution with HGMDN + 0.4 M KC1. Approximate protein peak concentrations were assessed by Coomassie brilliant blue staining. The peak protein fractions were pooled, adjusted to 0.2 M KCl with HGMDN, and applied to a 40 ml column of DEAE cellulose. RNase P was eluted in a single step with HGMDN buffer containing 0.4 M KCl.

The pooled RNase P activity was applied to a Mono Q H/R 5/5 (1 ml) column in HGMDN + 0.275 M KCl. A 20 ml gradient of 0.0275–0.625 M KCl was used for elution, and 0.5 ml fractions were collected and tested for RNase P activity, protein content, and the presence of specific RNAs by northern analysis. In addition to the activity peaks observed with fractions 28 and 32/33 (0.47 and 0.49 M KCl) (Fig. 5), another peak of RNase P activity was found in fractions 37 and 38 (0.52–0.53 M KCl). This coincided with a peak of mitochondrial RNase P, as analyzed by northern blot hybridization to an oligonucleotide probe comple-mentary to mitochondrial RNase P RNA, 5′-GGACTCCTGCG-GGGTCCGCCCGCG-3′ [16].

RNase P reactions

RNase P activity from chromatographic separation was assayed using a mitochondrial pre-tRNA^{Asp} substrate [58], [α-^{32}P]UMP-labeled as described for the *RPR1* as probe. The substrate was purified and assayed described [59].

RNase P activity was assayed as previously described for RNase MRP [34] using a yeast pre-rRNA substrate and 0.5–1 μl of the Mono Q fractions. This pre-rRNA substrate is defined by the PCR primers P1 and P5 (see Figs 3B and 8) which were used to synthesize this region by PCR amplification from the yeast rDNA repeat from plasmid pRibH7 (contains the 2.7 kb *Hin*dIII fragment in pBR322, gift of H. Federoff & J. Warner). This template was transcribed from the T7 promoter included in primer P1 and purified. The *in vitro* transcribed pre-rRNA was 3′-end labeled by addition of [5′-^{32}P]cytidine 3′,5′-bis(phosphate) with RNA ligase [60]. Electrophoretic separations of all assay products were visualized using a PhosphorImager.

Micrococcal nuclease digestions

Sensitivity of RNase P to micrococcal nuclease digestion was tested by combining 0.5 μl of the peak fraction from FPLC Mono Q separation in a 5 μl reaction with 4 mM CaCl$_2$ and 2 U micrococcal nuclease with incubation at 37°C for 15 min. In some cases 1 μl 200 mM EGTA (ethylene glycol-bis(β-aminotheyl ether)-*N,N,N′,N′*-tetraacetic acid) was first added specifically to inhibit micrococcal nuclease before RNase P addition. Micrococcal nuclease digestion was stopped by adding 1 μl 200 mM EGTA to those reactions to which it had not been previously added. RNase P reactions were then performed using the pre-rRNA substrate.

RESULTS

Characterization of an RNase P Mutant

Our laboratory has examined the effects of simultaneous mutations at highly conserved positions in *S.cerevisiae* nuclear
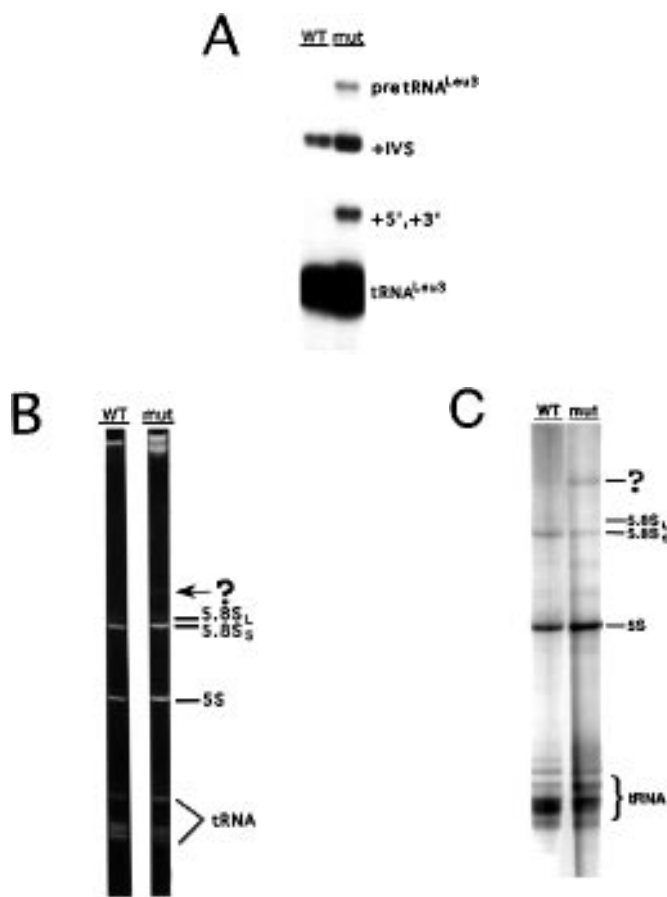
Figure 2. (**A**) Precursor accumulation of tRNA from the *RPR1*-mutated strain. Precursors of the tRNA^{Leu3} isoacceptor were identified in comparison to previous analysis by northern blot hybridization (19). RNAs are detected of the appropriate size for the primary transcript (pre-tRNA^{Leu3}), an end-processed species with its intron (+IVS), a transcript that has been spliced only (+5′,+3′), and fully processed tRNA^{Leu3}. (**B**) Ethidium stained denaturing polyacrylamide gel of the small RNAs from the wild-type and T$_{315}$ΔT$_{307}$ mutant. An unusual accumulated RNA species larger than 5.8S$_L$ from the *RPR1*-mutated strain is indicated with a question mark (?). (**C**) RNA from the same strains, ^{32}P pulse-labeled for 5 min. The aberrant RNA is designated as '?'.

Figure 3. (**A**) The yeast rRNA transcription unit. An enlargement of the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S rRNA sequence is shown below the drawing of the full transcription unit. The locations of the primers and probes used in this study are indicated. Previously characterized *in vivo* processing sites are shown below (47). The location of the 3′-end of the aberrant rRNA is marked as '?'. (**B**) Processing of rRNA in *S.cerevisiae*. Precursors are identified by size at left and right. The 18S, 5.8S and 25S mature rRNAs are shown below. Nomenclature for processing sites is the same as in (A).

RNase P RNA. Filled circles on the secondary structure model of the RNA in Figure 1 highlight one region of conserved nucleotides that was targeted for randomization of the sole copy of the *RPR1* gene, encoding the RNA component of yeast nuclear RNase P. One of these variants, T$_{315}$ΔT$_{307}$ (Fig. 1), was found to accumulate a previously unidentified RNA. Growth of the mutant is reduced at 25°C as well as 37°C, allowing analysis of non-lethal RNA processing defects at 25°C. To confirm the presence of a nuclear RNase P functional defect, a northern blot prepared with total RNA from both the mutant and wild-type strains was hybridized with a probe to tRNA^{Leu3} (Fig. 2A). Examination of this single tRNA population revealed defects in the tRNA^{Leu3} steady-state population consistent with results from a previously characterized RNase P mutant (19,61,62). RNA isolated from both the wild-type and mutant strains showed products corresponding in size to the pre-tRNA^{Leu3} containing only its intron and the fully-processed, mature tRNA^{Leu3}. In the RNA from the mutant, the probe identified additional RNAs consistent with an accumulation of the primary transcript (intron
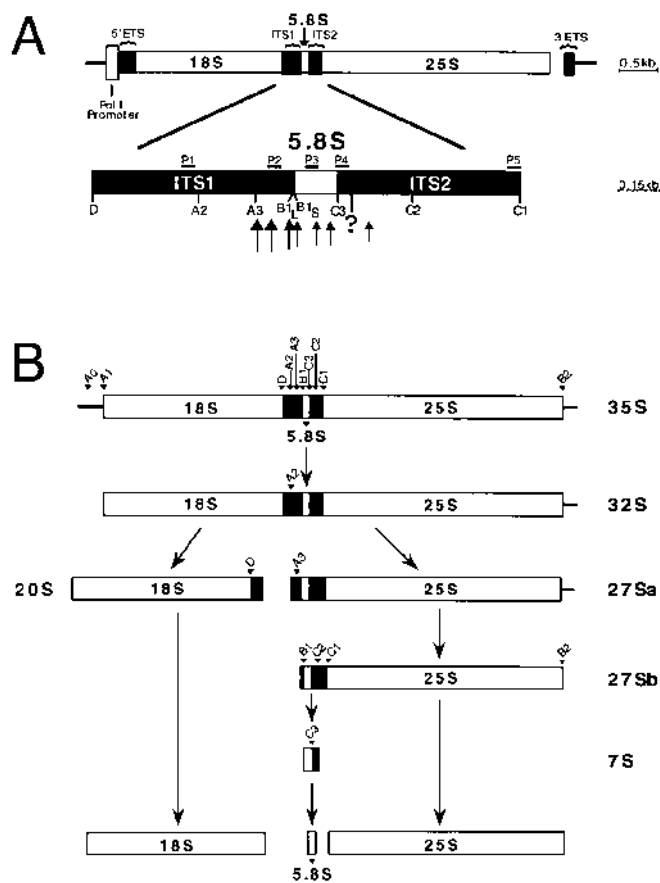
with 5′- and 3′-ends) and a spliced tRNA^{Leu3} carrying its 5′ leader and 3′ trailing sequences. This lack of 3′-end processing in an RNase P mutant has been observed previously (19) and may be due to extensive pairing between the 5′ leader and 3′ trailing sequences. The appearance of pre-tRNA^{Leu3} which is spliced, but not end-matured, suggests that maturation of termini before splicing is not obligatory (19).

When wild-type and mutant RNAs were subjected to electrophoresis in a denaturing polyacrylamide gel and stained with ethidium bromide, a novel RNA (denoted '?' in Fig. 2B), slightly larger than 5.8S, accumulated in the mutant. In repeated experiments, the total tRNA population was only slightly shifted to larger sizes, consistent with a modest defect in RNase P function. To visualize more clearly short-lived RNAs in this size range, newly synthesized RNA was pulse-labeled for 5 min with ^{32}P. Pulse-labeled RNAs from the mutant included an unknown RNA the same size as the novel band observed on ethidium-stained gels (Fig. 2C).

The abundance of the new '?' RNA, as well as its size relative to 5.8S rRNA, suggested that this might be an aberrant processing product of the ribosomal RNA genes. To test this hypothesis, a northern blot of RNA from both the mutant and wild-type strains
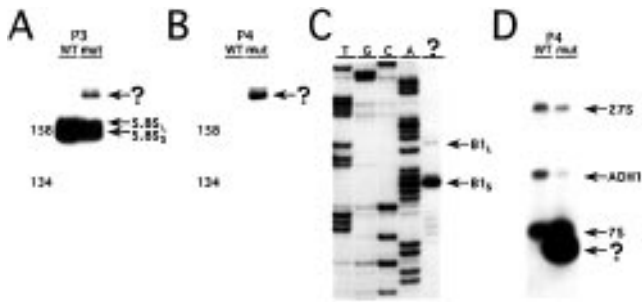
Figure 4. (**A**) Northern blot hybridization identifying small rRNAs containing 5.8S sequences using probe P3 (see Fig. 3A). (**B**) Northern blot analysis of the small rRNAs hybridized to probe P4, which is complementary to sequences 3′ of 5.8S. (**C**) Primer extension of gel isolated '?' rRNA for identification of its 5′-end using primer P3. (**D**) Northern analysis of pre-rRNAs after agarose gel electrophoresis to visualize large rRNAs. The probe is P4.
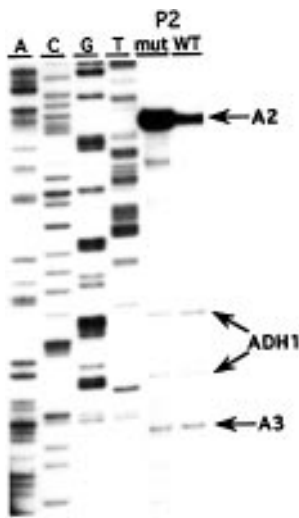

Figure 5. Quantitation of relative levels of rRNA precursors from ITS1 *in vivo* by primer extension. The rRNA processing sites, A2 and A3, are indicated at right as compared to primer extension sequencing using primer P2 shown in Figure 3A.

was probed with an oligonucleotide, P3, complementary to 5.8S RNA (Fig. 3A). The 5.8S hybridization signal in wild-type RNA corresponded to the sizes of the two major forms of the mature 5.8S rRNA, $5.8S_S$ and $5.8S_L$, that differ by an additional 7 nt at their 5′ termini (Fig. 4A) (63). An additional RNA was identified by this probe only in the RNA isolated from the mutant. This novel RNA appeared larger than the $5.8S_L$ rRNA by ~30 nt, corresponding to the size of the '?' RNA. Mapping of this previously uncharacterized RNA used antisense oligonucleotides annealing 5′ and 3′ to the 5.8S coding sequences to determine which end of the 5.8S rRNA included additional nucleotides. Hybridization to probe P4 (Fig. 3A) revealed a 3′ extension of the 5.8S rRNA in the aberrant RNA (Fig. 4B). To map the 5′-end more precisely, the aberrant RNA was isolated from a denaturing polyacrylamide gel and used in primer extension. The 5′- ends were shown to be the same as the mature 5′-ends of the $5.8S_S$ and $5.8S_L$ rRNAs (Fig. 4C). This 5′ heterogeneity could account for the apparent size heterogeneity of the aberrant RNA (Figs 2C, 4A and B), although multiple 3′ termini might also exist. The

approximate length of additional sequence beyond the mature 3′-end of $5.8S_S$ was 35 nt (±4 nt) (indicated on the sequence in Figs 8 and 3A).

To examine the larger precursor rRNAs (pre-RNAs) for changes in the mutant, northern blot analysis was performed using the mutant and wild-type total RNA on a 1.2% agarose gel with probe P4 (Fig. 4D). The strongest hybridizing and fastest migrating RNA is the aberrant species that was also observed in Figure 4A and B. A modest increase in 7S precursor varied from 1.3 to 5 times the level of wild-type in the mutant RNA in different experiments. No significant difference was seen in the level of the 27S precursor (27Sa and 27Sb) in the mutant compared with the wild-type RNA (Fig. 4D) or in the level of 35S precursor when levels were normalized to ADH1 mRNA levels. Attempts to identify other processing products, with an additional ITS2 oligonucleotide probe 115 nt downstream of the 5.8S rRNA sequence, did not reveal detectable accumulation of a novel pre-25S rRNA species with ITS2 sequences that might arise from cleavage at the '?' position (not shown).

Quantitation of pre-rRNAs with their 5′-ends at site A2

Although the path to production of the major aberrant RNA is unclear, we tested whether the RNase P defect might cause more subtle problems in maturation of the 5.8S 5′-end after the A2 cleavage (Fig. 3B). Primer extension was performed using primer P2 (Fig. 3A) for quantitation of the relative levels of the precursors with 5′-ends at A2 and A3. ADH1 mRNA was used for normalization of signals. Levels of transient pre-rRNAs with 5′-ends at A3 appear unchanged while the pre-rRNAs with their 5′-ends at A2 are increased 5-fold in the mutant (Fig. 5). The product seen below site A2 is attributable to a primer extension pause site as seen on longer exposure of the wild-type reaction. Increase in the levels of pre-rRNA with 5′-ends at A2 suggests that the mutant may be partially deficient in its ability to convert the precursors cleaved at A2 to those with mature 5.8S termini. It is unclear why this difference in rRNAs with 5′-ends at A2 is not manifest when the pre-rRNA is quantitated in a northern analysis (see above; Fig. 4D). It is possible that only the level of the relatively minor 27Sa intermediate (5′-end at A2) is increasing and the level of the more abundant 27Sb (5′-ends at $B1_S$ and $B1_L$) is not changing. Alternatively, the RNAs that give rise to the increase in 5′-ends at site A2 might have quite heterogeneous 3′-ends and be spread over multiple bands in Figure 4D that are not visible individually.

Partial purification of RNase P

To test whether nuclear RNase P was capable of cleaving rRNA *in vitro*, we partially purified the RNase P holoenzyme from wild-type and mutant yeast. The results from RNase P assays using a pre-tRNA$^{\text{Asp}}$ substrate and the most highly purified FPLC Mono Q fractions of wild-type enzyme are shown in Figure 6A. The RNase P activity produced two peaks centered in fractions 28 and 32/33. To determine the location of the RNA subunit of RNase P, samples were deproteinized and separated on a denaturing polyacrylamide gel (Fig. 6B). Two species of RNA were detected with an *RPR1* probe: the 369 nt fully processed *RPR1* RNA and the previously characterized ~453–483 nt precursor (19). The strongest hybridization signal, corresponding to the size of the 369 nt mature *RPR1* RNA, was seen in fraction 28. Fraction 32 RNA showed another peak of hybridization with both the mature
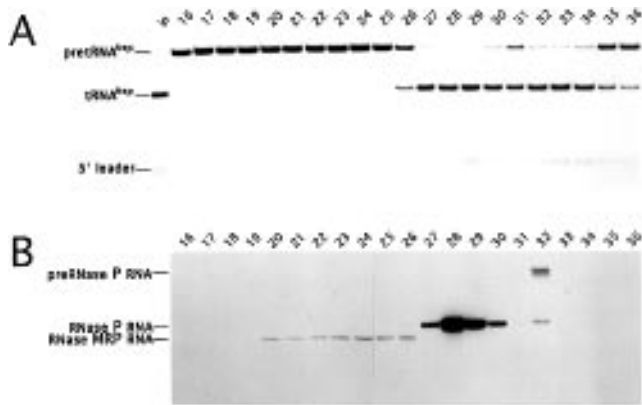
Figure 6. Mono Q chromatographic isolation of nuclear RNase P. (**A**) RNase P activity of wild-type holoenzyme from Mono Q gradient fractions was assayed by pre-tRNA^Asp cleavage, liberating the 5′ leader from the primary transcript. Mono Q fraction numbers are indicated above each lane of the gel. (**B**) Northern blot analysis of RNA isolated from the Mono Q fractions by sequential hybridization with RNase P and RNase MRP specific probes. 'preRNase P RNA' indicates the position of a previously characterized precursor (19) of the mature RNase P RNA.

RPR1 RNA and its precursor. The first peak of RNase P activity corresponds to the first peak of mature *RPR1* RNA and the second activity peak corresponds to the *RPR1* precursor-containing fractions. A third peak of pre-tRNA cleavage activity was seen in fractions 37 and 38, which was found to contain intact mitochondrial RNase P RNA by hybridization with a mitochondrial RNase P RNA probe (not shown).

To investigate the possibility that the related rRNA processing enzyme, RNase MRP, was present in our Mono Q fractions and separated from RNase P, a ^{32}P-labeled antisense RNase MRP RNA

was hybridized to the same northern blot (Fig. 6B). RNase MRP RNA was detected mainly in fractions 20–26, eluting at a lower salt concentration than most of the RNase P RNA. A fractionation scheme derived from these observations has also been used by others to reproduce this separation for analysis of RNase MRP in the absence of RNase P (37). The physical separation of these enzymes indicates that the eukaryotic nuclear RNase P enzyme does not require an association of both RNAs in a complex for activity *in vitro*.

Assay for cleavage of an rRNA substrate *in vitro*

To determine if RNase MRP was able to cleave a pre-rRNA substrate, a 603 nt pre-rRNA substrate was transcribed from a PCR template (primers P1 and P5 shown in Figs 3A and 8). The substrate spanned much of ITS1, 5.8S, and all of ITS2. In assays using this 3′-end labeled pre-rRNA substrate we were unable to find any evidence of endonucleolytic cleavage with fractions corresponding to the RNase MRP RNA (Fig. 6B). Instead, a set of discrete cleavage products were detected in those fractions coinciding with nuclear RNase P (Figs 6 and 7A). However, this does not discount the possibility of an undescribed activity as responsible for these rRNA cleavages. No similar activity corresponding to the mitochondrial RNase P was detected using the pre-rRNA substrate. In a further effort to identify RNase MRP activity in these fractions, a previously characterized *in vitro* substrate for yeast RNase MRP was used in a cleavage assay (not shown) (64). Little activity was detected in any MRP fractions, suggesting activity did not survive well under these purification methods, although full-length RNase MRP RNA could be detected as separate from the RNase P RNA peaks (Fig. 6B).

To provide additional evidence that the activity responsible for cleavage of the pre-rRNA substrate is dependent on an RNA component, a test for sensitivity to micrococcal nuclease
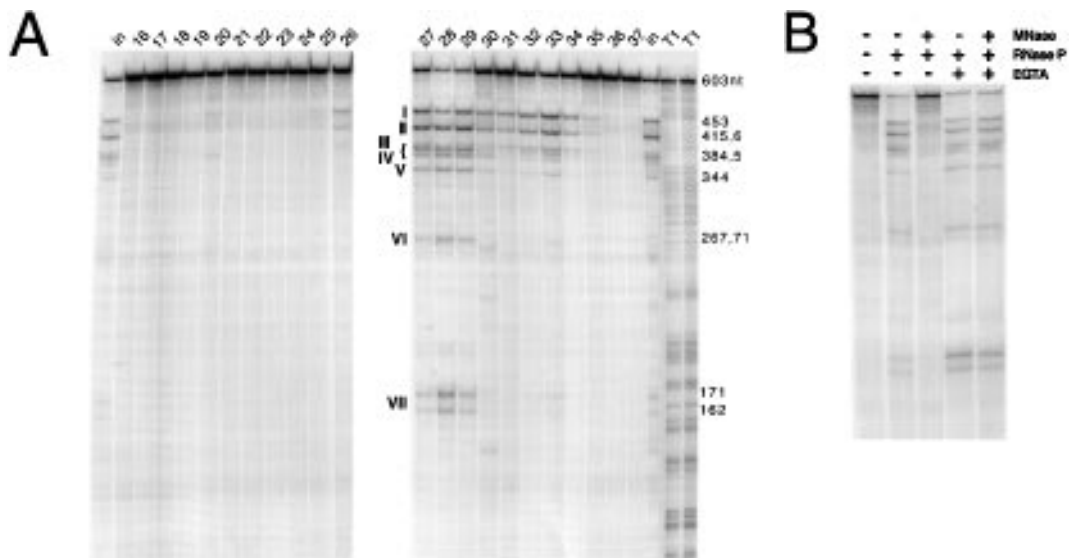

Figure 7. (**A**) Cleavage assay of a 3′-end labeled rRNA substrate spanning ITS1, 5.8S and ITS2 sequences in the presence of enzyme from the Mono Q fractions. The extent of the substrate is defined by the primers P1 and P5, shown in Figures 3 and 8. The Mono Q fractions used in assays are shown above each lane and are the same ones used in Figure 6. Cleavage products are labeled at the center with Roman numerals. The size of each product is indicated at right according to distance from the 3′-end as mapped by primer extension and comparison to the T1 nuclease digestion pattern (last 2 lanes). Assay of column input activity is indicated as 'in'. (**B**) Micrococcal nuclease (MNase) sensitivity of the activity responsible for rRNA substrate cleavage. A '+' indicates addition of MNase, RNase P and EGTA to a pre-incubation step prior to the cleavage reaction. EGTA was added to all samples before addition of substrate.
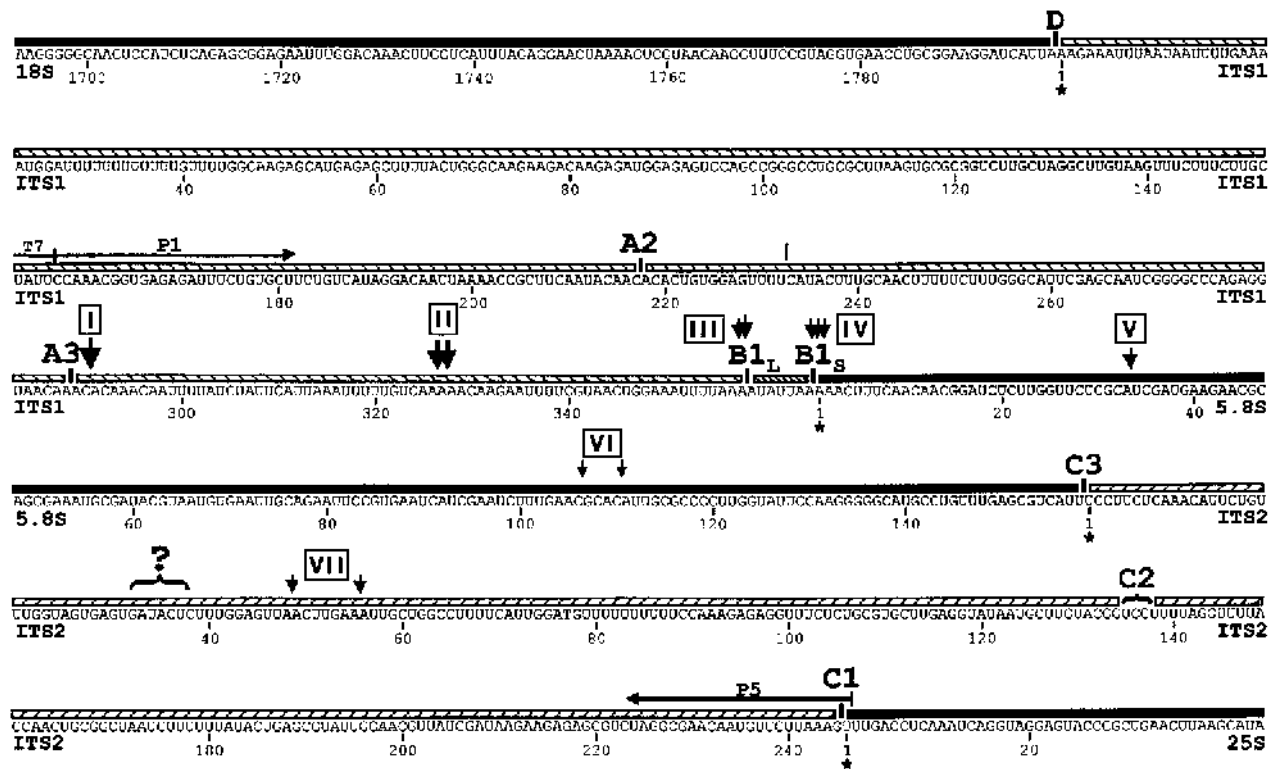
Figure 8. Cleavage sites in the rRNA substrate mapped relative to characterized rRNA processing sites *in vivo*. The cleavage sites are indicated by the roman numeral assigned in Figure 7A. The sequence shown includes the region surrounding 5.8S rRNA: the 3′ sequence of 18S (■); all of ITS1 (□); 5.8S (■); ITS2 (□); and the 5′ sequence of 25S rRNA (■). PCR primer P1 (including a T7 promoter) and primer P5 delineate the extent of the rRNA substrate used in the cleavage assays. An asterisk indicates the first nucleotide in ITS1, 5.8S, ITS2 and 25S sequences. '?' represents the approximate limits of the 3′ end of the aberrant rRNA identified in the *RPR1* mutant strain.

(MNase) inactivation was performed. RNase P Mono Q fraction 28 was pre-incubated with MNase either in the presence or absence of EGTA (Fig. 7B). RNase P is sensitive to MNase treatment in a pre-tRNA cleavage reaction (20) and MNase pre-digestion also renders the RNase P fraction unable to cleave the pre-rRNA substrate. In a control reaction, it was shown that addition of EGTA to inhibit the micrococcal nuclease before RNase P is added prevents the MNase from inactivating the pre-rRNA cleavage activity. These results suggest involvement of an RNA moiety in the activity responsible for pre-rRNA cleavage; reducing the chance that a minor contaminating nuclease was responsible for the activity.

Mapping sites of rRNA cleavage

Sites of cleavage of the pre-rRNA substrate were mapped more precisely by primer extension of the unlabeled substrate treated with fraction 28 RNase P (data not shown). The cleavage sites are indicated relative to the rDNA sequence in Figure 8 and relative to the region in Figure 3A. One of the prominent cleavages maps 2 nt downstream of the A3 site (Fig. 3) which is near the postulated site of action of RNase MRP (65). Another major cleavage site maps between sites A3 and B1 of ITS1. Sites that are less favored (Fig. 7A), are located at $B1_L$ and $B1_S$ (the mature 5′-ends of $5.8S_L$ and $5.8S_S$), within the 5.8S sequences, and within ITS2. None of these *in vitro* cleavage sites map immediately downstream of 5.8S rRNA, where failure to cleave in the RNase P mutant might lead directly to accumulation of the

3′-extended 5.8S rRNA seen in Figures 2 and 4. Possible indirect routes for accumulation of this rRNA are explored in the Discussion.

DISCUSSION

An RNase P mutant accumulates an aberrant rRNA

In addition to alterations in the population of the tRNA precursors in a yeast nuclear RNase P RNA mutant, there was an accumulation of a novel, abundant RNA slightly larger than 5.8S rRNA. The 5′-end of the aberrant RNA was shown to correspond to the mature 5′-ends of the $5.8S_L$ and $5.8S_S$ rRNAs, but the 3′-end extended 35 nt beyond the 3′-end of the mature 5.8S rRNA into ITS2. The accumulation of this novel species of rRNA suggests that the RNase P holoenzyme is required either directly or indirectly for proper formation of the mature 5.8S rRNA 3′-end.

Another strain with a mutation in the RNA subunit of RNase MRP also accumulates an rRNA species containing 5.8S sequences. However, in that strain the aberrant 5.8S rRNA has a normal 3′-end, but the 5′-end is extended by 149 nt (33–36). The accumulation of this 5′-extended 5.8S species along with a dramatic shift in the ratio of the mature forms toward the $5.8S_L$ form, led to the conclusion that RNase MRP is involved in proper $5.8S_S$ 5′-end maturation. Also accumulated in the conditional RNase MRP mutant is the proposed precursor to $5.8S_S$ whose 5′-end lies at A2 in ITS1 (Fig. 3B), supporting a direct precursor to product relationship for RNase MRP maturation of the 5′-end of $5.8S_S$ rRNA (65). The data presented

here suggest that RNase P may affect maturation of the 5.8S rRNA in a way that is different from the proposed role for RNase MRP in 5.8S$_S$ 5′ processing.

Another yeast enzyme involved in 3′ processing of 5.8S has been reported recently to be a 3′→5′ exonuclease activity, Rrp4p (66). A strain carrying the mutant allele, *rrp4-1*, accumulates 5.8S rRNAs with 3′ extensions. One of the species characterized in *rrp4-1* and wild-type RRP4 is the approximate length of the 3′-extended form of the 5.8S rRNA described here, suggesting that this pre-rRNA may be a previously undescribed rRNA precursor resulting from the characterized processing pathway or from an alternative pathway. It remains possible that the aberrant RNA that accumulates in the mutant is a cleavage product derived from the larger 27S pre-rRNAs earlier in the processing pathway, rather than from the 7S precursors.

Alteration in pre-rRNA processing following cleavage at A2

Examination of large rRNA precursors of wild-type and mutant RNase P RNAs did not reveal large changes in the normalized levels of the 27S (Fig. 4D) and 35S (not shown) rRNAs, nor was there an appearance of any aberrant rRNAs other than the 3′-extended 5.8S species. However, a more careful examination (Fig. 5) of the levels of the 27Sa major precursor to 5.8S and 25S rRNA (Fig. 3) by primer extension analysis revealed an increase in the levels of precursor with 5′-ends at processing site A2 in the RNase P mutant. RNase P appears to exert at least some of its influence after A2 cleavage, possibly participating in an uncharacterized pathway that, if interrupted, leads to the accumulation of the observed rRNA with additional 3′ sequence.

Activity of wild-type RNase P toward an *in vitro* rRNA substrate

Although RNA folding algorithms did not predict a tRNA-like RNase P substrate structure at the 3′-end of 5.8S rRNA, an attempt was made to use RNase P to cleave a pre-5.8S rRNA substrate *in vitro*.

The nuclear RNase P cleavage positions are indicated in Figure 8 relative to the nucleotide sequence of ITS1, 5.8S and ITS2. The simplest result from these experiments would have been the existence of a strong cleavage site at the end of 5.8S rRNA *in vitro*, thus easily explaining why 3′-extended 5.8S rRNA accumulates in the RNase P mutant. No such cleavage was observed, consistent with predicted folds not forming a structure appropriate for an RNase P substrate (67–69).

The two most prominent RNase P cleavage sites, | and ||, map to ITS1 (between 290 and 291 and between 326 and 328) within 75 nt of the 5′-end of 5.8S$_S$ sequences. Site | is 2 nt downstream of the proposed processing site A3. Cleavage at A3 is required for production of the mature 5′-end of 5.8S$_S$ rRNA and is the site of RNase MRP cleavage *in vivo* (37,65). Common substrate recognition for RNase P and RNase MRP has been shown previously *in vitro* (70) and may be a reflection of the evolutionary relatedness of RNase P and RNase MRP (14,43,46,47,70). Site || does not correspond to any proposed site *in vivo*, but may be a site that is rapidly processed further *in vivo* and has not been described. Clearly, other sites that map at positions within the 5.8S sequence are unlikely to be physiologically relevant and emphasize that the *in vitro* cleavage assay results must be viewed with caution. The extent of the pre-rRNA, the lack of

proteins, the non-physiological folding conditions, and other missing components of rRNA biogenesis are all potential problems.

Involvement of RNase P in rRNA processing

It is quite possible that RNase P does not directly cleave pre-rRNAs at all *in vivo*, but rather that RNase P mutations affect rRNA processing indirectly. RNase P might be part of an rRNA processing structure known as the 'processome' (9), a large complex in the nucleolus composed of snoRNPs and other associated proteins. Association of RNase P RNA with at least one protein that also associates with RNase MRP RNA was shown by co-immunoprecipitation of the RNA subunits of both enzymes by antibodies to a Protein A–Pop1 protein. RNase P RNA may influence the structure of a complex containing RNase MRP and the shared Pop1 protein, thereby interfering with proper 5.8S 3′-end processing through misassembly or disruption of the complex. Alternatively, some other cleavage product of RNase P, such as a snoRNA, might be required for proper folding or cleavage of the pre-rRNA.

An evolutionary and functional relationship between RNase P and RNase MRP has been suggested to explain a cleavage at site A3 for RNase MRP (47). In this scenario, RNase P originally functioned to cleave a tRNA with a conserved positioned in the equivalent 16S/23S spacer region of the rRNA operons from both Bacteria and Archaea to ensure separation of the rRNAs. It was postulated that RNase MRP evolved from RNase P in eukaryotes to cleave this region whose tRNA was subsequently lost during the course of evolution. While RNase P continues to process pre-tRNAs, it is possible that RNase P also remained a member of this complex retaining a functional role in rRNA maturation, but a role that is now distinct from RNase MRP.

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