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Identification of a functional core in the RNA component of RNase MRP of budding yeasts

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ABSTRACT

RNase MRP is an endonuclease participating in ribosomal RNA processing. It consists of one RNA and at least nine protein subunits. Using oligonucleotide-directed mutagenesis, we analyzed the functional role of five of the hairpins in the secondary structure of the RNA subunit of *Saccharomyces cerevisiae* RNase MRP. Deletion of an entire hairpin was either lethal or resulted in very poor growth. However, peripheral portions constituting up to 70% of a hairpin could be deleted without effects on cell growth rate or processing of rRNA. To determine whether these hairpins perform redundant functions, we analyzed mutants combining four or five benign hairpin deletions. Simultaneous removal of four of these hairpin segments had no detectable effect. Removing five created a temperature- and cold-sensitive enzyme, but these deficiencies could be partially overcome by a mutation in one of the RNase MRP protein subunits, or by increasing the copy number of several of the protein subunit genes. These observations suggest that the peripheral elements of the RNA hairpins contain no structures or sequences required for substrate recognition, catalysis or binding of protein subunits. Thus, the functionally essential elements of the RNase MRP RNA appear to be concentrated in the core of the subunit.

INTRODUCTION

RNase MRP is an endoribonuclease involved in ribosomal RNA processing (1–4). It cleaves at the A3 site within internal transcribed spacer 1 (ITS1) of the rRNA primary transcript to generate the precursor of $5.8S_S$ RNA (5,6). Under normal conditions, two forms of mature 5.8S RNA are found in ribosomes: the long form ($5.8S_L$) is 7 nt longer at the 5′ end than the short form ($5.8S_S$) (1,2). In the yeast *Saccharomyces cerevisiae*, the ratio between $5.8S_L$ and $5.8S_S$ is about 1:5. A typical phenotype of RNase MRP mutants is an increased $5.8S_L$ to $5.8S_S$ ratio and aberrant accumulation of a very long 5.8S rRNA, which is 149 nt longer at the 5′ end than the $5.8S_S$ rRNA (1,4) and corresponds to the product of cleavage at a site (A2) upstream of the RNase MRP cleavage site A3. RNase MRP has also been implicated in primer formation for mitochondrial DNA replication (7) and cell cycle control (8–10).

RNase MRP is a ribonucleoprotein particle, which in *S.cerevisiae* is composed of one RNA molecule and at least nine proteins (11–16). The RNA molecule is encoded by the single copy gene *RRP2* (Ribosomal RNA Processing 2, also termed *NME1*: Nuclear Mitochondrial Endonuclease 1) (1,3,4,17). RNase MRP is functionally and structurally similar to another ribonucleoprotein, RNase P, suggesting that they are evolutionarily related (18). The RNA molecules of both site-specific endoribonucleases share a similar 'cage-shaped' secondary structure (19) and they have eight protein components in common, with Snm1p unique to RNase MRP and Rpr2p unique to RNase P (11,16). In spite of their striking similarities, the two enzymes have different substrate specificities.

In eubacterial and archaeal species, RNase P RNA has been found to be active in tRNA processing *in vitro* in the absence of protein, suggesting that both catalytic and specificity domains reside on the RNA molecule (20,21). In eukaryotes, RNase P has a much higher protein to RNA ratio than that in prokaryotes, and the RNA has not been shown to cleave pre-tRNA by itself under the conditions tested (22). However, since eukaryotic RNase P RNAs have all the 'Critical Regions' conserved in eubacteria and Archaea (23) and RNase MRP RNA shows structural similarities to RNase P RNA (19,24), both RNAs may still have residual ribozyme functions, perhaps with certain activities taken over by the protein components.

Previous genetic studies were performed to identify functionally essential regions of the *S.cerevisiae* RNase MRP RNA subunit (25). However, phylogenetic comparisons of 18 species in the Saccharomycetaceae family and structural probing of *S.cerevisiae* RNase MRP RNA recently resulted in a revised secondary structure model for yeast RNase MRP RNA (24). Since deletion mutations removing only one side of a stem can produce unintentional rearrangement of the structure outside the mutated region, we re-examined structure–function relationships, constructing mutants based on the new model designing mutations to avoid complicating structural rearrangements. Furthermore, we systematically parsed each hairpin to determine which part, if any, is essential for assembly and/or function. Unexpectedly, we found that the termini of all six hairpins in the MRP RNA are dispensable. Furthermore, two mutants combining several of these deletions are described. One such mutant exhibits normal growth rate and rRNA processing at a wide range of temperatures. The other mutant

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exhibits cold- and temperature-sensitive growth, and its function in rRNA processing is rapidly inactivated at 37 or 16°C. Our results demonstrate that only the core of the RNase MRP RNA is required for assembly and function of the RNase MRP protein–RNA particle.

MATERIALS AND METHODS

Plasmids, strains and mutant construction

Plasmid pDK49, a derivative of YCplac22 (containing the *TRP1* gene), was constructed from pDK38 (26) by deleting a short segment of the multi-cloning site remaining in the vector sequence. It contains a functional *RRP2* gene [alias: *NME1*; (3)] including 184 bp upstream and 206 bp downstream. Mutations were introduced into *RRP2* by QuikChange mutagenesis (Stratagene), using primers containing 20 nt on each side of the mutation. After sequencing to confirm the correct mutation, plasmids carrying the *RRP2* mutants were transformed into strain YLL302 (*MATa ade2-1 his3Δ200 ura3-52 leu2-3,112 trp1-Δ1 rrp2Δ::HIS3*/pTop4) (26). Plasmid pTOP4 contains a wild-type *RRP2* gene and the *URA3* gene (4,26). Two independent isolates from each transformation were streaked on SD-Trp+5-fluoroorotic acid (5-FOA) plates to eliminate the residential pTOP4 plasmid and to test the ability of the mutant gene to support growth at different temperatures (27). The *pop4-8* gene cloned on the *URA3* CEN4 plasmid Ycp*lac*33 (28) has been described previously (14). A high-copy plasmid with *POP4* was constructed by subcloning into the 2μ *URA3* plasmid Ycp*lac*195 (28). High-copy plasmids carrying *SNM1* and *POP3* were obtained by PCR-amplification from chromosomal DNA and cloning into YCplac195. These four plasmids were individually transformed into YLL302 containing the Mini2 MRP RNA plasmid and cured of pTOP4.

RNA analysis

RNA isolation and northern blotting methods were essentially as described before (26). The probe for RNase MRP RNA was an oligonucleotide complementary to nucleotides 299–319 (Oligo O633); the probe for 5.8S was an oligonucleotide complementary to nucleotides 24–48 of $5.8S_S$ rRNA (Oligo O29).

RNA from the conditional mutants was isolated from cells grown overnight at 25°C until OD600 was 0.4–0.6 (corresponding to $\sim 10^7$ cells/ml) and then shifted to 37°C for 6 h. For temperature shift-up kinetic experiments, cells were grown overnight at 30°C until the OD600 reached ~0.6, when half of the culture was shifted to 37°C. Samples were removed immediately after the shift, then 2, 6 and 21 h later. Cultures were diluted once after the 6 h point to avoid their reaching stationary phase. For the shift to lower temperature, cells were grown overnight at 30°C until the OD600 was 0.4–0.6, and then half of the culture was shifted to 16°C. Samples were removed immediately before and then 17 and 37 h after the shift. Cultures were diluted after 17 h and again after 24 h to avoid their reaching stationary phase. Oligonucleotide probes were labeled at the 5′ end with [γ-^{32}P]ATP according to standard protocols (29). After probing, the northern blots were analyzed on a phosphorimager (Molecular Dynamics) to quantify the signals in long and short 5.8S rRNAs.

RESULTS

Isolation of mutations in the RNA of RNase MRP

Phylogenetic comparisons of sequences from the RNase MRP RNA within the Saccharomycetaceae family demonstrated the existence of six hairpins, called P3, ymP5, ymP6, ymP7, ymP8 and eP19 (24). Figure 1 illustrates the structure model for *S.cerevisiae*. Previous experiments showed that deletion of the entire P3 hairpin of MRP RNA is lethal (26) and that deletion of the terminal hairpin of P3 causes a temperature-sensitive phenotype (25). However, deletion of eight consecutive U residues at the end of hairpin P3 did not cause any defect (26), indicating that the terminal loop of P3 is dispensable. To map essential structural elements in other hairpins in RNase MRP RNA, extensive site-directed mutagenesis was carried out on the *RRP2* gene. Mutations were designed based on the predicted MRP RNA secondary structure (24) to minimize perturbation of the overall folding.

Plasmids encoding the resulting mutant MRP RNAs were introduced into strain YLL302, deleted of the chromosomal copy of *RRP2* but carrying an *URA3*-plasmid with a complementing wild-type MRP RNA gene (see Materials and methods). Using a plasmid shuffling technique (27), each plasmid carrying a mutated *RRP2* gene was tested for its ability to support growth by growing the resulting strains on 5-FOA plates containing glucose as a carbon source. Since 5-FOA kills cells carrying the wild-type *URA3* gene and since functional MRP RNA is essential for cell survival, only cells that have lost the original plasmid (*URA3 RRP2*) but have obtained a plasmid carrying a functional *RRP2* mutant will form colonies. The MRP RNA mutations are shown in Figure 1. Their growth phenotypes at various temperatures are shown in Figure 2. We also plated the mutants on 5-FOA plates with glycerol; with one exception (described below), the growth phenotypes were essentially the same on both carbon sources.

Analysis of MRP RNA hairpin mutations

Our results indicate that hairpin ymP5, which has a highly conserved sequence within the Saccharomycetaceae family (24), is important for RNase MRP activity. Deletion of the whole hairpin was lethal (P5-Δ1), as was a deletion removing only the part beyond the 'CAA' bulge (P5-Δ2). When we deleted about half of the hairpin and added a GAAA tetraloop (30) and a closing G–C base pair to stabilize the truncated stalk, the resulting mutant (P5-Δ3T) exhibited very slow growth at low temperatures and no growth above 30°C. Substitution of the CAA nucleotides in the bulge in hairpin ymP5 (P5-149,150,151) also caused a severe growth defect. Interestingly, deletion of the terminal loop plus 3 bp of the adjacent helix did not show any growth defect (P5-Δ4). The dispensability of this sequence is consistent with the lack of phenotype of an A to T transversion at position 131 (26). Together, these results suggest that the primary sequence, length and/or shape of the half of the ymP5 stem closest to the core of the RNA molecule are important for RNase MRP function.

Deletion of the entire ymP6 hairpin (P6-Δ1) was lethal. However, deleting nearly all of the hairpin did not cause any growth defect (P6-Δ2), suggesting that only the short stem at the base of the hairpin is essential for function. Indeed,

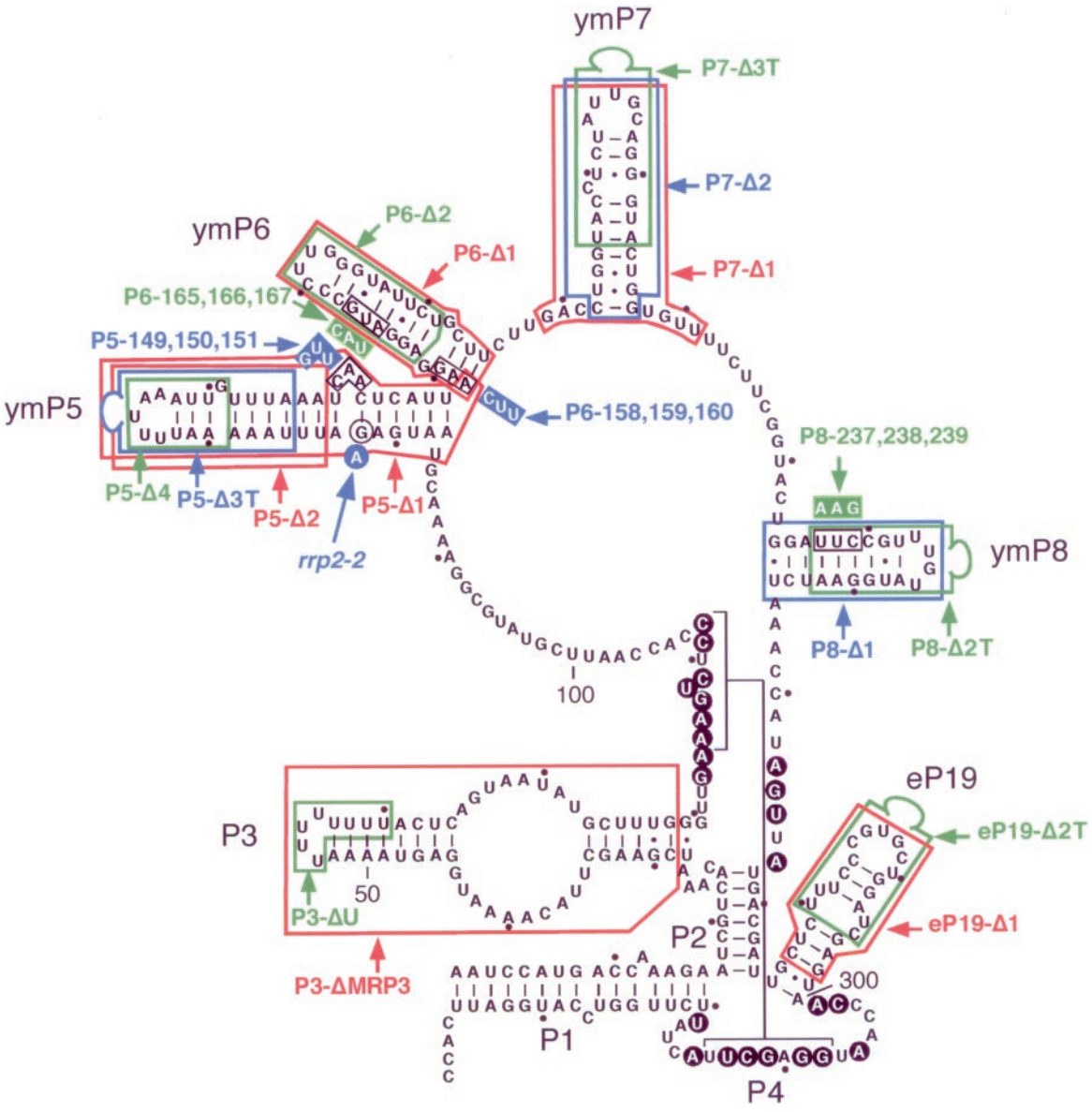

Figure 1. Summary of mutant MRP RNA phenotypes. The secondary structure model for MRP RNA was determined as described previously (24). The P1, P2, P3 and P4 hairpins are found in all RNase P and RNase MRP RNA structures. Hairpin eP19 is found in eukaryotic RNase P and RNase MRP RNA, while hairpins ymP5, ymP6, ymP7 and ymP8 are found in RNase MRP RNA from the Saccharomycetaceae family [for details see (24)]. Deleted bases have been boxed; bases substituted are also boxed with the replacement sequence indicated next to the box. Deletion mutants with an inserted tetra-loop (CGAAAG) are indicated by the suffix T and a loop at the end of the boxed bases. Mutations indicated in green have no significant effect on growth. Mutations in blue have a conditional phenotype, not growing at high temperature and, with the exception of P6-158,159,160, growing very slowly at lower temperatures. Mutations in red are lethal. Bases conserved in all currently known MRP sequences are indicated with black circles. The deletions in hairpin P3 have been described previously (26). The single base change in hairpin ymP5 (*rrp2-2*) has also been described before (4).

substitution of 3 nt to disrupt the loop end of the P6 helix (P6-165,166,167) had no effect on growth, but three base changes at the base of the helix (P6-158,159,160) resulted in a temperature-sensitive phenotype.

Deletion of the entire ymP7 hairpin (mutant P7-Δ2) resulted in very poor growth at low temperatures, and no growth above 30°C. An even larger deletion in the ymP7 region, extending several nucleotides into the flanking sequences (P7-Δ1), was lethal. However, a mutant with a shorter deletion and a terminal tetra-loop (P7-Δ3T) grew very well. Previously described deletions of up to 14 nt in what is now known to be ymP7 also grew well (4). Collectively, the mutants in ymP7 suggest that only a hairpin and no other specific structural features are required for activity.

As for ymP7, deletion of the entire P8 hairpin (P8-Δ1) resulted in poor growth at low temperature and no growth above 30°C. On the other hand, a shorter deletion in ymP8 in conjunction with insertion of a terminal tetra-loop (P8-Δ2T) grew very well. Also, substitution of 3 nt in the middle of the P8 helix (P8-237,238,239) had no discernible effect on growth. A mutant with the same deletion as P8-Δ2T but no stabilizing tetra-loop at the end was temperature-sensitive

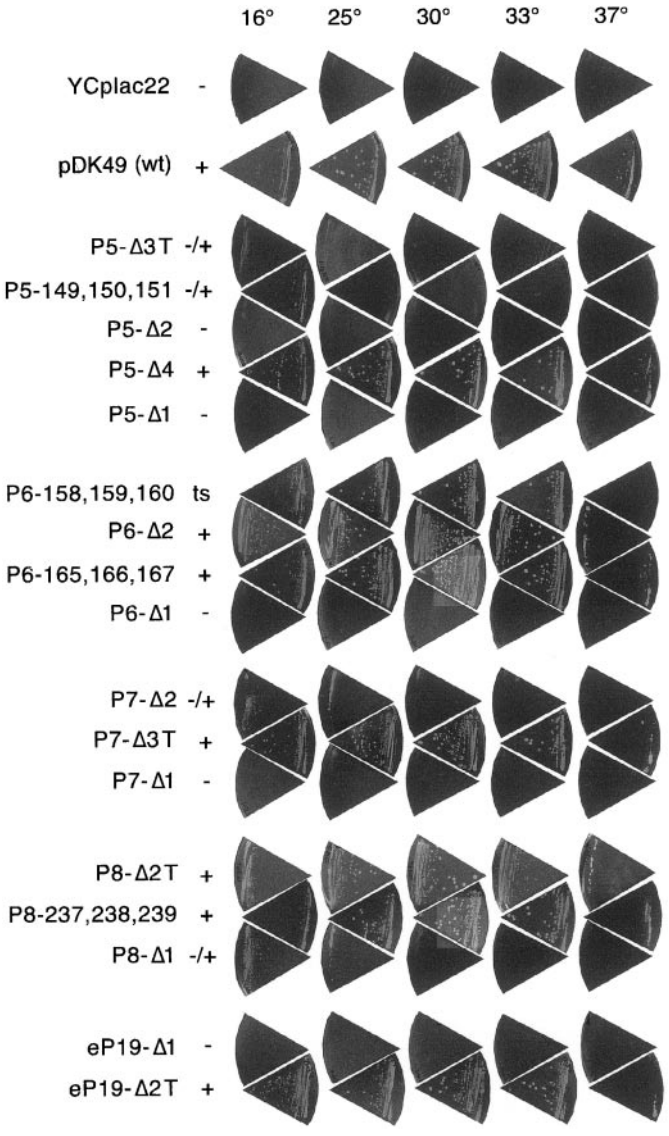

Figure 2. Growth phenotype of MRP RNA mutants. The indicated plasmids were transformed into strain YLL302 (see Materials and methods). Resulting transformants were streaked onto SD-Trp+5-FOA plates to select for loss of the wild-type *RRP2* gene, and incubated at the indicated temperatures for 4–6 days. For a description of the mutants, see Figure 1.

(25), suggesting that a stable hairpin structure is needed at the position of ymP8.

Hairpin eP19 is one of the common hairpins shared by RNase MRP and eukaryotic RNase P (19,22). A deletion removing most of the hairpin was lethal on glucose plates with FOA (eP19-Δ1). However, a mutant with a deletion that is 3 bp shorter and contains a capping tetra-loop did not display any discernable growth defect (eP19-Δ2T). Interestingly, the eP19-Δ1 mutant grows on a medium with glycerol as the carbon source [data not shown; (25)].

Stability of mutant MRP RNAs

The stability of the mutant MRP RNAs from the lethal and conditional deletion mutants was characterized by northern blot analysis, using a radioactive oligonucleotide specific to MRP RNA. Since the strains expressed both wild-type and mutant MRP RNAs, and since there was a size difference between the two RNAs, accumulation of the mutant RNA could be compared with that of the wild-type RNA. Most of the lethal and conditional deletion mutants accumulated mutant MRP RNA to essentially the same level as wild-type MRP RNA (Figure 3A). These results suggest that their growth defect is not the result of significantly reduced levels of mutant RNase MRP RNA. They also suggest that, while the function of RNase MRP is impaired, the deletion RNAs are still protected from ribonuclease digestion. Assuming that RNAs seriously defective in protein binding would be unstable, the mutant RNAs appear to have retained the ability to bind at least some proteins. Only mutant eP19-Δ1 showed a significantly reduced accumulation: in this strain, the deletion MRP RNA represented <20% of the total accumulated RNase MRP RNA (Figure 3A).

Conditional mutants and mutants with wild-type growth rates were tested after growth on 5-FOA plates to eliminate the wild-type *RRP2* gene. As expected, these strains expressed only the mutant MRP RNA (Figure 3B, top panel), which accumulated in abundance very similar to the wild-type strain (data not shown).

Function of mutant RNase MRP RNAs

Mutations that decrease the activity of RNase MRP result in an increased ratio of long to short 5.8S rRNAs (1,3,4,6). The function of RNase MRP in 5.8S rRNA processing was therefore tested for mutants that fully or partially supported growth in the absence of a wild-type *RRP2* gene. The conditional mutants were grown at 25°C and shifted for 6 h to 37°C; mutants supporting normal growth were grown at 30°C. As expected, the ratios of $5.8S_L$ to $5.8S_S$ RNA correlated, though not perfectly, with the effect on growth (Figure 3B, bottom panel). The mutants with wild-type growth rates had essentially the same long to short ratio as the wild-type strain. The conditional mutants that showed poor growth even at low temperatures (P5-Δ3T, P5-149,150,151, P7-Δ2 and P8-Δ1) had relatively higher $5.8S_L$ levels both at 25 and 37°C. And the temperature-sensitive mutant P6-158,159,160, which grew well at 25°C but not at all at 37°C, showed an increased ratio at 37°C compared to 25°C.

Since the lethal deletion mutants grow only in the presence of the wild-type gene for RNase MRP RNA, we could not directly assay their activity in 5.8S rRNA processing. However, cells carrying both mutant and wild-type MRP RNAs exhibited a normal growth rate and normal ratio of long and short 5.8S rRNAs (data not shown), suggesting that none of the mutations were dominant.

Construction and characterization of minimal MRP RNAs

Our mutagenesis results showed that the termini of all the MRP RNA hairpins are not essential when tested individually. Similar genetic studies of the U1 small nuclear RNA showed that growth defects were not apparent until mutations were combined, apparently because the U1 snRNP activity is not rate-limiting for growth (31). To determine if the same might be true for RNase MRP, we combined deletion mutations to create two 'minimal' MRP RNAs: Mini1 MRP contains mutations P5-Δ4, P6-Δ2, P7-Δ3T and eP19-Δ2T, and Mini2 MRP

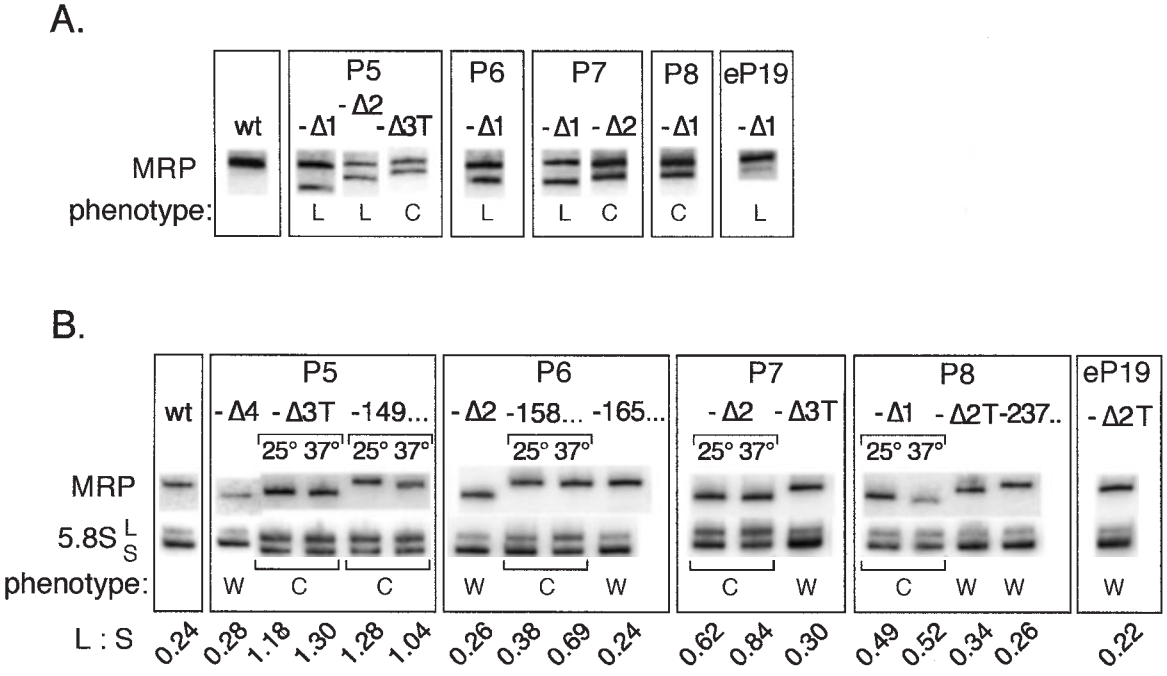

Figure 3. MRP RNA stability and 5.8S rRNA processing. (**A**) Total RNA was isolated from cultures grown in SD-Trp media, i.e. expressing both wild-type and mutant MRP RNAs. The RNA was fractionated on an 8% urea-polyacrylamide gel, transferred on to a nylon filter, and probed with oligo O633, complementary to MRP RNA. Phenotypes are indicated as follows: L, lethal; C, conditional (strain carrying only the mutant *rrp2* plasmid does not grow at high temperature; see Figure 2). (**B**) Total RNA was isolated from strains that had been selected for loss of the wild-type plasmid (i.e. expressing only mutant MRP RNA) by growth on SD-Trp+5-FOA plates, and probed with an MRP RNA probe as described for (A), or with oligo O29, specific for 5.8S rRNA. Phenotypes of the strains carrying only the mutant *rrp2* gene are indicated as follows: C, conditional; W, wild-type growth. Values below the 5.8S RNA panels indicate the calculated ratio of long to short 5.8S rRNA.

contains those deletions plus mutation P8-Δ2T. The two RNAs were first tested for their ability to support growth on 5-FOA plates. As shown in Figure 4A, Mini1 MRP could fully support growth at all five temperatures tested. However, cells carrying the Mini2 MRP RNA grew well at 25 and 30°C but showed strong temperature- and cold-sensitive phenotypes.

The Mini1 and Mini2 MRP RNAs were also tested for their accumulation and their activity in processing 5.8S rRNA. At 30°C (permissive temperature for Mini2), both MRP RNAs accumulated to roughly the same level as the wild-type MRP RNA (Figure 4B, top panel). There was no discernable defect in 5.8S rRNA formation for Mini1 MRP RNA, but there was a 2-fold increase in the relative level of $5.8S_L$ for the Mini2 (Figure 4B, bottom panel), as well as an increased accumulation of $5.8S_{VL}$ (data not shown), a 5.8S processing intermediate that has a 5′ end at the A2 cleavage site (1). In any case, these deletion studies indicate that the portion of MRP RNA essential for function is restricted to the center core region.

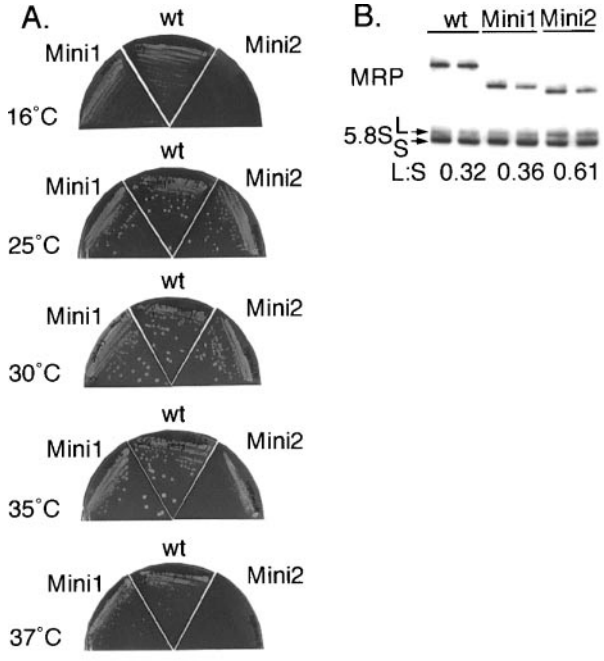

Figure 4. Characterization of the Mini1 and Mini2 MRP RNAs. (**A**) Growth phenotypes. Cells carrying a plasmid encoding the indicated MRP RNA were streaked on 5-FOA plates and incubated at the indicated temperatures for 5 days. (**B**) Northern blot analysis of RNA isolated from cells expressing Mini1 or Mini2 MRP RNA, probed with oligo O633, complementary to MRP RNA (upper panel) or oligo O29, specific for 5.8S rRNA (lower panel). Cells were grown at 30°C. Two independent isolates from each strain were tested. The ratio of long to short 5.8S rRNA for each type of MRP RNA is indicated.

Ribosomal RNA processing in the conditional Mini2 MRP RNA strain

To characterize further the function of the Mini1 and Mini2 MRP RNAs, 5.8S processing was analyzed in cells expressing only the mutant RNA after a shift from permissive (30°C) to non-permissive (37°C) temperature. As can be seen in Figure 5A, cells expressing either wild-type or Mini1 MRP RNA showed no significant change in the 5.8S composition after the temperature shift. However, for cells expressing Mini2 MRP RNA, the $5.8S_L$ to $5.8S_S$ ratio changed from

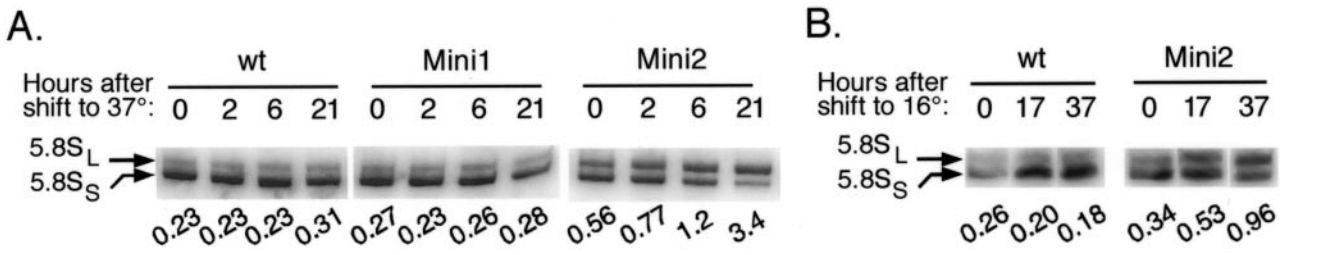

Figure 5. Effect of temperature shift on accumulation and activity of Mini1 and Mini2 MRP RNAs. (**A**) Effect of shift to 37°C. Cells carrying a plasmid encoding the Mini2 MRP RNA were shifted from 30 to 37°C. Samples were taken at 0, 2, 6 and 21 hours after the temperature shift. Total RNA was analyzed by northern blot analysis, probing for 5.8S rRNA as described in the legend to Figure 3. (**B**) Effect of shift to 16°C. Cells carrying a plasmid encoding the wild-type (W) or Mini2 (M) RNA were shifted from 30 to 16°C. Samples were taken at 17 and 37 h after the shift. Total RNA was analyzed by northern blot analysis as described for (A). The ratio of long to short 5.8S rRNA for each RNA sample is indicated.

1:2 to 1:1 within 6 h after the shift; by 21 h, the ratio was inverted, with three times more long than short 5.8S rRNA. We also observed an increased accumulation of $5.8S_{VL}$ (data not shown). A similar increased ratio in long to short 5.8S rRNA and increased $5.8S_{VL}$ was observed for Mini2 MRP RNA after a shift to 16°C (Figure 5B and data not shown). Again, the wild-type and Mini1 RNA-containing cells were not affected (Figure 5B and data not shown). Probing the blots for RNase MRP RNA showed no significant change in the MRP RNA levels after either temperature shift (data not shown).

After the shift to 37°C, we observed a weak signal in the Mini2 mutant representing a new band running slightly below $5.8S_{VL}$ (data not shown). Primer extension mapping of the 5′ end of this RNA suggested that it corresponds to 5.8S RNA, previously described in a *rrp5* mutant by Eppens *et al*. (32), with a 5′ extension terminating ~110 bases upstream of the 5′ end of mature 5.8S. This site in ITS1, called A4, is located between the A2 and A3 sites.

Suppression of the Mini2 defect

The temperature- and cold-sensitive growth and processing of 5.8S rRNA in Mini2 RNA-containing cells could be due to defective RNA folding and/or assembly of protein components of the enzyme at the non-permissive temperature. Alternatively, the reduced size of the RNase MRP RNA could affect the enzyme's interaction with the rRNA substrate or its catalytic activity. We addressed this problem using previously characterized suppressors of the *rrp2-2* mutation (G122A; Figure 1). The POP4 gene was discovered based on the ability of a dominant mutation in Phe-207 of the Pop4p subunit of RNase MRP (*pop4-8*) to partially suppress the temperature-sensitive growth and 5.8S rRNA processing defect of mutant *rrp2-2* (14). In addition, the *SNM1* gene was discovered as a high-copy suppressor of the same mutation (16), and increased copy number of *POP3* (13), originally identified in a synthetic lethal screen, also suppresses the temperature-sensitive growth of *rrp2-2*. It is not known if these suppressors are allele-specific or have a more general effect on RNase MRP deficiencies. To test if the suppressors have an effect on the phenotype of the Mini2 mutant, we transformed the Mini2 strain with plasmids carrying the *pop4-8*, *POP3* or *SNM1* suppressor genes, and tested for growth at low and high temperature. All suppressors restored growth of the Mini2 strain at 16°C, although they did not suppress the defect at 37°C (data not shown).

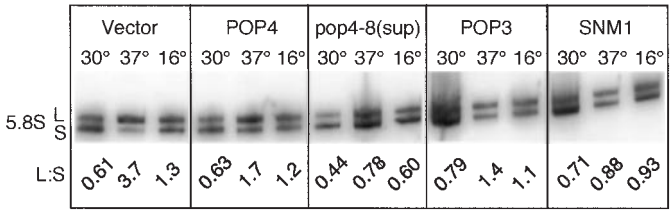

Figure 6. Effect of suppressors of Mini2 MRP RNA. Cells carrying plasmids with the indicated genes were grown at 30°C, and then shifted for 24 h at 37°C or for 48 h at 16°C. Total RNA was analyzed by northern blot analysis as described in the legend to Figure 3.

We examined the suppression in more detail by determining the 5.8S rRNA composition in Mini2 RNA-containing cells with or without the suppressors. The cells were grown at 30°C, and then shifted to 16°C for 48 h or to 37°C for 24 h. The Pop4-8 suppressor protein changed the 5.8S L/S ratio to nearly wild-type values at 16 and 37°C (Figure 6). Increased copy numbers of the wild-type *POP4*, *POP3* and *SNM1* genes also significantly reduced the L/S ratio, although not to the same degree as the *pop4-8* mutant gene. Thus, RNase MRP containing Mini2 RNA and mutant Pop4p appears to be capable of near normal processing of pre-rRNA even at high and low temperature.

Processing of 5.8S rRNA was suppressed equally at 16 and 37°C although no growth was seen at 37°C. This confirms previous observations that there is not a strict correlation between growth and RNase MRP-mediated processing of pre-rRNA (4). This discrepancy has led to the suggestion that RNase MRP has other functions in the cell. In particular, a correlation between RNase MRP activity and cell division has been reported previously (8–10). We therefore compared the morphology of cells carrying the Mini2 mutation, or Mini2 and the *pop4-8* suppressor, with wild-type cells after a shift to 37°C. After 4 h, there was essentially no effect. After 21 h, we observed that the morphology of many buds in the Mini2 strain changed to an elongated cylindrical or baseball-bat-like shape, although the fraction of Mini2 cells with buds was not changed (data not shown). This trend was partially suppressed by *pop4-8*. Since this change in morphology was not observed until well after RNase MRP activity was reduced as estimated from the accumulation of short and long 5.8S rRNA (Figure 5), we cannot determine if the effect of the temperature shift on cell morphology in the Mini2 mutant is a direct or indirect result of reduced RNase MRP activity.

We conclude that a suppressor mutation in Pop4p or increased supply of wild-type Pop4p, Pop3p or Snm1p can partially compensate for the deletions in the Mini2 RNA. These results suggest that the Mini2 RNase MRP RNA contains the elements necessary for catalysis and substrate recognition, and that the temperature sensitivity and decreased activity in 5.8S rRNA processing are caused by defective RNA folding and/or complex assembly. Moreover, our results indicate that the three suppressors we analyzed are not allele-specific and thus are most likely to accomplish their effects by facilitating assembly or stability of the RNase MRP particle.

DISCUSSION

The mutagenesis studies described here show that deletions removing entire hairpins in the RNA of RNase MRP result in no (ymP5, ymP6 and eP19) or very poor (ymP7 and ymP8) growth. Previous experiments demonstrated that hairpin P3 is also essential (25,26,33). These results suggest that there is no extensive domain in the RNA that is dispensable for RNase MRP function. We were somewhat surprised that the termini of all six hairpins are dispensable [(25,26), and this report], since terminal loops can contribute to protein binding (34) or play an important role in three-dimensional folding of RNA (35). Nevertheless, our finding that the termini of the hairpins are dispensable is consistent with the variability of sequence, length and helix irregularities in these portions of the RNase MRP RNAs from the Saccharomycetaceae family (24).

The ymP5 hairpin is the most conserved hairpin in the RNase MRP RNAs of members of the Saccharomycetaceae family (Figure 7). More than half of this hairpin is required, perhaps related to the well-conserved three nucleotides bulge in this hairpin. A mutation in this bulge (P5-149,150,151) results in very poor and temperature-sensitive growth. Furthermore, a G to A transition at position 122 in the base pair adjacent to the bulge generates the temperature-sensitive *rrp2-2* mutant (4). The bulge could play an important role in protein binding or in the formation of a long distance structural constraint in the RNA molecule, since helices distorted by this kind of irregularities can serve as protein-binding areas (34). No equivalent of hairpin ymP5 with a three nucleotides bulge has been identified in the MRP RNA of *Schizosaccharomyces pombe* or higher eukaryotes (24). Interestingly, a 5 nt bulge implicated in binding of the Est1 telomerase protein to the telomerase RNA also appears to be conserved in yeast species closely related to *S.cerevisiae* but not in higher eukaryotes (36). Further experiments are needed to clarify the degree to which protein–RNA interactions have been conserved in these and perhaps other nuclear RNPs.

It is interesting that all of hairpin ymP7 or ymP8 could be deleted, and the cells could still grow, albeit slowly. Moreover,

Figure 7. Consensus structure of hairpin ymP5 of the Saccharomycetaceae. The structure was based on the predicted structures of the members of the family described in (38). Bases conserved in all Saccharomycetaceae are shown by letters. Non-conserved nucleotides are shown as solid black circles.

deletions removing almost all of those two hairpins or hairpin ymP6 had no detectable effect on cell growth or 5.8S rRNA processing. The requirement of just a few base pairs at the base of these hairpins suggests that they may contribute to proper folding of the RNase MRP RNA, but that they may not contain specific targets for any of the nine protein components of the enzyme.

More of hairpin eP19 was required, but, still, no more than 5 bp at the base were necessary for wild-type growth. Hairpin eP19 exhibits interesting characteristics. The growth of all other mutants was affected similarly on both glucose (fermentable) and glycerol (non-fermentable) media, but eP19-Δ1 grew on glycerol but not on glucose [this report; (25)]. The mechanistic background for this effect of carbon source is not known, but may be related to assembly. For example, the relative rates of synthesis of components of RNase MRP in glycerol might be more conducive to assembly of eP19-Δ1 RNA-containing particles.

Having observed that the termini of MRP RNA are not essential when deleted individually, we combined deletions to determine the minimal sequence required for enzyme function. Mini1 MRP RNA, missing the termini of hairpins ymP5, ymP6, ymP7 and eP19 (66/339 bases = 19%), had an activity that was indistinguishable from the wild-type RNA. The Mini2 RNA mutant, lacking the termini removed in the Mini1 MRP RNA plus the terminus of ymP8 (82/339 bases = 24%), could support good growth at 30°C, although it could not grow at low (16°C) or high (37°C) temperatures.

Since neither the P8-Δ2T deletion nor the Mini1 collection of deletions by themselves display any defect, the temperature-sensitivity of the Mini2 cells must be caused by their additive effects. We hypothesize that, while not required individually, these hairpins cooperate to maintain the overall three-dimensional structure of the MRP RNA, which in turn is necessary for proper protein binding and/or enzymatic activity. This conclusion is corroborated by the finding that the functionality of the Mini2 RNase MRP is improved by manipulating RNase MRP protein genes. Although these experiments provide no direct information about the mechanism behind the suppressor effects, they support our conclusion that the central core remaining in the Mini2 RNA subunit is the only part of the RNA subunit required for substrate recognition and catalysis. The Mini2 RNA core structure is shown in Figure 8.

The Mini2 RNA contains only 275 nt (including bases introduced for the three tetra-loops), so one wonders how all nine proteins with a collective molecular weight of 2.8×10^5 could be arranged on such a small RNA molecule. It is very likely that some proteins are not bound directly to RNA, but rather, require protein–protein interactions. Indeed, two- and three-hybrid studies are consistent with this idea (37).

When shifted to a non-permissive temperature, cells synthesizing only Mini2 MRP RNA showed a significant increase in the accumulation of the long form of 5.8S rRNA. Since the $5.8S_L$ and $5.8S_S$ rRNAs are both processing end products, the ratio between the short and long forms changes only as the rRNAs produced *before* the temperature shift are diluted with products processed *after* the temperature shift. For this reason, the change in the ratio of long to short 5.8S rRNAs already visible by 2 h after the shift to 37°C suggests that pre-existing Mini2 MRP RNA-containing

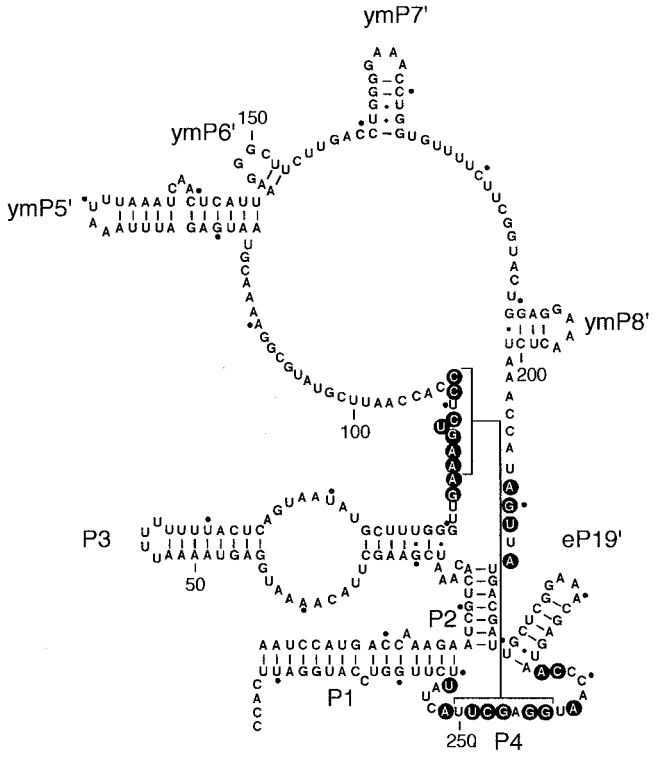

Figure 8. Predicted structure of RNase MRP RNA core remaining in the Mini2 mutant. Local RNA folding of the remaining portions of hairpins ymP5, ymP6, ymP7, ymP8 and yeP19 was predicted using mfold (39).

enzyme is rapidly inactivated after a shift to non-permissive temperature. At 16°C the change in the 5.8S rRNA composition took longer, as expected since all biological processes are severalfold slower at this lower temperature. To our knowledge, this is the first RNase MRP mutation that exhibits a strong temperature-dependent defect in 5.8S rRNA processing. Other mutants that show temperature-sensitive growth exhibit a defect in $5.8S_S$ processing that is more or less independent of the growth temperature [Figure 3B and the *rrp2-2* mutant described previously in (4)].

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