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An essential protein-binding domain of nuclear RNase P RNA

WILLIAM A. ZIEHLER, JONATHAN MORRIS, FELICIA H. SCOTT, CHRISTOPHER MILLIKIN, and DAVID R. ENGELKE

Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0606, USA

ABSTRACT

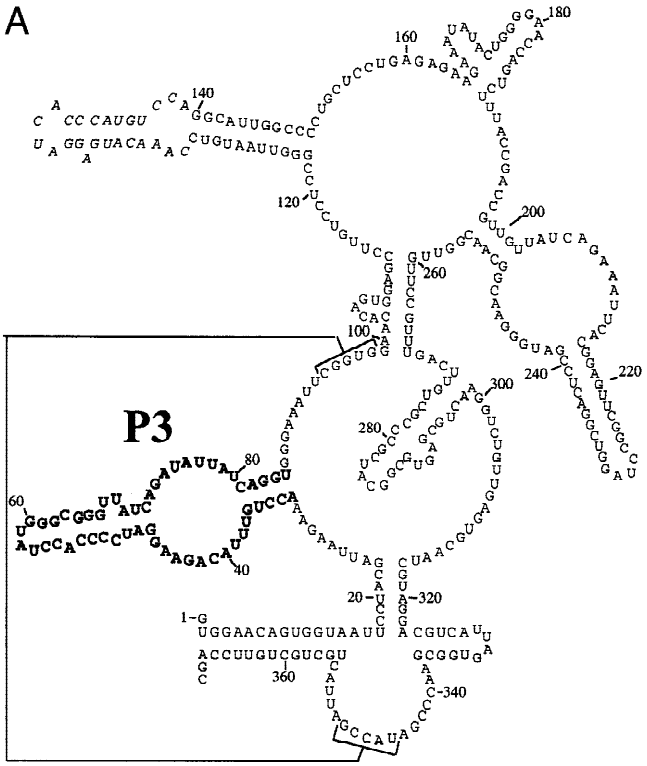
Eukaryotic RNase P and RNase MRP are endoribonucleases composed of RNA and protein subunits. The RNA subunits of each enzyme share substantial secondary structural features, and most of the protein subunits are shared between the two. One of the conserved RNA subdomains, designated P3, has previously been shown to be required for nucleolar localization. Phylogenetic sequence analysis suggests that the P3 domain interacts with one of the proteins common to RNase P and RNase MRP, a conclusion strengthened by an earlier observation that the essential domain can be interchanged between the two enzymes. To examine possible functions of the P3 domain, four conserved nucleotides in the P3 domain of *Saccharomyces cerevisiae* RNase P RNA (*RPR1*) were randomized to create a library of all possible sequence combinations at those positions. Selection of functional genes in vivo identified permissible variations, and viable clones that caused yeast to exhibit conditional growth phenotypes were tested for defects in RNase P RNA and tRNA biosynthesis. Under nonpermissive conditions, the mutants had reduced maturation of the *RPR1* RNA precursor, an expected phenotype in cases where RNase P holoenzyme assembly is defective. This loss of *RPR1* RNA maturation coincided, as expected, with a loss of pre-tRNA maturation characteristic of RNase P defects. To test whether mutations at the conserved positions inhibited interactions with a particular protein, specific binding of the individual protein subunits to the RNA subunit was tested in yeast using the three-hybrid system. Pop1p, the largest subunit shared by RNases P and MRP, bound specifically to *RPR1* RNA and the isolated P3 domain, and this binding was eliminated by mutations at the conserved P3 residues. These results indicate that Pop1p interacts with the P3 domain common to RNases P and MRP, and that this interaction is critical in the maturation of RNase P holoenzyme.

Keywords: P3; protein; RNase MRP; RNase P

INTRODUCTION

RNase P is the endoribonuclease responsible for removing the 5′ leader of tRNA primary transcripts (Altman et al., 1989; Pace & Smith, 1990; Chamberlain et al., 1996b). In yeast, the nuclear holoenzyme is composed of an RNA subunit, which is indispensable for enzyme activity, and at least nine protein subunits. This protein content is surprisingly complex, given that the analogous bacterial RNase P contains only one small protein in addition to its RNA subunit. In bacteria, the protein subunit functions in helping the RNA subunit to fold and in interactions with the pre-tRNA substrate. Eighteen-fold more protein is present in the nuclear enzyme, and at the moment there is little evidence as to what the increased protein content might be used for. Although the nuclear holoenzyme has additional interactions with the pre-tRNA substrate (Ziehler et al., 2000), the huge increase in protein content suggests that the proteins are serving entirely new functions in the eukaryotic nucleus. The question of subunit function is further complicated by the fact that another nuclear RNA processing enzyme, RNase MRP, has evolved from RNase P. RNase MRP has an essential RNA subunit that is evolutionarily related to *RPR1* RNA, and the two enzymes also appear to have eight of the nine protein subunits in common (Gold et al., 1989; Schmitt & Clayton, 1992; Lygerou et al., 1994, 1996; Morrissey & Tollervey, 1995; Chu et al., 1997; Dichtl & Tollervey, 1997; Stolc & Altman, 1997; Chamberlain et al., 1998; Schmitt, 1999). RNases P and MRP contain a homologous subdomain in their RNA subunits, designated P3, that is conserved in both secondary structure and sequence (Fig. 1A). The P3 domain secondary structure is a helix-internal loop-helix and contains highly conserved residues primarily in the initial helix and internal loop region. The terminal helix varies in length and nucleotide identity between species (Forster & Altman, 1990; Schmitt et al., 1993; Tranguch &

Reprint requests to: David R. Engelke, Department of Biological Chemistry, University of Michigan, 1301 East Catherine Street, Ann Arbor, Michigan 48109-0606, USA; e-mail: engelke@umich.edu.



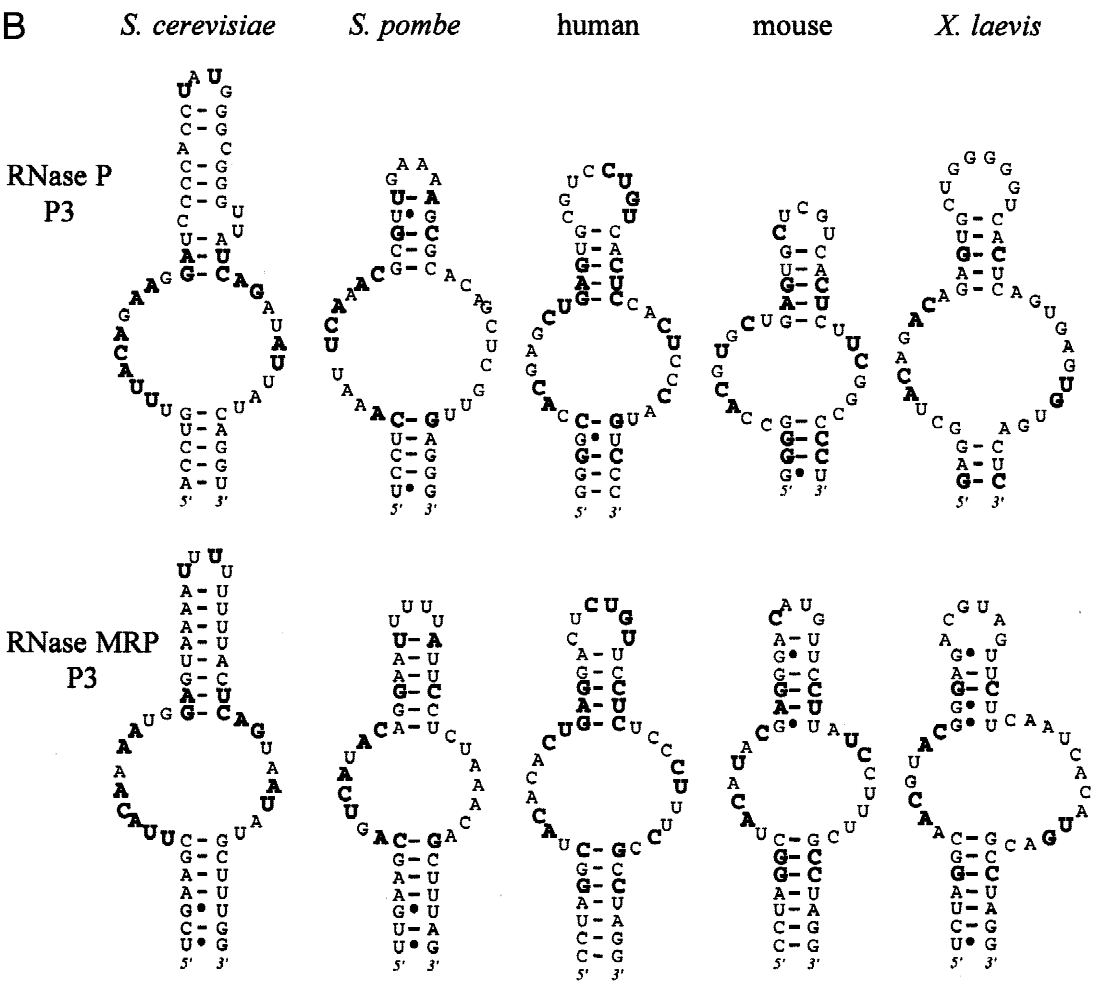


FIGURE 1. P3 domain comparisons from RNase P and RNase MRP RNAs. **A:** Secondary structure of *S. cerevisiae* RNase P RNA based on phylogenetic and biochemical analysis (Tranguch et al., 1994). The location of the P3 RNA domain is shown in bold. The location and sequence of the inserted hybridization tag (Bertrand et al., 1998) between positions 133 and 139 is shown in italics. **B:** P3 RNA domains from RNase P and MRP are shown from five eukaryotes, with intraspecies nucleotide conservations in bold. *S. cerevisiae* and *S. pombe* RNase P sequence is from Tranguch & Engelke (1993) and Krupp et al. (1986); human, mouse, and *X. laevis* RNase P is from Pitulle et al. (1998); *S. pombe* RNase MRP is from Paluh and Clayton (1995); *S. cerevisiae*, human, mouse, and *X. laevis* RNase MRP is from Schmitt et al. (1993).

Engelke, 1993; Paluh & Clayton, 1995; Pitulle et al., 1998). The P3 domains from *Saccharomyces cerevisiae* RNases P and MRP have been shown to be functionally equivalent, because swapping domains between enzymes was permitted (Lindahl et al., 2000).

Minimization experiments using domain deletions from yeast RNase P RNA have shown the P3 domain to be essential for generating a functional enzyme (Pagan-Ramos et al., 1994). Recent studies have further identified the P3 domain, in both human RNase P and RNase MRP, as required for binding the To antigen, a 40-kDa protein immunoprecipitated by human autoimmune sera (Reddy et al., 1983; Liu et al., 1994; Eder et al., 1997). Microinjection experiments using synthetic RNase MRP RNA result in nucleolar localization of the RNA, consistent with the role of RNase MRP in processing pre-rRNA (Jacobson et al., 1995). Microinjected RNase P RNA alone localized to the nucleolus only transiently (Jacobson et al., 1997). Transient nucleolar localization of RNase P RNA was dependent on the P3 domain, suggesting a potential role for P3 in nucleolar localization. It is currently not clear whether RNase P is also a nucleolar enzyme, and to what extent this is consistent among species. Yeast nuclear RNase P appears largely nucleolar (Bertrand et al., 1998), and in situ studies of mammalian RNase P have indicated either transient nucleolar association (Jacobson et al., 1997) or limited steady-state localization to a "perinuclear compartment" (PNC) located on the nucleolar periphery (Matera et al., 1995; Lee et al., 1996). It has not been clear from these data whether the role of the P3 domain in localization is to direct holoenzyme assembly, which in turn causes localization, or whether the RNA interacts directly with some nonholoenzyme cellular components.

To better understand the function of the P3 domain an extensive set of point mutations in the domain has been created and analyzed for function in vivo. The results suggest that significant sequence variation at highly conserved positions is tolerated for viability, but that most non-wild-type sequences lead to apparent defects in RNase P maturation and function. Further investigation showed that Pop1p, the largest protein subunit, is likely to be responsible for binding directly and specifically to the P3 domain of the RNA subunit, and that mutations at the conserved nucleotides disrupt this interaction.

RESULTS

The P3 domain of RNases P and MRP is composed of a conserved secondary structure with minimal conserved primary sequence

Phylogenetic analyses of eukaryotic RNase P and RNase MRP RNA subunits have established refined secondary structure models for the entire RNase P RNA (Tranguch & Engelke, 1993) and somewhat less extensive models for RNase MRP RNA (Schmitt et al., 1993; Pitulle et al., 1998). A consensus structure for *S. cerevisiae* RNase P RNA is shown in Figure 1A. One structural aspect common to the RNase P and MRP models is the existence of the P3 stem with an internal loop early in the RNA (nt 32–85 in *S. cerevisiae* RNA). Sequence comparison of RNase P and RNase MRP RNAs from a variety of species has identified residues that are widely conserved within species. In contrast, there is little or no sequence conservation between the P3 internal loops of even the same enzyme in distant species. Several nucleotide positions on the 5′ side of the P3 internal loop are conserved between the RNase P and RNase MRP RNAs within a species. Bold letters in Figure 1B indicate the positions that appear to be conserved between the RNase P and RNase MRP P3 domains within each species. It had previously been noted (Tranguch & Engelke, 1993) in yeast that the lower P3 helix positions were more tightly conserved among RNase P RNAs than they appear to be between RNase P and RNase MRP. Thus, it is possible that the loop and stem regions of the P3 domain serve different functions in RNase P. The conservation of the P3 internal loop sequences between RNases P and MRP is entirely consistent with a specific interaction with one or more proteins common to RNase P and MRP. If the same protein(s) bound to the P3 loop in both enzymes, the protein making direct contact would probably need to coevolve with both RNA subunits.

Viable P3 variants display limitations in mutational tolerance

Four of the P3 internal loop positions (U38, U39, C41, and A44) are the most tightly conserved among different yeast RNase P RNAs. A library of genes was created in which these four positions were randomized to give all of the 256 possible sequence combinations. Library screening for viable clones showed that only a fifth of the possible variants are able to produce functional enzyme. Identity, frequency, and growth phenotype for 89 sequenced clones are listed in Figure 2. Although the screen was not saturating and probably did not provide an exhaustive list of permissible sequences, several trends are apparent in the viable mutants. First, nearly one-third of the clones contained wild-type sequence. This preference for the wild-type sequence suggests that sequence deviation is unfavorable, which is predicted by the highly conserved nature of these positions. Second, no triple or quadruple mutants were isolated, possibly due to an additive effect in disrupting favorable contacts between the P3 RNA domain and the bound protein. Third, double mutants at positions 38 and 39 are all temperature sensitive. The adjacent double mutants might disrupt local structure to such a degree that an elevated temperature might

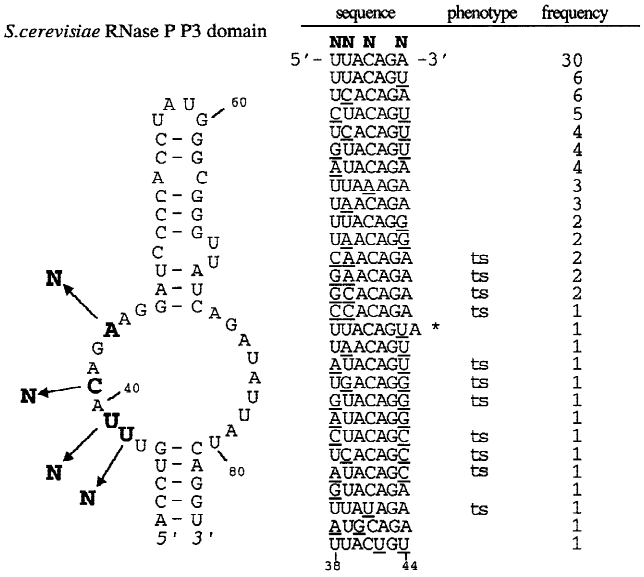


FIGURE 2. P3 RNA domain library location and identity, frequency, and phenotype of sequenced clones. The randomized positions in the mutated *RPR1* gene library are indicated by "N" relative to the P3 subdomain sequence. Clones exhibiting temperature-sensitive growth are denoted by "ts"; otherwise growth is similar to wild type. The sequence denoted by an asterisk contains a single nucleotide insertion mutation.

abolish the RNA–protein interaction. Nucleotides 38, 39, and 44 exhibited the full range of possible mutations. Position U38 was the least particular, varying relatively equally between changes to A, G, and C. Conversely, changes at position U39 were to C over 50% of the time, and position 44 favored a mutation to U 67% of the time. Nucleotide C41 was changed to A only three times and to U once. There were no C41 to G mutants present in the sequenced clones. Finally, nucleotide C41 was the least mutable; only 4 out of 89 clones had mutations at this position, and all were single nucleotide changes. A C41-to-U change was the only temperature-sensitive single mutation observed. Although detailed interpretations of the nucleotide identity requirements are not possible without a more exhaustive analysis of viable versus nonviable sequences, these results are consistent with an important role for the P3 internal loop sequences. This result is also consistent with mutagenesis of the corresponding region in RNase MRP RNA (Shadel et al., 2000), which suggested that the P3 loop sequences might be essential even if the distal stem was not.

Conditional mutations in the P3 domain of yeast RNase P result in an accumulation of 5′-unprocessed tRNA precursors

P3 mutations were investigated for their effect on RNase P activity in vivo. Relative RNase P activity can be determined from the profile of pre-tRNA species in the cell. Previous defects in RNase P enzymatic activity have shown an accumulation of tRNA species with unprocessed 5′ ends (Lee et al., 1991b). Similar results were observed for the conditional P3 mutants at both the permissive (30 °C) and nonpermissive (37 °C) temperatures, with precursor accumulation being only slightly more elevated at nonpermissive temperature. Examples of the RNA hybridization blots probed with an anti-tRNA^{Leu3} oligomer are shown in Figure 3A. The effects include an accumulation of primary tRNA transcript and an unusual intermediate in which splicing precedes terminal maturation (+5′, +3′, intron spliced tRNA). Accumulation of both of these precursors is consistent with a reduction in RNase P activity (Lee et al., 1991b). There is an absence of 5′ cleaved, 3′ exonuclease-trimmed tRNA at the nonpermissive temperature, as well. Clones without conditional growth phenotypes displayed RNase P activity ranging from near wild-type to defects similar to the conditional clones (Fig. 3B).

Conditional mutations in the P3 domain of yeast RNase P result in an accumulation of precursor RNase P RNA (*RPR1* RNA)

Many of the mutants were found to accumulate precursor RNase P RNA subunit (*RPR1* RNA), in addition to the accumulation of pre-tRNA substrates. *S. cerevisiae RPR1* RNA is an RNA polymerase III transcript containing an 84-nt 5′ leader (Lee et al., 1991a, 1991b). This precursor is processed by an uncharacterized nucleolytic pathway to produce mature RNase P RNA. Previous observations during purification of RNase P (Chamberlain et al., 1996a) showed that this precursor is assembled into an RNP complex that is similar in size and chromatographic properties to the mature RNase P, suggesting assembly precedes processing of the *RPR1* RNA subunit. Defects in RNase P holoenzyme assembly, including depletion of individual protein subunits, are believed to prohibit this processing of *RPR1* RNA (Pagan-Ramos et al., 1994; Chamberlain et al., 1998). In contrast, mutations in catalytically important residues in *RPR1* RNA do not cause pre-RPR1 accumulation (Pagan-Ramos et al., 1996a, 1996b). Therefore, observed reductions in mature RNase P levels relative to precursor RNase P RNA suggest a role for a functional P3 domain in RNase P assembly and processing, rather than a primary defect in catalytic competence. Figure 4A shows a representative blot of RNA from temperature-sensitive clones at permissive and nonpermissive temperatures probed using an anti-*RPR1* RNA DNA oligomer.

P3 library clones that did not exhibit conditional growth phenotypes (Fig. 4B) had pre-*RPR1* RNA/mature *RPR1* RNA ratios ranging from near wild-type levels to accumulation of pre-*RPR1* RNA similar to the temperature-sensitive clones (Fig. 4A). Clones with wild-type levels

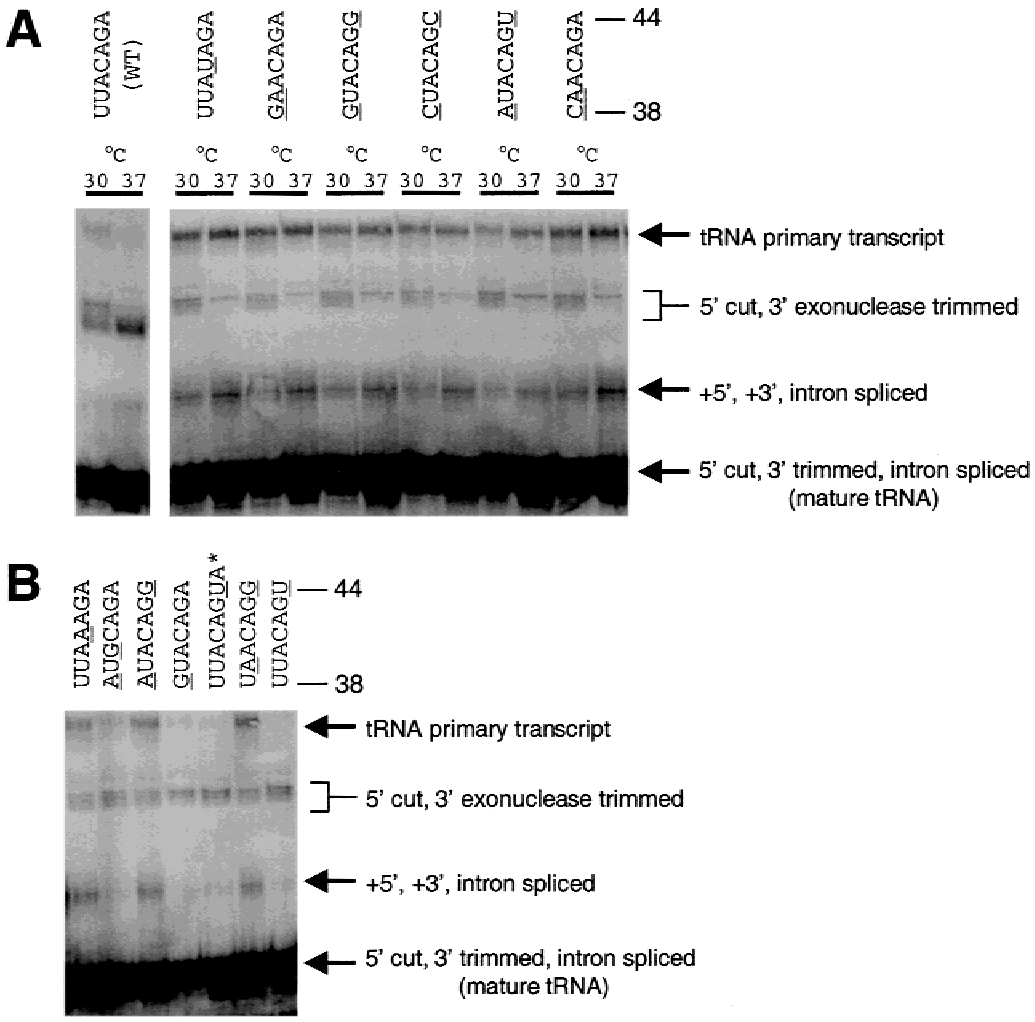


FIGURE 3. Anti-tRNA^{Leu3} blot hybridization. **A:** RNA from temperature-sensitive P3 mutant clones harvested at permissive (30 °C) and nonpermissive (37 °C) conditions were probed with radiolabeled anti-tRNA^{Leu3} DNA oligomer. A subset of mutant RNAs are shown, with sequence listed above each pair of lanes and mutations underlined. Identities of the various pre-tRNA intermediates are indicated by arrows and have been determined previously (Lee et al., 1991b). **B:** RNA from P3 mutants with wild-type growth were analyzed by the same blotting methods after growth at 30 °C. The sequence denoted by an asterisk contains an insertion mutation.

of mature *RPR1* RNA also had more normal pre-tRNA levels (compare Figs. 3B and 4B). This is consistent with the notion that reduced levels of mature *RPR1* RNA result in reduced RNase P enzyme activity.

Pop1p, a subunit common to RNases P and MRP, interacts with the P3 domain in a sequence-specific manner

If the P3 domain nucleates assembly of the holoenzyme ribonucleoprotein, it is expected that one or more of the protein subunits should interact specifically with the conserved sequences. This would be consistent with the proposed interaction of the bacterial protein subunit with the analogous P3 region of *Escherichia coli* RNase P RNA (M1 RNA) (Morse & Schmidt, 1993). Both genetic and biochemical analyses were considered for determining specific protein–RNA interactions. Unfortunately, the RNase P protein subunits were, for the most part, highly insoluble or unstable when expressed individually in a variety of ways. This made direct biochemical assessment of binary subunit interactions difficult to interpret. Instead, the yeast three-hybrid system (SenGupta et al., 1996) was employed to determine which of the *S. cerevisiae* RNase P protein subunits bind specifically to the P3 domain. The three-hybrid system is outlined in Figure 5A. "Bait" RNAs were tethered to the artificial promoter regions of two reporter genes in yeast, *HIS3* and the β-galactosidase coding region. Protein coding regions for the RNase P proteins or controls were then fused to activation domains and tested for whether interaction between the RNase P protein subunit and the bait RNA could bring the activator domain into position to induce expression of both reporter genes. Complete

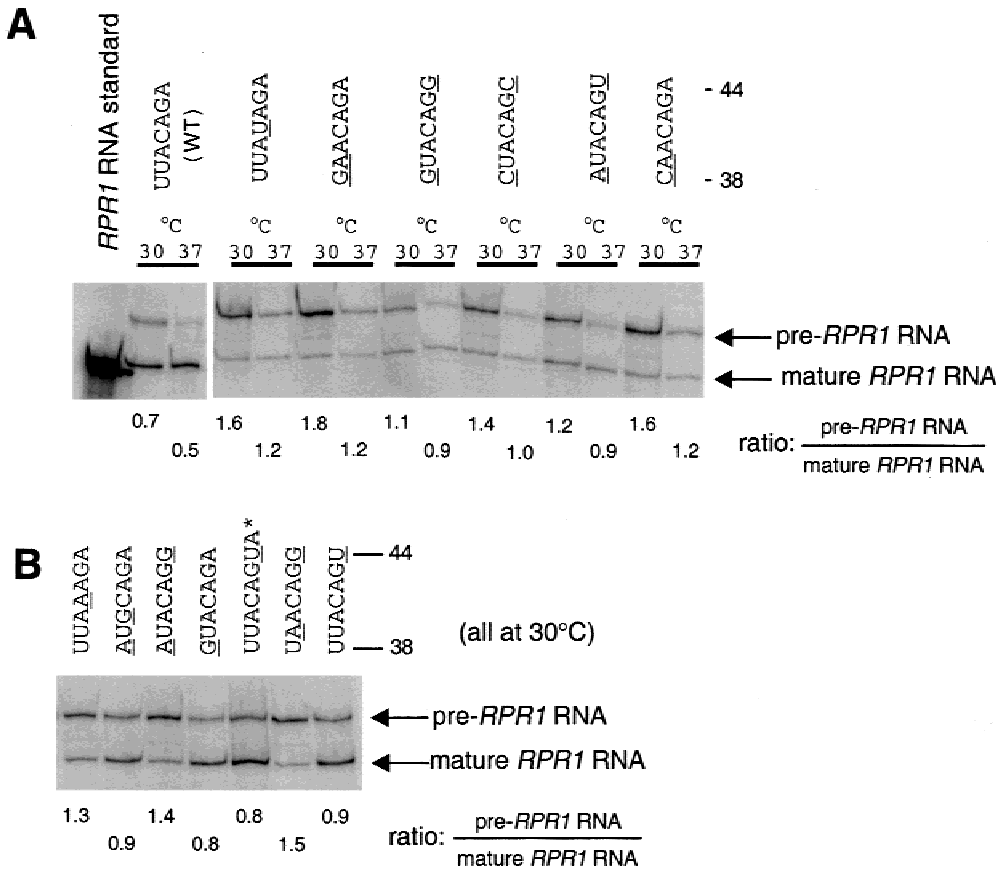


FIGURE 4. Anti-*RPR1* RNA blot hybridization. **A:** RNA from temperature-sensitive P3 mutant clones harvested at permissive (30 °C) and nonpermissive (37 °C) conditions probed with radiolabeled anti-*RPR1* DNA oligomer. P3 loop sequence is listed above each pair of lanes, with mutations underlined. Locations of pre- and mature *RPR1* RNAs are designated with arrows. **B:** RNA from P3 mutants with wild-type growth harvested at 30 °C. The sequence denoted by an asterisk is an insertion mutation.

analysis of protein–RNA subunit analysis through three-hybrid interactions will be published elsewhere, along with the analysis of protein–protein interactions through the two-hybrid system (F.H. Scott, S. Xiao, C. Millikin, J.M. Zengel, L. Lindahl, and D.R. Engelke, in prep.). For the purposes of this work, it was observed in the larger set of experiments that deletion of the entire P3 domain caused loss of Pop1p binding. None of the other protein subunits displayed P3-dependent, specific binding to the *RPR1* RNA subunit. We therefore tested the ability of Pop1p to bind to the P3 RNA subdomain alone, and the effects of conserved position mutations on P3-dependent binding of Pop1p to the full *RPR1* RNA subunit. Growth in the absence of histidine is one indication of stable interaction between the test protein and the bait RNA. This data is shown for Pop1p in Figure 5C, and was completely compatible with expression of the other, β-galactosidase gene reporter (not shown). The expression of the reporter genes for different RNA baits in the presence or absence of Pop1p are summarized in panel D of Figure 5.

Bait RNAs consisted of whole wild-type *RPR1* RNA, the *RPR1* antisense sequence (control), the wild-type P3 domain alone, and the *RPR1* subunit with point mutation in all four conserved P3 positions (Fig. 5B). The four simultaneous point mutations (A38, A39, G41, and U44) were expected to strongly disrupt P3 function, because no triple or quadruple mutants were recovered from the in vivo library screen and the least permissible nucleotide was used at each position. We have subsequently determined that the quadruple point mutation does not produce functional RNase P (data not shown). Bait RNA plasmid was also used that had no inserted sequences in addition to the MS2 binding sequences that tether the RNA to the promoter. None of these RNAs caused expression of the reporter genes in the absence of Pop1p fused to the activator domain (not shown), nor did Pop1p fused to the activator cause reporter expression without part of the *RPR1* sense sequence fused to the bait RNA. Pop1p gave substantial expression of both reporters when the bait contained the whole wild-type *RPR1* or the P3 domain alone. Antisense *RPR1* sequences or quadruple point mutations in *RPR1* RNA eliminated reporter expression. This was not due to instability of these RNAs, because they were expressed at levels comparable to

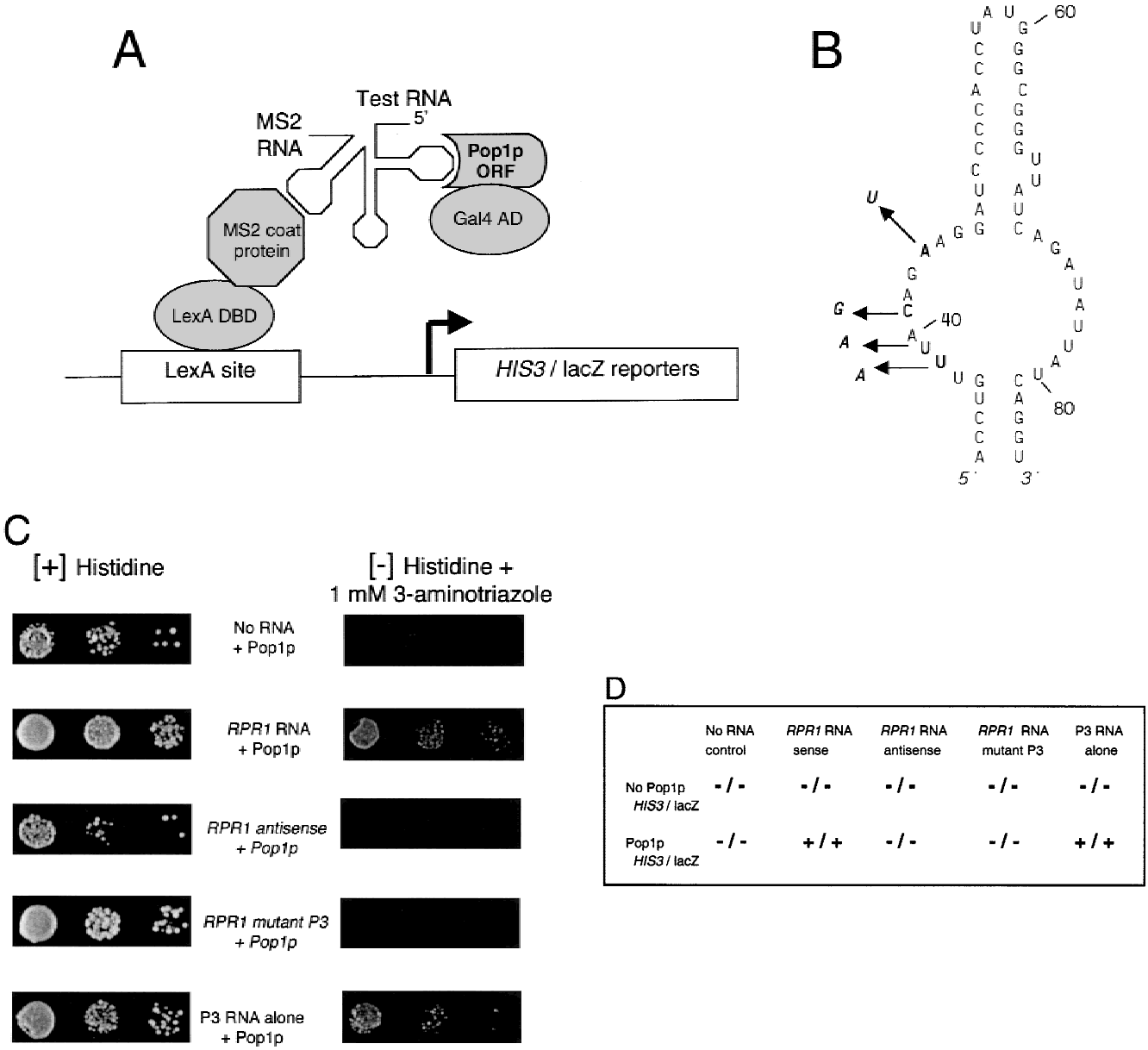


FIGURE 5. Testing whether the Pop1p RNase P protein subunit interacts with the P3. **A:** The three-hybrid test for protein–RNA interactions is shown schematically. The test RNA (*RPR1* RNA, P3 RNA, or controls) are tethered near the promoters of two reporter genes, *HIS3* and the lacZ ORF, by fusing the test RNA to MS2 RNA. The MS2 RNA is bound by the MS2 binding protein, which is fused to the LexA DNA-binding domain (LexA DBD), which binds to LexA DNA sites in the promoters. When a test protein fused to the Gal4 activation domain (Gal4 AD) binds to the test RNA, the cells are His+ and produce β-galactosidase. **B:** The wild-type sequence of the P3 subdomain is shown, with the identity and location of quadruple point mutations ("*RPR1* mutant P3" in **C** and **D**) indicated by arrows. "P3 RNA alone" test RNA contains only the wild-type P3 sequence as shown. **C:** Three-hybrid test strains containing the indicated bait RNAs and the Pop1p-activation domain fusion were plated as serial dilutions on growth media containing or lacking histidine. All bait RNAs gave His^- phenotypes if only the activation domain was tested in the absence of the Pop1p fusion (not shown). His+ phenotypes occurred only when sense, nonmutated P3 domain was contained in the bait RNA. **D:** Three-hybrid test results are summarized for the *HIS3* expression and β-galactosidase expression assays, which gave mutually consistent results. Results are given for the indicated RNA baits using both Pop1p fused to the activation domain and the activation domain alone.

the wild-type *RPR1* RNA (F.H. Scott, S. Xiao, C. Millikin, J.M. Zengel, L. Lindahl, and D.R. Engelke, in prep.). We therefore conclude that Pop1p binds selectively to P3 RNA in a manner requiring the positions in the P3 subdomain that are conserved between RNases P and MRP.

DISCUSSION

The library screen for viable P3 mutants identified a set of rules governing permissible nucleotides at positions 38, 39, 41, and 44 of this RNA domain. The four

positions each have different degrees of mutational tolerance. The more discriminating positions might provide essential structural elements for P3 function, or they might directly interact with protein partners. Because neither triple nor quadruple mutations were recovered, it is likely that detrimental effects are additive.

Nucleotide U38 is found to mutate to each of the other three bases rather randomly and without obvious preference. Despite the variable nature of position 38, mutations at this position have an additive effect when coupled with a change at its neighbor, nt 39. When paired with a proximal mutation in position 39, the double mutant is temperature sensitive for all such clones sequenced. Mutations found at U39 also encompass all three nucleotide possibilities, although over half of all recovered clones contained a cytosine at position 39, indicating a preference for pyrimidines.

The fewest variations were observed at nucleotide C41; three clones were changed to adenosine and one to uridine. These four mutants did not have any second changes at positions 38, 39, and 44. In the rare instance C41 is changed, no other changes appear to be tolerated. The C41-to-U change was the only temperature-sensitive single mutation. All other temperature-sensitive clones were double mutations. The wild-type cytosine is likely contributing an important element for P3 function, such that its loss is equivalent to two mutations in the other three randomized positions. Three C41-to-A clones were recovered, and although not temperature sensitive, they showed an accumulation of pre-*RPR1* RNA and concomitant defects in pre-tRNA processing (Figs. 3B and 4B).

Changes in the identity of nucleotide A44 are partially restricted. All possibilities are represented in the sequenced viable clones, and this position is frequently changed as part of double mutant clones, but 67% of all changes at position 44 are to uridine. There is no nucleoside conservation, as in the preferred pyrimidine change in position 39, or similarity in orientation of hydrogen bonding potential. However, this could be reconciled by the following scenario. The adenosine at position 44 might not normally participate in the function of the P3 domain, such as binding protein, but when changed to uridine, the position may now provide a favorable interaction. This is supported by the fact that only 1 out of 16 U44 mutants with a secondary mutation are temperature sensitive, and G44/C44 mutants with a second mutation are almost always temperature sensitive. U44 is therefore a relatively innocuous mutation, and may even facilitate the function of the P3 domain in some contexts. The function of the P3 domain is either independent of A44-to-U mutations, effectively making double mutants with an A44-to-U change behave as single mutants, or the A44 to U contributes to the function of the P3 domain enough to prevent temperature-induced defects. A U44 insertion mutation (U inserted between wild-type G43 and A44 positions), likely a PCR artifact generated during library construction, was also isolated. The U44 insertion behaves similarly to the wild-type construct with respect to both *RPR1* RNA levels and pre-tRNA processing, further demonstrating the absence of negative effects of an A44-to-U mutation or U insertion.

Temperature-sensitive phenotypes permitted investigation into the function of the P3 domain in context of RNase P biosynthesis and catalysis. The RNA blot hybridizations determined the effects of P3 mutations on RNase P activity and RNase P RNA maturation. Processing of pre-tRNA substrates is decreased, possibly the consequence of increased precursor RNase P RNA and decreased mature RNase P populations. The increase in pre-RNase P RNA suggests possible holoenzyme assembly and maturation defects, as previously described (Pagan-Ramos et al., 1996a). These effects were consistent for all temperature-sensitive clones examined. Even several P3 mutants with wild-type growth characteristics exhibited some defects in pre-tRNA and pre-*RPR1* RNA processing. Pre-*RPR1* RNA is known to assemble into a ribonucleoprotein (RNP) complex very similar, if not identical, to the mature holoenzyme RNP prior to processing of the *RPR1* RNA (Chamberlain et al., 1996a). The temperature-sensitive P3 mutants appear defective in forming mature RNase P RNA, although it is not clear whether this is due to problems with protein subunit association or the actual endonucleolytic processing of the pre-*RPR1* RNA. It is apparent, however, that a critical event in assembly of the functional yeast RNase P involves a functional P3 domain.

The yeast three-hybrid results indicate that the largest protein subunit, Pop1p, interacts with RNase P RNA in a sequence-specific manner. Because the three-hybrid system is performed in yeast, and the proteins and RNAs being screened are from yeast, various explanations can account for the three-hybrid results. There could theoretically be adapter proteins, bridging between Pop1p and the RNA domain that result in the observed interaction. However, this adapter would probably be titrated out of endogenous complexes, because the protein subunits are not normally abundant, but Pop1p and the RNA bait are expressed from high copy number plasmids. In addition, three-hybrid analyses show that only Pop1p, out of all the known RNase P protein subunits, binds selectively to the P3 domain or is sensitive to mutations at conserved positions (data not shown). The most direct interpretation of these results is therefore that Pop1p is binding directly to P3. This interaction is likely to be a critical step in assembling a functional holoenzyme, and probably occurs prior to processing of the pre-*RPR1* RNA to the mature size. It should be noted that this data does not exclude the possibility that Pop1p might contact other positions in the RNA subunit (and other proteins), even though those contacts are not absolutely essential to specific binding.

It is not clear what relation this data has to previous reports that the P3 domain of human RNase MRP interacts either with a 40-kDa component of the To autoantigen (Yuan et al., 1991; Liu et al., 1994) or with at least two smaller proteins (Pluk et al., 1999). Both studies relied on relatively crude assembly of RNPs, in which human Pop1 protein might have played a role in assembly, but not contacted the RNA in such a way as to crosslink. Indeed, in the latter study it was concluded that Pop1 protein bound in a way that required the P3 domain, but that there was no evidence that the interaction was direct. The present data suggests that Pop1 can interact directly with the RNA subunit in a fashion that requires conserved P3 positions. One way to reconcile these data with the previous MRP experiments is to propose that both Pop1p and smaller subunits interact with P3, but that Pop1 does not crosslink well to the RNA. Alternatively, it is possible that protein subunits do not interact in a directly analogous manner between the yeast RNase P RNA and the human RNase MRP RNA. Whichever is the case, the ability of Pop1p to specifically bind the RNA subunit suggests that this recognition might be a key early element in assembling the full RNP complex. Continued efforts are in progress to use biochemical reconstitution of the both enzymes from purified components to address these issues.

MATERIALS AND METHODS

Phylogenetic analysis of RNase P and RNase MRP P3 RNA domains

The P3 RNA domains from RNase P and RNase MRP were compared to provide a consensus structure. P3 domains from the following species were used: *S. cerevisiae* and *Schizosaccharomyces pombe* RNase P (Tranguch & Engelke, 1993); human, mouse, and *Xenopus laevis* RNase P (Pitulle et al., 1998); *S. pombe* RNase MRP (Paluh & Clayton, 1995); *S. cerevisiae*, human, mouse, and *X. laevis* RNase MRP (Schmitt et al., 1993). Secondary structure is conserved throughout all species P3 domains in RNase P and RNase MRP; however there is very little primary sequence conservation among distant species (Fig. 1B). Most intraspecies conservation between P3 domains from RNases P and MRP is located in the 5′ side of the internal loop. In the case of *S. cerevisiae*, there are seven internal loop P3 nucleotides that are identical in RNase P and RNase MRP (Fig. 1B). However, four of the seven (U38, U39, C41, and A44) are also conserved between six different yeast species RNase P P3 domains previously sequenced (Tranguch & Engelke, 1993), suggesting the identity of these four nucleotides is important for the function of the P3 domain in yeast.

Generation of P3 *RPR1* library by directed degenerate DNA oligomers

The *S. cerevisiae* RNase P RNA gene (*RPR1*) was randomized at positions U38, U39, C41, and A44 using the polymerase chain reaction and DNA oligomers degenerate at the four nucleotide positions. The starting wild-type *RPR1* clone contains a phenotypically silent insert of 20 extra nucleotides at the end of the stem loop between positions 133 and 139 that was used as a hybridization tag for mutated RNAs. This silent insertion has been tested previously (Bertrand et al., 1998) and is shown in Figure 1. The library was constructed in a single copy (CEN) vector with a *LEU* selectable marker as previously described (Pagan-Ramos et al., 1996b) and transformed into the haploid yeast strain JLY1 (MATa *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 RPR1::HIS3*). JLY1 has the essential *RPR1* chromosomal gene deleted and replaced by *HIS3* (Lee et al., 1991b) and a wild-type *RPR1* on a counterselectable *URA3*-marked plasmid (Pagan-Ramos et al., 1996b). Transformation of this strain with the mutated *RPR1* library was followed by screening for clones that were viable when the wild-type *RPR1* gene was removed by growth media containing 5-fluororotic acid (Pagan-Ramos et al., 1996b). The four randomized positions allow for 256 possible variants; 1,000 clones were screened yielding 193 viable P3 library clones. Each viable clone was tested for temperature (37 °C) and cold (18 °C) sensitivity. Temperature sensitivity was scored as zero growth under nonpermissive conditions. There were 37 temperature-sensitive clones, two of which were also cold sensitive. Sequence, frequency, and temperature sensitivity of 89 viable clones are listed in Figure 2. A wild-type *RPR1* gene carried on the identical plasmid used to produce the library provided a control for growth and RNA profile characterizations.

RNA blot hybridization of pre-tRNA^{Leu3}

Effects of P3 mutations on RNase P enzymatic activity were determined by probing whole cellular RNA with a radiolabeled anti-tRNA^{Leu3} DNA oligonucleotide. Total cellular RNA was isolated from temperature-sensitive clones grown at permissive (30 °C) or nonpermissive temperatures (37 °C) for 4 h (Kohrer & Domdey, 1991). Concentration of isolated RNA was determined by measuring UV absorbance at 260 nm and 6-μg sample quantities were run on denaturing polyacrylamide gels, transferred to Nytran membrane and probed using a radiolabeled DNA oligonucleotide complementary to Leu3 tRNA (5′-^{32}P-CTTAGACCGCTCGGCCAAAC-3′). A radiolabeled DNA oligonucleotide complementary to signal recognition particle RNA (*SCR1* RNA, 5′-^{32}P-GGCGTGCAAT CCGTGTCT-3′) was also used to probe the blots to normalize hybridization signal. RNA blot hybridization profiles were examined for changes characteristic of RNase P enzymatic activity defects (Lee et al., 1991b). These changes include an accumulation of 5′ unprocessed pre-tRNA, 5′ unprocessed with mature 3′ end, and 5′ unprocessed with spliced intron tRNA species.

RNA blot hybridization of RNase P RNA

Whole cellular RNA from temperature-sensitive mutants grown at permissive and nonpermissive conditions was probed with a radiolabeled anti-RPR1 DNA oligonucleotide. The probe is complementary to nt 310–327 of *S. cerevisiae* RNase P RNA (5′-GACGTCCTACGATTGCAC-3′). Electrophoresis and hybridization were as previously described (Pagan-Ramos et al.,

1996a). Holoenzyme assembly defects result in an accumulation of precursor RNase P RNA as previously described (Pagan-Ramos et al., 1996a). The same radiolabeled anti-*SCR1* RNA DNA oligonucleotide used in the pre-tRNA^{Leu3} blots was used as a hybridization control.

Three-hybrid test for RNA–protein interactions

The three-hybrid test was performed as previously described (SenGupta et al., 1996). The wild-type and mutant forms of *RPR1* RNA were cloned into the *Sma*I restriction site of pIIIA-MS2-2 in which an RNA polymerase III leader/promoter (from the *RPR1* gene) directs transcription of the hybrid RNA containing two tandem MS2 sites and the *RPR1* terminator. The pIIIA-MS2-2 plasmid is a modified version of pIIIMS2-2 containing the *ADE2* gene. The open reading frame of Pop1p was cloned between the *Xma*I and *Xho*I restriction sites of the pACTII plasmid, which produces a hybrid protein linked to the GAL4 activation domain. The L40-coat yeast strain (*Mat***a**, *ura3-52*, *leu2-3*, *his3*Δ*200*, *trp1*Δ*1*, *ade2*, *LYS2*::(lexAop)-*HIS3*, *ura3*::(lexA-op)-*lacZ*, *LexA-MS2 coat(TRP1)*) was transformed with the pIIIA-MS2-2 plasmid containing no RNA insert or wild-type or mutant forms of the *RPR1* RNA and pACTII-Pop1p ORF. Double transformants were selected for their ability to grow on SD-ura-lys-trp-leu media. Transformants were then tested for their ability to grow on SD-ura-lys-trp-leu-his media in the absence or presence of 1-mM, 5-mM, 10-mM, and 20-mM 3-aminotriazole (Sigma). Double transformants were also tested for their ability to produce β-galactosidase in the presence of 5-bromo-4-chloro-3-indolyl β-galactoside by colony color assay on nitrocellulose filters. The pIIIA-MS2-2 and pACTII plasmids as well as the L40-coat yeast strain were the kind gifts of the M. Wickens laboratory.

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REFERENCES

Altman S, Baer MF, Bartkiewicz M, Gold H, Guerrier Takada C, Kirsebom LA, Lumelsky N, Peck K. 1989. Catalysis by the RNA subunit of RNase P—A minireview. *Gene 82*:63–64.

Bertrand E, Houser-Scott F, Kendall A, Singer RH, Engelke DR. 1998. Nucleolar localization of early tRNA processing. *Genes & Dev 12*:2463–2468.

Chamberlain JR, Lee Y, Lane WS, Engelke DR. 1998. Purification and characterization of the nuclear RNase P holoenzyme complex reveals extensive subunit overlap with RNase MRP. *Genes & Dev 12*:1678–1690.

Chamberlain JR, Pagán-Ramos E, Kindelberger DW, Engelke DR. 1996a. An RNase P RNA subunit mutation that affects ribosomal RNA processing. *Nucleic Acids Res 24*:3158–3166.

Chamberlain JR, Tranguch AJ, Pagan-Ramos E, Engelke DR. 1996b. Eukaryotic nuclear RNase P: Structures and functions. *Prog Nucleic Acid Res & Mol Biol 55*:87–119.

Chu S, Zengel JM, Lindahl L. 1997. A novel protein shared by RNase MRP and RNase P. *RNA 3*:382–391.

Dichtl B, Tollervey D. 1997. Pop3p is essential for the activity of the RNase MRP and RNase P ribonucleoproteins in vivo. *EMBO J 16*:417–429.

Eder PS, Kekuda R, Stolc V, Altman S. 1997. Characterization of two scleroderma autoimmune antigens that copurify with human ribonuclease P. *Proc Natl Acad Sci USA 94*:1101–1106.

Forster AC, Altman S. 1990. Similar cage-shaped structures for the RNA components of all ribonuclease P and ribonuclease MRP enzymes. *Cell 62*:407–409.

Gold HA, Topper JN, Clayton DA, Craft J. 1989. The RNA processing enzyme RNase MRP is identical to the Th RNP and related to RNase P. *Science 245*:1377–1380.

Jacobson MR, Cao LG, Taneja K, Singer RH, Wang YL, Pederson T. 1997. Nuclear domains of the RNA subunit of RNase P. *J Cell Sci 110*:829–837.

Jacobson MR, Cao LG, Wang YL, Pederson T. 1995. Dynamic localization of RNase MRP RNA in the nucleolus observed by fluorescent RNA cytochemistry in living cells. *J Cell Biol 131*:1649–1658.

Kohrer K, Domdey H. 1991. Preparation of high molecular weight RNA. *Methods Enzymol 194*:398–405.

Krupp G, Cherayil B, Frendewey D, Nishikawa S, Söll D. 1986. Two RNA species co-purify with RNase P from the fission yeast *Schizosaccharomyces pombe*. *EMBO J 5*:1697–1703.

Lee B, Matera AG, Ward DC, Craft J. 1996. Association of RNase mitochondrial RNA processing enzyme with ribonuclease P in higher ordered structures in the nucleolus: A possible coordinate role in ribosome biogenesis. *Proc Natl Acad Sci USA 93*:11471–11476.

Lee JY, Evans CF, Engelke DR. 1991a. Expression of RNase P RNA in *Saccharomyces cerevisiae* is controlled by an unusual RNA polymerase III promoter. *Proc Natl Acad Sci USA 88*:6986–6990.

Lee JY, Rohlman CE, Molony LA, Engelke DR. 1991b. Characterization of *RPR1*, an essential gene encoding the RNA component of *Saccharomyces cerevisiae* nuclear RNase P. *Mol Cell Biol 11*:721–730.

Lindahl L, Fretz S, Epps N, Zengel JM. 2000. Functional equivalence of hairpins in the RNA subunits of RNase MRP and RNase P in *Saccharomyces cerevisiae*. *RNA 6*:653–658.

Liu MH, Yuan Y, Reddy R. 1994. Human RNaseP RNA and nucleolar 7-2 RNA share conserved 'To' antigen-binding domains. *Mol Cell Biochem 130*:75–82.

Lygerou Z, Allmang C, Tollervey D, Séraphin B. 1996. Accurate processing of a eukaryotic precursor ribosomal RNA by ribonuclease MRP in vitro. *Science 272*:268–270.

Lygerou Z, Mitchell P, Petfalski E, Seraphin B, Tollervey D. 1994. The *POP1* gene encodes a protein component common to the RNase MRP and RNase P ribonucleoproteins. *Genes & Dev 8*:1423–1433.

Matera AG, Frey MR, Margelot K, Wolin SL. 1995. A perinucleolar compartment contains several RNA polymerase III transcripts as well as the polypyrimidine tract-binding protein, hnRNP I. *J Cell Biol 129*:1181–1193.

Morrissey JP, Tollervey D. 1995. Birth of the snoRNPs: The evolution of RNase MRP and the eukaryotic pre-rRNA-processing system. *Trends Biochem Sci 20*:78–82.

Morse DP, Schmidt FJ. 1993. Suppression of loss-of-function mutations in *Escherichia coli* ribonuclease P RNA (M1 RNA) by a specific base-pair disruption. *J Mol Biol 230*:11–14.

Pace NR, Smith D. 1990. Ribonuclease P: Function and variation. *J Biol Chem 265*:3587–3590.

Pagan-Ramos E, Lee Y, Engelke DR. 1996a. A conserved RNA motif involved in divalent cation utilization by nuclear RNase P. *RNA 2*:1100–1109.

Pagan-Ramos E, Lee Y, Engelke DR. 1996b. Mutational analysis of *Saccharomyces cerevisiae* nuclear RNase P: Randomization of universally conserved positions in the RNA subunit. *RNA 2*:441–451.

Pagan-Ramos E, Tranguch AJ, Kindelberger DW, Engelke DR. 1994. Replacement of the *Saccharomyces cerevisiae RPR1* gene with heterologous RNase P RNA genes. *Nucleic Acids Res 22*: 200–207.

Paluh JL, Clayton DA. 1995. *Schizosaccharomyces pombe* RNase MRP RNA is homologous to metazoan RNase MRP RNAs and may provide clues to interrelationships between RNase MRP and RNase P. *Yeast 11*:1249–1264.

Pitulle C, Garcia-Paris M, Zamudio KR, Pace NR. 1998. Comparative structure analysis of vertebrate ribonuclease P RNA. *Nucleic Acids Res 26*:3333–3339.

Pluk H, van Eenennaam H, Rutjes SA, Pruijn GJ, van Venrooij WJ. 1999. RNA–protein interactions in the human RNase MRP ribonucleoprotein complex. *RNA 5*:512–524.

Reddy R, Tan EM, Henning D, Nohga K, Busch H. 1983. Detection of a nucleolar 7-2 ribonucleoprotein and a cytoplasmic 8-2 ribonucleoprotein with autoantibodies from patients with scleroderma. *J Biol Chem 258*:1383–1386.

Schmitt ME. 1999. Molecular modeling of the three-dimensional architecture of the RNA component of yeast RNase MRP. *J Mol Biol 292*:827–836.

Schmitt ME, Bennett JL, Dairaghi DJ, Clayton DA. 1993. Secondary structure of RNase MRP RNA as predicted by phylogenetic comparison. *FASEB J 7*:208–213.

Schmitt ME, Clayton DA. 1992. Yeast site-specific ribonucleoprotein endoribonuclease MRP contains an RNA component homologous to mammalian RNase MRP RNA and essential for cell viability. *Genes & Dev 6*:1975–1985.

SenGupta DS, Zhang B, Kramer B, Pochart P, Fields S, Wickens M. 1996. A three-hybrid system to detect RNA–protein interactions in vivo. *Proc Natl Acad Sci USA 93*:8496–8501.

Shadel GS, Buckenmeyer GA, Clayton DA, Schmitt ME. 2000. Mutational analysis of the RNA component of *Saccharomyces cerevisiae* RNase MRP reveals distinct nuclear phenotypes. *Gene 245*:175–184.

Stolc V, Altman S. 1997. *Rpp1*, an essential protein subunit of nuclear RNase P required for processing of precursor tRNA and 35S precursor rRNA in *Saccharomyces cerevisiae*. *Genes & Dev 11*:2414–2425.

Tranguch AJ, Engelke DR. 1993. Comparative structural analysis of nuclear RNase P RNAs from yeast. *J Biol Chem 268*: 14045–14055.

Tranguch AJ, Kindelberger DW, Rohlman CE, Lee JY, Engelke DR. 1994. Structure-sensitive RNA footprinting of yeast nuclear ribonuclease P. *Biochemistry 33*:1778–1787.

Yuan Y, Tan E, Reddy R. 1991. The 40-kilodalton T0 autoantigen associates with nucleotides 21 to 64 of human mitochandrial RNA processing/7-2 RNA in vitro. *Mol Cell Biol 11*:5266–5274.

Ziehler WA, Day JJ, Fierke CA, Engelke DR. 2000. Effects of 5′ leader and 3′ trailer structures on pre-tRNA processing by nuclear RNase P. *Biochemistry 39*:9909–9916.