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Characterization of *RPR1*, an Essential Gene Encoding the RNA Component of *Saccharomyces cerevisiae* Nuclear RNase P

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RNA components have been identified in preparations of RNase P from a number of eucaryotic sources, but final proof that these RNAs are true RNase P subunits has been elusive because the eucaryotic RNAs, unlike the procaryotic RNase P ribozymes, have not been shown to have catalytic activity in the absence of protein. We previously identified such an RNA component in *Saccharomyces cerevisiae* nuclear RNase P preparations and have now characterized the corresponding, chromosomal gene, called *RPR1* (RNase P ribonucleoprotein 1). Gene disruption experiments showed *RPR1* to be single copy and essential. Characterization of the gene region located *RPR1* 600 bp downstream of the *URA3* coding region on chromosome V. We have sequenced 400 bp upstream and 550 bp downstream of the region encoding the major 369-nucleotide *RPR1* RNA. The presence of less abundant, potential precursor RNAs with an extra 84 nucleotides of 5′ leader and up to 30 nucleotides of 3′ trailing sequences suggests that the primary *RPR1* transcript is subjected to multiple processing steps to obtain the 369-nucleotide form. Complementation of *RPR1*-disrupted haploids with one variant of *RPR1* gave a slow-growth and temperature-sensitive phenotype. This strain accumulates tRNA precursors that lack the 5′ end maturation performed by RNase P, providing direct evidence that *RPR1* RNA is an essential component of this enzyme.

RNase P from both procaryotic and eucaryotic sources is an endonuclease that cleaves pre-tRNA substrates to yield mature 5′ termini. In procaryotes, both the RNA and protein components of the ribonucleoprotein enzymes are required for in vivo activity (15, 27, 29, 33, 43, 49, 57, 58), but the RNA component alone is capable of efficiently catalyzing the correct reaction under some conditions in vitro (3, 18, 21, 22, 52). Extensive phylogenetic sequence comparisons of these RNAs (30), combined with folding energy calculations and cleavage sensitivity studies (6, 19, 51, 52), suggest a conserved, highly ordered secondary structure. Most if not all of the key contacts with the substrates depend on this structure, with the protein contributing to efficiency through secondary effects such as charge shielding between RNA chain phosphate backbones. The mechanism by which pre-tRNA substrates are recognized is not clearly understood, however, since there are no obvious regions of required Watson-Crick base pairing between the enzyme and substrate RNAs (20, 36).

Studies of eucaryotic RNase Ps have suggested that they also contain essential RNA subunits, although the role of the RNA components has not been firmly established. RNAs that copurify with both nuclear and organelle RNase Ps have been characterized (1, 7, 9, 11, 12, 14, 24, 32, 34, 35, 37, 38, 40, 42) and shown to have sequence and structural similarities to each other and to a lesser degree to the procaryotic RNAs (5, 37). The identification of the eucaryotic RNAs as essential RNase P subunits has not been rigorously confirmed, however, since there are no reports of the eucaryotic RNAs having enzyme activity in the absence of protein and some studies report RNase Ps with no essential RNA component discernible (9, 63).

We previously reported the sequence of a 369-nucleotide RNA that copurifies with nuclear RNase P activity from *Saccharomyces cerevisiae*. In this report, a single-copy, essential gene encoding this RNA is identified and characterized. Disruption of this gene, termed *RPR1* (RNase P ribonucleoprotein 1), and complementation with a plasmid-borne variant defective for growth leads to accumulation of tRNA precursors lacking the 5′ RNase P cleavage. This observation confirms the identity of *S. cerevisiae RPR1* RNA as an essential component of RNase P and suggests that similar RNAs isolated from other eucaryotes are also bona fide RNase P components.

MATERIALS AND METHODS

Strains and genetic methods. *S. cerevisiae* W3031A (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) and the isogenic strain W3031B (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) were obtained from E. Esposito (originally R. Rothstein) and used for gene disruption, complementation analysis, and Southern and Northern (RNA) analyses. Strains BJ411 (*MATα his1 prb1-1122 pre1-126*), previously referred to as EJ101 (31; gift of E. Jones), and PP1002 (*MATα ade2 leu2-3,112 pep4-3 rna3 rna82*) (46; gift of P. Piper) were used for preparation of RNase P and end analysis of the *RPR1* RNA transcript. Strain YPH149 (*MATα ade his7 lys2 ura3-52 trp1-d1::TRP1*$^+$) was obtained from P. Hieter and used for chromosome mapping. Yeast transformation was performed by the lithium acetate procedure (28). Sporulation and tetrad analysis were performed by using standard genetic methods (59). Cells were grown in selection medium which contained 0.67% yeast nitrogen base without amino acids, 2% dextrose and amino acids for selection, or YPD medium (2% dextrose, 2% peptone, 1% yeast extract).

Cloning of the *RPR1* gene. A yeast genomic DNA library in centromere-containing plasmids (YCp50 library) was obtained from D. Thiele (54; originally from D. Botstein and G. Fink) and was screened by colony hybridization (2). The probe used in this screening was prepared by in vitro transcription of an antisense *RPR1* cDNA clone with T7

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RNA polymerase in the presence of [α-^{32}P]UTP (10). Several clones were chosen from 23 positive clones and were subsequently used for restriction mapping and subcloning. A 1.3-kb *Bgl*II-*Hin*dIII fragment was subcloned in pUC19, and both strands were sequenced by the dideoxynucleotide chain termination method, using modified T7 DNA polymerase (26) and oligonucleotide primers that hybridize at intervals across the *RPR1* region (37). T7 RNA polymerase (10) and modified T7 DNA polymerase (62; gift of J. Huibregtse) were prepared as described previously. Primers for sequencing were prepared by the Center for Molecular Genetics Core Facility at the University of Michigan School of Medicine.

Gene disruption and Southern analysis. A 1.75-kb *Bam*HI fragment containing the *HIS3* gene was isolated from YCp407 (39) by using agarose gel electrophoresis and electroelution (41). The isolated fragment was then inserted into the *Bam*HI site of *RPR1* genomic clone. W303 diploid cells were transformed with the linearized *HIS3*-interrupted *RPR1* genomic DNA clone, and transformants were selected on defined medium lacking histidine (55). The resulting transformants were sporulated and dissected for tetrad analysis. DNAs were isolated from the two haploids (W3031A and W3031B), the parental diploid (W303), and the heterozygous diploid, with a single *RPR1* disruption (59). The DNAs were digested with *Stu*I, separated in a 1% agarose gel, and transferred to a nylon membrane (41). The membrane was then probed with a [α-^{32}P]UTP-labeled antisense *RPR1* RNA transcript as previously described (37, 59).

Mapping of the *RPR1* gene. Strain YPH149 was grown to an optical density at 650 nm (OD_{650}) of 0.4. Cells were harvested and embedded in agarose gel to make a chromosomal DNA plug as described by Southern et al. (60). Chromosomes were separated in a 1.0% agarose gel at 14°C by pulsed-field electrophoresis, using a Bio-Rad CHEF-DRII apparatus. The switch time was 70 s for 15 h and 120 s for an additional 12 h. Seventeen chromosomes were visible after staining with ethidium bromide. Chromosomes were partially digested in the gel by soaking twice in 0.25 M HCl for 15 min and then transferred to a nylon membrane by the standard capillary method (41). The membrane was then probed with labeled antisense *RPR1* RNA (37, 59). The *RPR1* probe hybridized only to chromosome V (data not shown). It was subsequently found by Riles and Olson that the *RPR1* gene was localized to the restriction fragment containing the *URA3* gene on chromosome V (52a). Comparison of the restriction map of the *RPR1* 8-kb genomic DNA clone with that published for the *URA3* region (53) and sequencing of the *RPR1* clone downstream of the *Hin*dIII site confirmed the relative positions of the two genes (Fig. 1).

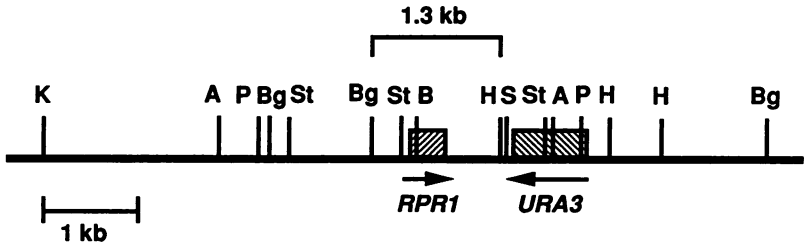
Plasmid construction. The 400-nucleotide 5′-flanking region and 550-nucleotide 3′-flanking region were amplified by the polymerase chain reaction using *Taq* polymerase (56) and inserted into the *Bam*HI and *Hin*dIII sites of the single-copy centromere vector YCp50 or the 2μm-based multicopy vector YEp24. The resulting constructs contained an *Eco*RI site at the junction of the two flanking regions. *RPR1* cDNA, *Escherichia coli* RNase P M1 cDNA (50), human RNase P H1 cDNA (4; gift of S. Altman), *S. cerevisiae* mitochondrial RNase P RNA cDNA, or the *RPR1* cDNA dimer was first ligated with *Eco*RI linker and inserted into this *Eco*RI site. *S. cerevisiae* mitochondrial RNase P RNA cDNA was prepared by amplification with two synthetic primers and *Taq* polymerase (56) on a genomic clone (42; gift of C. Wise and N. Martin). In an attempt to obtain conditionally expressed clones, various RNase P RNA cDNAs were inserted adjacent to a *GAL10* promoter or between a *CUP1* promoter and a *CYC1* terminator in either a single-copy centromere vector or a 2μm-based multicopy vector. These plasmid constructs were used to test for complementation of the disrupted chromosomal *RPR1* gene through transformation of the diploid strain heterozygous for the disruption and tetrad analysis of the resulting transformants.

Northern analysis of *RPR1* RNA and tRNA. The wild-type haploid strain (W3031A), an *RPR1*-disrupted haploid strain complemented with multiple copies of *RPR1* cDNA construct, and an *RPR1*-disrupted haploid strain complemented with multiple copies of *RPR1* cDNA dimer construct were grown in YPD medium overnight at 30°C. Fresh YPD medium was then inoculated with these overnight cultures to an initial OD_{650} of 0.05 and grown at 30 and 38°C. Cell growth was monitored spectrophotometrically every 2 h to determine doubling time. One milliliter (approximately 1.2×10^8 cells) of the cultures was harvested at an OD_{650} of 1.0 and used for preparation of total RNA (23). All RNA samples were separated in a 6% denaturing acrylamide gel and transferred to a nylon membrane, using a semidry electroblotter (Gelman Sciences, Inc.) under conditions specified by the manufacturer. The RNA was then cross-linked to the membrane by UV exposure (Fotodyne UV light mode 3-3000) for 3 min. The membrane was probed with [α-^{32}P]UTP-labeled antisense *RPR1* RNA or labeled antisense $tRNA^{Leu3}$ (37, 59). For in vitro digestion of RNA with nuclear RNase P, a highly purified fraction from sucrose gradient centrifugation was used as previously described (37).

5′ end analysis. The 5′ end of *RPR1* transcripts was determined by using primer extension from a labeled antisense oligonucleotide that hybridized to nucleotides 33 to 48 of the *RPR1* coding region (Fig. 1). Both total yeast RNA and purified RNase P holoenzyme RNA were tested and gave essentially the same results: a major product corresponding to the mature 5′ end and a minor product at −84 corresponding to a potential precursor (data not shown). This is consistent with our previous results showing that a small amount of a longer *RPR1* RNA copurifies with the major 369-nucleotide RNA and RNase P holoenzyme. Total yeast RNA (10 μg) was combined with reverse transcriptase buffer, and extensions were performed as previously described (25). Both primer annealling and the extension reactions were carried out at 44°C. Stop mix (4 μl of 2% sodium dodecyl sulfate, 0.1 M EDTA, 1 mg of proteinase K per ml) was added, and the mixture was incubated for 10 min at 44°C. After precipitation, the reaction was resuspended in formamide loading buffer, heated at 95 to 100°C for 2 min, and separated on an 8% denaturing acrylamide gel. The positions of the primer extension stops were determined by direct comparison with dideoxynucleotide chain termination reactions on a linearized *RPR1* genomic clone, using the same labeled primer (26).

S1 analysis. A probe for determination of the 3′ end of the *RPR1* transcripts through protection from S1 nuclease was generated from an *RPR1*-containing *Rsa*I fragment cloned into the *Eco*RI polylinker site of pUC19 with the use of *Eco*RI linkers. The DNA was linearized at the unique *Sty*I site in the coding region of the *RPR1* gene, and the 5′ overhangs were end filled with [α-^{32}P]dCTP, using standard techniques (41). The 330-bp 3′ end probe containing the antisense strand labeled at the *Sty*I site was then removed from the pUC19 vector by cleavage with *Eco*RI. The radiolabeled strand was isolated by electrophoresis through a denaturing 6% polyacrylamide gel and electroelution (41). S1

A



B

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     BglII                                          SpeI
-407 agatctgcca attgaacata acatggtagt tacatatact agtaatatgg ttcggcacac

-347 attaaaagta taaaaactat ctgaattacg aattacatat attggtcata aaaatcaatc

-287 aatcatcgtg tgttttatat gtctcttatc taagtataag aatatccata gttaatattc
                                                HpaI
-227 acttacgcta ccttttaacc tgtaatcatt gtcaacaggt atgttaacga cccacattga
                                          NaeI
-167 taaacgctag tatttctttt tcctcttctt attggccggc tgtctctata ctcccctata
                                    StuI          A Box
-107 gtctgtttct tttcgtttcg attGTTTTAC GTTTGAGGCC TCGTGGCGCA CATGGTACGC
                                                1
 -47 TGTGGTGCTC GCGGCTGGGA ACGAAACTCT GGGAGCTGCG ATTGGCAGTG GAACAGTGGT
                                    BamHI
  14 AATTCCTACG ATTAAGAAAC CTGTTTACAG AAGGATCCCC ACCTATGGGC GGGTTATCAG

  74 ATATTATCAG GTGGGAAATT CGGTGGAACA CAGTGGAGCC TTGTCCTCCG GGTTAATGTC

 134 GCTTTTGGCA TTGGCCCCTG CTCCTGAGAG AAGAAATATA CTGGGGAACC AGTCTTTACC
                                    StyI  B Box
 194 GACCGTTGTT ATCAGAAATT CACGGAGTTC GGCCTAGGTC GGACTCCGAT GGGAACGGCA

 254 ACGGTTGTTC CGTTTGACTT GTCGCCCGCT ACGGCGTGAG CGTCAAGGTC TGTTGAGTGC
          AatII                                 B Box  369
 314 AATCGTAGGA CGTCATTAGT GGCGAACCCG ATACCGATTA CTGCTGCTGT TCCAGCCCAT

 374 ATCCAACTTC CAATTTAATC TTTCTTtttt aattttcact tatttgcgat acagaaagaa

 434 aaaagcgata gtaactattg aattttgttt ggatttggtt agattagata tggtttctct

 494 ttatatttac atgctaaaaa tgggctacac cagagataca taattagata tatatacgcc
                                    StyI
 554 agtacacctt atcggcccaa gccttgtccc aaggcagcgt tttgttcttg gaaacgctgc

 614 cctacacgtt cgctatgctt caagaacttt tctgagcact tcatgatgca tgtttgttcc

 674 ttattggtta gctttgatgt tgtgaagtca ttgacacagt ctgtgaaaca tctttctacc

 734 agattagagt acaaacgcat gaaatcctc atttgctttt gttccactac tttttggaac

 794 tcttgttgtt ctttcggagt tcaatgcgtc catctttaca gtcctgtctt attgttcttg

 854 atttgtgccc cgtaaaatac tgttacttgg ttctggcgag gtattggata gttccttttt
          HindIII
 914 ataaaggcca tgaagctt
```

FIG. 1. Location and sequence of the *RPR1* gene region. (A) Location of the *RPR1* gene. The *RPR1* gene was located on chromosome V immediately adjacent to *URA3* as described in Materials and Methods. A partial restriction endonuclease map of the region and the relative orientations of the *RPR1* and *URA3* coding regions are shown. Restriction sites: A, *Apa*I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I; St, *Stu*I. (B) Sequence of the *RPR1* region sense strand between the *Bgl*II and *Hin*dIII sites. Positions are numbered relative to the first nucleotide in the major *RPR1* RNA (+1). The sequence corresponding to the major RNA is underlined, and the sequence corresponding to the longest of the less prevalent longer RNAs (Fig. 6) is indicated by capital letters (Fig. 2). Sequences corresponding to suspected RNA polymerase III A box and B box internal promoters are indicated by overlining (see Discussion). The *Sty*I site used to prepare the 3′ end S1 probe is indicated.

nuclease analysis was then performed as previously described (2). Size markers for the S1-protected fragments were unrelated dideoxynucleotide chain termination reactions in adjacent lanes (not shown).

Nucleotide sequence accession number. The GenBank accession number for the *RPR1* region sequence is M27035.

RESULTS

Antisense transcripts of the cDNA encoding *S. cerevisiae* RNase P-associated RNA (37) were used to screen an *S. cerevisiae* genomic library in YCp50 by filter hybridization (2). Twenty-three positive clones that encompassed the cDNA sequence were identified. A partial restriction endonuclease map of a 6-kb region surrounding the gene is shown in Fig. 1A. In preparation for obtaining the genetic map location of the locus, referred to subsequently as *RPR1* (RNase P ribonucleoprotein 1), the cDNA was mapped to chromosome V by probing chromosomal blots after pulsed-field gel electrophoresis (60; data not shown). Before classical genetic mapping of the clone could be accomplished, however, localization by hybridization to chromosomal restriction digest blots indicated that *RPR1* was close to *URA3* (52a). Comparison of the restriction map of the genomic *RPR1* clones with the published map of the *URA3* (53) locus suggested that the two genes might be adjacent, a possibility which has been confirmed by extending the sequence of the *RPR1* region (Fig. 1B; see below) downstream past the *Hin*dIII site and encountering the previously published sequence downstream of *URA3* (53).

The sequences of both strands of DNA in the region shown in Fig. 1B were obtained by direct analysis of a pUC19 subclone containing a 1.3-kb *Bgl*II-*Hin*dIII fragment, using sequential oligonucleotide primers and dideoxynucleotide chain termination methods for double-stranded templates (26). The positions corresponding to the major *RPR1* RNA are underlined. A larger RNA that also copurifies with RNase P in relatively small quantities and hybridizes to the *RPR1* probes (37) has also been characterized (Fig. 2), and the extent of the larger RNA is indicated by capital letters in Fig. 1B. Previous work indicated that the *RPR1* RNA and DNA sequences were colinear (37), suggesting that the larger RNA was longer at the 5′ or 3′ terminus than the 369-nucleotide major RNA and might be a precursor. The 5′ and 3′ termini of the larger RNA were mapped by primer extension and S1 nuclease analysis, respectively, in total cellular RNA from several *S. cerevisiae* strains used in this and a previous study (37). Extension was performed from a radiolabeled primer that anneals 32 nucleotides downstream of the 5′ terminus of the smaller RNA (Fig. 1B). In all three strains, there were multiple extension stops corresponding to the slightly heterogeneous 5′ end of the major 369-nucleotide RNA and also a significant amount of extension product corresponding to a transcript with 84 nucleotides of additional 5′ sequence (Fig. 2). S1 nuclease mapping of the 3′ end of the RNA showed a major product corresponding to the unique 3′ end of the 369-nucleotide RNA but also lesser amounts of RNAs ranging from 16 to 30 nucleotides longer (Fig. 2). In strains BJ411 and W3031A, the longer products appeared as clusters roughly 16 to 19 nucleotides longer than the major 3′ terminus of the 369-nucleotide RNA. We are unable to tell whether the appearance of multiple bands reflects RNA heterogeneity or is an artifact of the S1 assay. Strain PP1002, however, harbors an *rna82* lesion that causes aberrant 3′ end processing in 5S and tRNA RNA polymerase III transcripts (46–48). In this strain, a faint signal corresponding to an additional 28 to 30 nucleotides past the 369-nucleotide signal was seen in some but not all experiments. The data chosen for Fig. 2, for example, show no obvious longer RNA, whereas the data in Fig. 6 do.

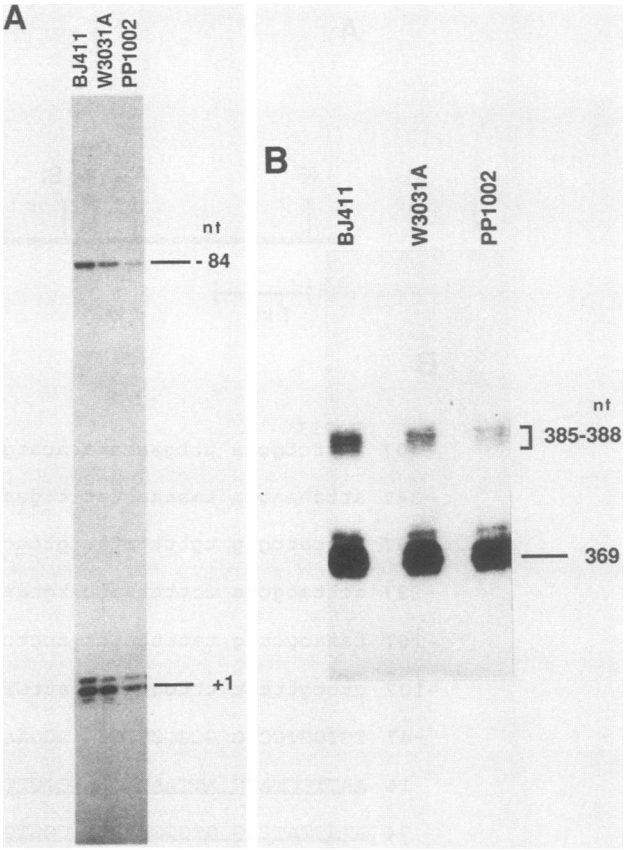

FIG. 2. 5′ and 3′ end analysis of *RPR1* RNAs. (A) Primer extension analysis was performed as described in Materials and Methods with 10 μg of RNA from each of the indicated haploid yeast strains and a radiolabeled primer that anneals to positions 33 to 48 in the *RPR1* sequence. (B) S1 nuclease analysis was performed with a DNA fragment labeled on the antisense strand at the *Sty*I site as described in Materials and Methods. The exact 3′ terminus of the major RNA was determined previously (37). (Low levels of slightly longer 3′ termini were observed in some experiments [e.g., Fig. 6] but were not dependent on the concentration of S1 nuclease within an experiment.) nt, Nucleotides.

We have not yet been able to demonstrate that the longer RNAs are the primary transcripts of the *RPR1* gene or that they are precursors of the major form. If such a conversion is taking place, however, the lack of stable intermediates at the 5′ end suggests that a single step might be involved, whereas multiple 3′ reactions are suggested by the results with PP1002 and alternate constructs described below (see Fig. 6). This pattern is interesting in that it resembles the pattern of pre-tRNA processing, and there is preliminary evidence that RNase P RNAs are synthesized by RNA polymerase III (4; see Discussion). Given this possibility, potential A box and B box intragenic promoter elements for RNA polymerase III (16) have been indicated in Fig. 1B and will be discussed below. Each has about 80% sequence similarity to the corresponding promoter consensus (16).

Gene disruption was performed by the strategy outlined in Fig. 3 to confirm that the gene is single copy, to examine

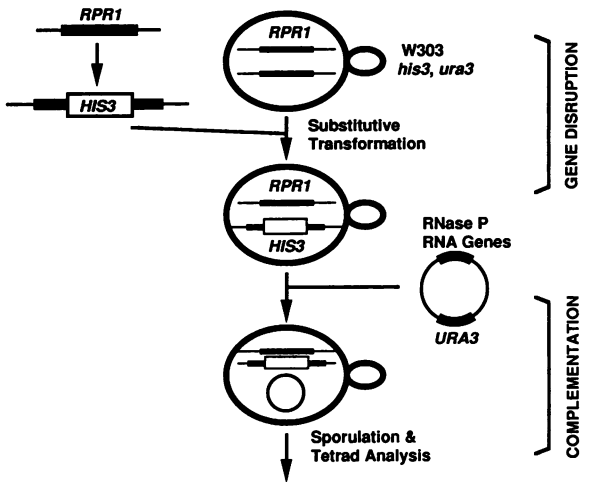

FIG. 3. Scheme for *RPR1* gene disruption and complementation. Procedural details are described in Materials and Methods.

whether the gene product is essential, and to prepare an *rpr1* mutant background in which the phenotype of defective *RPR1* gene constructs could be tested. *HIS3* insertion into diploids was selected by growth on medium lacking histidine, followed by sporulation and tetrad dissection. Only two spores per tetrad were viable (Fig. 4A), and viable spores were not His^+ (not shown), indicating that *HIS3*

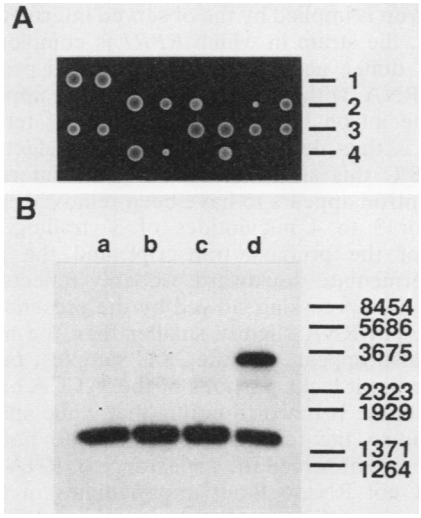

FIG. 4. Analyses of diploids with *RPR1* gene disruptions. Diploids selected for *URA3* plasmid acquisition in the *RPR1* gene disruption scheme (Fig. 3) were sporulated, and tetrads were dissected. (A) Growth of dissected spores on rich medium showed 2:2 segregation expected from disruption of an essential gene. 1, 2, 3, and 4 refer to the positions of the four spores from each of the eight tetrads shown. (B) Southern analyses were performed with an antisense *RPR1* RNA probe and DNAs from the wild-type haploid strains [lane a, W3031A(**a**); lane b, W3031B(α)], a wild-type diploid strain (lane c, W303), and a diploid strain heterozygous for an *RPR1* gene disruption (lane d) after cleavage with *Stu*I. Sizes of the *RPR1* fragments were 1.5 kb in the two wild-type haploid strains (lanes a and b) and wild-type diploid strain (lane c) and 1.5 and 3.25 kb in the strain heterozygous for an *RPR1* gene disruption (lane d). Size markers (indicated in nucleotides) were lambda phage DNA digested with *Bst*EII and stained with ethidium bromide (not shown).

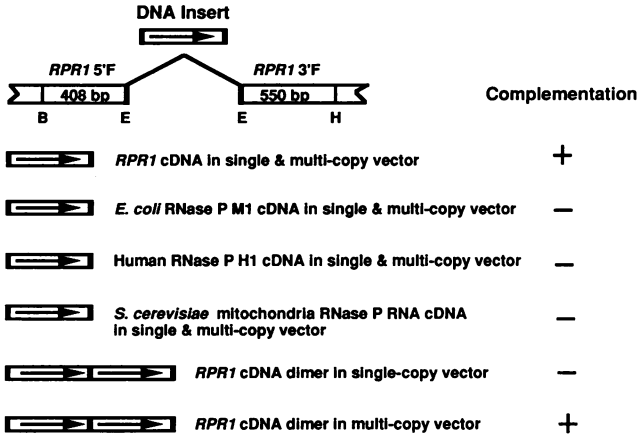

FIG. 5. Complementation of disrupted chromosomal *RPR1* gene by various RNase P RNA cDNAs. cDNA for each mature RNase P RNA was inserted to replace the 369-nucleotide major *RPR1* RNA coding region by addition of *Eco*RI linker as described in Materials and Methods. These plasmids were used to transform a diploid heterozygous for an *RPR1* gene disruption. Spores were obtained from transformants and dissected to determine complementation. +, All four spores were viable as the result of complementation by a plasmid-borne RNase P RNA gene; –, only two spores were viable as the result of no functional complementation by the plasmid.

inserted at an essential locus. Evidence that the substitutive transformation site was the *RPR1* gene was provided by Southern analysis of DNA from the parental diploid, a diploid heterozygous for the disruption, and viable haploids probed with *RPR1* antisense RNA (Fig. 4B). Only in the case of the heterozygous disrupted His^+ diploid did *Stu*I digestion produce two *RPR1* fragments, one the normal size and a second larger by an amount corresponding to the 1,750-bp *HIS3* fragment. Taken together, these results indicate that *RPR1* is single copy and essential.

Several experiments were conducted to test whether variants of the *RPR1* cDNA sequences or heterologous RNase P RNA component cDNAs could complement the disrupted *RPR1* gene. These experiments were conducted for two reasons. First, there is precedent for mixed RNase P protein and RNA components from distantly related bacteria (21) or from bacteria and human cells (17) to be able to reconstitute functional holoenzyme in vitro, and it was of interest to determine whether the *RPR1* product was sufficiently related to the analogous RNAs from *E. coli*, human cells, or yeast mitochondria (42) to reconstitute mixed holoenzymes in vivo. Second, repeated attempts to produce partially defective or conditionally lethal mutations in *RPR1* had been unsuccessful, and it was hoped that one of the heterologous or altered cDNA constructs would give such a defect with a molecular phenotype identifiable as an RNase P lesion. At first, heterologous cDNAs or mutated *RPR1* cDNAs were placed under the control of inducible *S. cerevisiae* RNA polymerase II promoters in plasmids, but no such construct including bona fide *RPR1* cDNA would complement (not shown), suggesting that the RNase P RNA could not be properly synthesized by RNA polymerase II.

The YCp50 and YEp24 plasmid constructs described in Materials and Methods and in Fig. 5 were therefore devised to drive the expression of the cDNAs from the *RPR1* 5′- and 3′-flanking regions. In each case, the cDNA corresponding to the indicated mature RNase P RNA was inserted to

TABLE 1. Doubling time in YPD medium

Strain	Doubling time (h)	
	30°C	38°C
Wild type	1.27	1.60
RPR1 cDNA	1.36	1.70
RPR1 cDNA dimer	2.60	5.80[a]

[a] 3.2 h for approximately three generations after temperature shift, followed by 5.8 h.

replace the 369-nucleotide major *RPR1* RNA coding region by the addition of *Eco*RI linkers. After use of these plasmids to transform a heterozygous *RPR1*-disrupted diploid, spores were obtained and dissected to determine complementation. The mature *RPR1* cDNA served as a control for defects due to the linker insertions in YCp50 and YEp24 and showed no growth defects. However, none of the heterologous cDNAs from *E. coli*, human cells, or yeast mitochondria gave any detectable complementation. It is not clear at this time whether this finding is due to defects in transcription, RNA processing, assembly and transport, or function. Several variants of the *RPR1* gene also did not complement (not shown) until a serendipitous insertion of an *RPR1* cDNA direct tandem repeat was tested. In single copy, this construct also did not complement, but diploids carrying the dimer on a multicopy plasmid gave two normal spores (nondisrupted chromosomal *RPR1* copies) and two slowly growing spores when sporulated. Growth curves for the normal and defective spores (Table 1) show that the difference in doubling was twofold at 30°C and increased to more than threefold at 38°C.

To characterize the defect caused by the direct cDNA repeat *RPR1* dimer, expression of both *RPR1* RNA and tRNA was examined at 30 and 38°C. Figure 6 shows Northern blot analysis and 5′ and 3′ end analysis of *RPR1* in the parent strain, a disrupted *RPR1* haploid strain complemented with multiple copies of the single *RPR1* cDNA construct (Fig. 5), and a disrupted haploid complemented with multiple copies of the cDNA dimer construct. The wild-type RNA displayed the normal pattern of 369-nucleotide RNA, with lesser amounts of the larger RNA containing longer 5′ and 3′ termini. The cDNA monomer clone in multiple copies accumulated considerably more of the larger RNA and somewhat more of the smaller 369-nucleotide RNA with the correct 5′ and 3′ termini. If the longer RNA is in fact a precursor of the 369-nucleotide form, it thus appears that it can be correctly processed at both the 5′ and 3′ ends without regard for the exact sequence flanking the mature termini. Accumulation of the presumptive precursor would indicate that some step in its processing is unable to keep up with the increased synthesis, either through overloading the assembly and processing machinery or through slowing the rate of some limiting step(s). Since the 5′ termini of the larger RNAs are discrete and extend beyond the wild type by approximately the size of the added linker (Fig. 6B), the multiple longer products seen in the cDNA Northern analyses are probably extended at the 3′ ends, consistent with the longer products seen with the S1 3′ analyses. It is not known whether these longer products arise from inefficient transcription termination or inappropriately processed longer transcripts that are not stable enough to be seen with the wild-type gene.

The *RPR1* cDNA dimer construct was also transcribed to give a large percentage of accurate 5′ termini on the longer RNA, but very little of the smaller RNA 5′ end appeared (Fig. 6B). Longer heterogeneous primer extension products might be due to primer annealing to the second copy of the RNA in dimeric transcripts. Similarly, the 3′ termini of the dimeric transcripts appeared in roughly the same pattern observed for the cDNA monomer insert (Fig. 6C) except that there was less of the 3′ signal corresponding to the small *RPR1* RNA. Thus, the most likely interpretation of the pattern of dimer clone RNAs in Fig. 6A is that transcripts are initiating at the normal position upstream of the first cDNA copy. The heterogeneous pattern of long RNAs arises either from multiple terminations in and past the second copy or from termination at the correct site after the dimer insert followed by aberrant processing. In either case, little RNA of normal size is made in this growth-defective strain at either 30 or 38°C.

By using the same RNA samples as in Fig. 6, the steady-state levels of mature and precursor tRNA were examined by probing duplicate Northern blots with an antisense RNA probe to $tRNA^{Leu3}$. This tRNA is synthesized as a precursor with a 10-nucleotide leader, a 32-nucleotide intron, and 6 to 7 nucleotides of 3′ trailing sequence (44, 61). The terminal sequences are normally removed before the intron (61), and in vitro removal of the 3′ sequences by yeast lysates is quite slow before the 5′ leader has been removed by RNase P (unpublished observations). The partial processing products in Fig. 7, assigned by direct size comparison to sequenced in vitro products (not shown), show that this pattern was followed in both the parental strain and the disrupted strain carrying the single cDNA insert, with somewhat more primary transcript accumulating at the higher temperature in both. Ordered removal of the 5′ end, then the 3′ end, and then the intron is implied by the observed intermediates (61). In contrast, the strain in which *RPR1* is complemented by the cDNA dimer accumulated mostly near-primary-transcript-size RNA, with very little intermediate appearing that contains the intron but lacks the 5′ and 3′ termini. End processing is thus defective in the *RPR1*-defective strain. Also, at 38°C this strain accumulated an intermediate in which the intron appears to have been removed, but not the 5′ leader or 3 to 4 nucleotides of 3′ trailing sequence. Smearing of the primary transcript and the apparently spliced intermediate downward probably reflects partial 3′ exonucleolytic processing slowed by the presence of the 5′ leader (12). An RNA slightly smaller than the mature-size $tRNA^{Leu3}$ also appeared in the 38°C samples, but it is not known whether it lacks portions of the 3′ CCA or has some other deficiency. It is worth noting that while spliced products containing the terminal sequences do not normally accumulate, we observed the appearance of RNA the size of spliced but not RNase P-cut intermediates in the *RPR1*-defective strain, indicating that the normal ordering is probably not obligatory in vivo. The occurrence of tRNA splicing before end maturation has also recently been observed by others (45).

Because size analyses of tRNA only suggest which precursors accumulate, purified total RNA from the defective strain grown at 38°C was digested with isolated RNase P (37) and blotted along with the other samples shown in Fig. 7. The accumulated $tRNA^{Leu3}$ primary transcript was converted to the size of intron-containing precursor with slight size heterogeneity consistent with variable amounts of remaining 3′ trailer. Identical size reductions were observed for the lesser amounts of primary transcripts accumulated by the wild-type and *RPR1* cDNA monomer strains (not shown). The amount of spliced intermediate was also de-

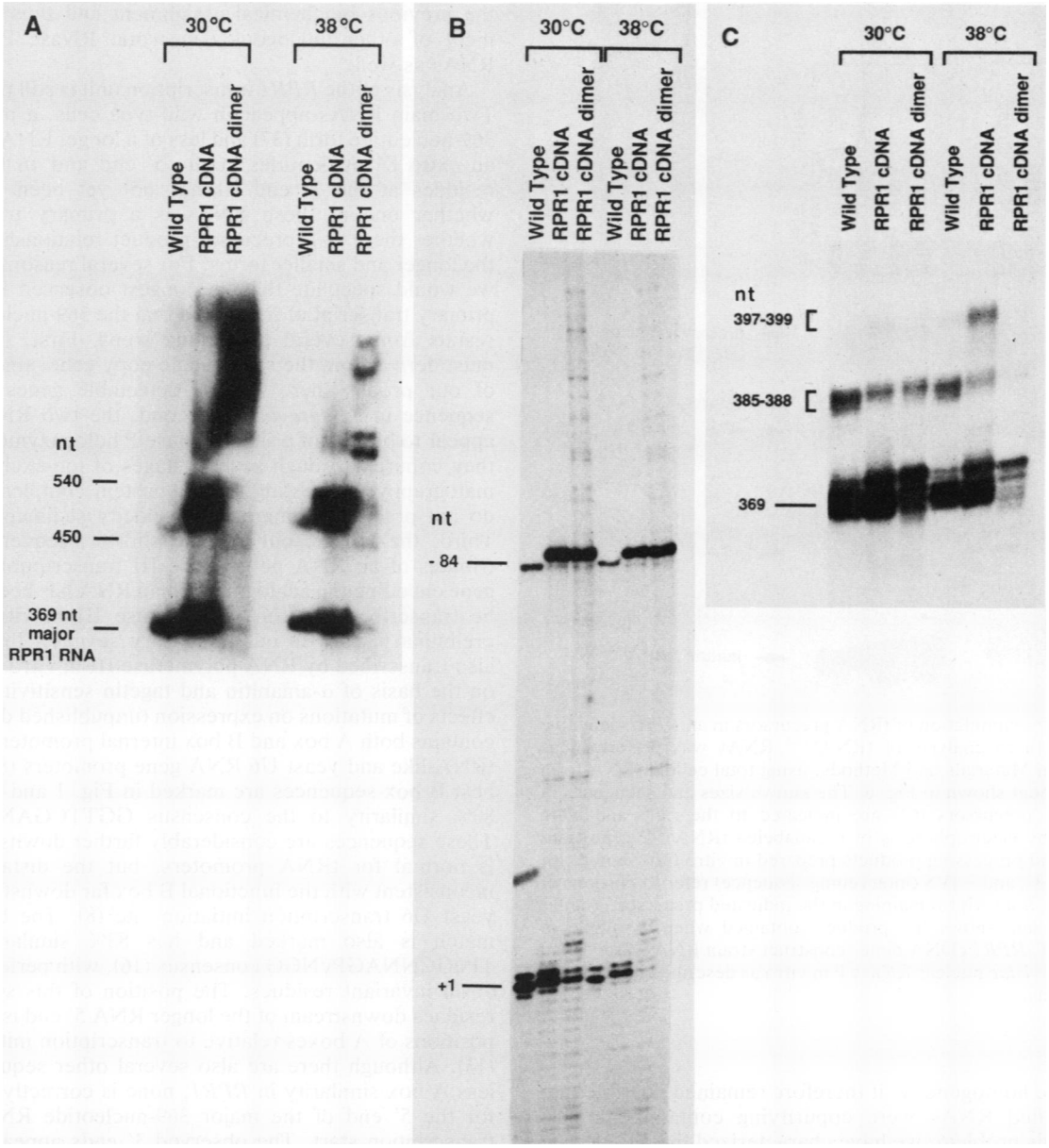

FIG. 6. *RPR1* expression from monomer and tandem dimer cDNA clones. Northern and end analyses were performed on RNA isolated from the parental haploid strain ('Wild Type'), W3031A, and disrupted haploids complemented with either the monomer or a tandem dimer *RPR1* cDNA insert between the *RPR1* flanking regions as described in the legend to Fig. 4 and the text. Analyses were performed as described in the legend to Fig. 2 and the text with total cellular RNA from cultures grown at either 30 or 38°C. (A) Northern analysis using antisense *RPR1* RNA. The positions of the known 369-nucleotide (nt) RNA and longer markers are indicated. (B) 5′ end analysis by primer extension. The cDNA construct termini corresponding to the 369-nucleotide RNA are the same as in wild-type cells, while the RNAs initiating further upstream are longer by 5 bp corresponding to the added *Eco*RI linker. (C) 3′ end analysis by S1 nuclease protection. The major 3′ termini are discussed in the text. Positions are numbered according to Fig. 1B.

creased, presumably through conversion to mature-size tRNA by removal of the 5′ terminus. Thus, the major accumulated pre-tRNAs in the *RPR1*-defective strain have not yet been cleaved with RNase P, even though they are appropriate substrates for the enzyme.

DISCUSSION

RNAs that copurify with RNase P activity have now been isolated from a number of eucaryotic sources (1, 7, 9, 11, 12, 14, 24, 32, 34, 35, 37, 38, 40, 42). The sequence similarities among the nuclear RNase P RNAs and their potential structural similarities to the analogous procaryotic ribozymes (5, 37) strongly suggested that the identification of these RNAs as RNase P components was correct, but formal proof has been lacking. Unlike the procaryotic ribozymes, the eucaryotic RNAs have not been shown to have catalytic activity when purified or synthesized in vitro, nor have sequence-specific attacks with RNase H been shown to destroy RNase P activity. As with any enzyme not purified

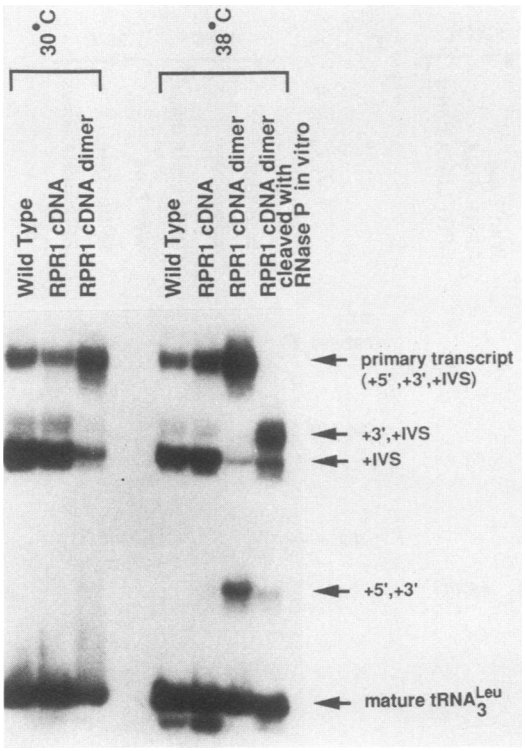

FIG. 7. Accumulation of tRNA precursors in an *RPR1*-defective strain. Northern analysis of tRNA^{Leu3} RNAs was performed as described in Materials and Methods, using total cellular RNA from the experiment shown in Fig. 6. The known sizes and sequences of the normal precursors (61) are indicated to the right and were confirmed by electrophoresis of radiolabeled tRNA^{Leu3} gene transcription and processing products prepared in vitro (not shown, 13, 61). +5′, +3′, and +IVS (intervening sequence) refer to portions of the primary transcript remaining in the indicated precursor. The far right-hand lane shows the products obtained when a sample of purified 38°C *RPR1* cDNA dimer construct strain RNA was cleaved with *S. cerevisiae* nuclear RNase P in vitro as described in Materials and Methods.

to absolute homogeneity, it therefore remained possible that the identified RNAs were copurifying contaminants. To address this problem, we have characterized the *RPR1* gene for a yeast nuclear RNase P-associated RNA and have used a defective variant to determine whether RNase P function is impaired.

Disruption of the single *RPR1* gene on chromosome V was lethal, and only one of a number of *RPR1* variants tested for complementation gave a defective growth phenotype short of complete lethality. This variant, a tandem cDNA dimer inserted between the *RPR1* flanking regions, made little correct-size *RPR1* RNA and accumulated tRNA precursors that had not undergone cleavage by RNase P. It is formally possible that the defect in *RPR1* is not actually in an RNase P subunit but rather causes this accumulation indirectly through undetermined interference with some other stage of tRNA or RNase P RNA maturation. This seems unlikely, however, considering that (i) *RPR1* RNA cofractionates with highly purified *S. cerevisiae* RNase P (37), (ii) similar RNAs copurify by different methods with other RNase Ps from other eucaryotes, and (iii) RNase P cleavage appears to be a very early step in tRNA maturation. The genetic evidence presented here therefore represents an essential validation of the previous biochemical assignment and thus the assignment of other analogous eucaryotic RNase P-associated RNAs as well.

Analysis of the *RPR1* transcription unit is still preliminary. Two main RNAs appear in wild-type cells, a predominant 369-nucleotide form (37) and less of a longer RNA containing an extra 84 nucleotides at the 5′ end and 16 to 30 extra residues at the 3′ end. It has not yet been determined whether one of these RNAs is a primary transcript or whether there is a precursor-product relationship between the longer and smaller forms. For several reasons, however, we would speculate that the longest observed RNA is the primary transcript of *RPR1* and that the 369-nucleotide form results from several processing steps. First, both RNAs must derive from the same single-copy gene, since with use of our probes there are no detectable genes of similar sequence in *S. cerevisiae*. Second, the two RNAs do not appear to be part of a single RNase P holoenzyme. Although they copurify through several stages of ion-exchange chromatography, suggesting similar protein complements, they do not precisely comigrate in velocity sedimentation (37). Third, the longer, but not the shorter, sequence has attributes of an RNA polymerase III transcription unit. The gene encoding the analogous human RNA has been shown to be transcribed by RNA polymerase III in vitro (4), and preliminary work in our laboratory suggests that *RPR1* is also transcribed by RNA polymerase III in vitro and in vivo on the basis of α-amanitin and tagetin sensitivities and the effects of mutations on expression (unpublished data). *RPR1* contains both A box and B box internal promoters typical of tRNA-like and yeast U6 RNA gene promoters (8). The two best B box sequences are marked in Fig. 1 and have about 80% similarity to the consensus GGTTCGANTCC (16). These sequences are considerably farther downstream than is normal for tRNA promoters, but the distance is not inconsistent with the functional B box far downstream of the yeast U6 transcription initiation site (8). The best A box match is also marked and has 83% similarity to the TPuGCNNAGPyNGG consensus (16), with perfect matches at all invariant residues. The position of this sequence 20 residues downstream of the longer RNA 5′ end is correct for positions of A boxes relative to transcription initiation sites (13). Although there are also several other sequences with less A box similarity in *RPR1*, none is correctly positioned for the 5′ end of the major 369-nucleotide RNA to be a transcription start. The observed 3′ ends appear heterogeneous and might represent multiple processing events, since the longest extend into a T_6 region downstream that would be an appropriate RNA polymerase III terminator. A reasonable hypothesis for *RPR1* RNA production is therefore synthesis of a precursor (indicated by underlining in Fig. 1) followed by processing at both the 5′ and 3′ ends to give the 369-nucleotide mature RNA.

The disruption of the chromosomal *RPR1* gene opens the possibility of examining the effects of incorporating variant RNase P RNAs into holoenzyme in vivo, thus circumventing the inactivity of the RNA alone and our inability to reconstitute significant activity from separated RNA and protein components in repeated experiments. In the studies described here, we attempted to substitute several heterologous RNase P cDNAs for the mature *RPR1* cDNA to investigate whether they would function in the yeast holoenzyme, as has been demonstrated previously for reconstituted heterologous components. Unfortunately, all such attempts were lethal recessive, and it could not be unambiguously determined whether the defect was in processing,

assembly, stability, activity, or transcription (e.g., due to lack of internal promoters). It is expected, however, for reconstitution of RNase P holoenzyme in vivo, that future studies evaluating both heterologous RNAs and mutations in *RPR1* will be facilitated by the *RPR1*-disrupted and -defective strains.

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