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Replacement of the *Saccharomyces cerevisiae RPR1* gene with heterologous RNase P RNA genes

Eileen Pagán-Ramos, Anthony J.Tranguch, David W.Kindelberger and David R.Engelke*
Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109-0606, USA

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ABSTRACT

Phylogenetic studies of yeast nuclear RNase P RNA genes have shown a striking conservation of secondary structure for the *Saccharomyces* and *Schizosaccharomyces* RNase P RNAs, yet much of the primary sequence and many substructures vary among the RNAs examined. To investigate which sequences and structural features can be varied and still allow function in a heterologous organism, RNase P genes from several yeast species were tested for the ability to substitute for the *Saccharomyces cerevisiae* RNA. The RNase P genes from *Saccharomyces carlsbergensis* and *Saccharomyces kluyveri* could act as the sole source of RNase P RNA within *S.cerevisiae* cells, whereas the genes from *Saccharomyces globosus* and *Schizosaccharomyces pombe* could not. Although heterologous RNase P RNAs were synthesized by the cells in all cases, the RNAs that complemented tended to be processed from longer precursor transcripts into mature-sized RNase P RNA, while the RNAs that did not complement tended to accumulate as the longer precursor form. The results identified sequences and structures in the RNA that are not essential for interaction with species-specific proteins, processing or localization, and suggested other positions that may be candidates for such processes.

INTRODUCTION

RNase P is a ribonucleoprotein enzyme that cleaves precursor tRNA molecules to generate mature 5′ termini (reviewed in ref. 1). RNase P activity has been identified from numerous prokaryotic and eukaryotic sources including eubacteria, archaebacteria, fungi, vertebrates and mitochondria (2–11). In eubacteria, both the protein and RNA components are required for function *in vivo*, but the RNA moiety has been shown to retain catalytic activity *in vitro* in the absence of the protein subunit (12). Conversely, the RNAs from other sources have not been shown to exhibit catalytic function by themselves. The relative roles of the eukaryotic RNA and protein components in substrate binding and catalysis remain unclear.

The nature of the interaction between RNase P RNA and its tRNA substrates has been studied in the eubacterial enzyme (reviewed in ref.13). The combination of tRNAs' divergent primary sequence, conserved tertiary structure, and lack of sequence complementarity with RNase P RNAs suggests that substrate binding occurs through recognition of higher order pre-tRNA structure by a complex RNase P RNA structure. Phylogenetic comparative analysis has been used to investigate RNase P RNA secondary structures from various sources. Such analyses performed in eubacteria resulted in the discovery of a conserved common RNA core structure (14,15).

Recently, a phylogenetic analysis of yeast nuclear RNase P RNA genes resulted in an eukaryotic secondary structure model (16). We had previously identified the RNA component of *S.cerevisiae* nuclear RNase P (7) and characterized its gene, *RPR1* (17). *RPR1* was shown to be an essential single-copy gene and to be transcribed by RNA polymerase III *in vivo* (18). Comparative sequence analysis of nuclear RNase P RNAs from six *Saccharomyces* species revealed a consensus RNA secondary structure which also fit the RNAs from more distant *Schizosaccharomyces* species (16,19). Comparison of this consensus core to the conserved eubacterial structure and to vertebrate RNase P RNAs from HeLa cells (8) and *Xenopus laevis* (9) revealed a number of secondary structural similarities, suggesting that eukaryotic RNase P RNAs from evolutionarily divergent sources share a core of conserved structural features, many of which have counterparts in eubacterial RNase P RNA (16).

The RNase P RNA from *S.cerevisiae*, as from other non-eubacterial sources, has not yet been shown to be catalytic in the absence of protein, and our attempts to reconstitute the RNA and protein component(s) *in vitro* have also been unsuccessful. Sedimentation studies suggest that protein makes up a significant proportion of the eukaryotic enzymes (20), in agreement with the recent finding that the protein subunit from the yeast mitochondrial enzyme is significantly larger than that found in eubacterial RNase P (21). The protein complement of the eukaryotic nuclear enzymes has not yet been determined, although RNA footprinting studies suggest it covers a much larger percentage of the RNA subunit than is the case in the bacterial holoenzymes (A.Tranguch, submitted). To augment the phylogenetic analysis with information regarding which altered sequences and structures in heterologous RNAs do or do not allow function in *S.cerevisiae*, we tested the ability of RNase P RNA

*To whom correspondence should be addressed

from both closely and distantly related yeast species to substitute for the *S.cerevisiae* RNA *in vivo*. Results obtained from these complementation experiments reveal possible positions in the RNA that are not essential for protein interaction, processing or localization, as well as positions that might be essential for these functions.

MATERIALS AND METHODS

Strains and genetic methods

Plasmids were constructed and maintained in *Escherichia coli* strain DH5αF′ using standard techniques (22). *S.cerevisiae* strain W3031A (*MAT***a** *ade2*-1 *his3*-11,15 *leu2*-3,112 *trp1*-1 *ura3*-1 *can1*-100, gift of R.Rothstein), was used to construct the strain JLY1 which contains an *rpr1*::*HIS3* disruption (17). Since the *RPR1* gene has been shown to be essential (17), the haploid strain must be kept viable by a plasmid-borne *RPR1* gene.

Isolation of plasmid DNA from DH5αF′ strain was performed using standard techniques (22). Bacterial and yeast transformations were performed by electroporation as described (23,24), using a Bio Rad Gene Pulser and Pulse Controller.

Yeast cells were grown in two different selection media: SD-ura-his-trp (2% dextrose, 0.67% yeast nitrogen bases, amino acids supplements lacking uracil, histidine and tryptophan) for selection of plasmids with *URA3* and *TRP1* markers; or SD-his-trp+5-FOA (same as above but containing uracil and 1g/liter 5-fluoroorotic acid) to promote the loss of the *URA3*-marked YCp50-*RPR1* plasmid ('curing') and retention of the *TRP1*-marked plasmid. All media lacked histidine to prevent the loss of the *HIS3* gene that disrupts the chromosomal *RPR1* gene. Cells were incubated at 30°C unless otherwise specified.

Recombinant plasmids

YCp50-*RPR1*, a single copy plasmid containing a wild-type copy of the *RPR1* gene was constructed from a 1.3 kilobase (kb) *Bgl*II−*Hind*III fragment of *RPR1* cloned in the *Bam*HI−*Hind*III polylinker sites of pUC19 as described previously (17).

The high copy (2μ) plasmid pRS424-5′3′RPR was used as an expression vector for heterologous RNase P RNA cDNAs (Fig. 1A) and was constructed as follows. 408-base pairs (bp) of *RPR1* 5′ flanking sequences and 550-bp of *RPR1* 3′ flanking sequences were amplified by polymerase chain reaction (PCR) using Taq DNA polymerase (25) and inserted into the *Bam*HI and *Hind*III sites of the centromeric plasmid pBM950 (gift of M.Johnston). The resulting construct (5′3′*RPR1*pBM, unpublished data), contained an *Eco*RI site (created by addition of *Eco*RI linkers) at the junction of the two flanking regions. Plasmid 5′3′*RPR1*pBM was restricted with *Bam*HI and *Sal*I and the resulting fragment containing the *RPR1* flanking sequences was ligated into the *Bam*HI−*Sal*I sites of the *TRP1*-marked plasmid pRS424 (gift of P.Hieter; 26) to create pRS424-5′3′RPR (Fig. 1A). In the constructs used for complementation experiments, cDNAs representing the mature domain of the different yeast species' RNase P RNAs ('test genes') were inserted in the *Eco*RI site of the pRS424-5′3′RPR plasmid (Fig. 1A) between the *RPR1* promoter/leader region (84-bp contained within the 5′ flank sequences) and the terminator region (37-bp contained within the 3′ flank). Correct orientation of the test genes in pRS424-5′3′RPR was determined by PCR.

The *S.cerevisiae RPR1* cDNA used as a positive control in the complementation experiments was obtained as a 374-bp *Eco*RI fragment containing the *RPR1* mature domain coding region cloned into pGEM-3Z plasmid (Promega) as previously described (18). The heterologous cDNAs were synthesized by PCR amplification of the mature region of the RNase P genes. The

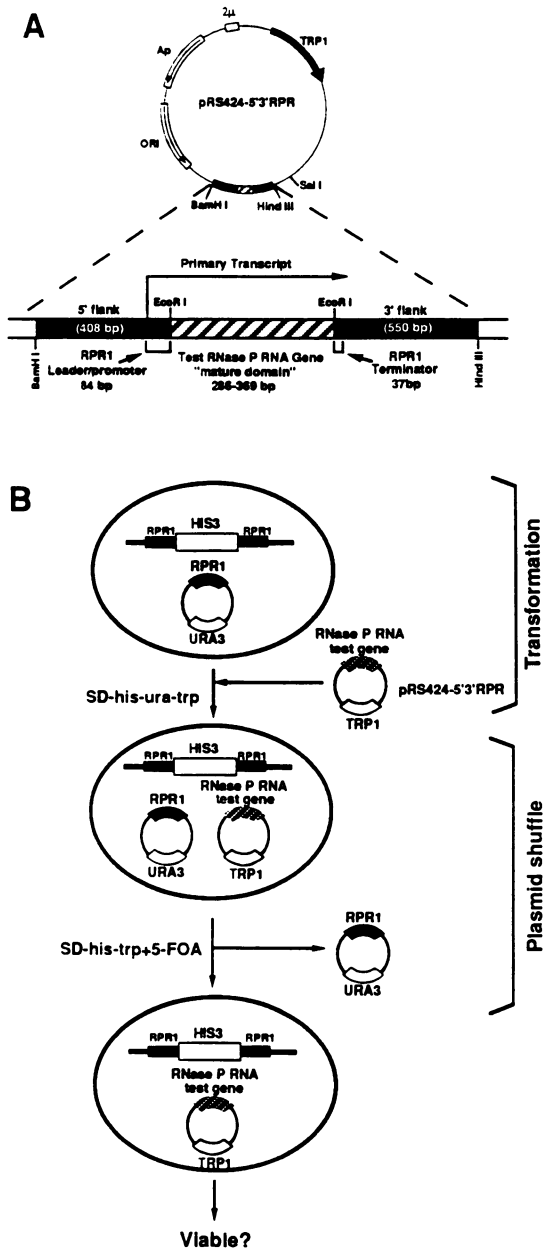


Figure 1. Experimental strategy to test for heterologous RNase P RNA complementation of the *RPR1* disruption. **(A)** Expression vector. The high-copy plasmid pRS424-5′3′RPR (*TRP1* marker) was used as an expression vector for heterologous RNase P RNA cDNAs. This vector contained 5′ and 3′ *RPR1*-flanking sequences. The 5′ flank included 84-bp comprising the promoter/leader sequences and the 3′ flank included 37-bp containing the terminator. cDNAs of the mature domain of RNase P RNAs from *S.cerevisiae* (369 nt ; positive control), *S.carlsbergensis* (359 nt), *S.kluyveri* (336 nt), *S.globosus* (339 nt) or *S.pombe* (286 nt) were inserted into the *Eco*RI site of the vector to create the final constructs used in the complementation experiments. **(B)** Complementation strategy. *S.cerevisiae* strain JLY1, carrying the *RPR1* wild type gene in the *URA3*-marked plasmid YCp50-*RPR1*, was transformed with the plasmid pRS424-5′3′RPR carrying the 'test RNase P genes'. To test the ability of these genes to complement the *RPR1* disruption, the YCp50-*RPR1* plasmid was eliminated from the transformed cells by plating on 5-FOA medium lacking histidine and tryptophan. The ability of the test genes to complement the disruption was determined by the viability of cells on this medium.

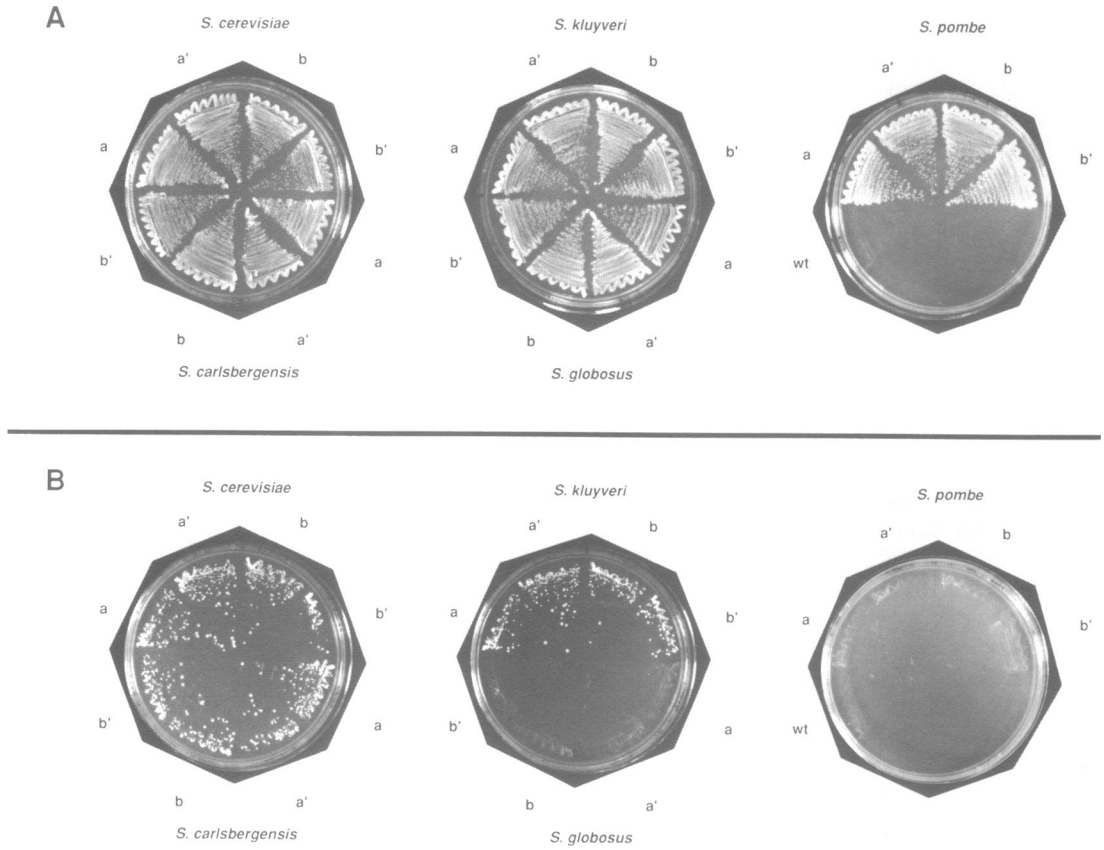


Figure 2. Complementation of *RPR1* disruption by RNase P genes from evolutionary divergent yeast species. **(A)** JLY1 strain carrying both the *RPR1* and the test genes were plated on SD-ura-his-trp medium to serve as a positive control for growth and number of colonies plated. Two independent clones were selected for each RNase P gene (**a** vs. **b**) and two individual transformants with each clone were tested (denoted as **a**, **a'** and **b**, **b''**). **(B)** The same transformants as in panel A are shown, but plated on SD-his-trp+5-FOA medium to remove the wild type RNase P RNA gene. Only the cells transformed with the *S.cerevisiae* (positive control), *S.carlsbergensis* or *S.kluyveri* RNase P cDNA constructs were viable, demonstrating the ability of these test genes to complement the *RPR1* disruption. The cells containing the *S.globosus* or *S.pombe* genes were not viable.

templates used for PCR amplification were genomic DNA clones of *S.carlsbergensis*, *S.kluyveri* and *S.globosus* (16), and a genomic DNA clone of *S.pombe* (6). Primers with sequences specific for the 5′ or 3′ ends of the mature domain of each RNase P RNA gene were used for the PCR amplification. The primers also introduced *Eco*R I sites at each end of the cDNA to allow subcloning in the *Eco*R I site of pRS424-5′3′RPR plasmid. The hybridization positions of the primers were the following: Eco5′carlsbergensis (+1 to +18) and Eco3′carlsbergensis (+339 to +359), Eco5′kluyveri (+1 to +17) and Eco3′kluyveri (+316 to +336), Eco5′globosus (+1 to +18) and Eco3′globosus (+319 to +339), Eco5′pombe (+1 to +25) and Eco3′pombe (+256 to +286). The numbers of RNase P RNA positions are relative to the mature RNA 5′ end as described earlier (16). PCR conditions were 25 rounds of: 94°C for 1 min, 42°C for 30 s and 72°C for 1 min.

RNA isolation and analysis

RNA was isolated from exponentially growing yeast cultures (27). Approximately 10μg per lane were used for Northern blot analysis. RNA was normalized by measuring absorbance at 260 nm and by ethidium bromide staining to visualize ribosomal RNA bands after electrophoresis in agarose gels (Figures 3 and 4), and by quantitating U6 RNA levels as an internal control (Figure 4). The RNA used for the Northern blots was isolated from yeast cells containing both the single-copy plasmid carrying the *RPR1* gene and the high-copy plasmid containing the heterologous test RNase P RNA genes.

Northern blot analyses were performed essentially as described previously (16). [^{32}P]-radiolabeled antisense oligodeoxynucleotides were used as probes to detect specific RNAs on the Northern blots. The probes' signal was roughly the same in all cases. For the Northern blot analysis of the *S.cerevisiae RPR1* RNA (Fig. 4A), a 16-nucleotide probe that hybridizes between nucleotides +33 and + 48 of *RPR1* RNA was used. U6 RNA (28) was detected by a 17-nucleotide probe that hybridizes between nucleotides +62 and +88. For Northern blot analyses of RNase P RNA from the other four yeast species (Fig. 3), probes that hybridize specifically to the 3′ end of each heterologous RNase P RNA were used (described under Recombinant plasmids). These probes did not show cross-hybridization to the *RPR1* RNA under the conditions used for the experiments. Radiolabeled RNA size markers used to determine the sizes of RNA products were prepared as described previously (16). Quantitation of RNA from *S.cerevisiae* Northern blot (Fig. 4) was performed by exposing the blot to a Molecular Dynamics PhosphorImager and by performing volume integration with the ImageQuant program (Molecular Dynamics Corp.). The values obtained after volume integration are expressed in arbitrary units. For comparison of the levels of *RPR1* RNA, the numbers obtained

from the volume integration of RNAs from individual clones were averaged for each isolate.

RESULTS

Complementation of the *RPR1* disruption by RNase P genes from evolutionarily divergent yeast species

The ability of RNase P RNAs from both closely and distantly related yeasts to complement the disrupted *S.cerevisiae RPR1* gene was tested for several reasons. First there is precedent for mixed RNase P protein and RNA components from distantly related bacteria being able to reconstitute functional holoenzyme *in vitro* (12). Additionally, *in vivo* complementation of an RNase P RNA gene deletion in *E.coli* by genes from distantly related eubacteria has been demonstrated (29). Second, results from phylogenetic studies of RNase P RNAs from several yeast species showed a striking evolutionary conservation of RNA secondary structure. The purpose of the present study is to identify sequences and structures in the yeast RNA that are essential for function within an individual organism, even though they are variable between species. The yeast species used in these experiments and the percent sequence identity of their RNase P RNA with *S.cerevisiae RPR1* RNA were: *Saccharomyces carlsbergensis* (86%), *Saccharomyces kluyveri* (70%), *Saccharomyces globosus* (65%) and *Schizosaccharomyces pombe* (51%) (16).

The plasmid constructs and the experimental strategy for the complementation experiments are outlined in Figure 1. The cDNAs of the RNase P RNA coding sequences from the four yeast species were cloned in a high copy (2μ) expression plasmid between the leader/promoter and terminator of the *S.cerevisiae RPR1* gene. The RNA is expected to be synthesized as a precursor containing the *RPR1* leader and terminator sequences, with subsequent removal of those sequences, as was shown to be the case when the *S.cerevisiae* mature region cDNA is cloned into this high copy vector (17). By using this expression system, we were able to test whether heterologous RNase P RNAs' mature coding regions were functional without concern over whether the heterologous transcriptional promoters were functional in *S.cerevisiae*. The plasmids carrying the RNase P cDNA inserts will be referred to as 'test RNase P genes' (Fig. 1). Four to six

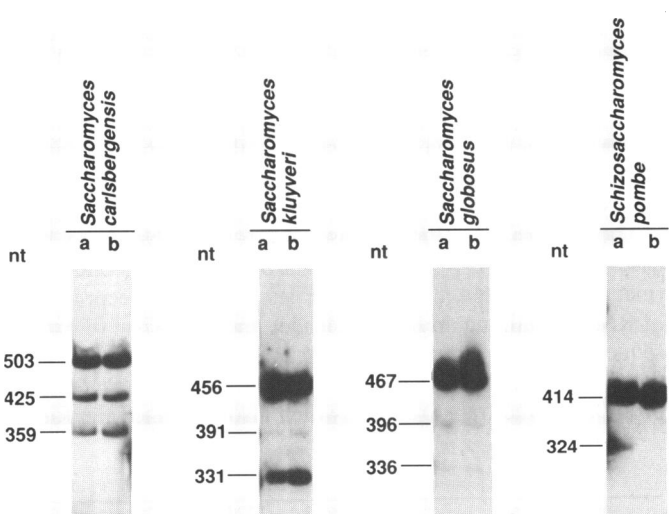


Figure 3. Expression of heterologous RNase P RNAs in *S.cerevisiae*. Total RNA isolated from cells containing both the *RPR1* gene and a 'test gene' was subjected to Northern blot analysis using antisense probes specific to each species' RNA (see Materials and Methods). Two individual positive clones of each RNase P were inspected (**a** and **b**). *Estimated* sizes of hybridizing RNAs (+/−10 nt) relative to markers are indicated at the *left* of each blot.

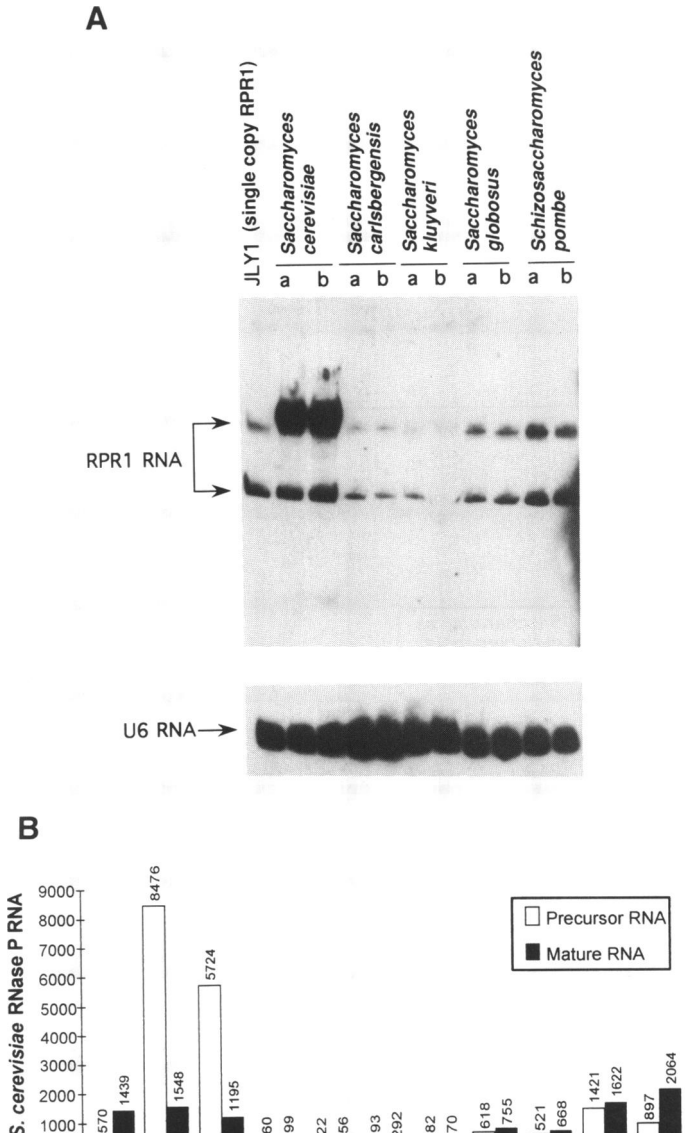
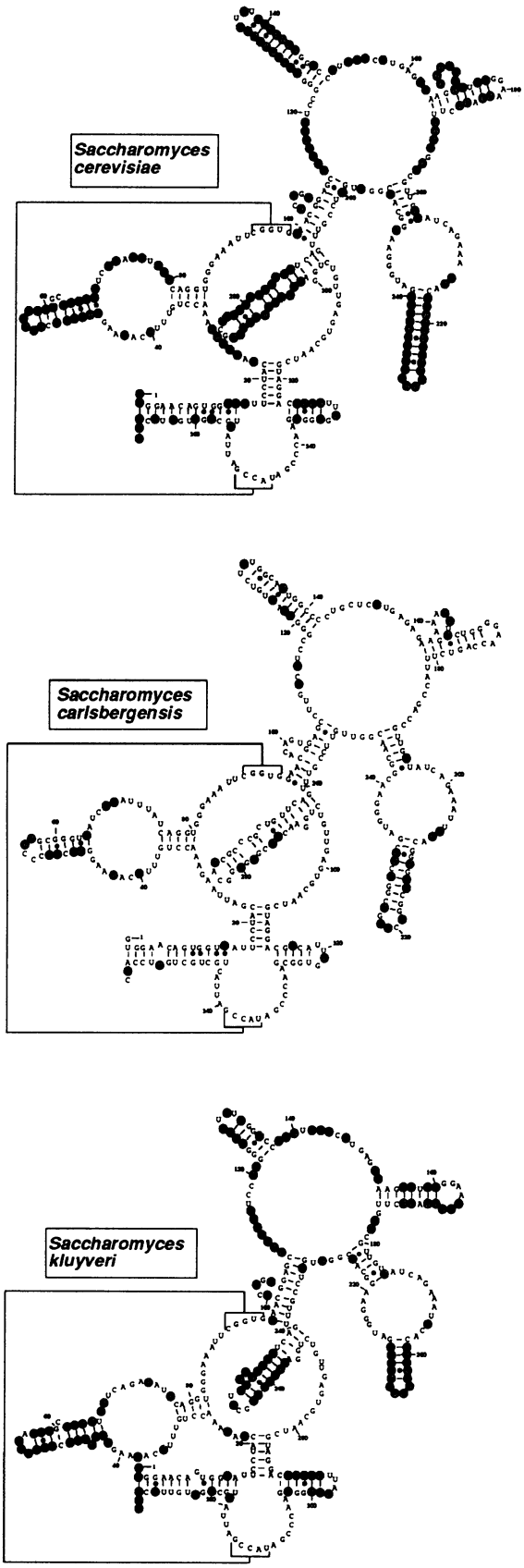


Figure 4. Expression of *RPR1* RNA in *S.cerevisiae* cells that also contain the heterologous RNase P RNA genes. **(A)** Total RNA isolated from cells containing both the *RPR1* gene and one of the 'test genes' was subjected to Northern blot analysis using an antisense probe specific for the *RPR1* RNA. The first lane (JLY1) shows RNA obtained from the strain containing only a single copy of *RPR1*. The other pairs of lanes show RNAs from cells containing a single copy of *RPR1* and also high copy of the indicated test gene. Two clones from each species were inspected (**a** and **b**). Both the precursor (*upper*) and mature (*lower*) forms of *RPR1* RNA are indicated. A U6 RNA probe was used as an internal control for RNA levels. **(B)** The relative levels of the precursor and mature *RPR1* RNAs shown in Figure 4A were quantitated using a PhosphorImager (Molecular Dynamics) and expressed as arbitrary units using the ImageQuant program. The levels of RNA were normalized to U6 RNA levels. The amounts of *S.cerevisiae* precursor and mature RNA are given when each test gene is also present in high-copy. *RPR1* RNA from JLY1 cells shows the levels with no test gene.



independent clones of each cDNA in the correct orientation were chosen and transformed into the *RPR1*-disrupted yeast strain JLY1. Multiple isolates were tested in all cases to ensure that phenotypes in yeast were reproducible and not due to any unexpected mutations acquired in the cloning process. To test the ability of the heterologous RNase P genes to complement the *RPR1* disruption, the YCp50-*RPR1* plasmid containing the wild type *RPR1* gene was eliminated from the transformed yeast cells by plating on 5-FOA medium lacking histidine and tryptophan. Growth on this medium induces the loss of the *URA3*-marked plasmid, YCp50-*RPR1*, and viability of the cells becomes dependent on the ability of the test RNase P gene to function as the source of RNase P RNA.

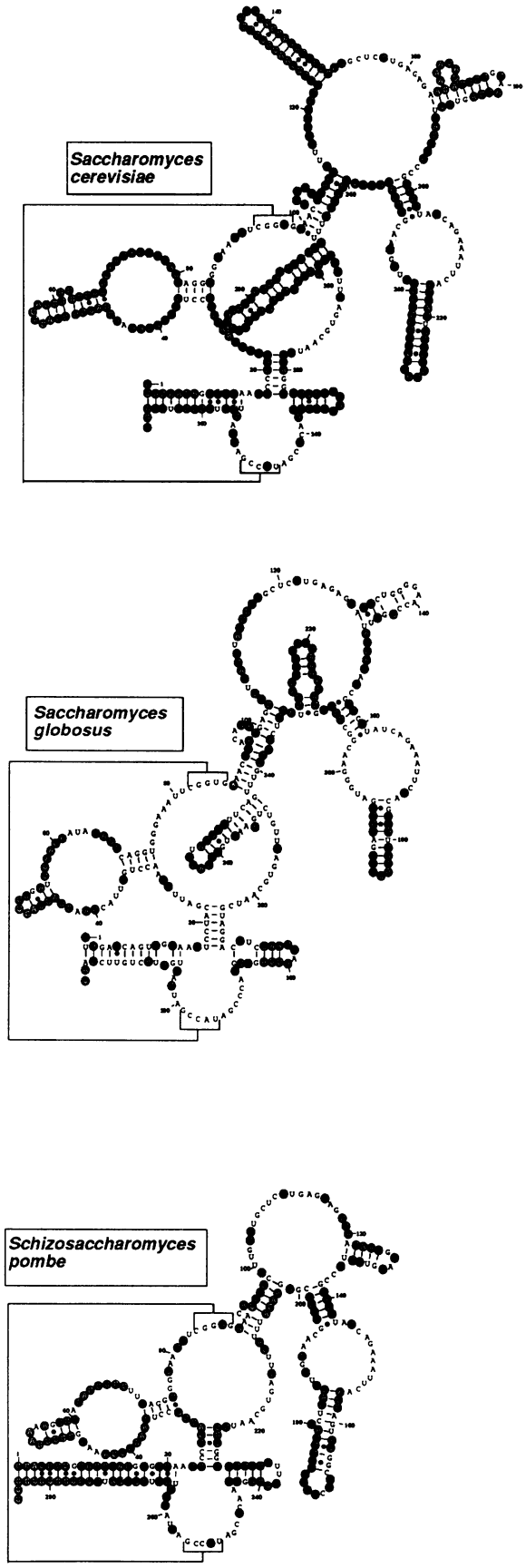
The results of the complementation experiments are shown in Figure 2. Eight single colonies derived from transformation with each of the RNase P test gene plasmids were plated on 5-FOA medium to test for complementation. In Figure 2, two independent plasmids (**a** and **b**) and two independent yeast transformants with each plasmid clone are shown (**a**, **a′**; **b**, **b′**). Cells were streaked on medium retaining the YCp50-*RPR1* plasmid as control for growth and for the number of cells plated (Fig. 2, panel A). Growth of colonies on 5-FOA medium (Fig. 2, panel B) indicates complementation. The genes from *S.cerevisiae* (positive control), *S.carlsbergensis* and *S.kluyveri* were able to complement the *RPR1* disruption, but the *S.globosus* and *S.pombe* genes were not. Non-complementing transformants were also found to be non-viable at 25°C (data not shown).

Expression of heterologous RNase P RNAs in *S.cerevisiae*

We investigated whether the lack of complementation of the *RPR1* gene deletion in *S.cerevisiae* by RNase P genes from *S.globosus* and *S.pombe* might be due to lack of expression or to instability of the foreign RNA in *S.cerevisiae*. Northern blot analyses were performed on total RNA extracted from JLY1 cells containing both the plasmid-borne wild type *RPR1* gene and plasmids carrying the RNase P cDNAs from the other yeast species (Figs. 3 and 4). Figure 3 shows the results where species-specific probes were used to detect the test RNase P RNAs. The RNase P RNAs from all the species, including those that did not complement the *RPR1* disruption, are expressed in *S.cerevisiae*. This is in contrast to reports from *in vivo* complementation experiments performed in bacteria which showed that in most cases, the inability to complement an RNase P RNA gene deletion correlated with the absence of stable heterologous RNase P RNA (29).

The pattern of *RPR1* RNase P RNA on Northern blots, described previously (18), consists of a 369-nucleotide RNA (mature *RPR1* RNA), and a longer less abundant precursor form of RNA containing an 84-nt 5′ leader and 27−37-nt of extra 3′ sequences. This pattern can be observed in Figure 4 (panel A, first lane) where *RPR1* RNA was detected using an *RPR1*-specific probe in all the transformants which also contained the foreign

Figure 5. Sequence and structural comparison of *S.cerevisiae*' s RNase P RNA with the heterologous RNase P RNAs that are able to complement the *RPR1* disruption. The sequences and phylogenetically-derived secondary structures (16) of the RNase P RNAs from *S.carlsbergensis* and *S.kluyveri* were compared to that from *S.cerevisiae* RNA. On the *S.carlsbergensis* or *S.kluyveri* RNA nucleotides that differ from the *S.cerevisiae* RNA are denoted by filled circles. These positions are combined on the *S.cerevisiae* RNA.



RNase P gene. The estimated sizes of the RNA products in Figure 3 match (within error for estimating the sizes) the predicted RNase P RNA products. The expected RNA sizes for the precursor and mature form of RNase P RNAs from the other yeast species in Figure 3 are respectively: 490-nt and 359-nt for *S.carlsbergensis*, 462-nt and 336-nt for *S.kluyveri*, 467-nt and 339-nt for *S.globosus* and 417-nt and 286-nt for *S.pombe* (16). The RNA pattern of the species that complemented the *RPR1* disruption, *S.carlsbergensis* and *S.kluyveri*, showed a distribution similar to that of *RPR1* RNA where the smallest RNA is the size of the expected mature form and the largest RNA is the size expected to contain the extra 5′ and 3′ sequences. These two heterologous RNAs also showed a third intermediate-sized form that is less prevalent for *S.kluyveri* RNA and slightly more prevalent than the mature form for *S.carlsbergensis* RNA. A phenomenon similar to this one has been previously observed for *RPR1* RNA, where multiple RNAs larger than the fully processed one are detected on Northern blots (17). It is possible that in *S.carlsbergensis* and *S.kluyveri* the intermediate-sized RNA could also be an intermediate with 3′ or 5′ unprocessed ends.

For *S.globosus* and *S.pombe*, the RNase P RNAs that were not able to complement the *RPR1* disruption, there is accumulation of the precursor RNA form. The intermediate-sized and the fully processed RNAs are either very faint or absent. These results suggest that these two RNase P RNAs might not be recognized by the assembly and/or processing machinery. This interpretation is consistent with the effect of the different heterologous RNAs from high copy genes on the stability of *RPR1* from a single copy gene in the same cell (see below).

Effects of heterologous RNase P RNAs on endogenous *RPR1* RNA expression

The levels of expression of the *RPR1* RNA were also inspected by Northern blot analysis in cells containing both the wild type *RPR1* gene in single copy and a test RNase P gene in high copy (Fig. 4). Figure 4B shows the quantitation of the *RPR1* RNAs from the Northern blot in Figure 4A. The levels of both the precursor RNA and mature RNA forms were normalized to U6 RNA levels, used as an internal control. Cells transformed with the native *RPR1* cDNA in multiple copies accumulate considerably more of the larger precursor RNA and only somewhat more of the smaller mature 369-nt RNA, indicating a possible overloading of the assembly and processing machinery (17, Fig. 4). In particular, it is possible that the RNase P protein subunit(s) become limiting for assembly, and that without assembly into a nucleoprotein particle processing of the RNase P RNA does not proceed. The hypothesis that full assembly of the holoenzyme occurs before terminal RNA processing is supported by co-purification of the larger and smaller RNA forms as nucleoproteins on multiple ion exchange resins (7).

Accumulation of precursor RNA is observed in Figure 4 (*S.cerevisiae* a and b lanes) showing RNA from JLY1 cells

Figure 6. Sequence and structural comparison of *S.cerevisiae*'s RNase P RNA with the heterologous RNase P RNAs that are not able to complement the *RPR1* disruption. The sequences and phylogenetically-derived secondary structures (16) of the RNase P RNAs from *S.globosus* and *S.pombe* were compared to *S.cerevisiae*'s RNase P RNA. Nucleotides that differ from the *S.cerevisiae* RNA are highlighted by filled circles and combined on the *S.cerevisiae* RNA.

transformed with high copy *RPR1* cDNA. In these *S.cerevisiae* lanes, two RNA products are actually present in the precursor region (not obvious on this exposure). The largest, most intense RNA corresponds to the *RPR1* cDNA expression construct precursor containing extra nucleotides from the *Eco*R I linkers, whereas the less abundant RNA corresponds to the wild type gene's primary transcript. Steady state levels of both precursor and mature *RPR1* RNAs are severely reduced in cells that also contain RNase P RNAs that complement the *RPR1* disruption (*S.carlsbergensis* and *S.kluyveri*). The levels of *RPR1* RNA in the cells containing the non-complementing RNase P RNA genes from *S.globosus* or *S.pombe* were similar to those produced in cells containing only the single copy wild type *RPR1* gene (first lane Fig. 4). The reduction in the levels of *RPR1* RNA in cells containing the *S.carlsbergensis* and *S.kluyveri* were 3 to 4-fold for the precursor RNA and 6 to 8-fold for the mature RNA compared to the expression of wild type *RPR1*. In cells containing the *S.globosus* gene there was a small reduction (2-fold) in the level of the mature RNA whereas the level of the precursor was close to the control. The RNA levels in the cells containing *S.pombe* gene did not show any reduction. Taken together with the data in Figure 3, these results suggest that the heterologous RNase P RNAs that fail to complement neither process to mature form nor compete for limiting determinants of maturation. It is not clear whether these limiting determinants are the protein subunits, but the failure to assemble a mature holoenzyme is a possible cause of the inability of the RNAs to functionally complement.

DISCUSSION

Since the eukaryotic nuclear RNase P RNAs have not yet been shown to have catalytic activity when purified from holoenzyme preparations or when synthesized *in vitro*, it is necessary to characterize the effect of any changes made to the RNA component in the context of the holoenzyme. The availability of the *S.cerevisiae* strain with an *RPR1* disruption has made it possible to perform studies of RNase P structure and function *in vivo* by substituting heterologous or mutated RNase P RNA genes. This approach has been used to study RNase P in eubacteria (29, 30), but its use has been limited in eukaryotic systems.

In this work, RNase P RNA genes from evolutionarily divergent yeast species were introduced into the *RPR1*-disrupted yeast strain in an attempt to identify sequences and structural domains in the *S.cerevisiae* RNA that might be important for enzyme function *in vivo*. In order to function in *S.cerevisiae*, the heterologous RNase P RNAs need to not only be synthesized, but also to fold properly, bind the protein components, be processed, and localize correctly within the cells. The RNAs from *S.globosus* and *S.pombe* are clearly not intrinsically defective for substrate binding and catalysis, since they function in their normal cellular environment. Their inability to complement correlates with their failure to be processed from precursor to smaller RNAs, but it is not clear that the inability to be processed *per se* causes loss of function. Suppression of wild type *RPR1* levels by overexpression of the complementing RNAs *S.carlsbergensis* and *S.kluyveri* suggests that these compete for some component that stabilizes RNase P RNA, perhaps limiting amounts of RNase P protein. The lack of such suppression by the non-complementing *S.globosus* and *S.pombe* RNAs might conversely suggest that these RNAs are unable to compete successfully for the *S.cerevisiae* protein components. This lack of protein binding could then lead to both lack of RNA maturation and lack of function.

The sequence and proposed secondary structure for *S.cerevisiae* RNase P RNA (16) were compared with the structures of the RNAs that complemented the *RPR1* disruption (Fig. 5) and with those that did not complement (Fig. 6). In each case, nucleotides that are not conserved between *S.cerevisiae* and the heterologous RNAs are represented as filled circles surrounding white letters. For purposes of the discussion, the regions of the RNA will be referred to by nucleotide position numbers in the *S.cerevisiae* model. When comparing the sequences of the *S.cerevisiae* RNA with the sequences of the *S.carlsbergensis* and *S.kluyveri* counterparts, it is clear that there are some structural domains in which sequences are not conserved (Fig. 5). The fact that these RNAs are able to complement the *RPR1* disruption suggest that there might not be sequence recognition between these regions of the RNA and protein component(s). The regions that showed less conservation of sequence are the hairpin stems Sce 47–54/59–68, Sce 123–134/139–150, Sce 216–226/231–241 and part of Sce 269–282/289–303, four extra nucleotides in the region of Sce 23–31, and the 6-nt bulge Sce 167–172 (absent in *S.kluyveri*) (Fig. 5). There are also several sequence patches not part of obvious secondary structures that are not conserved, e.g., nucleotides in the regions of Sce 69–80, Sce 113–119 and Sce 190–197. For entire stems, nucleotide sequence may not be important, but the presence of the structure may be. However, initial mutational studies guided by this work confirm that independent deletions of stems Sce 123–134/139–150 and Sce 117–226/231–241 leave functional RNase P RNAs (manuscript in preparation).

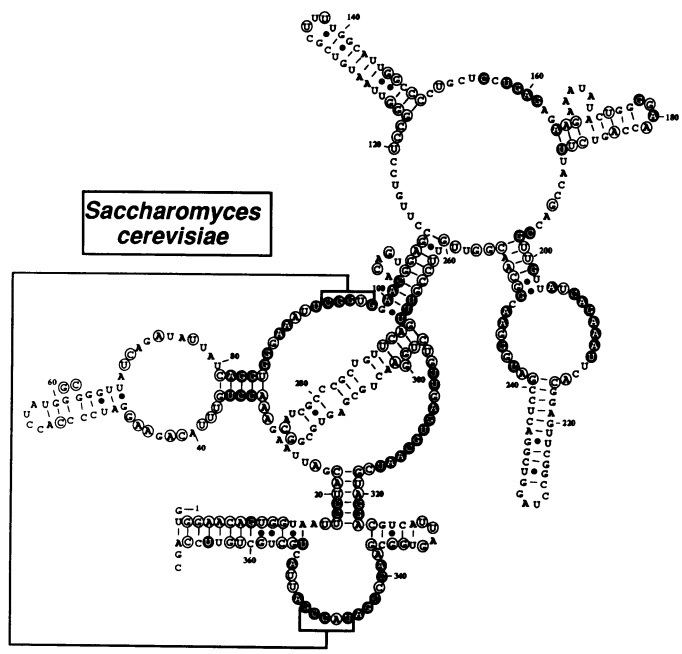


Figure 7. Possible important nucleotides of *S.cerevisiae* RNase P RNA. Nucleotide positions of *S.cerevisiae* RNase P RNA that are conserved in complementing RNAs, but not conserved in non-complementing RNAs are summarized to indicate positions that might be critical for recognition by *S.cerevisiae* proteins or processing machinery. These positions are denoted by open circles. Dark circles with white letters indicate nucleotides that are invariant among yeast (16) and therefore presumed to be functionally important.

When the *S.cerevisiae* RNA is compared to the RNA species that did not complement the *RPR1* disruption, numerous sequence and structural differences are observed (Fig. 6). Some of the most obvious differences are the presence of the stem 212–218/223–229 in *S.globosus* RNA, the absence of stem Sce 269–282/289–303 in *S.pombe* and absence of stem Sce 123–134/139–150 in both species. Some of the differences in Figure 6 overlap the set of non-essential sequences shown in Figure 5.

Positions that are conserved in complementing RNAs, but not conserved in non-complementing RNAs are shown as open circles in Figure 7. In addition, dark circles with white letters in Figure 7 show positions that are invariant among yeast (16) and as such also suspected of playing essential functional roles. Many of the sequences denoted by open circles in the *S.cerevisiae* structure cluster near nucleotides that are invariant among yeast (dark circles). Most of the open circles correspond to nucleotides that are conserved within the *Saccharomyces* RNAs but changed in *Schizosaccharomyces*. These highlighted positions in Figure 7 are part of nucleotide regions that have been identified as highly conserved according to phylogenetic studies (16). Some of these highly conserved regions are the terminal stem Sce 2–14/355–367, stem Sce 17–22/318–323, Sce 31–36, Sce 38–44, Sce 81–98, Sce 100–111/262–272, Sce 204–216/241–249, Sce 299–317 and Sce 337–354 (Fig. 7). Interestingly, there is a group of nucleotides that were conserved only between *S.cerevisiae* RNA and the RNAs that complemented, *S.carlsbergensis* and *S.kluyveri*, suggesting candidate positions to more specific interactions of the RNA, possibly with the protein component(s). Some of these positions are Sce 37,42,45,46,53,62,63, several nucleotides in the region Sce 70–77, Sce 120–125, 146–147, 149–150, and Sce 165,166,184,187, 194, 199, 200, 253, 255, 256, 259, 260, 329, 330, 332 and 335 (Fig. 7).

Previous comparisons of eukaryotic RNase P RNAs identified sequences that are highly conserved and therefore possibly essential for function (16,31). This study serves a slightly different function in that complementing clones are used to sort variable sequences by whether they either *are not* or *might be* essential for function with other RNase P components. Thus it is possible to identify positions that are crucial for protein binding or localization, but that can vary because of compensatory mutations in protein partners. These studies will serve as guides for directed mutagenesis experiments to determine structure–function relationships in the holoenzyme.

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