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Phylogenetic analysis of the structure of RNase MRP RNA in yeasts

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ABSTRACT

RNase MRP is a ribonucleoprotein enzyme involved in processing precursor rRNA in eukaryotes. To facilitate our structure–function analysis of RNase MRP from *Saccharomyces cerevisiae*, we have determined the likely secondary structure of the RNA component by a phylogenetic approach in which we sequenced all or part of the RNase MRP RNAs from 17 additional species of the *Saccharomycetaceae* family. The structure deduced from these sequences contains the helices previously suggested to be common to the RNA subunit of RNase MRP and the related RNA subunit of RNase P, an enzyme cleaving tRNA precursors. However, outside this common region, the structure of RNase MRP RNA determined here differs from a previously proposed universal structure for RNase MRPs. Chemical and enzymatic structure probing analyses were consistent with our revised secondary structure. Comparison of all known RNase MRP RNA sequences revealed three regions with highly conserved nucleotides. Two of these regions are part of a helix implicated in RNA catalysis in RNase P, suggesting that RNase MRP may cleave rRNA using a similar catalytic mechanism.

Keywords: RNA catalysis; RNA processing; rRNA

INTRODUCTION

Ribonuclease MRP (RNase MRP) and RNase P are both enzymatically active ribonucleoproteins with essential RNA subunits (Frank et al., 2000; Xiao et al., 2001). RNase P is present in all three primary branches of life and is the universal enzyme for generating the mature 5′ end of tRNAs. RNase MRP, which has been found only in Eucarya, has a role in processing precursor rRNA, cleaving in the internal transcribed spacer 1 (ITS1) between the 18S and 5.8S rRNA moieties (Schmitt & Clayton, 1993; Chu et al., 1994; Lygerou et al., 1994). The enzyme may also be involved in formation of mitochondrial DNA replication primers (Lee & Clayton, 1997) and other, as yet unidentified, reactions (Cai et al., 1999; van Eenennaam et al., 2000). Whereas the RNA subunits from bacterial and archaeal RNase P have enzymatic activity in the absence of protein subunits, RNase MRP and eukaryotic RNase P RNAs are not catalytically active without associated protein subunits (Frank et al., 2000; Xiao et al., 2001).

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RNase MRP and RNase P are of clinical importance, because the Th/To antigens found in both enzymatic particles have been identified as targets for human autoimmune antibodies (Reimer et al., 1988; Reimer, 1990). In addition, mutations in the RNA subunit of RNase MRP have recently been recognized as a cause of cartilage-hair hypoplasia (Ridanpaa et al., 2001).

RNase MRP and RNase P have distinct enzymatic specificities (Lygerou et al., 1996). Nevertheless, two lines of evidence suggest that these two enzymes are evolutionarily related. First, the two enzymes in *Saccharomyces cerevisiae* share eight of nine protein subunits; only one protein subunit is unique to each of the enzymes (Lygerou et al., 1994; Schmitt & Clayton, 1994; Chu et al., 1997; Dichtl & Tollervey, 1997; Stolc & Altman, 1997; Chamberlain et al., 1998; Stolc et al., 1998). Second, the RNA subunits of RNase MRP and RNase P have been proposed to share a similar structural domain (Forster & Altman, 1990), suggesting that the RNA subunit-encoding genes are paralogues (Morrissey & Tollervey, 1995). Significantly, a portion of the shared domain (helix P4) is similar to the presumptive active site of bacterial RNase P, suggesting that RNase MRP and eukaryal RNase P retain vestiges of the RNA-mediated catalysis originating from an ancestor shared

with bacterial RNase P (type A). Beyond the structural similarities of the eukaryal and bacterial RNase P RNAs, support for RNA-based catalysis in yeast RNase P also has been recently provided by the finding that sulphur substitution of the bond targeted for cleavage of pre-tRNA strongly inhibits the reaction in the same manner as for the bacterial RNA (Thomas et al., 2000). Finally, at least one hairpin can be exchanged between the RNase P and RNase MRP RNAs without loss of enzymatic activity, suggesting that the two hairpins have coevolved, and likely interact with one or more of the common protein subunits (Lindahl et al., 2000; Ziehler et al., 2001).

Phylogenetic comparisons have offered a powerful approach to structure analysis of RNA molecules. This approach was used to define the relationships between eukaryal, archaeal, and bacterial RNase P RNAs (Haas et al., 1996; Chen & Pace, 1997; Pitulle et al., 1998; Frank et al., 2000; Xiao et al., 2001). However, due to the limited number of RNase MRP RNA sequences available for comparison, it has not been possible to devise accurate structure models for RNase MRP RNA and fully assess the extent to which the RNase MRP and RNase P RNAs are similar. To address this problem, we have used two approaches to acquire additional RNase MRP RNA sequences. First, we designed PCR primers based on conserved regions within previously isolated RNase MRP genes, and amplified portions of RNase MRP RNA encoding genes in a variety of yeasts in the *Saccharomycetaceae* family. Second, we used libraries for genetic complementation and colony hybridization to identify complete genes of RNase MRP RNA from several yeasts. Phylogenetic comparisons of 18 species have produced a substantial revision of the secondary structure of yeast RNase MRP RNA. Furthermore, a detailed comparison between RNase MRP and RNase P RNAs from these yeasts has provided insight into the molecular evolution of these two RNA-containing enzymes.

RESULTS AND DISCUSSION

Phylogenetic analysis

Because the two available yeast RNase MRP RNA sequences (from *S. cerevisiae* and *Schizosaccharomyces pombe*) were not easily aligned, we could not identify regions of consensus long enough for design of PCR primers. Instead, we compared the proposed secondary structures of these two yeast RNAs and those of metazoan RNase MRP and fungal and metazoan RNase P RNAs to identify conserved structural elements. We reasoned that regions comparable in secondary structure would also be conserved in sequence among species more closely related to *S. cerevisiae* than is *S. pombe*. Based on this analysis, we identified two regions as suitable for design of PCR primers: (1) the highly conserved P4 helix that is present in all known RNase P and RNase MRP RNAs, and (2) the more phylogenetically diverse P1 helix and part of the neighboring P2 helix (Fig. 1). Several forward and reverse primers were synthesized for each of these sequences based on the *S. cerevisiae* sequence. Using various combinations of these primers (see Materials and Methods), partial RNase MRP RNA genes were PCR amplified. Additional primers were then designed, taking this new sequence information into account, and more RNase MRP RNAs were amplified. All together, we acquired MRP sequences from 17 species belonging to the genera *Arxiozyma*, *Kluyveromyces*, *Saccharomyces*, *Torulaspora*, and *Zygosaccharomyces* (Table 1, Fig. 2).

To capture the entire RNase MRP RNA gene sequence from selected species, we constructed libraries of size fractionated *Eco*RI-digested genomic DNA from *Kluyveromyces lactis*, *Saccharomyces globosus*, and *Torulaspora delbrueckii*, and selected clones carrying the full RNase MRP RNA gene by genetic complementation of a deletion of the genomic RNase MRP RNA gene in *S. cerevisiae*. This strategy was successful for *K. lactis* and *S. globosus*, but not for *T. delbrueckii*. However, we cloned the complete gene from *T. delbrueckii* by colony hybridization.

Two lines of evidence suggested that we had in fact cloned MRP genes. First, the 5′ and 3′ regions of the complete sequences from these species were consistent with structures of helices P1–P4 in other RNase MRP and RNase P RNAs (Figs. 1 and 2). Second, the genes from *S. globosus* or *K. lactis* were functional in *S. cerevisiae* as indicated by their support of growth of constructs in which the *S. cerevisiae* RNase MRP RNA gene had been replaced by the corresponding gene from either of the two species. Furthermore, the ratio between long and short 5.8S rRNA, which is an indicator of RNase MRP activity (Chu et al., 1994; Lygerou et al., 1994), was indistinguishable from wild-type *S. cerevisiae* for the strain carrying the *S. globosus* gene and only slightly elevated for the *K. lactis* gene (Fig. 3). These results indicate that the *S. globosus* and *K. lactis* RNase MRP RNAs work as well as, or nearly as well as, *S. cerevisiae* RNase MRP RNA when transplanted to *S. cerevisiae* cells.

We also analyzed the function of the *T. delbrueckii* RNase MRP RNA gene. Although we were unsuccessful in cloning this gene by genetic complementation, we were able to swap plasmids carrying the *T. delbrueckii* gene and the *S. cerevisiae* gene. The resulting strain carrying only the *T. delbrueckii* gene grew very slowly and had a significantly elevated ratio of long to short 5.8S rRNA (Fig. 3), indicating reduced RNase MRP activity. We reasoned that this phenotype might be caused by poor expression of the heterologous gene in *S. cerevisiae*. Accordingly, we replaced the sequences flanking the *T. delbrueckii* gene with *S. cerevisiae* se-

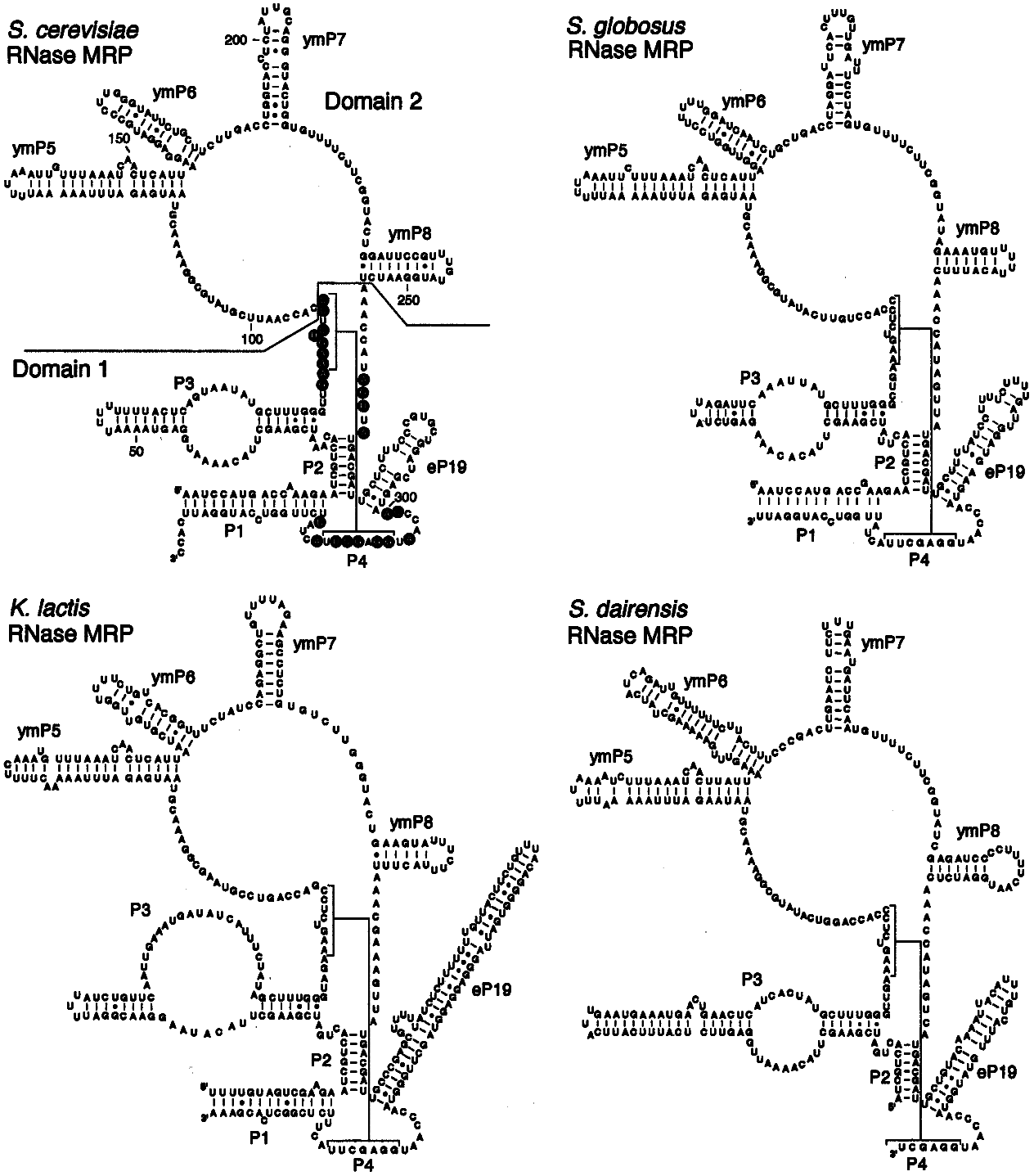

FIGURE 1. Secondary structures of RNA subunits of RNase MRP in yeasts of the *Saccharomycetaceae* family. Although not shown here, structures with the same set of helices can also be drawn for the other RNase MRP sequences shown in Figure 2. Nucleotide numbers are only shown in the *S. cerevisiae* structure, as we have not mapped endpoints of the mature RNA sequences of other species. It is likely that the new sequences have a few extra bases beyond P1, as is shown for *S. cerevisiae*. Nucleotides shown in reverse contrast in the *S. cerevisiae* structure are identical in all known RNase MRP RNA structures (see text).

quences to optimize expression of the heterologous gene. Indeed, this resulted in normal growth rate and normal ratio of the two forms of 5.8S rRNA, suggesting that *T. delbrueckii* RNase MRP RNA also works as well as *S. cerevisiae* RNase MRP RNA (Fig. 3).

The partial RNase MRP RNA genes are also homologs of the *S. cerevisiae* RNase MRP RNA gene (Fig. 2). Calculated for the region encompassed by the P4-specific primers YMRP1F and YMPR1R, this identity ranges from 64% (*Saccharomyces dairensis*) to 100%

TABLE 1. Yeast strains and RNase MRP RNA accession numbers.

Organism	Strain	Source[a]	Accession no.
Arxiozyma telluris	NRRL YB-4302	1	AY090593
Kluyveromyces africanus	NRRL Y-8276	1	AY090594
Kluyveromyces delphensis	NRRL Y-*2379*	1	AY090595
Kluyveromyces lactis	ATCC 48793	2	AY090596
Saccharomyces barnetti	NRRL Y-27223	1	AY090597
Saccharomyces bayanus	NRRL Y-17034	1	AY090598
Saccharomyces castellii	NRRL Y-12630	3	AY090599
Saccharomyces cerevisiae	YLL302	4	Z14231
Saccharomyces dairensis	NRRL Y-12639	1	AY090600
Saccharomyces diastaticus	NRRL Y-2044/ATCC 13006	1	AY090601
Saccharomyces globosus	ATCC 10600	5	AY090602
Saccharomyces kluyveri	NRRL Y-4288-26	1	AY090603
Saccharomyces norbensis	CG-H315	6	AY090604
Saccharomyces pastorianus	NRRL Y-1525	1	AY090605
Saccharomyces servazzii	NRRL Y-12661	1	AY090606
Saccharomyces unisporus	NRRL Y-1556	1	AY090607
Torulaspora delbrueckii	ATCC 10662	2	AY090608
Zygosaccharomyces rouxii	NRRL-Y229	3	AY090609

[a]1. C. Kurtzman; 2. American Type Culture Collection; 3. C. Guthrie; 4. Lindahl et al., 2000; 5. Tranguch and Engelke, 1993; 6. R. Mortimer.

(*Saccharomyces diastaticus*). It is interesting to compare the pairwise sequence identity among RNase MRP and P RNAs. The sequences between the two strands of the P4 helix are known for both RNase P and RNase MRP RNAs for 15 species. The mean sequence identity between the RNAs of each species and *S. cerevisiae* is 73% for RNase MRP RNA, but only 59% for RNase P RNA, indicating that within the fungi analyzed here, the RNase P RNA has undergone more extensive evolution than has RNase MRP RNA. Much of the variation in RNase P is due to sporadic presence of helices (Frank et al., 2000).

The structures in Figure 1 and the sequences shown in Figure 2 indicate that much of the sequence variation that exists between the RNA species is localized to putative helical elements, with variable lengths. Consequently, the sequences in Figure 2 are aligned only in regions where homology is evident by sequence similarities. Helical regions that vary in length are not necessarily aligned (e.g., helices ymP6, 7, 8, and eP19). Individual instances of the proposed structures of these variable length helices are depicted in Figure 1. Despite many base substitutions within the variable helices, compensatory mutations maintain the integrity of the proposed pairings. The sequence covariation found in both the complete and the partial MRP genes supports the existence of all MRP helices shown in Figures 1 and 2.

As expected, the structures of the four helices proposed in Domain 1 (labeled P1, P2, P3, and P4; Fig. 1) conform, in general, to previously proposed models for RNase MRP RNA (Forster & Altman, 1990; Karwan, 1993; Schmitt et al., 1993; Reilly & Schmitt, 1996). Indeed, homologous structures are also present in all known examples of cellular RNase P RNA, including those of Archaea and Bacteria (Chen & Pace, 1997). In keeping with the nomenclature used for RNase P RNA, we refer to these regions as P1–P4 (for paired regions 1–4). A fifth helix in Domain 1, eP19 (Fig. 1), was also proposed previously (Schmitt et al., 1993) and occupies a position equivalent to eP19 in eukaryotic RNase P RNA (Frank et al., 2000). This helix also occurs in some, but not all, RNase P RNAs from Bacteria and Archaea.

The structure of Domain 2 (Fig. 1) shows no similarity with the RNase P structure. Two of the proposed helices in this region, labeled ymP5 and ymP8 (Figs. 1 and 2), are also similar to hairpins previously proposed in the *S. cerevisiae* RNase MRP RNA (Schmitt et al., 1993). Compensatory changes among the species analyzed here support the ymP5 structure (Figs. 1 and 2). For example, the fourth base pair from the bottom of ymP5 is GC in *S. cerevisiae*, but AU in *S. dairensis* (Fig. 1). Apart from these changes, the sequence of ymP5 is remarkably conserved. Finally, we note that the ymP5 structure is also compatible with compensatory mutations in the *S. cerevisiae* RNase MRP RNA gene (Lindahl & Zengel, 1996).

The proposed structure for the remaining part of Domain 2 (Fig. 1) differs significantly from the previous model (Schmitt et al., 1993), which is incompatible with the newly expanded sequence set. This region contains two helices, ymP6 and ymP7, which are highly variable in primary sequence, but well supported by compensatory changes in most of the individual base pairs (Figs. 1 and 2). These two helices replace a very long helix in the old model, which has been proposed to protrude to one side of the molecule (Schmitt, 1999).

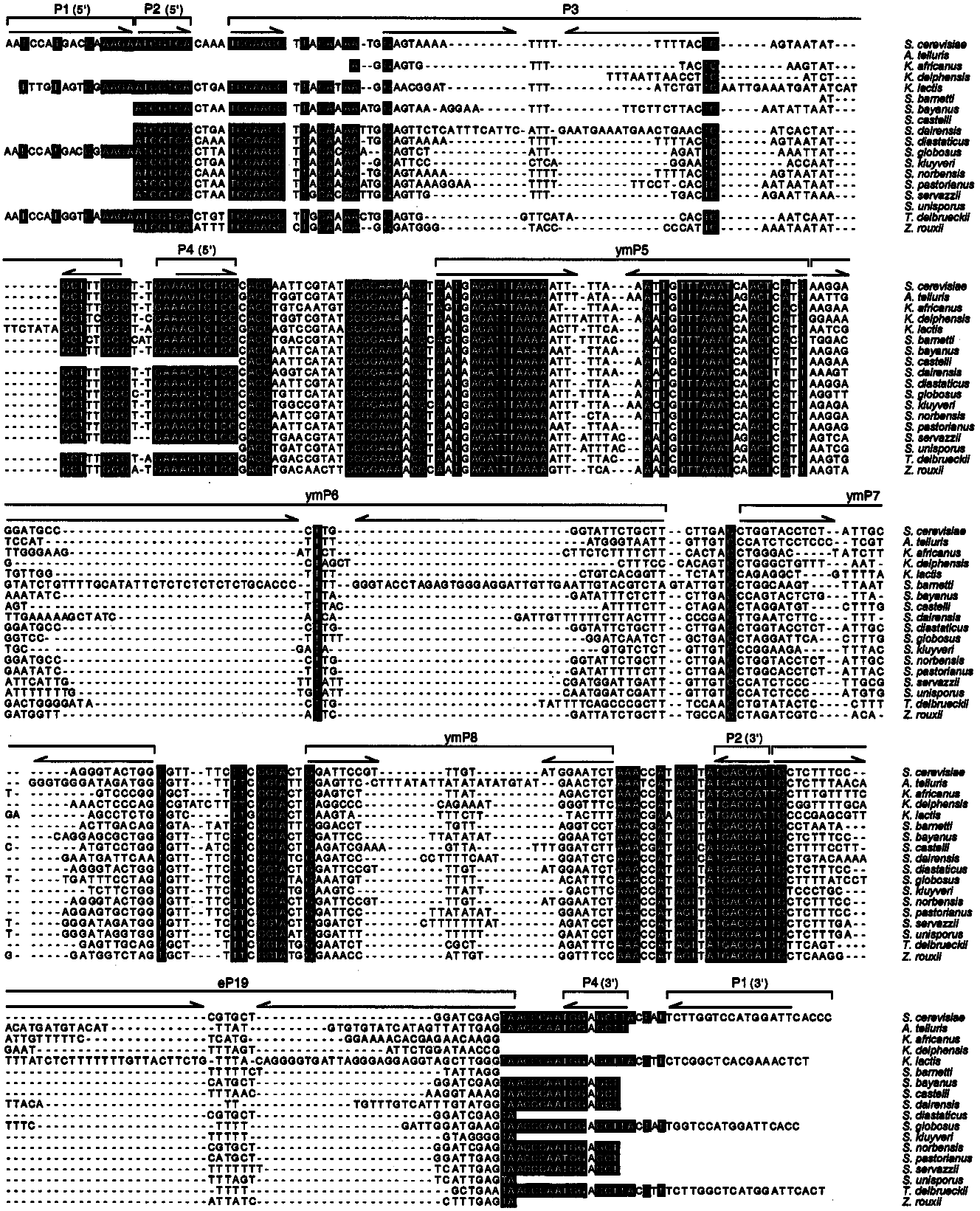

FIGURE 2. Sequences of RNA subunits of RNase MRP in yeasts of the *Saccharomycetaceae* family. Regions of sequence length variation are not necessarily aligned by homology. Instead, putative helical regions are defined by homologous flanking sequences. The *S. cerevisiae* sequence was published (Schmitt & Clayton, 1992). The remaining sequences were determined from DNA isolated from the strains listed in Table 1 (see text for details). Accession numbers are listed in Table 1.

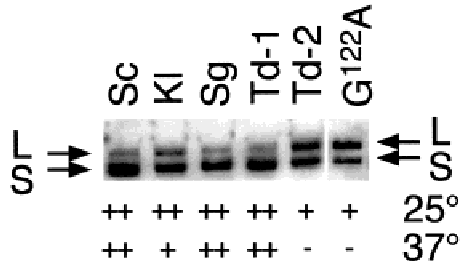

FIGURE 3. Northern analysis of 5.8S rRNA in strains of *S. cerevisiae* carrying heterologous RNase MRP genes. The chromosomal copy of the RNase MRP gene (*RRP2, alias NME1*) has been deleted in *S. cerevisiae* strain YLL302. Plasmid swapping was used as previously described (Lindahl et al., 2000) to replace the plasmid-borne copy of *S. cerevisiae RRP2* with the corresponding gene from *S. globosus* (Sg), *K. lactis* (Kl), or *T. delbrueckii* (Td). For *S. globosus* and *K. lactis*, both the gene sequence and the flanking sequences (i.e., promoter and transcription terminator) were heterologous, but for *T. delbrueckii*, we prepared RNA from two constructs. In one strain (Td-2), the flanking sequences from *T. delbrueckii* were kept, but in the other strain (Td-1) the flanking sequences were replaced with *S. cerevisiae* sequences. For comparison we also show RNA from YLL302 in which the *S. cerevisiae RRP2* gene has been maintained (Sc) and from the *S. cerevisiae* mutant *rrp2-2* in which base G^{122} has been replaced with A (Lindahl et al., 1992; Chu et al., 1994). The latter mutant has reduced RNase MRP activity, resulting in a reduced growth rate and increased ratio of long to short 5.8S rRNA. Blots were probed with a ^{32}P-labeled oligonucleotide complementary to positions 24–48 of the short version of 5.8S rRNA.

The replacement of this long helix with ymP6 and ymP7 in our new model also impacts the interpretation of some previously published deletion mutations in RNase MRP RNA (Shadel et al. 2000), because they remove part, but not all, of ymP6 and ymP7, and thus potentially could lead to refolding of the structure outside of the deletion itself.

Structure probing analysis

To further test the structure model, we performed chemical and enzymatic structure probing of the *S. cerevisiae* RNase MRP RNA. We were particularly interested in exploring the region where the proposed structure differs radically from the previously proposed model of the RNA (ymP6 and ymP7, Fig. 1). Total RNA isolated from *S. cerevisiae* or in vitro-synthesized RNase MRP RNA was subjected to limited digestion with RNase V1 and T2, as well as modification with dimethyl sulfate (DMS). RNase V1 is specific for regions of stacked bases (including helical regions), while the other two probes attack single-stranded regions (Ehresmann et al., 1987). Sites of attack in RNase MRP RNA were mapped by reverse transcription using primer YMRP3R (Table 2).

As shown in Figure 4, the digestion and modification patterns are compatible with the proposed structures of ymP6, 7, and 8. In particular, we found strong attacks by RNase T2 and DMS in the loop regions of ymP7 and ymP8. Two nucleotides in the ymP7 loop that were strongly attacked by RNase T2 are paired in the previously proposed model (Schmitt et al., 1993). Hence, the results of structure probing comply better with the new phylogenetic model proposed here than with previous suggestions for the RNase MRP RNA secondary structure. DMS probing of in vitro-synthesized RNase MRP RNA gave results virtually identical to the analysis of RNA isolated from cells (data not shown). Because the phylogenetically based structure outside the ymP6–7 region did not differ significantly from the previously proposed structures, we did not do a complete structure probing of these regions. However, available results (not shown) fully support the structure in Figure 1.

TABLE 2. Oligo nucleotides.

YMRP1F: 5′-AATCCATGACCAAAGA
YMRP2F: 5′-GCTTTGGGWTGAAAGTCTCC
YMRP3F: 5′-TGCTTTGGGTTGAAAGTCTCC
YMRP4F: 5′-CGAAGAATCGTCACTGATCGAAGC
YMRP1R: 5′-CATGGACCAAGAATAGTA
YMRP2R: 5′-TAGTAAGCTCCATTGGGT
YMRP3R: 5′-ATAGTAAGCTCCATTGGGTTA
YMRP4R: 5′-TAAGCCCCATTGGGTTAC
YMPR5R: 5′-CTCATTACGTTTTCCGCATACG

Potential determinants in RNase MRP RNA for protein binding

The *S. cerevisiae* RNase MRP and RNase P particles have a protein to RNA ratio between 2 and 3 (Chamberlain et al., 1998), substantially greater than the ratio in the bacterial RNase P, which is about 0.15. RNase MRP and eukaryotic RNase P are therefore much more proteinaceous than the bacterial enzyme. To begin to understand the function(s) of the extra proteins in the eukaryotic enzymes, it is important to know the distribution of proteins relative to structural features of the RNA subunit.

Recent three-hybrid experiments suggest that Pop1p, the largest protein common to RNase MRP and RNase P, interacts with helix P3 (Ziehler et al., 2001), an essential hairpin in both RNases (Pagan-Ramos et al., 1994; Lindahl et al., 2000; Shadel et al., 2000). In in vitro binding assays with human RNase MRP components, the P3 helix has also been crosslinked to smaller proteins, including the Th/To antigen (Yuan et al., 1991; Liu et al., 1994; Reddy & Shimba, 1996; Pluk et al., 1999). The idea that P3 serves as a protein-binding site is also compatible with the critical importance of this hairpin for nucleolar localization of RNase MRP RNA injected into rat epithelial cells (Jacobson et al., 1995).

In all cases where sequences for both RNase MRP and RNase P RNAs are known, several bases in the internal loop and flanking base pairs are conserved in the two P3 hairpins (Fig. 5; Ziehler et al., 2001). Indeed,

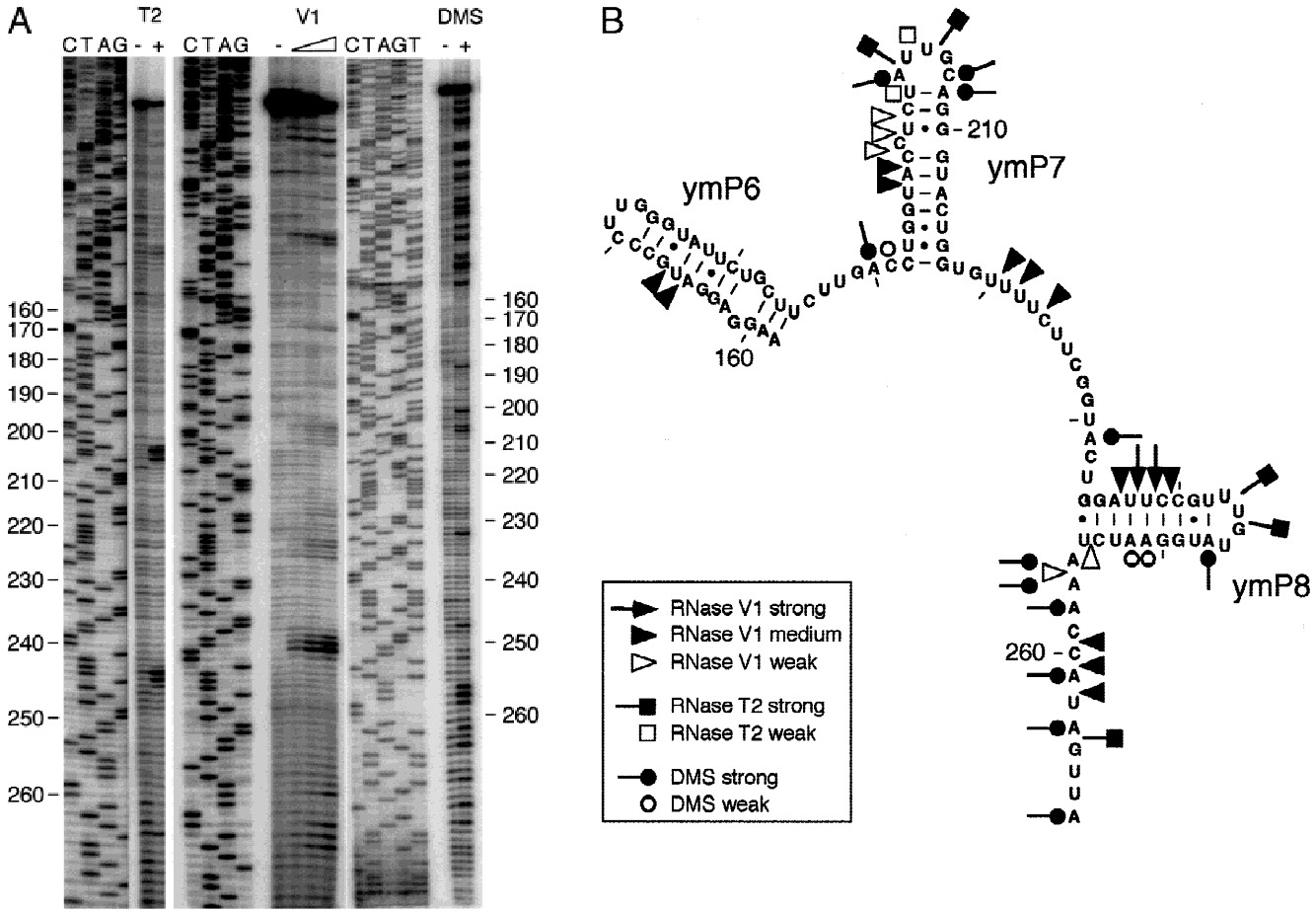

FIGURE 4. Structure probing of RNase MRP RNA from *S. cerevisiae*. Total RNA from *S. cerevisiae* was isolated and subjected to treatment with DMS, RNase T2, or RNase V1. Incubation time and the concentrations of the probing agent and RNA were adjusted such that at least 25–50% of the RNase MRP RNA was unaffected by the agent (to minimize multiple hits in a single RNA molecule). Sites accessible to each probe were mapped by reverse transcription using primer YMRP3. The products of the primer extension were analyzed by gel electrophoresis followed by phosphorimager analysis. Examples of gel images are shown in **A**. Summary of the accessible nucleotides are shown in **B**. Base numbers relative to the 5′ end of the RNA molecule are indicated.

we have shown that the P3 hairpins in RNases MRP and P in *S. cerevisiae* are functionally interchangeable (Lindahl et al., 2000; Ziehler et al., 2001). This suggests that one or more proteins may recognize features shared by the two hairpins, presumably because the P3 structures in the two RNAs and the proteins binding to this region of the RNAs have coevolved (Lindahl et al., 2000). Functionally critical conserved features of the P3 hairpin must also extend to the RNase MRP RNAs of other members of the *Saccharomycetaceae*, as RNase MRP RNA genes from *S. globosus*, *K. lactis*, and *T. delbrueckii* can substitute for the *S. cerevisiae* RNase MRP RNA gene (see above). Nevertheless, such determinants are not universally conserved, given that *S. cerevisiae* RNase MRP RNA in which the P3 hairpin of RNase MRP from *S. pombe* or human has been substituted for the natural P3 hairpin does not form an active RNase MRP enzyme (Lindahl et al., 2000). Interestingly, mutations in *S. cerevisiae* RNase MRP RNA that remove or alter the terminal loop of P3 have no phenotype (Lindahl et al., 2000), suggesting that this part of P3 does not contribute significantly to protein binding. Taken together, these observations suggest that bases in the P3 internal loop region may serve as a species-specific site for protein binding. However, inspection of P3 structures from different species does not provide obvious candidates for such critical features.

It is improbable that all nine protein subunits bind to P3. Some proteins may bind to other conserved regions, such as P4 (see below). Other proteins may not bind directly to RNA, but may be associated with proteins that have direct RNA contact. It also is plausible that the Snm1p and Rpr2p proteins, which are unique to RNase MRP and P, respectively, associate with the regions that differ structurally between the two RNA subunits (ymP5–8).

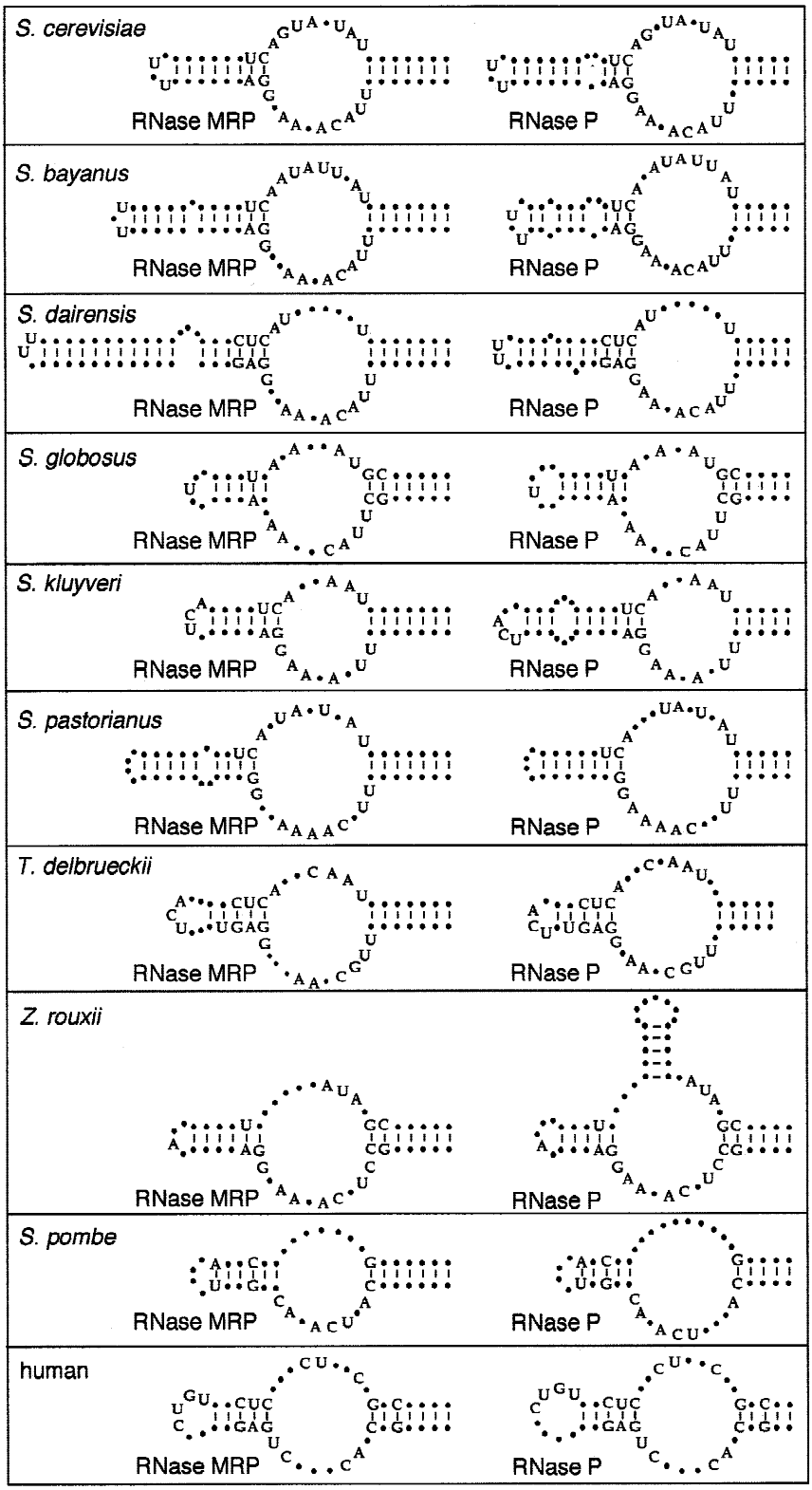

FIGURE 5. Comparison of secondary structures in P3 helices from RNase MRP and RNase P RNAs in various yeasts of the *Saccharomycetaceae* family. Sequence data were obtained from this paper, from Tranguch and Engelke (1993) and Frank et al. (2000), and from supplementary data to Frank et al. (2000) (http://pacelab.colorado.edu/Publications/publications.html).

Comparison of the RNase MRP and RNase P RNA structures in yeasts and higher eukaryotes

The region of yeast RNase MRP RNA containing helices P1 through P4 (Domain 1) is very similar to the structures previously reported for RNase MRP RNA from mammals, *Xenopus laevis*, and *S. pombe* (Schmitt et al., 1993; Paluh & Clayton, 1995). The sequence for *Arabidopsis thaliana* RNase MRP RNA (Kiss et al., 1992) is also compatible with this structure (not shown). Furthermore, comparisons of all known RNase MRP RNA

sequences reveal three regions of the secondary structure with universally conserved nucleotides (Fig. 1). Interestingly, these regions correspond in position in the secondary structure with three of the five "conserved regions" in RNase P RNA (Pagan-Ramos et al., 1996; Chen & Pace, 1997). Two of the conserved sequences contribute to the P4 helix. This helix has been implicated as part of the catalytic site in bacterial RNase P and is thus of special interest. Comparison of P4 in RNase P RNAs from species of all kingdoms shows a pattern of conserved bases (Fig. 6). A significant subset of these bases is also conserved in the P4 helix of known RNase MRP RNAs (Fig. 6), suggesting that P4 in RNase MRP may also contribute to the catalytic center of RNase MRP.

The remaining half of the RNase MRP RNA structure (Domain 2; Fig. 1) is much more divergent. As already mentioned, the structure of Domain 2 in any particular yeast RNase MRP RNA differs considerably from the structure of RNase P RNA in the same species. Furthermore, the sequences of RNase MRP RNAs in mammals, *Xenopus*, and *S. pombe* are not compatible with a structure homologous to the structure in "*S. cerevisiae*-like" yeasts that includes helices ymP5 through ymP8 (Fig. 1). We suggest that different groups of eukaryotes differ in Domain 2 structure.

The divergence of Domain 2 may reflect differences in the substrates. On the one hand, the highly conserved structure of all pre-tRNAs correlates with the high degree of structure conservation of Domain 2 among RNase P molecules from all kingdoms. On the other hand, the differences between Domain 2 from RNase P and MRP RNAs as well as between RNase MRP RNAs from different types of organisms correlate with (1) the apparent absence of secondary structure similarity between the two RNA substrates (pre-tRNA and the "A3 site" in ITS1 of rRNA), and (2) the striking variability among ITS1 structures from diverse organisms. This suggests that Domain 2 may contribute to substrate recognition whereas Domain 1 functions mainly as the catalytic engine. Such functional division between two independently folding domains has also been suggested by kinetic analysis of deletion derivatives of *Bacillus subtilis* RNase P (Loria & Pan, 2001).

MATERIALS AND METHODS

Strains

The yeast strains used, listed in Table 1, were obtained from Drs. C. Guthrie (University of California, San Francisco (UCSF)), C. Kurtzman (National Center for Agricultural Utilization Research), R. Mortimer (Berkeley Yeast Genetic Strain Collection (YGSC)), and D. Engelke (University of Michigan), as well as from American Type Culture Collection (Manassas, Virginia). Cultures were grown in YEPD medium or synthetic complete medium lacking tryptophan (Sherman et al., 1974). Plasmids were amplified in *Escherichia coli* DH5α.

DNA methods

Genomic DNA was isolated from yeast strains either by the protocol of Philippsen et al. (1991), or by use of the QIAmp kit (Qiagen). Partial RNase MRP genes were amplified by polymerase chain reaction (PCR) with primers designed from conserved regions of RNase MRP RNA (Table 2). The following primer pairs were used: YMRP1F/YMRP1R (*Saccharomyces bayanus*, *S. dairensis*, *Saccharomyces norbensis*, *Saccharomyces pastorianus*, *Saccharomyces servazzii*); YMRP1F/YMRP2R (*S. diastaticus*, *S. globosus*, *S. kluyveri*, *T. delbrueckii*, *Zygosaccharomyces rouxii*); YMRP2F/YMRP1R (*Arxiozyma telluris*, *Saccharomyces castellii*, *Saccharomyces unisporus*); YMRP3F/YMRP3R (*S. globosus*, *K. lactis*, *T. delbrueckii*); YMRP4F/YMRP4R (*Kluyveromyces delphensis*, *S. bayanus*), YMRP4F/YMRP3R (*Kluyveromyces africanus*, *S. barnetti, Z. rouxii*). Each 100-μL reaction contained 10–30 mM Tris-Cl, pH 8.3–9, 50 mM KCl, 1.5–4.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.05% NP40, 1 U Taq polymerase, 200–500 ng of each primer, and 100–1,000 ng genomic DNA. Amplification of RNase MRP RNA genes was carried out either by a standard regimen (e.g., 30 cycles, 92 °C 1 min, 50 °C 1–2 min, 72 °C 3–7 min; in some cases this regimen was preceded by 1 cycle 95 °C 10 min) or by "touchdown PCR" (20 cycles, 92 °C 30 s, 65 °C 30 s -1 °C/cycle, 72 °C 90 s; 20 cycles 92 °C 30 s, 45 °C 30 s, 72 °C 90 s). For standard PCR reactions, a range of annealing temperatures (45–55 °C) was assayed and products were isolated from the highest temperature that produced DNA fragments of the expected length upon agarose gel electrophoresis. If several bands were present, the band of the expected size was excised and purified by QIAquick gel extraction kit (Qiagen) following the manufacturer's instructions. PCR products were either sequenced directly, or after cloning using the TA Cloning or TOPO Cloning kits (Invitrogen) following the manufacturer's instructions.

The complete genes for *S. globosus* and *K. lactis* were cloned by first determining by southern analysis the size of the *Eco*RI fragment carrying the RNase MRP RNA gene using as probe PCR fragments generated as described above. A library of *Eco*RI fragments of the appropriate size was constructed in the plasmid vector YCplac22 (Gietz & Sugino, 1988) and transformed into YLL302 (Lindahl et al., 2000), selecting for tryptophan prototrophy. Finally, candidates for plasmids carrying complementing RNase MRP RNA genes were selected by plating on 5-fluoroorotic acid, which kills cells with a functional *URA3* gene (Boeke et al., 1987). The chromosomal RNase MRP RNA gene (*RRP2*) is deleted from YLL302, and the only *RRP2* gene in this strain is carried by a *URA3* plasmid. Hence, only colonies with a YCplac22-borne heterologous RNase MRP RNA gene capable of complementing the chromosomal deletion of the RNase MRP RNA gene in YLL302 survive the 5-fluoroorotic acid selection. Surviving colonies were confirmed as carrying the heterologous RNase MRP RNA gene by sequence analysis and comparison with the partial genes obtained by PCR amplification. We failed to clone the complete RNase MRP RNA gene from *T. delbrueckii* using this method. Instead, the RNase MRP RNA gene from this species was retrieved by colony hybridization of a size-selected library using as probe oligonucleotide YMPR5R, which is complementary to positions 101–122 of the *S. cerevisiae*

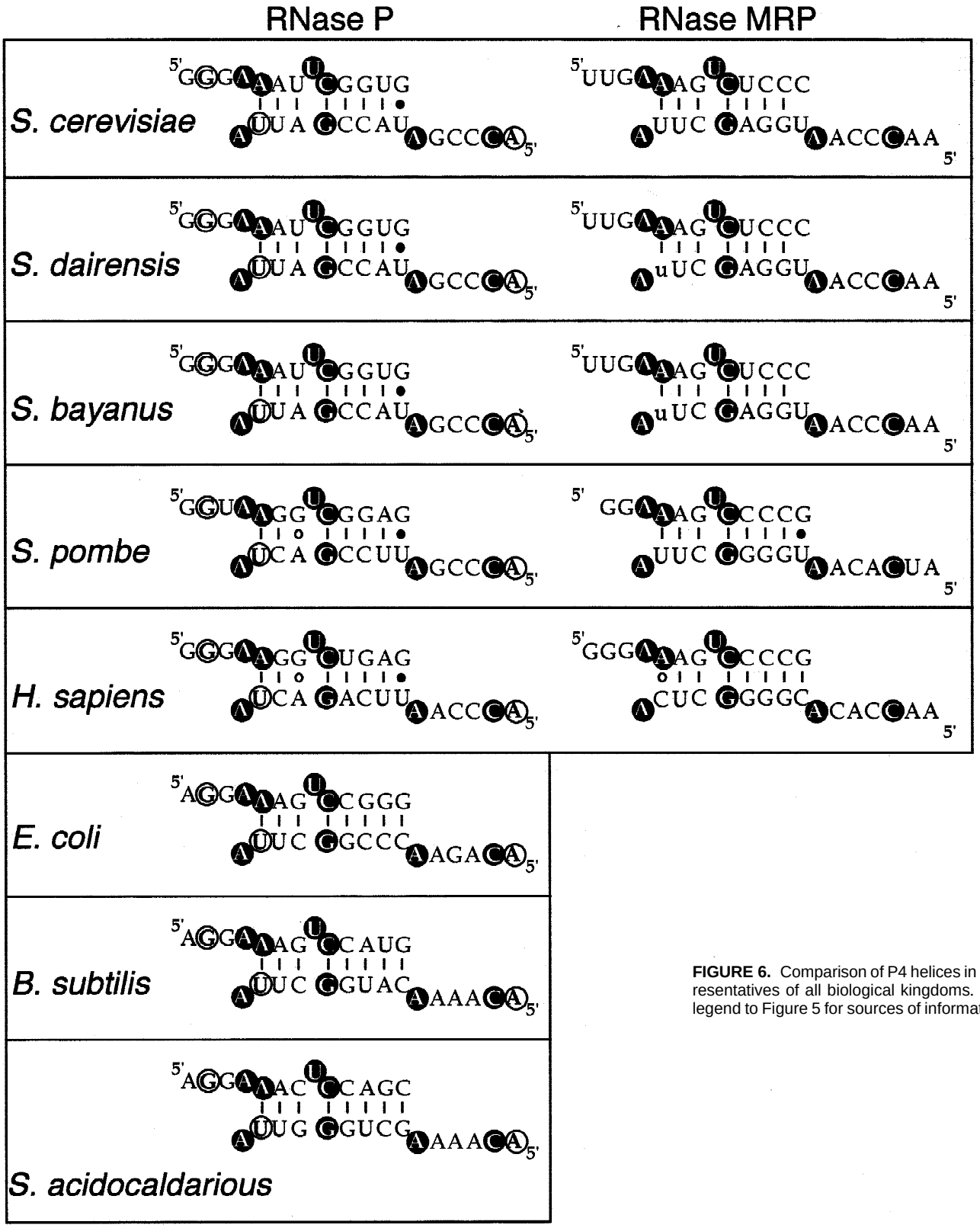

FIGURE 6. Comparison of P4 helices in representatives of all biological kingdoms. See legend to Figure 5 for sources of information.

RRP2 gene. A plasmid with the *T. delbrueckii* RNase MRP gene flanked by the sequences upstream and downstream of the *S. cerevisiae* RNase MRP RNA gene was constructed from plasmid pDK49, a derivative of YCp*lac*22 (Gietz & Sugino, 1988) carrying the *S. cerevisiae RRP2*, including 184 bp upstream and 206 bp downstream of this gene. The *S. cerevisiae* structural gene, but not the flanking regions, in pDK49 was replaced with a PCR fragment con-

taining the *T. delbrueckii* gene as described by Geiser et al. (2001).

Sequence analysis and alignment

RNase MRP RNA genes were sequenced on an ABI 373A or ABI 310 automated DNA sequencer. Newly determined sequences were deposited in GenBank (see Table 1 for accession numbers). The previously determined sequence of RNase MRP RNA in *S. cerevisiae* was obtained from GenBank (accession number Z14231). Sequences were manually aligned using the applications SeqApp (courtesy of Dr. Don Gilbert) or MegAlign (DNA Star). Possible RNA secondary structures were identified using M-fold (Mathews et al., 1999; Zuker et al., 1999) or visual inspection.

RNA structure probing

Total RNA was purified from YLL302 (Lindahl et al., 2000) and subjected to RNase V1 and T2 digestion and dimethylsulfate modification (DMS), using published procedures (Ehresmann et al., 1987; Shen et al., 1988) with minor modifications. Sites of cleavage or modification were identified by primer extension using a primer hybridizing to positions 299–319 of *S. cerevisiae* RNase MRP RNA.

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