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Pseudouridylation at Position 32 of Mitochondrial and Cytoplasmic tRNAs Requires Two Distinct Enzymes in *Saccharomyces cerevisiae**

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Cytoplasmic and mitochondrial tRNAs contain several pseudouridylation sites, and the tRNA:Ψ-synthase acting at position 32 had not been identified in *Saccharomyces cerevisiae*. By combining genetic and biochemical analyses, we demonstrate that two enzymes, Rib2/Pus8p and Pus9p, are required for Ψ32 formation in cytoplasmic and mitochondrial tRNAs, respectively. Pus9p acts mostly in mitochondria, and Rib2/Pus8p is strictly cytoplasmic. This is the first case reported so far of two distinct tRNA modification enzymes acting at the same position but present in two different compartments. This peculiarity may be the consequence of a gene fusion that occurred during yeast evolution. Indeed, Rib2/Pus8p displays two distinct catalytic activities involved in completely unrelated metabolism: its C-terminal domain has a DRAP-deaminase activity required for riboflavin biogenesis in the cytoplasm, whereas its N-terminal domain carries the tRNA:Ψ32-synthase activity. Pus9p has only a tRNA:Ψ32-synthase activity and contains a characteristic mitochondrial targeting sequence at its N terminus. These results are discussed in terms of RNA:Ψ-synthase evolution.

thases (pseudouridine synthases Pus1p, Pus3p, Pus4p, Pus5p, Pus6p, and Cbf5p). Pus1p and Pus3p are TruA-related proteins. Pus1p is a multisite-specific RNA:Ψ-synthase acting at seven different positions in various cytoplasmic tRNAs (positions 26, 27, 28, 34, 36, 65, and 67), as well as at position 44 in U2 snRNA (6–8). Pus3p catalyzes Ψ formation at positions 38 and 39 in the anticodon stem-loop of several cytoplasmic and mitochondrial tRNAs (9). Like the *E. coli* TruB enzyme, the related yeast Pus4p is responsible for the formation of the highly conserved Ψ residue at position 55 in both cytoplasmic and mitochondrial tRNAs (10). Probably as much as 40 Ψ residues in yeast cytoplasmic rRNAs are formed by a unique enzyme of the TruB family, Cbf5p, that is guided by H/ACA small nucleolar RNAs (11, 12). A unique Ψ residue is present in yeast mitochondrial rRNA, and its formation is catalyzed by Pus5p, a member of the RluA family (13). Pus6p, another member of the RluA family, is responsible for Ψ formation at position 31 in both cytoplasmic and mitochondrial tRNAs (14). Pus7p, which was not listed initially among the putative RNA:Ψ-synthases because of its divergent amino acid sequence, was identified as an U2 snRNA:Ψ-synthase (15). We recently demonstrated that it acts at position 13 in cytoplasmic tRNAs and position 35 in the pre-tRNA^{Tyr} (16).

Presently, only three of the putative RNA:Ψ-synthases identified by Koonin (2) have no assigned RNA substrate: Pus2p, a TruA-related protein, and two proteins of the RluA family. They are encoded by the *YDL036* and *RIB2* (*YOL066*) ORFs, respectively. In addition to its potential RNA:Ψ-synthase encoding capacity, the *S. cerevisiae* *RIB2* gene was described a long time ago as encoding a DRAP-deaminase. This enzyme catalyzes the deamination of the 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5'-phosphate (DRAP) into 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione 5'-phosphate in the riboflavin biosynthesis pathway (17). The implication of the *RIB2* gene in riboflavin biosynthesis was demonstrated by genetic approaches (18, 19). However, the deaminase activity of Rib2p was never demonstrated directly. On the other hand, the activity and function of the putative RNA:Ψ-synthase domain present in Rib2p remained obscure.

In yeast, only rRNAs, tRNAs, and UsnRNAs are known to be the substrates of RNA:Ψ-synthases. The *S. cerevisiae* cytoplasmic tRNAs contain altogether 15 pseudouridylation sites, and 8 additional ones were detected in mitochondrial tRNAs (20) (Fig. 1). Several of these modification sites are common to cytoplasmic and mitochondrial tRNAs, and the RNA:Ψ-synthases responsible for their formation have been identified (Fig. 1). Only the enzymes responsible for the formation of residue Ψ1 found in two cytoplasmic tRNAs (tRNA^{Arg}(ACG) and tRNA^{Lys}(UUU)), Ψ72 found in the mitochondrial tRNA^{Met}(CAU), and Ψ32, a frequent modification, present in

Pseudouridine (Ψ)¹ and 2'-O-methylated nucleotides are the most abundant and universally found modified residues in RNAs of all types of organisms. Pseudouridine residues are formed post-transcriptionally by a group of enzymes called RNA:Ψ-synthases (1, 2). For a long time, the *Escherichia coli* RNA:Ψ-synthases were the only ones to be identified (3–5).

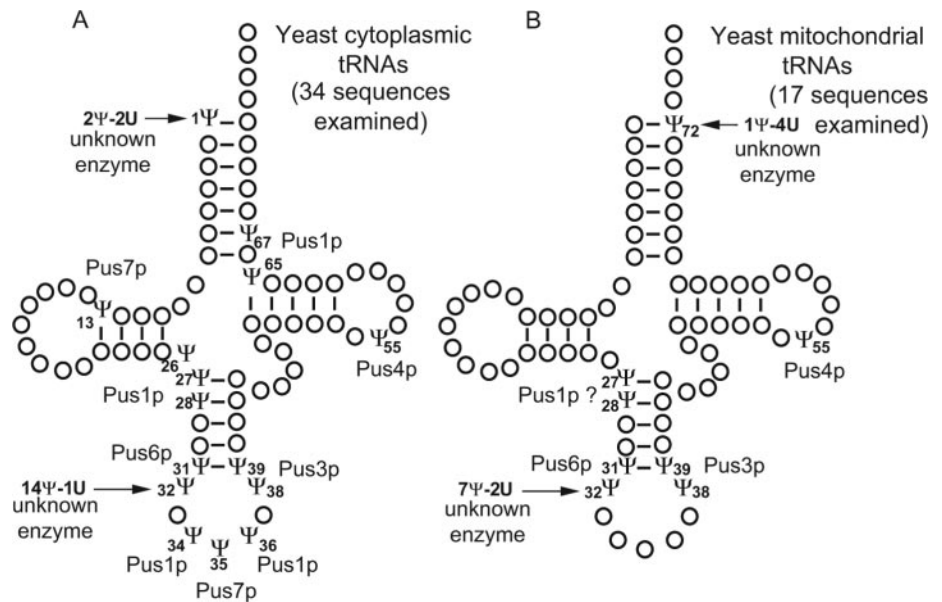
Search for homologs of *E. coli* RNA:Ψ-synthases in genomic data banks of Bacteria, Archaea, and Eukarya led to the identification of four families of RNA:Ψ-synthases (families TruA, TruB, RluA, and RsuA) (2). Using this sequence homology approach, nine genes encoding putative RNA:Ψ-synthases were identified in the *Saccharomyces cerevisiae* genome. Six of them correspond to the already characterized RNA:Ψ-syn-

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¹ The abbreviations used are: Ψ, pseudouridine; snRNA, small nuclear RNA; DRAP, 2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5'-phosphate; ORF, open reading frame; CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-*p*-toluenesulfonate; RT, reverse transcription; UsnRNA, U-rich small nuclear RNA.

FIG. 1. Pseudouridylation sites detected in the cytoplasmic (A) and the mitochondrial (B) tRNAs of *S. cerevisiae*. The tRNAs are schematically represented with their cloverleaf structures, and each of the positions, where a Ψ residue was detected in one or more of the cytoplasmic or mitochondrial tRNAs, is indicated by "Ψ." When identified, the enzymes acting at the indicated positions are given. For the other sites, the number of tRNAs containing a Ψ residue or an U residue is given. These data were compiled from the tRNA data base (20).



the anticodon loop of 14 cytoplasmic tRNAs and 7 mitochondrial tRNAs, have not been characterized yet. The presence of Ψ32 was shown to stabilize the three-dimensional structure of the anticodon loop of tRNA^{Gln} by its implication in a water bridge interaction (21, 22). It was thus important to look for the enzyme(s) responsible for Ψ32 formation in tRNAs. As far as the UsnRNAs are concerned, only the enzymes responsible for the formation of 2 of the 6 pseudouridines identified in these RNAs are known (8, 15). The enzymes that catalyze the U to Ψ conversion at positions 5 and 6 in U1 snRNA, 42 in U2 snRNA, and 99 in U5 snRNA remain to be characterized.

In this work, by use of strains with disrupted *RIB2* and/or *YDL036* ORF(s), and by production of the recombinant proteins, encoded by these two ORFs, we demonstrated that these two proteins, now renamed Rib2/Pus8p and Pus9p, respectively, are tRNA:Ψ32-synthases. Rib2/Pus8p acts on cytoplasmic tRNAs, whereas Pus9p modifies the same position in mitochondrial tRNAs. Accordingly, the corresponding ORFs are renamed as *RIB2/PUS8* and *PUS9*.

EXPERIMENTAL PROCEDURES

Deletions of the *PUS9*(*YDL036c*) and *RIB2/PUS8*(*YOL066c*) ORFs in *S. cerevisiae*—Deletions of the *PUS9*(*YDL036c*) and *RIB2/PUS8*(*YOL066c*) ORFs were performed in the diploid BMA64 (*MATa/α; ura3-52; trp1Δ2; leu2-3_112; his3-11; ade2-1; can1-100*) and haploid BMA64A (*Mata*) *S. cerevisiae* strains, by replacement of the entire coding regions by the *TRP1* auxotrophic marker that was amplified from plasmid pFL35 (23). Positive transformants (*pus9Δ::TRP1* and *rib2/pus8Δ::TRP1*) were selected on YNB plates lacking tryptophan. Independent deletion of the *RIB2/PUS8* ORF was also done by substitution for the *Kan^r* gene followed by selection on Geneticin G₄₁₈ (200 mg/liter). The *Kan^r* gene was amplified from plasmid pFA6a-kanMX2 (23). A haploid strain with simultaneous deletion of both the *PUS9* and *RIB2/PUS8* ORFs was also prepared by replacement of the *RIB2/PUS8* ORF by the *Kan^r* resistance gene in the *pus9Δ::TRP1* haploid strain. Positive transformants (*pus9Δ::TRP1/rib2/pus8Δ::Kan^r*) were selected in the presence of riboflavin (15 mg/liter) on YNB plates containing Geneticin G₄₁₈ (200 mg/liter), but lacking tryptophan. All gene disruptions were confirmed by PCR amplification on yeast colonies. Sequences of oligonucleotide primers used in this study can be obtained on request.

Complementation of the Disrupted Strains—To generate *S. cerevisiae* plasmids expressing proteins encoded by *PUS9* and *RIB2/PUS8* ORFs in a functional form, these ORFs were amplified by PCR from the genomic DNA of the *S. cerevisiae* BMA64 strain using oligonucleotides that generated *NheI* and *HindIII* restriction sites at the N and C termini, respectively. The amplified DNA fragments were subcloned at the *SmaI* site of plasmid pUC18. After sequencing, the resulting constructs (pUC18-*PUS9* and pUC18-*RIB2/PUS8*) were cleaved by the

NheI and *HindIII* restriction endonucleases, and the fragments containing the complete ORFs were inserted, downstream from the *GalS* promoter, between the *XbaI* and *HindIII* sites of plasmid p416GalS (24). Several mutants of these constructions were prepared: point mutations in the regions coding the putative RNA:Ψ-synthase active site of both ORFs were introduced by PCR-mediated site-directed mutagenesis (D238A, GAT → GCT for *PUS9* and D211A, GAC → GCC for *RIB2/PUS8*). By application of the megaprimer approach to plasmid pUC18-*RIB2/PUS8*, we also produced genes encoding N-terminally (amino acids 1–448) or C-terminally (amino acids 450–591) truncated Rib2/Pus8p. The truncated genes were PCR-amplified, cut by the *NheI* and *BamHI* restriction endonucleases, and inserted into plasmid p416GalS to generate the p416GalS-*RIB2/PUS8ΔPUS8* and the p416GalS-*RIB2/PUS8ΔDRAP* plasmids. For PCR amplification of the C-terminally truncated *RIB2/PUS8* gene, we used an oligonucleotide that created a UAA STOP codon. Deletion of the mitochondrial targeting sequence in the *PUS9* ORF (amino acids 8–30) was done by PCR amplification. Yeast transformations were performed by the standard lithium acetate procedure (25).

Recombinant Proteins—Plasmids pET28-*PUS9* and pET28-*RIB2/PUS8* were built for overexpression of the recombinant His₆-Pus9p and His₆-Rib2/Pus8p proteins in *E. coli*. To this end, the *NheI*-*HindIII* fragment of plasmid pUC18-*PUS9* or the *NheI*-*BamHI* fragment of pUC18-*RIB2/PUS8* was inserted into plasmid pET28b (Novagen), that was cut by the same enzyme pairs. Point mutations in the region coding the putative RNA:Ψ-synthase active site of both ORFs were introduced as described above (D238A, GAT to GCT for *PUS9* and D211A, GAC to GCC for *RIB2/PUS8*). The resulting constructs were used to transform *E. coli* BL21CodonPlus(DE3)RIL cells (Stratagene). Recombinant His₆-Pus9p and His₆-Rib2/Pus8p were overproduced in cells grown at 18 °C in LB medium containing 50 μg/ml kanamycin by using an isopropyl 1-thio-β-D-galactopyranoside concentration of 50 μM for the induction. The recombinant proteins were purified from *E. coli* cell extracts by adsorption chromatography on nickel-nitrilotriacetic acid-agarose (Qiagen, France), as described earlier (7, 26). The protein fractions eluted by 250 mM imidazole were diluted twice with 87% of glycerol and stored at –20 °C.

In Vitro Tests—Cell-free S10 extracts from yeast were prepared as described previously (27). The activities of RNA:Ψ-synthases acting at positions 1, 13, 32, and 72 in tRNAs were tested using T7 transcripts of the yeast tRNA^{Arg}(anticodon ACG), tRNA^{Asp}(anticodon GUC), and tRNA^{Met}(anticodon CAU), respectively (16). *In vitro* tests of the tRNA:Ψ32-synthase activities of the recombinant His₆-Pus9p and His₆-Rib2/Pus8p were performed on yeast tRNA^{His}(anticodon GUG) and tRNA^{Asp}(anticodon GUC) T7 transcripts, or on total RNA extracted from the mutated strains. The plasmids, pTFM-Asp and pTFM-His, used for *in vitro* transcription of tRNA^{Asp} and tRNA^{His} were kindly provided by C. Florentz (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France). For *in vitro* enzymatic assays, total yeast RNA (10 μg) or tRNA transcript (4 pmol) was incubated for 2 h with about 0.5 μg of recombinant protein in 10 μl of reaction mixture containing 100 mM

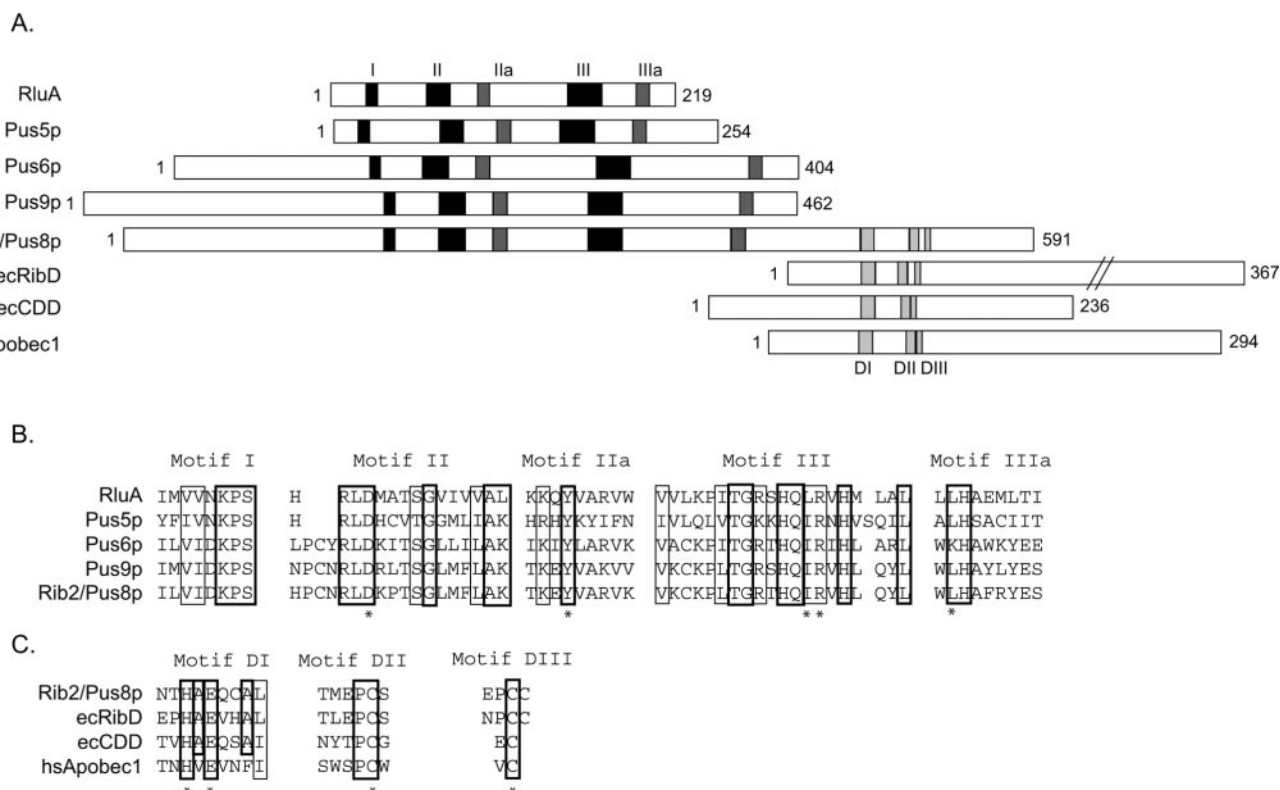


FIG. 2. The Rib2/Pus8p protein sequence contains both an RNA: Ψ -synthase and a deaminase domain. *A*, schematic representation of the sequence alignment of the yeast Pus5p, Pus6p, Pus9p, and Rib2/Pus8p RNA: Ψ -synthases, together with the *E. coli* RluA, RibD (*ecRibD*) and cytosine deaminase (*ecCDD*) enzymes and the human Apobec I deaminase (*hsApobec1*). The five conserved motifs I, II, Ila, III, and IIIa of RNA: Ψ -synthases are represented by black and dark gray rectangles (43). The three specific motifs (DI, DII, and DIII) of deaminases are represented by gray rectangles (44). *B*, the amino acid sequence alignment of motifs I, II, Ila, III, and IIIa of the RluA, Pus5p, Pus6p, Pus9p, and Rib2/Pus8p enzymes. Identical and semi-conserved residues are boxed. The thickness of the line corresponds to the level of conservation. The conserved residues (D, Y, I, R, and L) in the various RNA: Ψ -synthases active sites are indicated by asterisks. *C*, the amino acid sequence alignment of motifs DI, DII, and DIII of the Rib2/Pus8p, EcRibD, EcCDD, and hsApobec1 deaminases. As in *B*, identical and semi-conserved residues are boxed, and the thickness of the line corresponds to the level of conservation. The conserved residues (H, E, C, and C) in the deaminases active site are indicated by asterisks.

Tris-HCl, pH 8.0, 100 mM $\text{NH}_4\text{-OAc}$, 5 mM MgCl_2 , 2 mM dithiothreitol, and 0.1 mM EDTA. After incubation, the modified RNA was phenol-extracted, ethanol-precipitated, and used for CMCT/RT mapping of Ψ residues.

CMCT/RT Mapping of Ψ Residues in RNA—Preparation of total RNA from yeast strains and CMCT/RT mapping of pseudouridine residues were performed as described previously (8, 28). This method is based on the production of alkaline-resistant modification of Ψ residues by a water-soluble carbodiimide CMCT, followed by detection of the modified residues by primer extension. The oligonucleotides, used for analysis of the yeast snRNAs are described in Ref. 8. CMCT/RT mapping of Ψ residue at position 31 in cytoplasmic tRNA^{Met}(anticodon CAU) and mitochondrial tRNA^{Met}(anticodon CAU) was done with oligonucleotides complementary to residues 40–57 and 46–67, respectively. CMCT/RT mapping of Ψ residue at position 32 in cytoplasmic tRNA^{Gly}(anticodon GCC) and tRNA^{Val}(anticodon UAC) was performed with oligonucleotides complementary to residues 59–76 and 39–57, respectively. Mitochondrial tRNA^{Ser}(anticodon GCU) and tRNA^{Trp}(anticodon UCA) were also tested for Ψ 32 formation, using oligonucleotides complementary to residues 44–57 and to residues 58–76, respectively.

Sequence Analysis—Subcellular localization of Pus9p and Rib2/Pus8p was predicted by the PSORTII (Ref. 29 and psort.nibb.ac.jp) and NNPSL (Ref. 30 and predict.sanger.ac.uk/nnpsl/) prediction software. Screening of the non-redundant protein data base at the National Center for Biotechnology Information was performed with the BLASTP2 software (www.ncbi.nlm.nih.gov/BLAST/). Analysis of protein sequence was done by construction of multiple sequence alignments using the ClustalW software.

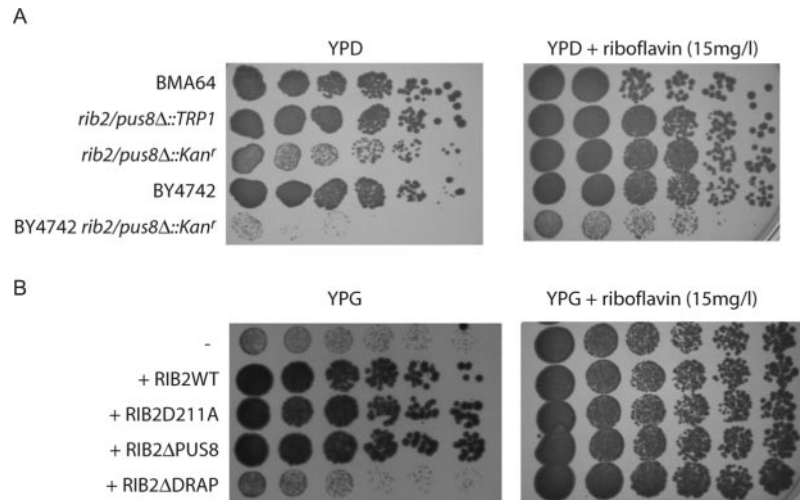
RESULTS

The RIB2/PUS8 and PUS9 ORFs Encode Proteins with Highly Homologous RNA: Ψ -Synthase Domains—Schematic representations of the organization and sequence homologies of

several proteins of the RluA RNA: Ψ -synthases family, including Pus5p, Pus6p, and the proteins encoded by the *PUS9* and *RIB2/PUS8* ORFs are presented in Fig. 2. The RNA: Ψ -synthase domains of Rib2/Pus8p and Pus9p are more similar to the one of Pus6p, than to that of Pus5p (Fig. 2). Because Pus5p is a mitochondrial rRNA: Ψ -synthase (13), whereas Pus6p is a tRNA: Ψ -synthase (14), this was a hint for possible activity of Rib2/Pus8p and Pus9p on tRNAs. In agreement with the finding that Rib2/Pus8p is implicated in riboflavin biosynthesis (19), it contains a long C-terminal extension (160 amino acids), which displays sequence similarity with members of the bacterial RibD family of deaminases. These enzymes are involved in riboflavin biosynthesis in bacteria (19) (Fig. 2). Altogether, the sequence alignments suggested the presence in Rib2/Pus8p of an N-terminal domain with a U to Ψ conversion activity and a C-terminal domain with a DRAP-deaminase activity. Analysis with the PSORTII (29) and NNPSL (30) software suggested that Rib2/Pus8p is a cytoplasmic enzyme, which was experimentally confirmed (see ygc.med.yale.edu/ygc-cgi/). In contrast, Pus9p was predicted to be targeted in mitochondria by its N-terminal sequence.

The PUS9 and RIB2/PUS8 ORFs Are Not Essential in *S. cerevisiae*—To study the functions of the Pus9p and Rib2/Pus8p proteins, each of the two corresponding ORFs was disrupted in the *S. cerevisiae* BMA64A strain by the one-step PCR-based approach (23). The *PUS9* ORF was replaced by the *S. cerevisiae* *TRP1* auxotrophic marker in the BMA64A strain. The resulting *pus9 Δ ::TRP1* strain showed no marked growth

FIG. 3. Growth properties of the WT and Δ pus9 and Δ rib2/pus8 *S. cerevisiae* strains. A, the growth properties of the WT BMA64A and BY4742 *S. cerevisiae* strains and their *rib2/pus8 Δ ::TRP1* and *rib2/pus8 Δ ::Kan^r* derivatives were tested on YPD medium with or without addition of 15 mg/liter riboflavin. Starting from a dilution of 1.5×10^7 cells/ml, 5-fold serial dilutions (5 μ l each) were spotted on YPD agarose plates and incubated for 48 h at 30 °C. In B, the BMA64A *rib2/pus8 Δ ::Kan^r* strain was transformed either with an empty p416GalS plasmid (-) or with p416GalS-derived plasmid encoding WT Rib2/Pus8p (*RIB2WT*), the Rib2/Pus8p variant (*RIB2D211A*), the N-terminally truncated Rib2/Pus8p (*RIB2 Δ PUS8*) or the C-terminally truncated Rib2/Pus8p (*RIB2 Δ DRAP*). Serial dilutions and incubations were done as described in the legend to A, except that YPG agarose plates with or without 15 mg/liter riboflavin were used.



phenotype, both in rich (YPD yeast extract, peptone, dextrose) and in minimal (YNB yeast nitrogen base) media, and this, at any of the tested temperatures (data not shown). The doubling times, measured at 30 °C in YPD medium for the WT and *pus9 Δ ::TRP1* strains were identical (about 90 ± 5 min). The *RIB2/PUS8* ORF was substituted either by the *TRP1* marker (*rib2/pus8 Δ ::TRP1* strain) or by a bacterial *Kan^r* resistance gene (*rib2/pus8 Δ ::Kan^r* strain). After verification by PCR amplification that the expected DNA recombination had occurred, growth phenotypes were analyzed. Unexpectedly, the growth phenotype generated by the *RIB2/PUS8* ORF substitution depended upon the kind of substitution. The *rib2/pus8 Δ ::TRP1* substitution had no detectable effect on growth in YPD medium (doubling time of 95 min, which is identical to the value obtained for the WT strain), whereas the *rib2/pus8 Δ ::Kan^r* replacement led to a marked decrease of growth rate (Fig. 3). The *rib2/pus8 Δ ::Kan^r* substitution even had a greater effect in the genetic background of the BY474 strain, because no growth was detected on YPD medium (see also www-deletion.stanford.edu/cgi-bin/deletion/search3.pl). We confirmed these data with a haploid strain (BY4742 *rib2/pus8 Δ ::Kan^r*) generated by A. P. Gerber (Fig. 3). Disruption of the *RIB2* DRAP-deaminase gene in the yeast *Pichia guilliermondii* was also found to be lethal, but growth could be restored by addition of riboflavin in the culture medium (31). We also found that a normal growth of the BMA64A *rib2/pus8 Δ ::Kan^r* strain on both YPD and YPG media is restored by addition of 15 mg/liter riboflavin (Fig. 3). To test whether expression of the DRAP-deaminase domain can also restore growth, the two Rib2/Pus8p domains were delineated with the Predict Protein program, and their coding regions were inserted in the p416GalS yeast expression vector. As shown in Fig. 3B, expression of the deaminase (p416GalS-*RIB2/PUS8 Δ PUS8*), but not of the RNA: Ψ -synthase domain (p416GalS-*RIB2/PUS8 Δ DRAP*), restored normal growth on YPG medium in the absence of riboflavin. To verify that the growth defect was not related to the absence of Rib2/Pus8p RNA: Ψ -synthase activity, we produced a *RIB2/PUS8* ORF mutated in the codon corresponding to the postulated essential Asp residue of the RNA: Ψ -synthase active site (*D211*, Fig. 2). Expression of the variant D211A protein in the *rib2/pus8 Δ ::Kan^r* strain restored normal growth on YPG medium in the absence of riboflavin (Fig. 3B). Altogether, the data demonstrated that, as previously found for the Pus1p, Pus4p, Pus5p, and Pus6p RNA: Ψ -synthases, the Pus9p and Rib2/Pus8p RNA: Ψ -synthase activities can be abolished without marked effect on cell growth. The different behaviors of

rib2/pus8 Δ ::TRP1 and *rib2/pus8 Δ ::Kan^r* strains suggest that the *Kan^r* gene product may interfere with some metabolic pathways in *S. cerevisiae*, leading to the requirement of an increased riboflavin concentration.

Only Ψ ₃₂ Formation in tRNAs Is Affected by Individual Disruption of the PUS9 and RIB2/PUS8 ORFs—Because several UsnRNA: Ψ -synthases remained to be identified, we first tested the effect of individual *PUS9* and *RIB2/PUS8* ORF disruptions on UsnRNA pseudouridylation. Total RNA was extracted from the BMA64A strain and its derivatives (*pus9 Δ ::TRP1* and *rib2/pus8 Δ ::Kan^r*), which will now be designated as Δ pus9 and Δ rib2/pus8 strains, for simplification. The presence of Ψ residues was analyzed by the CMCT/RT approach (8). Neither of the two ORF substitutions was found to alter the pattern of UsnRNA pseudouridylation (data not shown). Thus, Rib2/Pus8p and Pus9p are probably not involved in UsnRNA modification.

We then tested the possible activity of each enzyme at the tRNA positions, which had no assigned RNA: Ψ -synthases: namely, positions 1 and 32 in cytoplasmic tRNAs, and positions 32 and 72 in mitochondrial tRNAs (Fig. 1). The tRNA: Ψ 32-synthase activity of yeast extracts from the Δ rib2/pus8 and Δ pus9 strains was tested using as substrate an *in vitro* transcribed *S. cerevisiae* cytoplasmic tRNA^{Asp} labeled by incorporation of [α -³²P]UTP (7). In this tRNA, only Ψ 32 is located 5' to a U residue and thus can be labeled and detected after RNase T2 digestion. As a control we measured the tRNA: Ψ 13-synthase activity of the extracts, as described previously (16). Whereas the disruption of the *PUS9* ORF had no detectable effect on the tRNA: Ψ 32-synthase activity, the replacement of *RIB2/PUS8* ORF decreased this activity by a factor of about 2 (Table I and Fig. 4). Hence, Rib2/Pus8p might have a tRNA: Ψ 32-synthase activity that is partially redundant with another RNA: Ψ -synthase activity present in total yeast extract. The *PUS9*-encoded protein was a possible candidate. It might indeed be a tRNA: Ψ 32-synthase specific for the mitochondrial compartment. To test for this possibility, Ψ 32 formation in cytoplasmic and mitochondrial tRNAs of the Δ rib2/pus8 and Δ pus9 strains was tested by the CMCT/RT approach. Because of natural post-transcriptional modifications that impair primer extension analysis, the cytoplasmic tRNA^{Asp}, used in the above experiments, could not be analyzed by this method. Hence, the mitochondrial tRNA^{Ser} and the cytoplasmic tRNA^{Gly}, which both contain a Ψ residue at position 32, were selected for this experiment. As illustrated in Fig. 5A, Ψ 32 formation in the mitochondrial tRNA^{Ser} was completely abolished in the Δ pus9 strain, but remained unaffected in the Δ rib2/pus8 strain. The

TABLE I
Pseudouridine formation in selected yeast tRNA transcripts catalyzed by extracts of WT, Δ pus9, Δ rib2/pus8, and Δ rib2/pus8/ Δ pus9 yeast strains

Radiolabeled transcripts were incubated with yeast extract at 37 °C for 60 min, and the Ψ content was analyzed by two-dimensional TLC after RNase T2 digestion.

Position tested in tRNA	tRNA transcript/labeling	Wild-type strain	Δ pus9 strain	Δ rib2/pus8 strain	Δ rib2/pus8/ Δ pus9 strain
1	Yeast tRNA ^{Arg} /UTP	0.85	0.84	0.91	0.87
13	Yeast tRNA ^{Asp} /ATP	0.75	0.79	0.78	0.81
32	Yeast tRNA ^{Asp} /UTP	0.71	0.76	0.34	0.02
72	Yeast tRNA ^{Met} i/ATP	0.78	0.87	0.82	0.83

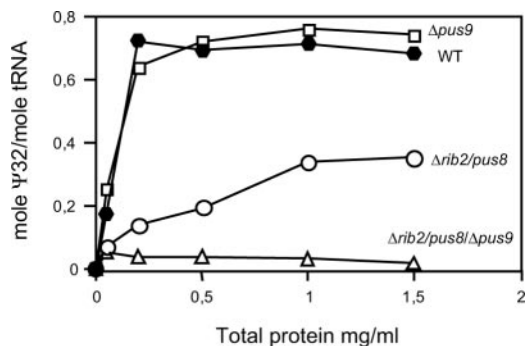


FIG. 4. Deletion of the *RIB2/PUS8* but not the *PUS9* ORF alters the tRNA:Ψ32-synthase activity of yeast extract. The figure represents time courses for formation of residue Ψ32 in the *in vitro* transcript of yeast cytoplasmic tRNA^{Asp} incubated at 37 °C with cell-free extracts of the WT, Δ pus9, Δ rib2/pus8, and Δ rib2/pus8/ Δ pus9 yeast strains. The uniformly [α -³²P]UTP-labeled tRNA^{Asp} transcript was incubated with the extracts, phenol-extracted, and completely digested by RNase T2. The resulting [$3'$ - α -³²P]mononucleotides were separated by two-dimensional TLC on cellulose plates. Quantification was done by using a PhosphorImager, and the amount of formed Ψ residue was calculated taking into account the nucleotide composition of the transcript.

same result was obtained for the mitochondrial tRNA^{Trp} (not shown). In contrast, the *PUS9* deletion had no significant effect on Ψ32 formation in the cytoplasmic tRNA^{Gly} (Fig. 5B, lanes 7 and 8) and tRNA^{Val} (not shown). These data strongly suggested that Pus9p catalyzes Ψ32 formation in mitochondrial tRNAs. A confirmation was obtained by complementation of the Δ pus9 strain by a plasmid bearing the WT or a mutated *PUS9* ORF (Fig. 6). Indeed, the p416GalS-*PUS9*WT plasmid expressing the active protein was able to restore Ψ32 formation in the mitochondrial tRNA^{Ser} (Fig. 6, lanes 7 and 8), whereas its derivative p416GalS-*PUS9*D238A, encoding a Pus9p with a D238A amino acid substitution in the GRLD motif of the RNA:Ψ-synthase domain (Fig. 2), did not restore the activity (Fig. 6, lanes 9 and 10). Hence, we concluded that the *S. cerevisiae* *PUS9* ORF encodes the tRNA:Ψ32-synthase responsible for modification of mitochondrial tRNAs.

The effects of the *RIB2/PUS8* and *PUS9* ORF deletions on the tRNA:Ψ1- and tRNA:Ψ72-synthase activities of cellular extracts were analyzed by the nearest neighbor approach using transcripts corresponding to the *S. cerevisiae* cytoplasmic tRNA^{Arg} and mitochondrial tRNA^{Met}i, respectively. The tRNA^{Arg} transcript was labeled with [α -³²P]UTP, because among the Ψ residues present in this tRNA, only residue Ψ1 is followed by an U residue. The tRNA^{Met}i transcript was labeled with [α -³²P]ATP, because in this tRNA residue Ψ72 is followed by an A residue. Comparison of the activities of the WT, Δ pus9 and Δ rib2/pus8 yeast extracts showed that neither Rib2/Pus8p, nor Pus9p is implicated in Ψ1 formation in cytoplasmic tRNAs or Ψ72 formation in mitochondrial tRNAs (Table I).

The Recombinant Pus9p and Rib2/Pus8p Both Possess a tRNA:Ψ32-synthase Activity in Vitro—The above experiments suggested that both Pus9p and Rib2/Pus8p have a tRNA:Ψ32-

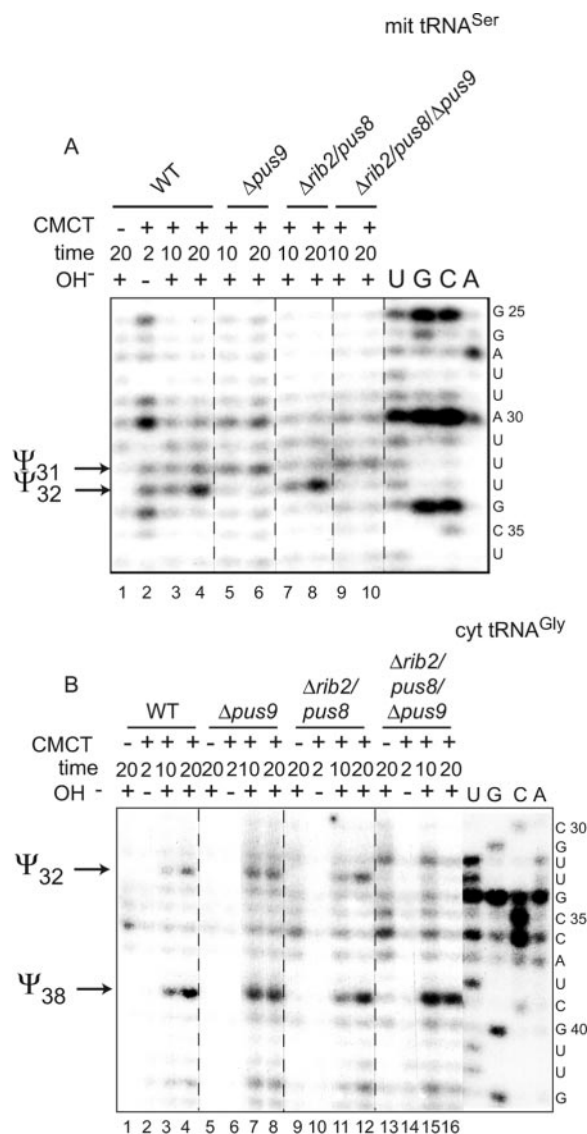


FIG. 5. Mapping of residue Ψ32 in the mitochondrial tRNA^{Ser} (A) and cytoplasmic tRNA^{Gly} (B) from the WT and mutated *S. cerevisiae* BMA64A strains. Total RNA was extracted from the WT, Δ pus9, Δ rib2/pus8, and Δ rib2/pus8/ Δ pus9 *S. cerevisiae* strains and modified by CMCT, for 2, 10, and 20 min with (+) or without (–) subsequent alkaline treatment (OH[–]). For both tRNAs a control experiment was performed in the absence of CMCT treatment. Lanes U, G, C, and A correspond to the sequencing ladders obtained with the same oligonucleotide. The reverse transcription stops, corresponding to residue Ψ32 or to residues Ψ31 and Ψ38, are indicated.

synthase activity. To complete this demonstration, the *E. coli* pET28b expression vector was used to produce recombinant His-tagged Rib2/Pus8p and Pus9p proteins, which were affinity-purified on nickel-nitrilotriacetic acid-agarose beads. We also produced Rib2/Pus8p and Pus9p proteins with an aspartic

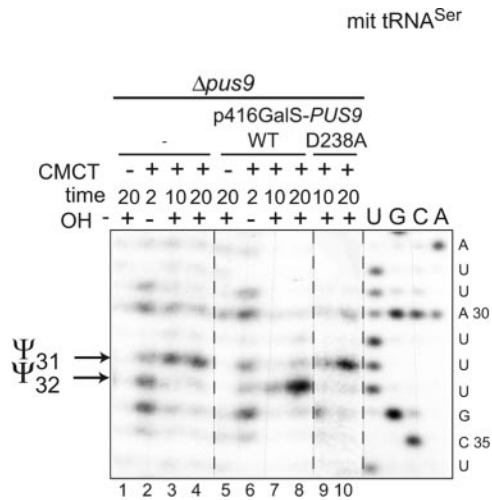


FIG. 6. The aspartic acid residue Asp-238 in the active site of Pus9p is essential for RNA:Ψ-synthase activity. The yeast *S. cerevisiae* Δ pus9 strain was transformed with p416GalS plasmids, bearing the WT (Asp-238) or mutated (D238A) PUS9 ORF. Total RNA was extracted from the two transformed strains, and the mitochondrial tRNA^{Ser} was analyzed by the CMCT/RT approach (shown in lanes 7–10). The reverse transcription stops, corresponding to residues Ψ31 and Ψ32 are indicated.

acid (Asp) to alanine (Ala) amino acid substitution in the GRLD motif of the active site. The RNA:Ψ-synthase activities of the four recombinant proteins were tested by the nearest neighbor approach using an *in vitro* transcribed *S. cerevisiae* tRNA^{Asp} labeled by [α -³²P]UTP incorporation. Thin layer chromatography (TLC) analysis demonstrated that the recombinant WT Pus9p and Rib2/Pus8p proteins both had a tRNA:Ψ32-synthase activity (Fig. 7, B and D), whereas the mutated proteins were inactive (Fig. 7, C and E). Using the CMCT/RT approach, we confirmed that the U to Ψ conversion occurred only at position 32 in tRNA^{Asp} (Fig. 7F). Taken together, our data confirmed the activity of both Rib2/Pus8p and Pus9p RNA:Ψ-synthases at position 32 in tRNAs.

Simultaneous Inactivation of the RIB2/PUS8 and PUS9 ORFs Impairs Ψ32 Formation in Both Cytoplasmic and Mitochondrial tRNAs—For a complete demonstration of the requirement of both Rib2/Pus8p and Pus9p for Ψ32 formation in all tRNAs, we disrupted the RIB2/PUS8 ORF by *Kan^r* marker in the *pus9Δ::TRP1* strain. The resulting strain, which will be designated as Δ rib2/*pus8*/ Δ pus9, was viable only in the presence of 15 mg/liter riboflavin in the growth medium. The absence of tRNA:Ψ32-synthase activity in the cell extract was demonstrated by the *in vitro* activity test based on the use of [α -³²P]UTP-labeled tRNA^{Asp}, as described above (Fig. 4). In addition, the absence of U to Ψ conversion at position 32 in the cytoplasmic tRNA^{Gly} and the mitochondrial tRNA^{Ser} extracted from the Δ rib2/*pus8*/ Δ pus9 strain was confirmed by CMCT/RT analysis (Fig. 5A, lanes 9 and 10 and Fig. 5B, lanes 15 and 16). Altogether the data clearly demonstrated that two enzymes (Rib2/Pus8p and Pus9p) are required for Ψ32 formation in both cytoplasmic and mitochondrial tRNAs. Also, as expected, expression of the WT, but not of the D112A-mutated Rib2/Pus8p protein, restored Ψ32 formation in the cytoplasmic tRNA^{Gly} of the Δ rib2/*pus8*/ Δ pus9 strain (Fig. 8).

As demonstrated by CMCT/RT analysis of the cytoplasmic tRNA^{Val} (Fig. 8, lanes 15 and 16), and the mitochondrial tRNA^{Ser} (data not shown), expression of the N-terminal RNA:Ψ-synthase domain of Rib2/Pus8p (p416GalS-RIB2/PUS8 Δ DRAP) was also able to restore Ψ32 formation in cytoplasmic tRNAs, but not in mitochondrial tRNAs. This observed complementation demonstrates that the presence of the deaminase domain of Rib2/

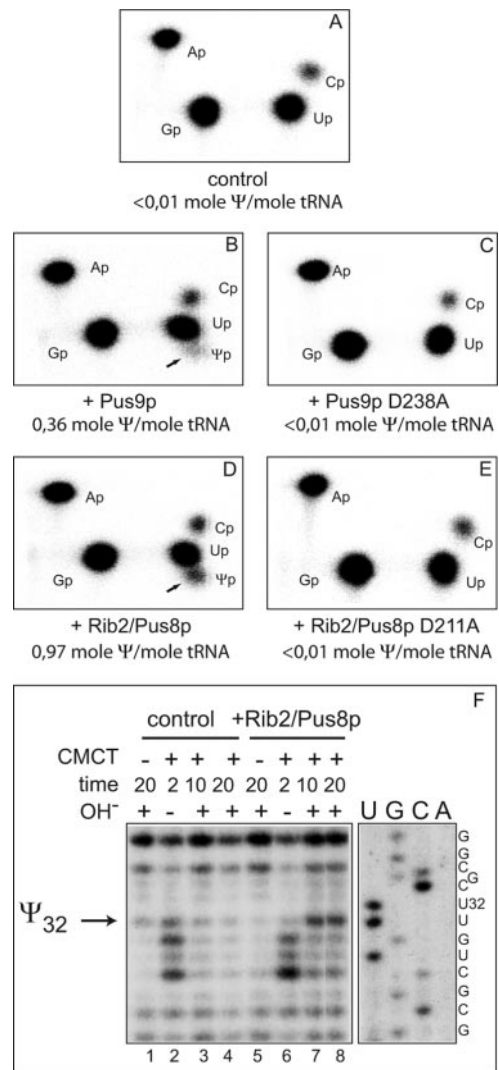


FIG. 7. The recombinant Pus9p and Rib2/Pus8p both have a tRNA:Ψ32-synthase activity. A tRNA^{Asp} transcript labeled with [α -³²P]UTP was incubated with the WT or variant D238A His₆-Pus9p recombinant protein (B and C) or with the WT or variant D211A His₆-Rib2/Pus8p recombinant protein (D and E). After RNase T2 digestion, the released 3'-mononucleotides were separated by two-dimensional TLC, and the autoradiograms of the plates are shown. A control digestion without prior incubation with recombinant enzyme is also shown (A). The ΨMP spot is indicated by an arrow. The N/R chromatographic system was described previously (45). To control that the ΨMP spot observed on the plates corresponds to modification at position 32 (F), CMCT/RT experiments were performed with unlabeled tRNA^{Asp} transcripts incubated or not with the recombinant WT His₆-Rib2/Pus8p protein.

Pus8p is required neither for folding of the N-terminal RNA:Ψ-synthase domain nor for its activity.

Because the tRNA:Ψ1- and tRNA:Ψ72-synthase activities of extract from the Δ rib2/*pus8*/ Δ pus9 strain were found to be identical to those of the WT strain (Table I), we concluded that some RNA:Ψ-synthases, other than Rib2/Pus8p and Pus9p, should be responsible for the formation of these two post-transcriptional modifications.

Cellular Localization of Pus9p in *S. cerevisiae*—Based on the presence of a characteristic Lys/Arg-rich sequence at its N terminus, a mitochondrial localization was predicted for Pus9p by the PSORTII and NNPSL software (29, 30). To test these predictions, we built a yeast plasmid (p416GalS-PUS9 Δ N) expressing a Pus9p variant lacking 23 amino acids (amino acids 7–30) in the N-terminal region. The CMCT/RT analysis of the cytoplasmic tRNA^{Val} and mitochondrial tRNA^{Ser} extracted

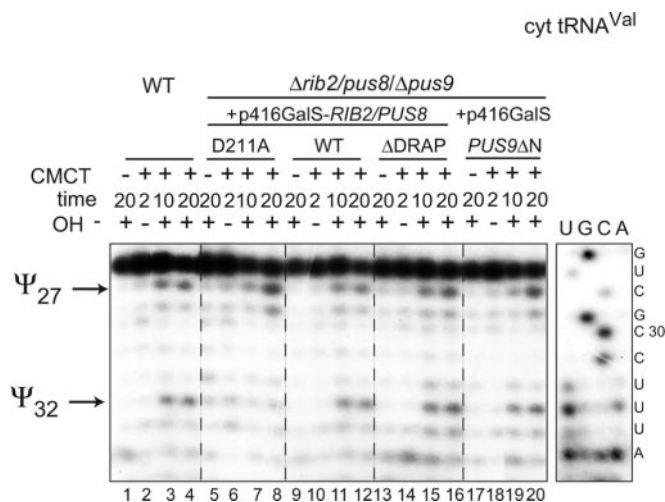


FIG. 8. The RNA:Ψ32-synthase activity of Rib2/Pus8p depends upon residue Asp-211 and is not linked to the deaminase domain. The yeast *S. cerevisiae* strain $\Delta rib2/pus8/\Delta pus9$ was transformed with plasmids coding the WT (lanes 9–12) or mutated (D211A) Rib2/Pus8p protein (lanes 5–8) or its truncated version (Δ DRAP lacking C-terminal DRAP-deaminase domain, lanes 13–16). Transformation of the same strain was also performed with the p416GalS-*PUS9* Δ N plasmid, coding the N-terminally truncated (Δ N) Pus9p variant (lanes 17–20). The presence of residue Ψ32 in the cytoplasmic tRNA^{Val}(UAC) extracted from the transformed strains was analyzed by the CMCT/RT approach (as described in the legend for Fig. 5). The reverse transcription stops, corresponding to residues Ψ27 and Ψ32 are indicated.

from the $\Delta rib2/pus8/\Delta pus9$ strain transformed with p416GalS-*PUS9* Δ N construct revealed the formation of Ψ32 in cytoplasmic (Fig. 8, lanes 19 and 20), but not mitochondrial tRNAs (Fig. 9, lanes 15 and 16). These data demonstrated that the N-terminal sequence of Pus9p is required for its mitochondrial targeting and confirmed the capacity of Pus9p to modify cytoplasmic tRNAs *in vivo*.

DISCUSSION

Two unusual features of the yeast *S. cerevisiae* tRNA modification machinery are revealed in this work: (i) two members of the RluA family of RNA:Ψ-synthases are required for U to Ψ conversion at position 32 in cytoplasmic and mitochondrial tRNAs and (ii) the cytoplasmic enzyme Rib2/Pus8p contains an additional domain with an unrelated activity.

Bacterial Members of the RluA Family Have Different RNA Specificities Compared with Their Yeast Counterparts—The RluA family of RNA:Ψ-synthases seems to be the most complex of the five identified RNA:Ψ-synthase families. Four members of this family are present in both *E. coli* (RluA, RluC, RluD, and TruC) and *S. cerevisiae* (Pus5p, Pus6p, Rib2/Pus8p, and Pus9p). However, while three of the four *E. coli* enzymes modify 23 S rRNA (RluC (32), RluD (33), and RluA, which also acts at position 32 in tRNAs (34)), only one of the four *S. cerevisiae* enzymes modifies rRNAs (Pus5p, which modifies the mitochondrial 21 S rRNA (13)). Indeed, despite its sequence homology with RluA, RluC, and RluD, the yeast Pus6p enzyme is responsible for pseudouridylation at position 31 in both cytoplasmic and mitochondrial tRNAs (14). This tRNA position is not modified in *E. coli*. We demonstrate here that the two remaining unidentified members of the RluA family in *S. cerevisiae*, Rib2/Pus8p and Pus9p, have the same activity as the *E. coli* RluA enzyme; they both act at position 32 in tRNAs. Accordingly, Rib2/Pus8p and Pus9p are more similar to RluA, than to TruC, which is responsible for Ψ65 formation in *E. coli* tRNAs (35). Formation of Ψ65 in *S. cerevisiae* is catalyzed by Pus1p, a member of the TruA family (7). Unexpectedly, presently identified Pus9p shows a higher similarity with the RluD *E. coli*

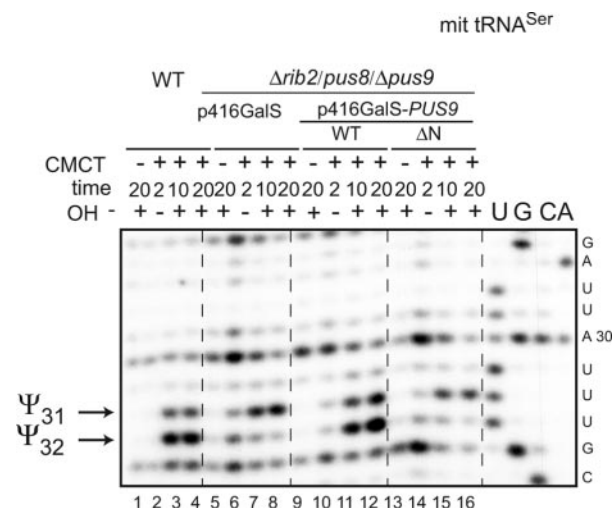


FIG. 9. The N-terminal sequence of protein Pus9p is required for its mitochondrial targeting. The $\Delta rib2/pus8/\Delta pus9$ strain was transformed with plasmid p416GalS expressing the WT (lanes 9–12) or Δ N variant (lanes 13–16) of Pus9p protein. The presence of residue Ψ32 in the mitochondrial tRNA^{Ser} of the transformed strain was analyzed by the CMCT/RT approach by using conditions described in the legend for Fig. 5. The reverse transcription stops, corresponding to residues Ψ31 and Ψ32 are indicated.

rRNA:Ψ-synthase than with the RluA *E. coli* tRNA:Ψ32-synthase. These observations suggest that RNA-substrate specificity of *S. cerevisiae* RluA-related RNA:Ψ-synthases is likely related to a different mechanism of rRNA modification in bacteria and eukarya. In eukarya, a unique RNA:Ψ-synthase Cbf5p (in yeast)/dyskerin (in human) associated with the guide H/ACA small nucleolar RNAs (11), is expected to form most of the pseudouridylations of cytoplasmic rRNAs. The Cbf5p/dyskerin enzyme belongs to the TruB family, so that members of the RluA family are not expected to be required for pseudouridylation of cytoplasmic rRNAs. However, it was recently proposed that the formation of one of the highly phylogenetically conserved 2'-O-methylations in yeast 25 S rRNA is catalyzed by both the Spb1 2'-O-methylase and the C/D snR52 small nucleolar ribonucleoprotein (36). Thus, one cannot exclude the possibility that some of the highly phylogenetically conserved Ψ residues in *S. cerevisiae* 25 S rRNA (namely Ψ2257 and Ψ2259) are formed both by an H/ACA small nucleolar ribonucleoprotein and/or one of the Pus5p, Pus6p, Rib2/Pus8p, or Pus9p enzymes. However, our data clearly show that the activities of the *S. cerevisiae* RluA-like enzymes are largely dedicated to tRNA modifications, whereas their *E. coli* counterparts are mainly involved in rRNA modification (32–34, 37). This extended activity on tRNAs in *S. cerevisiae* may be explained by the emergence of additional pseudouridylation sites in tRNAs compared with *E. coli* (position 31) and the need of distinct enzymes for the cytoplasmic and mitochondrial compartments. Taken together, these observations suggest that the evolution of RNA:Ψ-synthase substrate specificity is a rather rapid process at the scale of long term evolution. Appearance of the RNA-guided system during evolution probably resulted both in changes of RNA:Ψ-synthase specificities and disappearance of some of the protein catalysts.

Two Distinct tRNA:Ψ32-synthases Are Present in the Cytoplasmic and Mitochondrial Compartments of *S. cerevisiae*—Disruption of the *PUS9* ORF led to complete disappearance of Ψ32 formation in mitochondrial tRNAs, which is in perfect agreement with the computer prediction of a mitochondrial targeting signal in Pus9p and the preferential cytoplasmic localization of Rib2/Pus8p. Also in accord with the prediction of only a partial localization of Pus9p in mitochondria, cytoplas-

mic tRNAs were still modified at position 32 after disruption of the *RIB2/PUS8* ORF. Noticeably, deletion of the *RIB2/PUS8* ORF resulted in a reduced tRNA: Ψ 32-synthase activity of yeast extract as tested on the cytoplasmic tRNA^{ASP}. This could be due to a low activity of Pus9p on cytoplasmic tRNAs compared with mitochondrial tRNAs. However, a more likely explanation is the low level of mitochondrial enzyme recovery upon preparation of *S. cerevisiae* cell extract. Indeed, expression of the N-terminally deleted Pus9p variant, devoid of its mitochondrial localization signal, restored Ψ 32 formation in the cytoplasmic tRNAs from the Δ *rib2/pus8*/ Δ *pus9* strain.

The existence of independent enzymatic systems dedicated to cytoplasmic and mitochondrial modification also raises the question of the differential tRNA recognition by the Rib2/Pus8p and Pus9p tRNA: Ψ 32-synthases. The exact definition of their RNA recognition rules and identification of the required identity elements are attractive subjects for future studies in this field.

Rib2/Pus8p Probably Originates from a Gene Fusion—Previous studies on riboflavin biosynthesis in *S. cerevisiae* suggested the involvement of a Rib2p DRAP-deaminase activity (18, 19). Our data strongly reinforce this conclusion, because normal growth of the *rib2/pus8* Δ :*Kan*^r strain requires either the addition of riboflavin in the medium, or the expression of the DRAP-deaminase domain. Interestingly, this requirement was specific for the *rib2/pus8* Δ :*Kan*^r replacement. It was not observed for the *rib2/pus8* Δ :*TRP1* substitution, which points out the importance of the identity of the marker used for gene replacements. The *Kan*^r gene encodes an aminoglycoside phosphotransferase (38). This activity may somehow interfere with yeast metabolism. Therefore, the simultaneous defects generated by the foreign *Kan*^r gene expression and the disruption of the endogenous *RIB2/PUS8* gene lead to a growth phenotype in the BMA64A genetic context and to the absence of growth in the *S. cerevisiae* BY474 genetic context.

Inspection of the growth phenotypes of the strains produced in this study, in light of these considerations, demonstrates the presence of two independent domains in Rib2/Pus8p. The N-terminal domain has the tRNA: Ψ 32-synthase activity, and the C-terminal domain bears the DRAP-deaminase activity. Alignment of the Rib2/Pus8p C-terminal domain with the amino acid sequences of various deaminases revealed some homology with dCTP-deaminases and a more limited similarity with the Apobec1 deaminase that is involved in U to C conversion at position 6666 in the apolipoprotein B mRNA (Fig. 2). Because bi-functional proteins usually correspond to enzymes involved in the same metabolism, an attractive hypothesis is that the presence of the DRAP deaminase and RNA: Ψ -synthase in Rib2/Pus8p allows their successive actions on RNA substrate(s). If this was the case, C residues would be directly converted into Ψ residues. However, there is no observation in favor of C32 to Ψ 32 conversion, via a C-to-U deamination in tRNAs. Based on its well defined DRAP-deaminase activity, the Rib2/Pus8p C-terminal domain is likely dedicated to riboflavin biosynthesis. This conclusion is reinforced by the observation that the presence of both tRNA: Ψ 32-synthase and DRAP-deaminase domains in a given protein is restricted to species from the yeast Saccharomycetoidae subfamily. Genes encoding putative Rib2/Pus8p counterparts were detected in several species of the *Saccharomyces* genus and in species from the *Candida*, *Eremothecium*, and *Kluyveromyces* genera, but not in yeast species from the Schizoccharomycetoidae and Pezizomycotinaea subfamilies. Thus, the observed RNA: Ψ -synthase/DRAP-deaminase protein fusion may be the consequence of a DNA recombination event that occurred during yeast evolution. Gene fusions have already been described in *S. cerevisiae*. One

well studied example is the *URA2* gene, which corresponds to a fusion of the carbamylphosphate synthetase gene with the aspartate transcarbamylase gene (39). Likewise, the *THI6* gene encodes a fusion protein containing a thiamine phosphate pyrophosphorylase domain and a hydroxyethylthiazole kinase domain (40). In both cases, the fused genes encode proteins acting one after another in a given metabolic pathway, the pyrimidine pathway for the *URA2* gene and the thiamine pathway for the *THI6* gene, which is not the case for the *RIB2/PUS8* gene.

On the basis of sequence alignments, it is difficult to predict whether the presence of an RNA: Ψ 32-synthase/DRAP-deaminase protein fusion is systematically accompanied with the occurrence of a second tRNA: Ψ 32 synthase corresponding to the mitochondrial enzyme. However, because the DRAP-deaminase needs to act in the cytoplasm, fusion of the deaminase and RNA: Ψ -synthase activities in one protein may be the reason for the presence of a second RNA: Ψ 32-synthase dedicated to mitochondria. Indeed, this is the first time that two different enzymes are found to be required to generate modifications at the same position of tRNAs depending on their subcellular localization. In all other cases studied, the same nuclear gene provides activity for both cellular compartments (9, 10, 14, 41, 42), sometimes by alternative choice of AUG initiation codons (41, 42). This is also the first time that an RNA modification enzyme is found to be fused to an enzyme with an unrelated activity, and we propose that these two unusual properties are linked together.

*Only Two tRNA: Ψ -synthase Activities Remain to be Identified in *S. cerevisiae**—At the present stage of the *S. cerevisiae* RNA: Ψ -synthase analysis, only the proteins carrying the activities for Ψ 1 formation in the two cytoplasmic tRNAs (tRNA^{Arg}, (ACG) and tRNA^{Lys}(UUU)) and Ψ 72 formation in the mitochondrial tRNA^{Met}₁-(CAU) remain to be identified. The one or more substrates of only one of the putative RNA: Ψ -synthase (Pus2p) have not been defined. Thus, the possible activity of Pus2p at these two positions has to be tested. Neither *RIB2/PUS8* nor *PUS9* ORF disruption altered Ψ formation in UsnRNAs. Hence, the enzymes responsible for the formation of four Ψ residues in UsnRNAs (positions 5 and 6 in U1snRNA, 42 in U2snRNA, and 99 in U5snRNA) still also have to be identified. Like Pus7p, these enzymes might have a very low level of homology with known *E. coli* RNA: Ψ -synthases and thus have escaped detection by computer analysis using the conserved motif II as an RNA: Ψ -synthase signature.

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