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The yeast *Saccharomyces cerevisiae* YDL112w ORF encodes the putative 2′-*O*-ribose methyltransferase catalyzing the formation of Gm18 in tRNAs

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ABSTRACT

The protein sequences of three known RNA 2′-*O*-ribose methylases were used as probes for detecting putative homologs through iterative searches of genomic databases. We have identified 45 new positive Open Reading Frames (ORFs), mostly in prokaryotic genomes. Five complete eukaryotic ORFs were also detected, among which was a single ORF (YDL112w) in the yeast *Saccharomyces cerevisiae* genome. After genetic depletion of YDL112w, we observed a specific defect in tRNA ribose methylation, with the complete disappearance of Gm18 in all tRNAs that naturally contain this modification, whereas other tRNA ribose methylations and the complex pattern of rRNA ribose methylations were not affected. The tRNA G18 methylation defect was suppressed by transformation of the disrupted strain with a plasmid allowing expression of YDL112wp. The formation of Gm18 on an in vitro transcript of a yeast $tRNA^{Ser}$ naturally containing this methylation, which was efficiently catalyzed by cell-free extracts from the wild-type yeast strain, did not occur with extracts from the disrupted strain. The protein encoded by the YDL112w ORF, termed Trm3 (tRNA methylation), is therefore likely to be the tRNA (Gm18) ribose methylase. In in vitro assays, its activity is strongly dependent on tRNA architecture. Trm3p, the first putative tRNA ribose methylase identified in an eukaryotic organism, is considerably larger than its *Escherichia coli* functional homolog spoU (1,436 amino acids vs. 229 amino acids), or any known or putative prokaryotic RNA ribose methyltransferase. Homologs found in human (TRP-185 protein), *Caenorhabditis elegans* and *Arabidopsis thaliana* also exhibit a very long N-terminal extension not related to any protein sequence in databases.

Keywords: gene disruption; in vitro modification of tRNA; RNA modification; spoU; tRNA modifying enzyme; TRP-185

INTRODUCTION

Most cellular RNAs undergo a number of nucleoside modifications, the biological role of which is still often elusive (Agris, 1996). However, their importance has been clearly established in a few cases, essentially for tRNAs that contain the greatest variety and abundance of modified nucleosides (Björk, 1995), several of which appear to be involved in the optimization of tRNA function, not only in translation but also in other processes involving tRNAs, ranging from cell-cycle control to priming of retroviral replication (Persson, 1993; Björk, 1995; Fossé et al., 1998). The two most prevalent types of nucleoside modifications in prokaryotic and eukaryotic RNAs are pseudouridylations and 2′-*O*-ribose methylations, the precise role(s) of which remain(s) generally unknown, although they can both fine-tune the three-dimensional folding of RNA and its interactions with ligands (Lane et al., 1995).

Only a few RNA-modifying enzymes have been characterized as yet, particularly in eukaryotic cells. Formation of the numerous pseudouridines in yeast tRNAs is catalyzed by several tRNA:pseudouridine synthases, four of which have been recently identified (Simos et al., 1996; Becker et al., 1997a; Lecointe et al., 1998). Paradoxically, formation of the much larger number of pseudouridines present in yeast cytoplasmic rRNAs seems to involve a single pseudouridine synthase, Cbf5p (Lafontaine et al., 1998), in line with the finding that each site of pseudouridine formation is selected by base-

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pairing of a specific small nucleolar RNA guide belonging to the box H-ACA family (Ganot et al., 1997; Ni et al., 1997; Smith & Steitz, 1997). As for the scores of cytoplasmic rRNA ribose methylations, also site-specified by base-pairing of small nucleolar RNAs, belonging in this case to the box C/D family (Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Bachellerie & Cavaillé, 1997), the enzyme(s) catalyzing their formation remain(s) unknown. Ribose methylations of eukaryotic RNAs also include five different positions in tRNAs (Sprintz et al., 1998), nearly two dozen sites in vertebrate snRNAs (Massenet et al., 1998), and the two 5′cap-proximal nucleotides of mRNAs in higher eukaryotes (Narayan & Rottman, 1992).

Methylases form a large family of enzymes that catalyze methyl transfer from the ubiquitous S-adenosyl-L-methionine (AdoMet) to either nitrogen, oxygen, or carbon atoms in DNA, RNA, proteins, and small molecules. Although they use a common cofactor, methylases acting on different types of biological macromolecules exhibit very extensive sequence divergence. Even among the DNA methyltransferases, which belong to different classes defined by the position methylated, primary structures are quite different. Only the structural analysis revealed striking similarities pointing to the presence of a common catalytic domain structure in many (if not all) of them, with individual amino acids that have comparable functional properties identified by alignment of secondary and tertiary structures (Malone et al., 1995; Schluckebier et al., 1995). As for RNA 2′-*O*-methyltransferases, the amino acid sequences of two enzymes modifying cellular RNAs have been reported. *Saccharomyces cerevisiae* nuclear-encoded Pet56p methylates Gm2270 in mitochondrial 21S rRNA, corresponding to Gm2251 in *Escherichia coli* 23S rRNA (Sirum-Connolly & Mason, 1993). *Streptomyces azureus* tsr confers resistance to thiostreptron by methylating the ribose of a single nucleotide, A1068, in 23S rRNA of the antibiotic-producing organism (Thompson et al., 1982; Bibb et al., 1985; Cundliffe, 1989). Moreover, a third putative RNA ribose methylase encoded in the genome of a free-living organism has been characterized, spoU, which is essential for tRNA (Gm18) methyltransferase activity in *E. coli* (Persson et al., 1997). Finally, the structure of a viral RNA ribose methylase, VP39, has also been elucidated (Hodel et al., 1996). Pet56, tsr, and spoU, but not VP39, share three common short amino acid motifs, providing the basis for homology searches in databases (Koonin & Rudd, 1993; Gustafsson et al., 1996). Motif I is also present in an RNA-modifying enzyme, Rit1, that utilizes the 2′-hydroxyl of the tRNA ribose backbone as substrate and catalyzes the addition of a ribosyl phosphate onto A64 of yeast $tRNA^{fmet}$, suggesting that it could be involved in the recognition of the ribose (Aström & Byström, 1994; Persson et al., 1997). The function of motifs II and III is unknown, but motif II, which contains two conserved glycines following bulky hydrophobic amino acids (Koonin & Rudd, 1993; Gustafsson et al., 1996), is reminiscent of a conserved motif found in other methylases using AdoMet, including VP39, and thought to represent an AdoMet binding motif (Ingrosso et al., 1989; Kagan & Clarke, 1994). However, the crystal structure of methyltransferases complexed with their AdoMet cofactor indicates that this motif, although part of a consensus methyltransferase structure, is not involved in direct AdoMet contact (Djordjevic & Stock, 1997, and references therein).

The derivation of complete genomic nucleotide sequences has opened a new way for identifying the functions of uncharacterized open reading frames (ORFs), through homology comparisons of previously known proteins, which provides guidance to experimental analysis. By iterative searches of genomic databases starting with initial probes Pet56, tsr, and spoU, followed by direct experimental analysis, we have identified a yeast, *S. cerevisiae* ORF (YDL112w), that appears to encode a tRNA 2′-*O*-ribose methylase specific for one of the five nucleotide positions which are naturally ribose methylated in yeast tRNAs, G18. The structural substrate requirement for RNA ribose methylation activity has also been analyzed.

RESULTS

Detection of ORFs encoding putative RNA 2′-*O*-methylases

After an iterative search of genomic sequence databases starting with 3 probes spoU, tsr, and PET56, 56 complete and 4 truncated ORFs containing the three short amino acid motifs shared by the initial probes were retrieved, most of them from prokaryotic genomes (Table 1). In addition to the ten prokaryotic sequences previously identified as likely RNA 2′-*O*-methylases in similar genomic searches (Koonin & Rudd, 1993; Gustafsson et al., 1996), 32 eubacterial and 5 archaeal new positive ORFs were detected, ranging in size from 144 to 384 amino acids (Table 1). Five complete positive ORFs were also found in eukaryotic genomes (Fig. 1B)—one in *S. cerevisiae* (accession number YDL112w), one in the nematode *Caenorhabditis elegans* (U50191), two in the plant *Arabidopsis thaliana* (Z97343 and AF007270), and one in humans, TRP-185 protein (U38847)—together with two truncated ORFs, one also in *A. thaliana* (its sequence is included in Fig. 1B) and the other one in flatworm *Schistosoma mansoni*. In all prokaryotic positives, the segment encompassing motifs I–III accounts for most of the ORF length. In contrast, the newly found eukaryotic complete ORFs are dramatically enlarged (size range: 1203–1621 amino acids; Fig. 1C), except for one of the *A. thaliana* sequences (AF007270: 271 amino acids), which falls within the size range of the pro-

TABLE 1. ORFs encoding putative RNA 2′-O ribose methyltransferases.

	Organism	Acc. number (gene name)	ORF size (a.a.)
EUBACTERIA	*A. aeolicus*	AE000749 AE000761	211 240
	A. vinelandii	AF010139	257
	B. subtilis	Z99108 (CspR) Z75208 (ysgA) Q06753	160 248 249
	B. stearothermophilus	P32813 (ygl3)	157
	B. burgdorferi	AE001154 (BB0516) AE00118 (BB0052)	228 218
	B. circulans	Z46432 (563845)	144
	C. psittaci	L39892 (g666878)	156
	C. trachomatis	AE001313(yjfh) AE001324 L40369	269 151 truncated
	E. coli	P39290 (yjfh)* P33635 (yfif)* P37005 (lasT)* P33899 (yibk)* P19396 (spoU)* AE000339 (f246)	243 345 228 157 229 246
	H. influenzae	P44703 (HI0424)* P44868 (HI0766)* P44906 (HI0860)* P44676 (HI0380)*	351 160 246 241
	H. pylori	AE000569 (HP0553)	227
	M. capricolum	Z33076 (g530425)	223
	M. genitalium	P47588 (MG346) P47494 (MG252)	166 242
	M. leprae	Z97179 (MLCL383.24c)	169
	M. pneumoniae	P75424 P75257	242 166
EUBACTERIA	*M. tuberculosis*	Z85982 AL021931 Z92774 (MTCY06G11.26c) AL009198 (MTV004.23) Q10543 (CY31.09)	260 183 322 154 288
	S. marcescens	P37006*	truncated
	S. actuosus	P52391 (NHS)*	274
	S. azureus	P18644 (tsr)*	269
	S. laurentii	P52393 (TSNR)	270
	Synechocystis sp.	D64004 (slr0120) D90915 (slr0992) D90914 (slr0955) D90913 (slr1673)	240 153 384 274
	T. pallidum	AE001196 AE001190	292 319
EUCARYOTAE	*A. thaliana*	Z97343 AF007270 (A_IG002P16.25) U37699 (OBP33pep)	1402 271 truncated
	C. elegans	U50191 (T14B4.1)	1203
	H. sapiens	U38847 (TRP-185)	1621
	S. cerevisiae	P25270 (PET56)* YDL112w	412 1436
	S. mansoni	AF030962	truncated
ARCHAE	*M. thermoautotrophicum*	AE000937 (MTH1849)	240
	M. jannaschii	ORF 1476	230
	A. fulgidus	AE001110 (AF2399) AE000978	224 226
	Pyrococcus sp.	D86335	235

Names of organisms for which a complete genomic sequence is available are underlined. ORFs denoted by a star have been reported in previous genomic searches (Koonin & Rudd, 1993; Gustafsson et al., 1996). Accession numbers of the three initial probes are underlined.

karyotic sequences, like eukaryotic initial probe Pet56, which methylates mitochondrial rRNA. Over the C-terminal region spanning motifs I–III (Fig. 1B) amino acid sequences of the four long eukaryotic ORFs are more closely related to each other than to any other Table 1 positive and their fully divergent N-terminal extensions are unrelated to protein sequences in databases.

Disruption of the yeast *S. cerevisiae* ORF YDL112w

To test the function of the single positive ORF in the *S. cerevisiae* genome, gene disruption of YDLW112w was performed. The ORF was completely replaced by a *TRP1* marker in the haploid CMY133 strain (Fig. 2A,B). Recombinant clones were obtained by growth on a selective medium. The resulting yeast strain remained viable and did not exhibit any growth delay as compared to the parent wild-type strain in the various conditions of cell culture tested, that is, at various temperatures (37 °C, 30 °C, or 19 °C) in a rich medium (Fig. 2C), in a glucose-lacking medium, or in a minimum, or nonfermentable medium (not shown). To examine the effect of gene disruption on ribose methylation of rRNAs and tRNAs, the disrupted strain was grown for several hours in the presence of inorganic (^{32}P) phosphate. Ribosomal RNAs and tRNAs were purified by gel electrophoresis and their pattern of labeled 2′-*O*-methylated dinucleotides examined by digestion with RNase T2 followed by two-dimensional thin-layer chromatography (2D-TLC) (Fig. 3). RNase T2 cleaves all

phosphodiester linkages except those that are 2′-*O*-methylated, producing nucleoside 3′-monophosphates. In addition to the four major spots of unmodified mononucleotide 3′-monophosphates, a less intense spot of pseudouridine 3′-monophosphate was present in all digests (in *S. cerevisiae* pseudouridine amounts to about 1% of 18S and 25S rRNA nucleotides and 2% of tRNA nucleotides). For the three RNA fractions the digests also showed a complex pattern of much weaker spots, reflecting the presence of ribose methylations and modified bases other than pseudouridine. Given that *S. cerevisiae* 18S and 25S rRNAs contain only 4 and 6 base modifications other than pseudouridine, versus 18 and 37 2′-*O*-ribose-methylated nucleotides respectively, weak spots in rRNA digests mostly correspond to RNase T2-resistant, 2′-*O*-methylated dinucleotides. The disrupted and parent wild-type strain rRNA 2D-TLC patterns appeared identical, indicating that the YDL112w protein has a minimal, if any, role in ribose methylations of cytoplasmic rRNA (Fig. 3A–D). As for tRNA digests, the 2D-TLC patterns exhibited a single difference between the two yeast strains, even after prolonged autoradiographic exposure, with the complete disappearance in the disrupted strain (Fig. 3F) of the radioactive spot denoted by an arrow in Fig. 3E. The mobility of this spot was consistent with its corresponding to GmG, one of the few 2′-*O*-methylated dinucleotide expected to be generated by RNase T2 digestion of total *S. cerevisiae* tRNAs (ribose methylation is not a prevalent nucleotide modification in tRNAs).

One of the initial probes in our genomic search, Pet56, specifically catalyzes one of the two 2′-*O*-ribose methylations of *S. cerevisiae* mitochondrial rRNAs, Gm2270 in 21S rRNA (Sirum-Connolly & Mason, 1993; Sirum-Connolly et al., 1995). We observed, through primer extension at low dNTP concentration (Maden et al., 1995), that the other ribose methylation of yeast mitochondrial 21S rRNA, Um2791, which corresponds to universally conserved Um2552 in *E. coli* 23S rRNA, was not affected in the disrupted strain, ruling out an involvement of YDL112wp in yeast mitochondrial rRNA methylation (data not shown).

Identification of the tRNA ribose methylation defect in the disrupted strain

We first set out to confirm the identification of the spot that disappeared in 2D-TLC of the disrupted strain tRNA digest. An RNase T2 digest of wild-type tRNA was analyzed by 2D-TLC and the labeled material in the spot eluted and subsequently digested with spleen phosphodiesterase, which cleaves phosphodiester bonds even adjacent to a 2′-*O*-methylated ribose, generating nucleoside 3′-monophosphates. A 2D-TLC of the phosphodiesterase digest revealed the presence of two radioactive spots of equal intensities (Fig. 4A), in agreement with the notion that the spot detected in the RNase T2 digest corresponded to a ribose-methylated dinucleotide. As expected, one of the two spots generated by spleen phosphodiesterase digestion comigrated perfectly with the guanosine 3′-monophosphate marker, whereas the mobility of the other in both dimensions was almost identical to that of C 3′-monophosphate, that is, as predicted for a Gm 3′-monophosphate (Keith, 1995), thus confirming the identification of the ribose-methylated dinucleotide as GmG.

Among the *S. cerevisiae* cytoplasmic tRNAs, 2′-*O*-ribose methylations only occur at five nucleotide positions (Fig. 4B), most prevalently at invariant position G18, with a Gm18 in 12 out of 41 different tRNA sequences, always followed by a G19. Ribose methylations are also found at positions 4, 32. and 44, each one in only four tRNA species. At positions 32 and 44 the ribose-methylated nucleotide is always a Cm and a Um, respectively, and at position 4 it is either a Cm or an Am. Finally, ribose methylation is also found at position 34 in three tRNAs, once as Cm34 and twice as Gm34, within the two Gm34-containing tRNAs position 35 occupied by a A. As a result, the GmG dinucleotide in the RNase T2 digest of wild-type strain total tRNAs exclusively reflects 2′-*O*-ribose methylation of G18.

Formation of tRNA Gm18 in vivo depends on YDL112wp

To confirm that formation of the yeast tRNA Gm18 was dependent on YDL112wp, the disrupted strain was transformed with a centromeric plasmid allowing expression of YDL112wp under the control of its own promoter (Fig. 5). As expected, the GmG radioactive spot that selectively disappeared in the disrupted strain tRNA digests was fully restored by expression of YDL112wp (Fig. 5B). In addition to GmG, RNase T2 digestion of wild-type *S. cerevisiae* strain total tRNAs is expected to yield seven other ribose-methylated dinucleotides: CmU, CmC, CmA, CmG, UmG, GmA, and AmU, at levels likely to be much lower than GmG. An unambiguous assignment of all these dinucleotide spots was not possible in our experimental conditions, due to the presence of a complex, partially overlapping spectrum of base-modified tRNA nucleotides. However, the apparent identity of the wild-type and disrupted-strain patterns, except for the GmG spot, strongly suggests that most, if not all, other tRNA ribose methylations are not dependent on YDL112wp.

Characterization of the Gm18 tRNA ribose methylase activity in vitro

We used as a substrate a T7 transcript corresponding to an intronless the yeast $tRNA^{Ser}$. In addition to Gm18, this tRNA naturally contains a second 2′-*O*-ribose-methylated nucleotide, Um44, and several base mod-

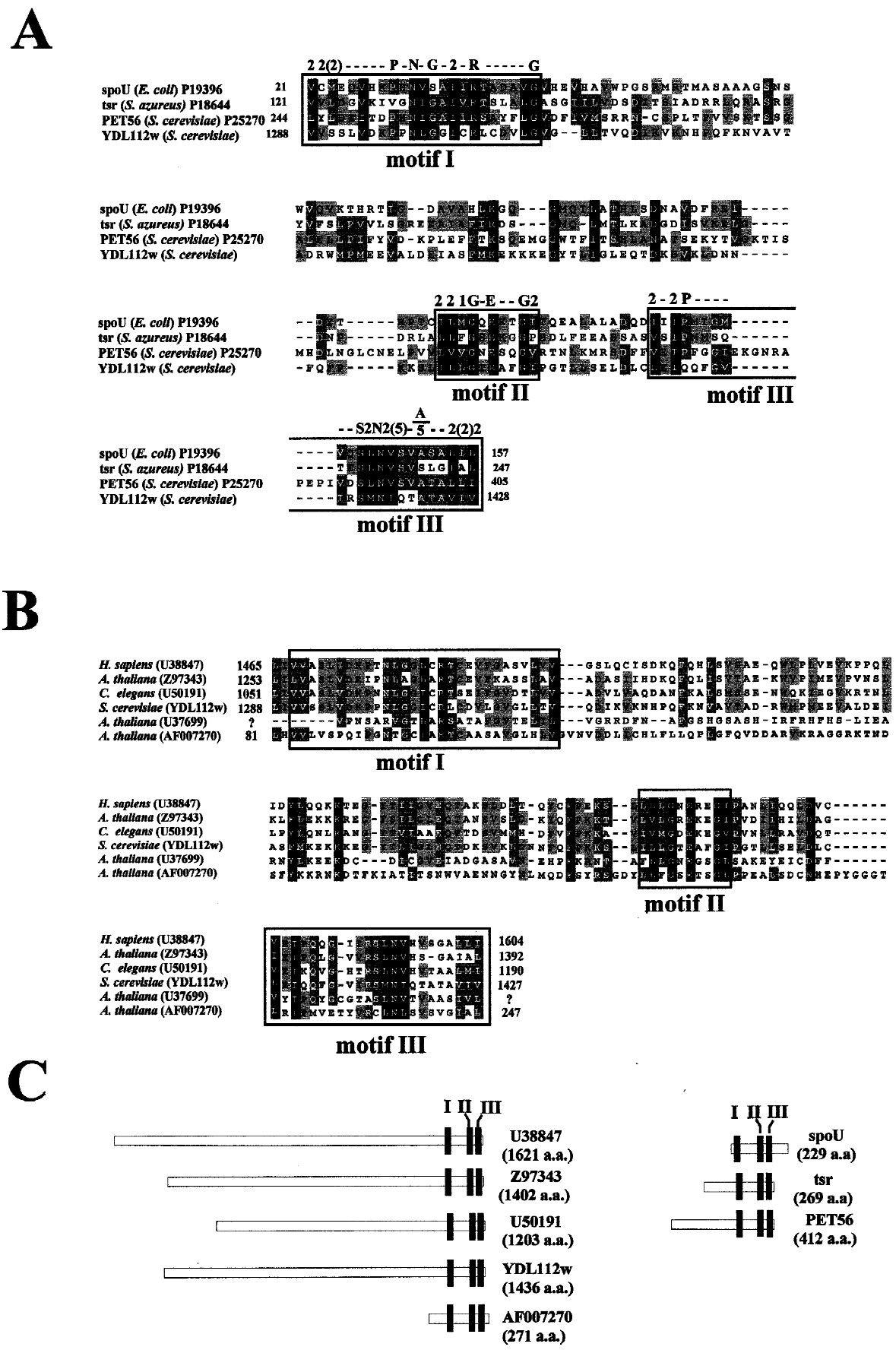

FIGURE 1. (*Legend on facing page.*)

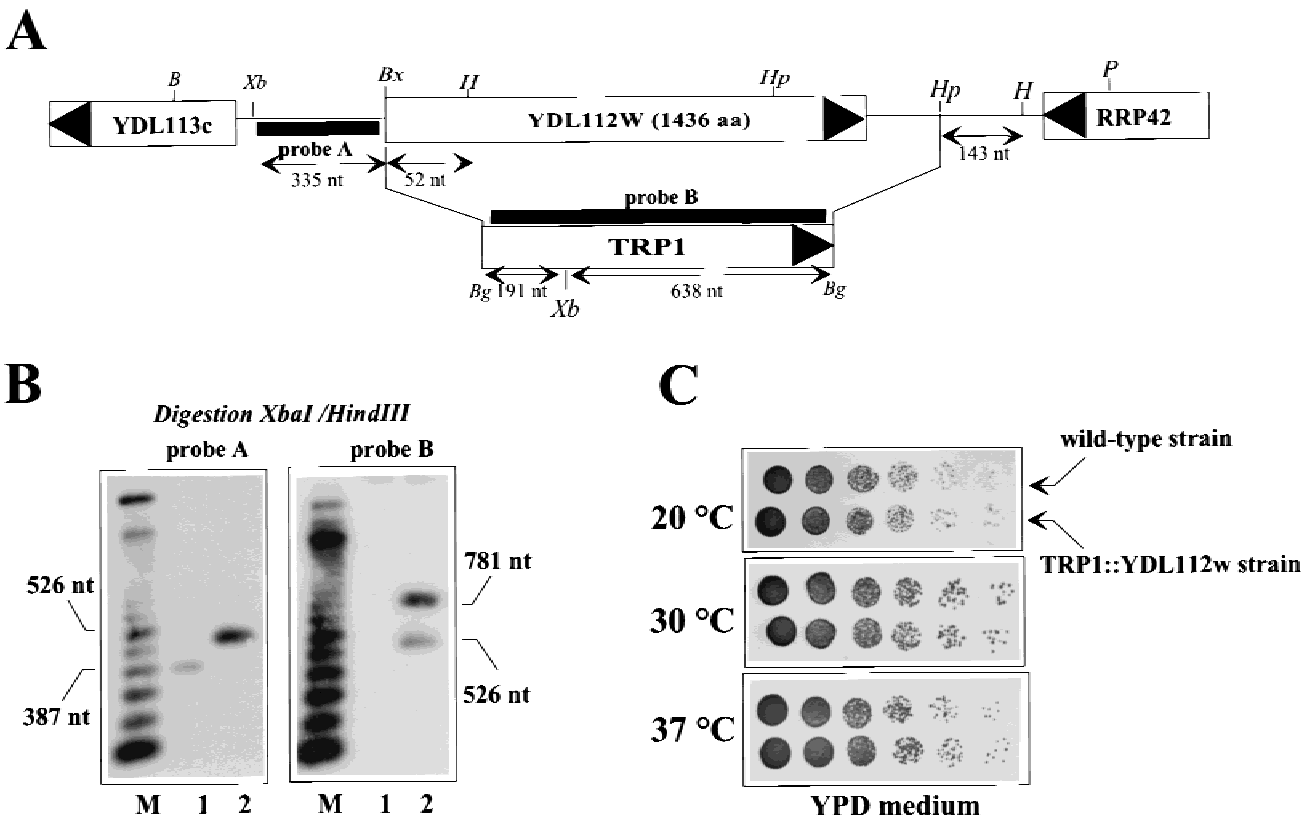

FIGURE 2. Disruption of the YDL112w ORF. **A**: Schematic restriction map of the YDL112w and *TRP1::YDL112w* loci. The two probes used for the Southern blotting analysis shown in **B** are denoted by filled bars. B: *Bam*HI; E: *EcoR*I: Hp: *Hpa*I; P: *Pst*I; Xb: *Xba*I; Bx: *Bst*XI; H: *Hind*III; Bg: *Bgl*II. **B**: Southern blotting analysis. Genomic DNAs extracted from the wild-type (lanes 1) or *TRP1::YDL112w* strains (lanes 2) were completely digested by *Xba*I/*Hind*III. After transfer the membrane was first hybridized with probe B then heat-denatured and rehybridized with probe A. The two DNA fragments serving as probes (filled bars) were obtained by restriction of plasmid DNA. They were labeled with α-^{32}P-dATP using the Megaprime DNA labeling system (Amersham). M: size marker (the 100 pb-DNA marker from Boehringer). **C**: Growth of the wild-type and disrupted *TRP1::YDL112w* strains. Starting from a dilution of 5×10^6 cells/mL, fivefold serial dilutions of CMY133/pFL39 and *TRP1::YDL112w* strain cultures (5 μL each) were spotted on YPD medium and incubated at the indicated temperature (20 °C, 30 °C, and 37 °C).

ifications (Fig. 6A). The transcript labeled with α-^{32}P-UTP was incubated for 60 min with the yeast S100 extract in the presence of AdoMet as the donor of methyl groups. After hydrolysis by RNase T2 or nuclease P1, tRNA digests were analyzed by 2D-TLC. RNase T2 digestion allowed us to identify the nucleotides that are 5′-adjacent to a uridine (or a modified uridine) in tRNA^{Ser}. Formation of Gm18 in the T7 transcript should therefore be reflected by the appearance of a GmG dinucleotide 3′-^{32}P-monophosphate. After incubation with the wild-type extract, a relatively intense spot migrating exactly as expected for GmGp—and as observed for

FIGURE 1. Detection of eukaryotic ORFs with amino acid sequence similarity to known 2′-*O*-ribose methylases. Alignment of the C-terminal amino acid sequences of the yeast *S. cerevisiae* ORF with (**A**) the three initial probes used for the genomic search and (**B**) the other newly found eukaryotic ORFs, including the truncated ORF found in *A. thaliana* genome (accession number U37699). **A** and **B**: The three conserved motifs I, II, and III shared by the three initial probes and related prokaryotic sequences identified in previous genomic searches (Koonin & Rudd, 1993; Gustafsson et al., 1996) are boxed. For each motif the improved consensus derived from the large set of positive sequences listed in Table 1 is shown above the alignment (amino acid codes: 1: bulky hydrophobic; 2: aliphatic; 3: FYWH; 4: KR; 5: ST; 6: EQ; 7: DN. The amino acid code symbol is in parentheses when the amino acid is conserved in 60–85% of the sequences, and without parentheses when conserved in more than 85% of the sequences). Positions occupied by amino acids belonging to the same subgroup are denoted by black boxes when conserved in the six sequences, by dark shading when conserved in five sequences, and by light shading when conserved in four sequences. For each ORF the numbering of the first and last amino acid positions in the aligned portion of the sequence is given at the alignment boundaries. **C**: Schematic representation of the primary structure of the newly found eukaryotic ORFs (left), by reference to the three initial probes (right). The three conserved motifs are depicted by vertical solid bars.

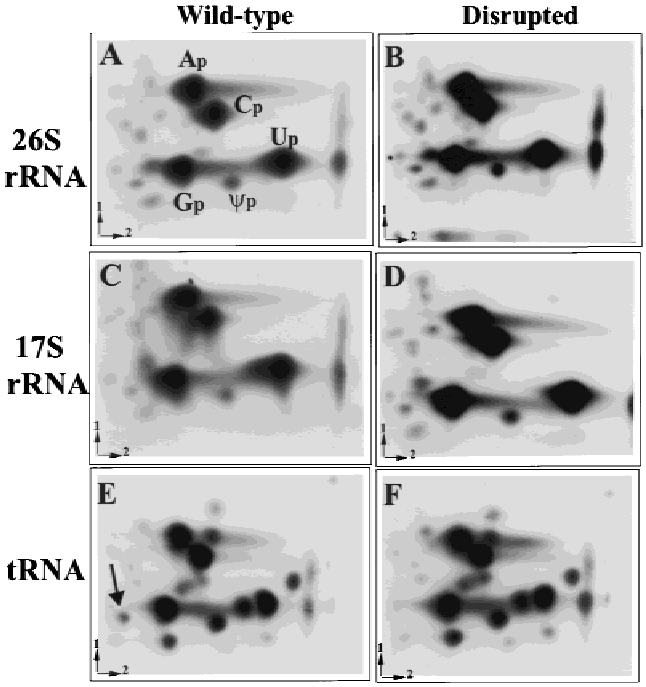

FIGURE 3. Genetic disruption of YDL112w affects a single type of tRNA nucleotide modification. In vivo ^{32}P-labeled mature 17S and 25S rRNAs and a tRNA sized-fraction were purified from wild-type CMY133 and null mutant *TRP1::YDL112w* strains and completely digested by RNAse T2. Digests of 26S rRNA (**A**, **B**), 17S rRNA (**C**, **D**) and the tRNA sized-fraction (**E**, **F**) from wild-type (**A**, **C**, **E**) and mutant (**B**, **D**, **F**) strains were analyzed by 2D-TLC with system B. The location of Ap, Cp, Gp, and Up (3′ monophosphates unmodified nucleotides) is indicated in **A**. The arrow points to the spot that disappears in the disrupted strain chromatogram.

the dinucleotide spot generated by RNase T2 hydrolysis of in vivo labeled total the yeast tRNAs (Fig. 3E, arrow)—was readily detected (Fig. 6B, top). Its identification was further confirmed by P1 nuclease digestion, which generated a spot coincident with Gm 5′-monophosphate (see below). Moreover, as expected, the GmGp spot was absent from the 2D-TLC pattern of in vitro transcripts of the yeast tRNA^{Phe}, which naturally contains a nonmodified G18G19 dinucleotide (data not shown). In addition to the GmGp spot, the 2D-TLC of the tRNA^{Ser} RNase T2 digest showed the presence of the various mononucleotide spots expected, including those reflecting efficient formation of modified uridines 5′-adjacent to a uridine in the tRNA^{Ser}, that is, a Ψp spot for Ψ32, a Dp spot for D20 and a Tp spot for T54. Quantification of the radioactive signals indicated that the extent of modification at these various sites ranged between 0.2 and 0.8 mole of modified nucleoside per mole of tRNA (Fig. 6B, top). Utilization of the disrupted strain extract resulted in complete and selective disappearance of the Gm18G radioactive spot, whereas the other nucleotide modifications (D20, ψ32, and T54) that could be monitored in these experimental conditions remained unaffected. Finally, incubation of the S100 extract from the disrupted the yeast strain transformed with the plasmid allowing expression of the YDL112w protein resulted in the reappearance of the Gm18G, although with a lower intensity than with the wild-type extract spot (Fig. 6B, top).

The selective deficiency of the disrupted strain extract in the catalysis of Gm18 formation was confirmed by utilization of a tRNA^{Ser} transcript, labeled this time by incorporation of α-^{32}P-GTP, followed by nuclease P1 digestion (results not shown). Nuclease P1 is not inhibited by 2′-*O*-methylation at the high concentrations used, generating nucleoside 5′-monophosphates. After incubation with the wild-type extract and nuclease P1 hydrolysis, a very substantial pGm signal was readily detected, pointing to the formation of about 0.2 moles of Gm18 per mole of tRNA, in full agreement with the previous results. After incubation with the disrupted strain extract, the pGm18 signal selectively disappeared, but it was restored, with a relative intensity very similar to that of the wild-type extract pattern, after incubation with the extract of the disrupted yeast strain expressing YDL112wp. This strongly suggests that the YDL112w ORF encodes the 2′-*O*-G18 methyltransferase, although the unlikely possibility that it encodes a regulator of the Gm18 methylase activity cannot be ruled out until the protein is purified. In contrast, the strong pm^2_2G spot (m^2_2G26 is the only other modified guanosine naturally present in tRNA^{Ser}) retained a similar intensity, whatever the origin of the extract.

Disruption of the YDL112w ORF does not affect formation of tRNA^{Ser} Um44 in vitro

Utilization of the α-^{32}P-UTP-labeled T7 transcript also allowed us to monitor formation of Um44 in tRNA^{Ser}. After nuclease P1 hydrolysis a pUm (2′-*O*-methylated uridine 5′-monophosphate) spot was detected (Fig. 6B, bottom left), reflecting a very significant formation of Um44. Formation of all other types of uridine modification naturally found in this tRNA was also observed. Thus, a relatively intense spot of Tp reflecting efficient production of T54 was again present, in full agreement with results of the previous experiment. Very substantial labelings of dihydrouridine and pseudouridine were also observed, consistent with a faithful formation of D16, D20, and D21 on the one hand, and Ψ32, Ψ39, and Ψ55 on the other hand, although individual extent of the reaction at each of these naturally modified positions of the yeast tRNA^{Ser} could not be determined in our conditions (the overall rate corresponds to an average of 0.2–0.3 moles of D or Ψ formed per mole of tRNA at each modifiable position). Remarkably, relative intensities of the various spots, including pUm, remained roughly similar whether the extract was prepared from wild-type or disrupted strain (Fig. 6B, bottom), showing that YDL112wp was not involved in Um44 formation.

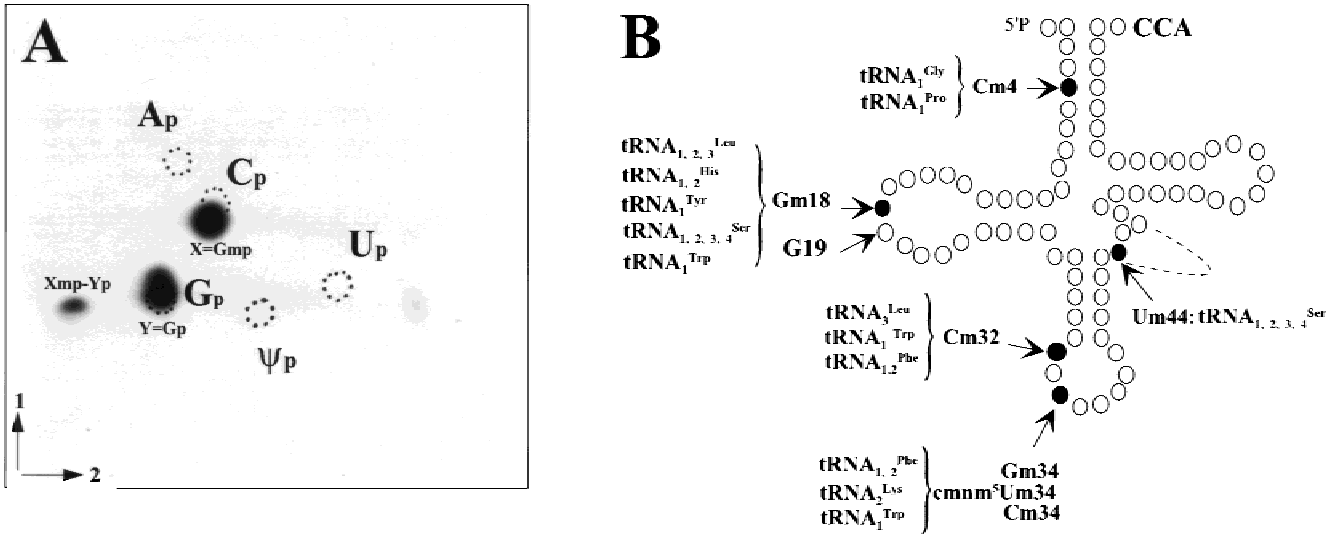

FIGURE 4. Identification of the tRNA nucleotide modification suppressed in the disrupted strain. **A**: Analysis of the 2D-TLC spot missing in the tRNA digest of the *TRP1::YDL112w* strain. The spot detected in a wild-type CMY133 tRNA digest was eluted from the TLC plate and the eluted material digested by spleen phosphodiesterase before analysis on 2D-TLC (system B). Unlabeled 3′ phosphate mononucleotides (Ap, Cp, Up, Gp, Yp) were used as markers. Undigested material: XmpYp. **B**: Location of the different 2′-*O*-ribose methylations in *S. cerevisiae* cytoplasmic tRNAs (all tRNAs containing the indicated ribose-methylated position are listed).

Formation of Gm18 in vitro is dependent on tRNA architecture

To gain an insight into the tRNA structural features required for in vitro formation of Gm18, we analyzed the effects of a few mutations in the RNA substrate (Fig. 7). The reaction was completely abolished not only by a G18C mutation but also by a G19C mutation. Interestingly, the formation of D20 was also completely blocked in both mutants, whereas formation of two other tRNA nucleotide modifications outside the D loop was only moderately, or not at all, affected (as shown in Fig. 7 for

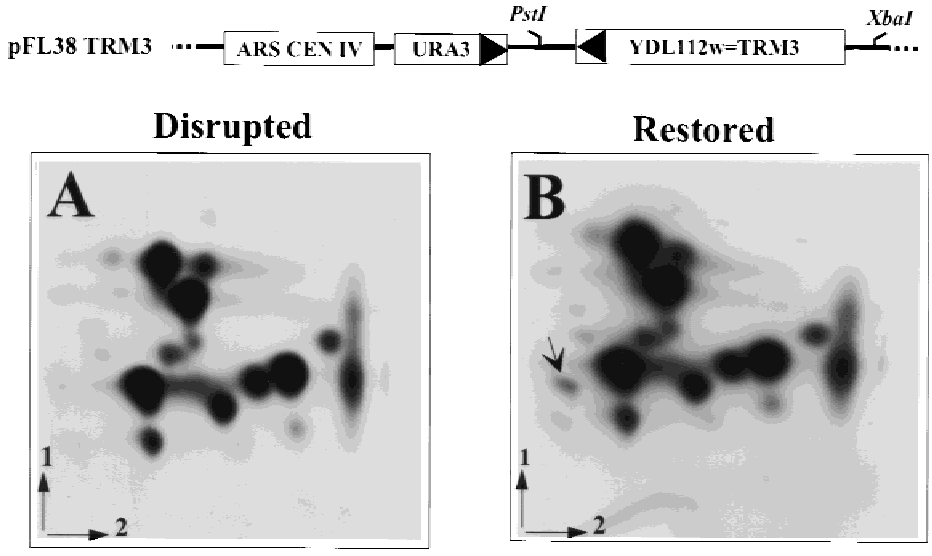

FIGURE 5. Formation of tRNA Gm18 in the disrupted strain is restored by expression of YDL112w protein. An in vivo ^{32}P-labeled, total tRNA fraction was purified from null mutant *TRP1::YDL112w* strain (**A**) and from the null mutant *TRP1::YDL112w* strain transformed with the pFL38TRM3 plasmid expressing YDL112wp (**B**) and RNAse T2 digests analyzed by 2D-TLC with system B. The unique radiolabeled spot missing in the tRNA chromatogram of the disrupted strain is indicated by an arrow in **B**. The structure of the construct allowing expression of YDL112wp is schematized on top.

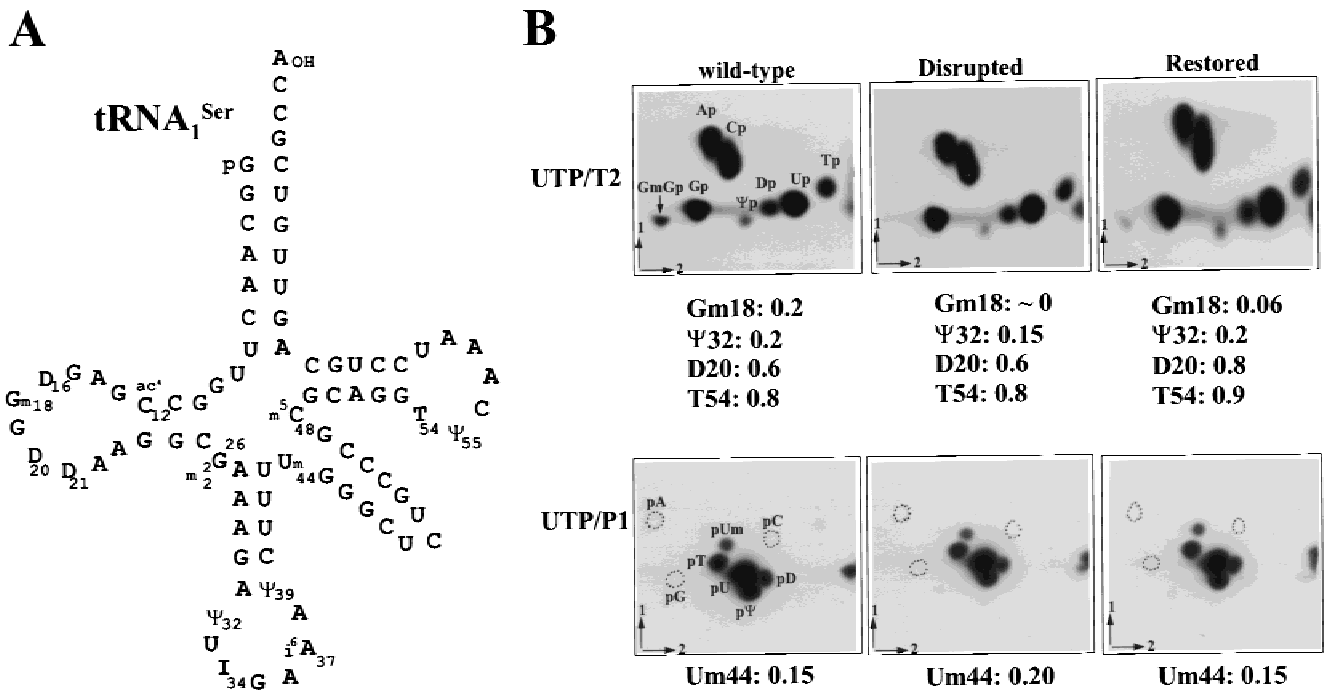

FIGURE 6. In vitro genetic restoration of tRNA^{ser} Gm18 biosynthesis. **A**: Schematic representation of tRNA^{Ser} secondary structure with indication of its natural nucleotide modifications. **B**: In vitro synthesized tRNA^{Ser}, labeled by incorporation of α-^{32}P-UTP, was incubated with S100 extract prepared from wild-type strain (left), *TRP1::TRM3* mutant (center), or *TRP1::TRM3* transformed with pFL38-PLJ1 expressing Trm3p (right). After incubation labeled tRNA was completely digested by either RNAse T2 (top) or nuclease P1 (bottom) and a two-dimensional TLC carried out in system B and A, respectively. The location of the unlabeled 5′ monophosphate nucleotide markers is indicated (dotted spots). The extent of modification at the particular positions that can be monitored in each assay is indicated below the chromatograms, expressed as moles of modified nucleosides per mole of tRNA (values were derived from a quantification of spot intensities by a Fuji Bas-1000 Imager).

Ψ32 and T54, respectively). In the crystals of all tRNAs investigated, the tertiary pair G19.C56 plays a key role in the three-dimensional structure, locking the D and TΨC loops (Dirheimer et al., 1995). G18 is also involved in a D loop-TΨC loop interaction through a tertiary pairing with Ψ55. To gain further insight into the significance of the effects of G18C and G19C mutations, we tested the effects of a C56 mutation preventing the formation of the G19.C56 tertiary pair. Mutation C56G completely blocked the formation of Gm18 during incubation with the S100 extract, strongly suggesting that the reaction depends on an intact three-dimensional structure of the tRNA substrate. Formation of D20 was also completely abolished in this mutant, also pointing to a three-dimensional structure dependence for this modification, in contrast to what was observed for Ψ32. As for T54, the formation of which is not dependent on an intact tRNA three-dimensional structure (Grosjean et al., 1996; Becker et al., 1997b), it was similarly blocked in the mutant transcript, probably as a result of an altered local conformation brought about by the nearby C56G mutation. We also tested the effects of a double C56G/G19C mutation that could maintain the potential for tertiary interaction between positions 19 and 56 through inverted pairing. However, formation of the three modifications in the D and TΨC loops monitored in our conditions, Gm18, D20, and T54, was still fully suppressed, suggesting that the precise initial three-dimensional conformation of tRNA was not fully restored in the double mutant. The potential importance for Gm18 formation of another tertiary base pairing in tRNA^{Ser} structure (Dock-Bregeon et al., 1989), U8–A14, was also examined through a U8C mutation. Only a moderate decrease in the formation of the two D loop nucleotide modifications, Gm18 and D20, was observed, possibly because an alternate reverse C8.A14 Hoogsteen pairing can take place in the mutant, and formation of T54 was not affected.

DISCUSSION

Given the previous characterization of the two yeast genes, *TRM1* and *TRM2*, that encode tRNA (base) methylases catalyzing formation of N^2,N^2-dimethylguanosine and ribothymidine, respectively (Hopper et al., 1982), the *S. cerevisiae* gene identified in this study has been named *TRM3* (for tRNA methylase). Formal demonstration of the enzymatic activity of Trm3p, the only putative eukaryotic tRNA ribose methylase re-

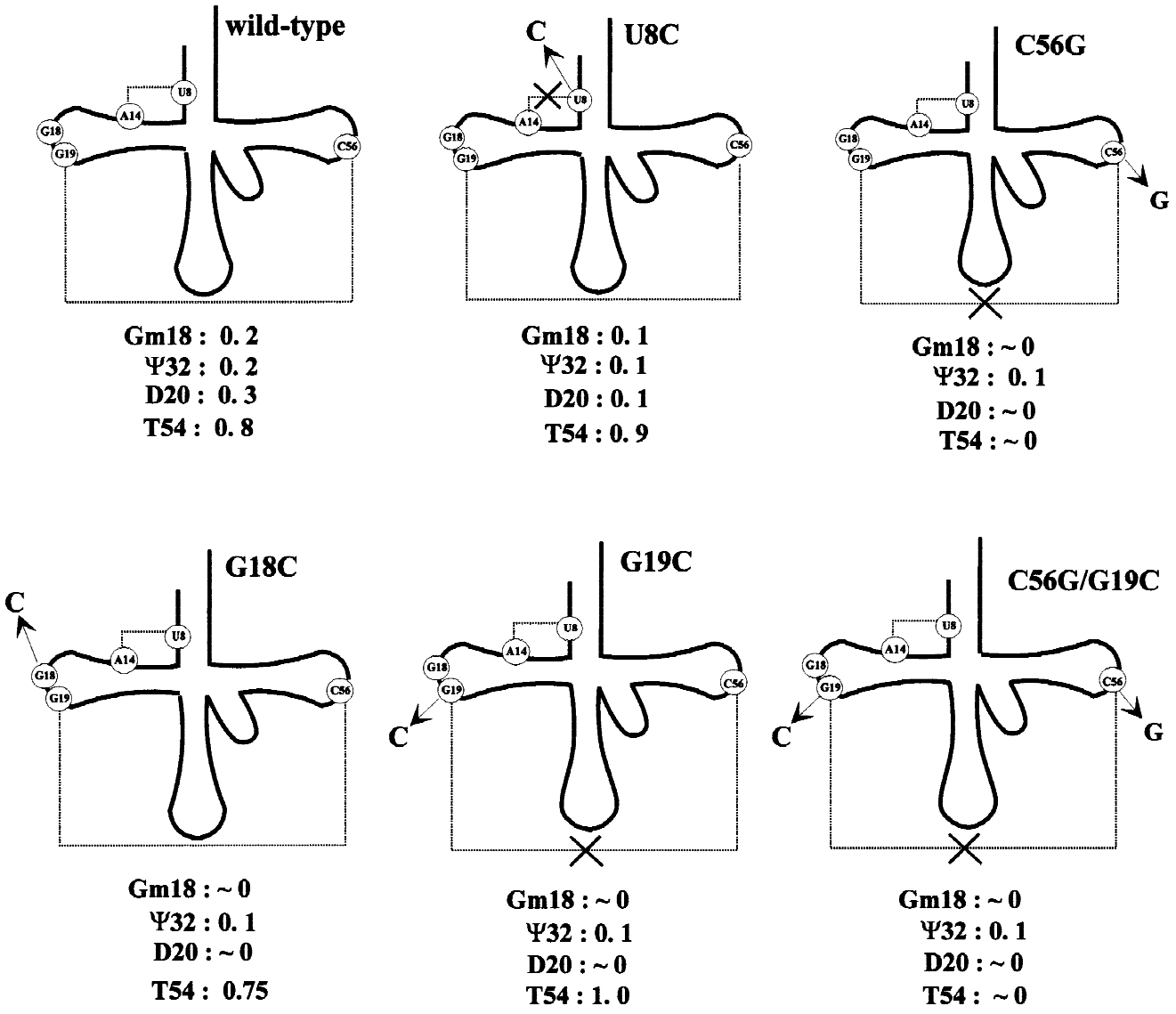

FIGURE 7. Mutations in tRNA^{ser} affecting Gm18 biosynthesis in vitro. Mutations preventing the formation of tertiary interactions (thin lines over the cloverleaf structure) were introduced in the T7 tRNA^{Ser} transcript and the modification levels (indicated below and expressed as moles of modified nucleosides per mole of tRNA) were monitored as in Figure 6. Nucleotides that have been mutated are indicated in circles. The transcript, labeled by incorporation of α-^{32}P-UTP, was incubated in the presence of wild-type strain extract and analyzed by 2D-TLC with system B after digestion with RNase T2.

ported as yet, will require purification of the protein. Required both in vivo and in vitro for a single tRNA ribose methylation, Gm18, *TRM3* corresponds to the the yeast homolog of *E. coli spoU*, required for the formation of this modification in all Gm18-containing *E. coli* tRNAs (Persson et al., 1997). The best studied RNA 2′-*O*-ribose methyltransferase is a viral protein, VP39, the crystal structure of which has been recently resolved (Hodel et al., 1996). VP39 is a bifunctional protein, which can also serve as a poly(A) polymerase processivity factor in addition to its role as 2′-*O*-methylase specific for the penultimate nucleotide of the mRNA 5′ cap structure (Shi et al., 1996). In the context of an organism with a rapid generation time, VP39, instead of acquiring an independent RNA binding domain, seems to have customized a methyltransferase catalytic domain for that purpose (Hodel et al., 1996), and its structure may substantially depart from a consensus for RNA ribose methylases of free living organisms. In line with this notion, the VP39 sequence was not retrieved in our genomic search. Likewise, the fact that YDL112w is the only ORF detected in the entire *S. cerevisiae* genome must reflect an extensive sequence divergence of the other, still unknown yeast 2′-*O*-ribose methyltransferases, thus illustrating the limitations of a homology-based approach. Sequence searches in less stringent conditions might, however, point to additional yeast candidates, although such anal-

yses might be considerably hampered by high numbers of false positives.

Substrate and site specificity of Trm3p

Formation of a given type of nucleotide modification at multiple sites of an RNA molecule frequently involves several distinct modifying activities, but some enzymes can modify different positions within an RNA substrate (Simos et al., 1996; Lecointe et al., 1998), and a dual substrate specificity can even be envisioned (Wrzesinski et al., 1995). Analysis of *TRM3*-disrupted yeast strain tRNAs and in vitro modification experiments of tRNA transcripts indicate that Trm3p has a very narrow substrate and site specificity, corresponding to the exclusive formation of Gm18 in all yeast cytoplasmic tRNAs naturally bearing this methylation. Several instances of a single nuclear gene encoding an enzyme modifying both cytoplasmic and mitochondrial tRNAs have been reported (Hopper et al., 1982; Becker et al., 1997a; Lecointe et al., 1998), and the possibility that Trm3p is also responsible for Gm18 formation in mitochondrial tRNAs cannot be ruled out. In *E. coli* RNA, a total of seven 2′-*O*-ribose-methylated nucleotides have been identified, including two other tRNA ribose methylations in addition to Gm18, Um32, and Cm32, the formation of which might be catalyzed by the same enzyme different from spoU. As for the four rRNA ribose methylations, one of which is on a guanosine (Smith et al., 1992), they do not depend upon spoU but are thought to each involve a distinct methylase (Gustafsson et al., 1996). Accordingly, we have detected 6 ORFs encoding putative RNA ribose methylases in the *E. coli* genome (Table 1).

While a pseudouridine synthase capable of forming ψ32 in tRNA^{Phe} in vitro also catalyzes specific formation of ψ746 in *E. coli* 23S rRNA (Wrzesinski et al., 1995), our data do not provide evidence for a dual substrate specificity of Trm3p. Since sites of cytoplasmic rRNA ribose methylations are exclusively specified by the RNA duplex structure involving a cognate small nucleolar RNA guide (Cavaillé et al., 1996; Kiss-Laszlo et al., 1996; Nicoloso et al., 1996; Bachellerie & Cavaillé, 1997) the involvement of only a few, if not a single rRNA ribose methylase, devoid of any site specificity and obviously distinct from Trm3p, seems likely. As for snRNAs, which contain phylogenetically conserved ribose-methylated nucleotides (including several guanosines) in vertebrates, their ribose methylation patterns in *S. cerevisiae* are not known (Massenet et al., 1998). However, given that Trm3p activity is strongly dependent in vitro on the three-dimensional conformation of mature tRNA, as discussed below, its involvement in snRNA ribose methylation seems unlikely. Finally, yeast mRNAs are devoid of ribose methylations adjacent to the 5′ cap, in contrast to vertebrate mRNAs (Narayan & Rottman, 1992).

Recognition signals for Trm3p

After injection in the *Xenopus* oocyte of mutated versions of yeast tRNA^{Phe}, which naturally contains Gm34, methylase activities catalyzing formation of Cm34, Am34, and Um34, in addition to Gm34, were detected, suggesting either that the enzyme modifying this anticodon position is not specific for the base or that several distinct methylases able to modify position 34 are present in the *Xenopus* oocyte (Droogmans et al., 1986). While Trm3p cannot modify in vitro a C introduced at position 18 in a tRNA^{Ser} transcript (Fig. 7), this could reflect primarily a dependence for an overall tertiary structure of the mature tRNA substrate rather than a particular base specificity. In line with this notion, Gm18 is formed only after intron excision, like several other modified nucleotides in yeast tRNAs (Grosjean et al., 1997). Among the tRNA modifying enzymes negatively dependent on the presence of an intron, some depend only on a local conformation of the tRNA substrate around the modification site whereas others depend on the global three-dimensional architecture of the tRNA molecule. Effects of mutations in the tRNA transcript on in vitro formation of Gm18 strongly suggest that Trm3p belongs to the second category of "intron-sensitive" tRNA modifying enzymes. Interestingly, while Gm18 is not naturally present in yeast tRNA^{Phe,} a thermostable tRNA (Gm18) methyltransferase purified from *Thermus thermophilus* can catalyze its formation in vitro (Kumagai et al., 1982). Unfortunately, the structure of this monomeric enzyme—with a molecular weight estimated at 20 kDa—that also recognizes the tertiary structure of tRNA (Matsumoto et al., 1987) has not been reported.

Peculiarities of the putative eukaryotic enzyme

The presence of an ~1,280-amino-acid-long N-terminal extension in Trm3p, as compared with spoU and other prokaryotic homologs, is intriguing. In contrast, the four pseudouridine synthases that modify yeast tRNAs are only moderately longer than their *E. coli* homologs, truA and truB (Simos et al., 1996; Becker et al., 1997a; Lecointe et al., 1998). Given that spoU and Trm3p appear to recognize the same three-dimensional architecture of mature tRNAs in *E. coli* or in yeast, the long N-terminal domain of Trm3p seems unlikely to be directly involved in the methylase catalytic activity or in substrate recognition. The intracellular location of Trm3p remains unknown. However, it is noteworthy that Pus1p, a tRNA pseudouridine synthase with an intron-dependent activity, is a nuclear protein interacting genetically with nuclear pore protein Nsp1p (Simos et al., 1996). Likewise, splicing of tRNA may be coupled to tRNA export through the nuclear pore complex, given that the tRNA endonuclease appears to be an integral nuclear membrane protein (Peebles et al., 1983) and

that the tRNA ligase is localized close to nuclear pores (Clark & Abelson, 1987). Moreover, Trm1p that catalyzes formation of tRNA N2, N2-dimethylguanosine localizes at or near the nuclear membrane (Rose et al., 1992, 1995). Since formation of Gm18, like a few other tRNA modifications, occurs only after intron excision, an association of Trm3p with the transport machinery and/or the nuclear pore complex through its N-terminal domain may be envisioned. Alternatively, Trm3p could reside in the cytoplasm and its long extension at the N-terminus could be required for its assembly into a macromolecular complex in much the same way as the aminoacyl-tRNA synthetase complexes in higher eukaryotes (Mirande, 1991; Kisselev & Wolfson, 1994).

The three other eukaryotic positive ORFs with very long N-terminal extensions, in human, *C. elegans*, and *A. thaliana*, do not necessarily represent orthologs of Trm3p. Intriguingly, the human ORF encodes a nuclear protein that binds with both high affinity and marked specificity to TAR RNA of HIV, hence its name of TRP-185 for 185-kDa TAR RNA protein (Sheline et al., 1991; Wu et al., 1991). Its binding is strongly dependent on the TAR RNA loop sequences and is stimulated by a set of cellular factors that also stimulate the binding of RNA polymerase II to HIV TAR RNA. Binding of TRP-185 and RNA polymerase II to TAR RNA is mutually exclusive and TRP-185 stimulates gene expression from the HIV LTR in vitro (Wu-Baer et al., 1995a, 1995b, 1996). Interestingly, defective assembly of large ribosomal subunits in *Pet*56 mutants seems caused by inactivation of the Pet56 protein rather than by the lack of 21S mitochondrial rRNA G2270 methylation normally catalyzed by this protein, suggesting an additional role for the Pet56 methylase, possibly as a rRNA chaperone (Mason et al., 1996). Likewise, although the presence of Dim1p, which catalyzes formation of the $m^6_2m^6_2A$ doublet at the 3′ end of 18S rRNA, is a critical factor for pre-rRNA processing, the absence of rRNA base-dimethylation itself does not result in any clear rRNA processing defect (Lafontaine et al., 1995). Moreover, the gene for tRNA m^5U54-methyltransferase is essential for viability in *E. coli*, although the known catalytic activity of the methylase is not (Persson et al., 1992), and the vaccinia virus VP39 2′-*O*-ribose methylase is a bifunctional protein (Shi et al., 1996), like several other polypeptides (Smith, 1994; Henikoff, 1987). In this context, the presence of a long N-terminal extension in the yeast tRNA (Gm18) methylase is consistent with Trm3p having a more complex role than that of a mere RNA modifying enzyme.

Role of Gm18

In *E. coli*, the absence of Gm18 in tRNAs has no significant effect on growth or on efficiency of decoding during translation (Persson et al., 1997). Likewise, Gm18 is not essential in *S. cerevisiae*, and disruption of the *TRM3* gene does not alter cell growth in the different conditions examined. Moreover, absence of Gm18 does not affect suppressor activity of $tRNA^{Tyr}$ SUP 40 (ochre) and $tRNA^{Tyr}$ SUP-RL1 (amber) suggesting that translation fidelity is not impaired (results not shown). However, the disrupted yeast strain is slightly but reproducibly more resistant than the wild-type strain to paromomycin, an aminoglycoside antibiotic that interferes with protein synthesis by inducing codon misreading through its binding to 30S ribosomal A-site RNA (Fourmy et al., 1996), and the effect is partially suppressed by expression of plasmid-encoded Trm3p (data not shown). While the molecular basis of the resistance to paromomycin is not understood, it is noteworthy that disrupted and wild-type strains exhibit the same dose-dependent sensitivity to translocation inhibitor cycloheximide (data not shown). Ribose methylation of G18 in yeast $tRNA^{Phe}$, which is naturally devoid of Gm18, has no detectable effect on amino acid acceptor activity and melting temperature of the tRNA (Kumagai et al., 1982). However, the complete lack of Gm18 observed in rat hepatoma cell $tRNA^{Ser}$ (codon IGA), which is the single difference as compared with normal rat liver $tRNA^{Ser}$, is related to extensive changes in elution profiles of this RNA, suggesting that the Gm18-devoid form has a substantially altered tertiary structure (Randerath et al., 1981). Intriguingly, in the rat, several tRNAs differ in the brain as compared with the liver in a specific lack of G18 ribose methylation (Rogg et al., 1977). Moreover, under tryptophan-limited growth conditions in yeast cells, a second important $tRNA^{Trp}$ species (isoacceptor B) accumulates that differs from isoacceptor A in one respect only: absence of ribose methylation of G18 (Stäheli et al., 1982). Finally, although the phylogenetically conserved Gm18 may play a part in the control of the D loop-TΨC loop interaction crucial for the stabilization of tRNA tertiary structure, its actual biological significance remains elusive, as do many other nucleoside modifications in tRNAs (Björk, 1995). None of the numerous yeast tRNA nucleoside modifications appears essential for yeast growth under laboratory conditions. Similar to the *TRM3*-disrupted strain, yeast cells lacking m^2_2G26, m^5U54, or several tRNA pseudouridines have no obvious physiological defects (Hopper et al., 1982; Ellis et al., 1986; Motorin et al., 1998). However, the lack of i^6A37 reduces the efficiency of nonsense suppression by a tyrosine-inserting suppressor tRNA and the lack of the 2′-*O*-ribosyl phosphate at position 64 in initiator tRNA, which prevents its discrimination from elongator tRNAs during protein synthesis, is related to a reduction of the growth rate (Aström & Byström, 1994). All organisms put considerable effort into modifying all their RNAs, not only tRNAs, suggesting that the modifications, although not essential, may have a crucial role in definite conditions of cell growth. The identification of the first putative nonorganellar RNA ribose methylase gene, in *S. cerevisiae*, should pave

the way for gaining new insights into the biosynthesis and function of RNA ribose methylations in eukaryotic cells.

MATERIALS AND METHODS

Unless otherwise noted, all techniques for cloning and manipulating nucleic acids were performed according to standard protocols (Sambrook et al., 1989).

Computer analyses

Searches were performed with version 1.4.9 of the BLAST program (Altschul et al., 1990), first on a bank containing all the translated ORFs available at the NCBI (National Center for Biotechnology Information), then on the complete genomes of *Haemophilus influenzae*, *Mycoplasma genitalium*, *Methanococcus jannaschii* and *S. cerevisiae*. Retrieved ORFs containing the three amino acid motifs shared by original probes Pet56, spoU, and tsr were used as probes in further iterative searches and the procedure repeated until no new occurrence was detected. All the finally retrieved ORFs represented an uninterrupted network of statistically significant connections with, in each case, a probability of fortuitous match of 10^{-8} or less, except for the truncated *A. thaliana* ORF ($P = 0.00037$ for an accidental match with *Bacillus subtilis* ORF Z75208). In a search of the *S. cerevisiae* complete genome, the YDL112w ORF has a probability of accidental match with spoU of 0.00041. Utilization of the Gapped BLAST program (Altschul et al., 1997) did not allow the detection of additional positives. Retrieved sequences were analyzed with the GCG software package (version 8.0), truncated sequence alignments obtained with the PILEUP program, and sequence profiles derived with the Profilemake program. Complete genomes were analyzed with the Profilesearch program. In the amino acid sequence of the *A. thaliana* ORF (Z97343), positions 1349–1402 (Fig. 1) differ from the database version because of the presence of a previously unnoticed 97-bp-long intron.

Chemicals, enzymes, and materials

α-^{32}P-nucleotide triphosphates (400 Ci/mmol) and ^{32}P-orthophosphate were from Amersham (UK), spleen phosphodiesterase and *Penicillium citricum* nuclease P1 from Boehringer Mannheim, and *Aspergillus oryzae* RNase T2 from BRL.

Media, strains, and plasmids

Saccharomyces cerevisiae strains were grown in rich (YPD) medium (yeast extract 1%, peptone 1%, and glucose 2%) or in minimum (YNB) medium (glucose 2%, 5 g/L NH_4SO_4, 1.7 g/L yeast nitrogen base without amino acids) supplemented by auxotrophic nutrients as specified (Sherman, 1991), or in nonfermentable (YPGE) medium (yeast extract 1%, peptone 1%, glycerol 3% (v/v), ethanol 3% (v/v)). The *TRM3* null mutant was constructed in the haploid yeast strain CMY133 (trp1-Δ, his3-Δ, ura3-52, lys2-801, Ade2-101, can +, Mat a). The genomic 5.8-kb *Bam*HI–*Pst*I DNA fragment encompassing *TRM3* coding sequence (i.e., YDL112w) was cloned in the pUC18 vector. The 4.3-kb *Bst*XI–*Hpa*I DNA fragment carrying the YDL112w ORF was replaced, after klenow treatment, with the 0.8-kb *Bgl*II DNA fragment carrying the *TRP1* auxotrophic marker from pFL39 (Bonneaud et al., 1991). The gel-purified 5-kb *Eco*RI–*Pst*I fragment was used to transform the CMY133 strain, recombinants were selected on minimum medium without tryptophan, and correct homologous recombination checked by Southern blot analysis. To express Trm3p in vivo, the 4.9-kb *Xba*I–*Pst*I DNA fragment spanning the entire YDL112w ORF and its two flanking genomic regions was cloned in the polylinker of centromeric pFL38 plasmid (GenBank accession number X70482) carrying the *URA3* gene as a selection marker, giving rise to pFL38PTRM3 recombinant (Fig. 5).

In vivo ^{32}P labeling of RNA

Wild-type strains transformed with the pFL39 plasmid and *TRP1::TRM3* strains were grown at 30 °C in YNB supplemented with auxotrophic nutrients until the exponential phase, washed twice, resuspended ($OD_{800} = 0.1$–0.2), and grown for 3 h in a rich medium lacking inorganic phosphate (Warner, 1991). Cultured cells (25 mL) were pelleted, washed, resuspended in 25 mL of rich medium devoid of inorganic phosphate, supplemented with 5 mCi of ^{32}P-orthophosphate, and grown for 12 h at 30 °C. Total RNA was then extracted (Tollervey & Mattaj, 1987) and analyzed by gel electrophoresis (1% agarose-formaldehyde gel for rRNAs, 8% acrylamide gel for tRNAs). RNAs were recovered by electroelution in dialysis tubing (2 h in 1×TBE) for rRNA , or by overnight incubation at 37 °C in 0.5 M NH_4 acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1% SDS for tRNA, and ethanol-precipitated.

In vitro tRNA modification assay

Enzymatic formation of modified nucleotides in tRNA, including site-specific ribose methylations, had been faithfully reproduced in vitro using cell-free yeast extracts and T7 transcripts of a synthetic yeast $tRNA^{Phe}$ gene (Jiang et al., 1997). Since yeast $tRNA^{Phe}$ (anticodon GAA), which naturally contains ribose-methylated nucleotides Cm32 and Gm34, is devoid of the Gm18 modification, we selected as a substrate for an in vitro study yeast $tRNA^{Ser}$ (anticodon IGA). In $tRNA^{Phe}$, formation of Cm32 and Gm34 in vitro was absolutely dependent on the absence of the intron in the tRNA transcript (Jiang et al., 1997). Likewise, in an in vitro transcription-splicing system allowing the production of the *S. cerevisiae* leucine-inserting amber suppressor tRNA gene SUP53, a $tRNA_3^{Leu}$ allele, the formation of Gm18 depended on mature tRNA structure (Strobel & Abelson, 1986). We therefore used as a substrate a T7 transcript corresponding to an intronless yeast $tRNA^{Ser}$. The plasmid expressing yeast $tRNA_1^{Ser}$ under the control of a T7 promoter (Dr. H. Himeno, Tokyo University, Japan) was linearized by *Bst*NI digestion. In vitro transcription was done as described (Jiang et al., 1997). The full-length transcript was purified by electrophoresis (8% acrylamide gel) and recovered by overnight elution (37 °C in 0.5 M NH_4 acetate, 10 mM Mg acetate, 1 mM EDTA, 0.1% SDS). Yeast S100 extract was prepared as described (Jiang et al., 1997) except that a “One-shot cell disrupter” (Constant Systems, UK; pressure = 2.7 kbar) was used instead of the

French Press. Efficiency of cell disruption was monitored by microscopic analysis. The in vitro RNA modification assay was performed as described (Jiang et al., 1997) except that 100–300 fmol of ^{32}P-labeled tRNA were heated (3 min, 85 °C) in the modification buffer and cooled slowly at room temperature before adding AdoMet (0.02 mM final). After incubation with S100 extract (60 min, 30 °C), full-length tRNA was gel-purified, ethanol-precipitated and digested by nucleases.

Analysis of modified nucleotides

In vivo- or in vitro-labeled RNAs were completely digested (24 h, 37 °C) with 0.1 mg/mL P1 nuclease, 0.05 U/mL RNase T2 ,or 0.02 mg/mL spleen phosphodiesterase. P1 nuclease and RNase T2 digestions were performed in 50 mM Na acetate (pH 5.2) and spleen phosphodiesterase digestion in 0.1 M NH_4 acetate (pH 5.7), 2 mM EDTA. Digests were analyzed by 2D-TLC on thin layer cellulose plates (20 cm × 20 cm, Merck) using chromatographic systems B and C (Filipowicz & Shatkin, 1983). Nucleotide 3′-monophosphates were routinely analyzed with system B—first dimension: isobutyric acid/NH_4OH/H_2O (577:38:385 by volume); second dimension: ter-butanol/HCl/H_2O (14:3:3 by volume), and nucleotide 5′-monophosphates with system A—first dimension: isobutyric acid/NH_4OH/H_2O (66:1:33 by volume); second dimension: 0.1 M sodium phosphate (pH 6.8)/ammonium sulfate/n-propanol (100:60:2 v/w/v). Unlabeled nucleotide markers added to labeled RNA digests were detected by UV irradiation (354 nm). Radiolabeled spots were identified by autoradiography using standard 2D-TLC maps (Hashimoto et al., 1975; Keith, 1995). The universal numbering system for tRNA positions is that of Sprinzl et al. (1998).

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