

Trm7p catalyses the formation of two 2'-O-methylriboses in yeast tRNA anticodon loop

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The genome of *Saccharomyces cerevisiae* encodes three close homologues of the *Escherichia coli* 2'-O-rRNA methyltransferase FtsJ/RrmJ, designated Trm7p, Spb1p and Mrm2p. We present evidence that Trm7p methylates the 2'-O-ribose of nucleotides at positions 32 and 34 of the tRNA anticodon loop, both *in vivo* and *in vitro*. In a *trm7Δ* strain, which is viable but grows slowly, translation is impaired, thus indicating that these tRNA modifications could be important for translation efficiency. We discuss the emergence of a family of three 2'-O-RNA methyltransferases in Eukaryota and one in Prokaryota from a common ancestor. We propose that each eukaryotic enzyme is located in a different cell compartment, in which it would methylate a different RNA that can adopt a very similar secondary structure.

Keywords: maturation/modification/RNA binding protein/RNA methyltransferase/three-dimensional structure

Introduction

One of the long-term goals of modern biology is to predict the function of a protein by analysing its amino acid sequence and, whenever possible, by inspection of its three-dimensional architecture (Burley, 2000). This can be achieved by a combination of structural genomics and bioinformatics approaches, followed by testing the predicted function *in vivo* and *in vitro*. With the completion of the sequencing projects for a growing number of organisms, it is now possible to carry out genome-wide searches for genes encoding putative proteins that share sequence motifs and thus potentially belong to the same family (Goffeau *et al.*, 1996; Koonin *et al.*, 1998). For instance, methyltransferases (MTases) that use S-adenosyl-L-methionine (AdoMet) as a cofactor exhibit conserved motifs that map onto the catalytic face of the common fold (Fauman *et al.*, 1999). Recently, a structural analysis has revealed that the heat shock protein FtsJ/RrmJ of

Escherichia coli exhibits the typical MTase fold (Bügl *et al.*, 2000). It has been demonstrated that FtsJ/RrmJ actually catalyses the formation of Um₂₅₅₂, which is located within a loop of five nucleotides present on domain V of the peptidyl transferase centre of the 23S rRNA (Caldas *et al.*, 2000a). *In vitro*, FtsJ/RrmJ is able to methylate the 23S rRNA when it is assembled into 50S ribosomal subunits, and it can also catalyse the formation of 2'-O-methylribose in *E. coli* tRNA, although the modified nucleotide(s) has not yet been mapped (Bügl *et al.*, 2000).

In yeast, almost all types of RNA molecules contain 2'-O-methylnucleotides: tRNA, snRNA, small nucleolar RNA (snoRNA) and rRNA (Rozenski *et al.*, 1999). Although transcription of these various RNAs takes place either in the nucleus (and the nucleolus) or in mitochondria, RNA maturation can be completed sequentially in every cellular compartment. For instance, several tRNA modifications occur after removal of the intron, probably after the tRNA has been transported to the cytoplasm (reviewed by Grosjean *et al.*, 1997). Since the formation of methylnucleotides in eukaryotes takes place in different compartments, it could either be achieved by the same enzymes distributed in various compartments or catalysed by different enzymes located in the nucleus, the cytoplasm and the mitochondria.

Remarkably, the yeast genome contains three open reading frames (ORFs) that exhibit significant sequence similarity to *E. coli* FtsJ/RrmJ: *YBR061c* (hereafter referred to as *TRM7*; see below), *YGL136c* (*MRM2*) and *YCL054w* (*SPB1*). Spb1p is a putative MTase from the yeast nucleolus that is involved in 60S ribosomal subunit synthesis (Pintard *et al.*, 2000). In a separate report, we demonstrated that Mrm2p is a mitochondrial site-specific 2'-O-ribose MTase that catalyses the formation of Um₂₇₉₁ on the peptidyl transferase centre of the 21S rRNA (Pintard *et al.*, 2002). Here, we report the results of experimental and bioinformatic analysis of Trm7p, which suggest that it is a new cytoplasmic 2'-O-RNA MTase.

In yeast tRNAs, 2'-O-methylriboses have been found at positions 4, 18, 32, 34 and 44 (Grosjean *et al.*, 1995; Sprinzl *et al.*, 1998). Although certain modifications occur on the pre-tRNA in the nucleus, methylation at positions 18, 32, 34 and 44 appears to occur after the removal of the intron (reviewed by Grosjean *et al.*, 1997). So far, Trm3p is the only putative yeast 2'-O-tRNA MTase that has been described. It is required for the site-specific formation of Gm at position 18 in the D-loop of intron-less tRNA (Cavaillé *et al.*, 1999). Two of the remaining four 2'-O-methylnucleotides are located within the anticodon loop, at positions 32 and 34, in tRNA^{Leu, Phe, Trp}. Modification of position 34, the wobble nucleotide of the anticodon, has been reported to be important for translation efficiency and/or fidelity (Curran, 1998; Satoh *et al.*,

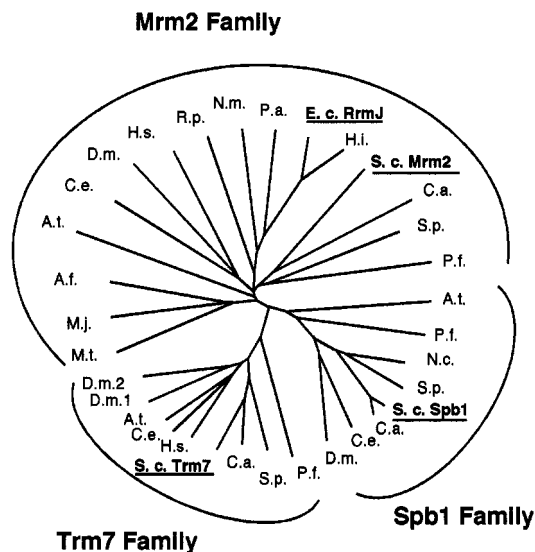


Fig. 1. Phylogenetic tree analysis reveals three putative orthologous lineages: the Trm7, Spb1 and Mrm2 families. The Mrm2 family is represented by Mrm2p from *S.cerevisiae* (P53123), FtsJ/RrmJ from *E.coli* (AAC76211) and other prokaryotic proteins [M.t., *Methanobacterium thermoautotrophicum* (G69103); M.j., *Methanococcus jannaschii* (Q58771); A.f., *Archaeoglobus fulgidus* (O28228); R.p., *Rickettsia prowazekii* (Q9ZE00); N.m., *Neisseria meningitidis* (AAF41212); P.a., *Pseudomonas aeruginosa* (AAG08138); H.i., *Haemophilus influenzae* (P45162)] and eukaryotic proteins [A.t., *Arabidopsis thaliana* (CAB69851); C.e., *Caenorhabditis elegans* (O62251); D.m., *Drosophila melanogaster* (Q9VDT6); H.s., *Homo sapiens* (AAF2488); C.a., *Candida albicans* (unfinished sequence from the Stanford Genome Technology Center, SGTC, <http://sequence-www.stanford.edu>); S.p., *Schizosaccharomyces pombe* (P78860); and P.f., *Plasmodium falciparum* 3D7 (unfinished sequence from the *P.falciparum* Genome Project–PlasmoDB, <http://www.plasmodb.org>)]. The Spb1 family is represented by Spb1p from *S.cerevisiae* (CAA42391) and seven other members [A.t., *A.thaliana* (CAB69851); P.f., *P.falciparum* (unfinished sequence from the PlasmoDB); N.c., *Neurospora crassa* (CAB88626); S.p., *S.pombe* (T37754); C.a., *C.albicans* (unfinished sequence from the SGTC); C.e., *C.elegans* (AAF39868); and D.m., *D.melanogaster* (AAF48557)]. The Trm7 family is represented by Trm7p from *S.cerevisiae* (CAA85004) and eight other members [P.f., *P.falciparum* (unfinished sequence from the PlasmoDB); S.p., *S.pombe* (Z98980); C.a., *C.albicans* (unfinished sequence from the SGTC); H.s., *H.sapiens* (HSA005892); C.e., *C.elegans* (Q22031); A.t., *A.thaliana* (CAB69851); and D.m.1. and D.m.2., *D.melanogaster* (AAF55380 and AAF55857)].

2000). We report here that Trm7p is a yeast MTase that catalyses the formation of 2'-O-methylribose at positions 32 and 34 in the anticodon loop of various yeast tRNAs. We propose that the three proteins Trm7p, Spb1p and Mrm2p form a subclass of 2'-O-ribose MTases that have emerged from a single common ancestor and have co-evolved with their three different RNA substrates, located in the cytoplasm, the nucleolus and the mitochondria, respectively.

Results

Trm7p belongs to a family of putative MTases from Eukaryota that are structurally related to the 2'-O-rRNA MTase FtsJ/RrmJ from E.coli

By searching the yeast genome, we identified three proteins exhibiting similarity to the bacterial 2'-O-rRNA MTase FtsJ/RrmJ (Caldas *et al.*, 2000a,b): Spb1p (Pintard *et al.*, 2000), Mrm2p (Pintard *et al.*, 2002) and Ybr061c

(referred to here as Trm7p; see below). We undertook the bioinformatic analysis to predict the structure of the three yeast proteins based on the data available for other MTases. The *E.coli* FtsJ/RrmJ sequence was used as a query in a PSI-BLAST search (Altschul *et al.*, 1997) of the non-redundant amino acid sequence database at NCBI (<http://www.ncbi.nlm.nih.gov/>) with a stringent expectation value cut-off of 10^{-10} . The search converged in the fifth iteration, yielding the alignment of highly conserved core segments. Sequences of 32 putative homologues of FtsJ/RrmJ were chosen and realigned to the PSI-BLAST core profile using Clustal_X (Thompson *et al.*, 1997). A distance-based phylogeny was reconstructed according to the neighbour-joining method (Saitou and Nei, 1987), revealing three evolutionary lineages with representatives from yeast that were named the Trm7, Spb1 and Mrm2 subfamilies (Figure 1). Mrm2 groups together with the FtsJ/RrmJ family members from the Eubacteria and Archaea, suggesting that its ancestor has been brought into the eukaryotic cell via the mitochondrial endosymbiont. The topology of the tree suggests that a gene duplication event leading to Trm7 and Spb1 occurred in a common ancestor of the Eukaryota.

The three-dimensional structures of the three putative MTases from yeast were homology modelled using Modeller (Sali and Blundell, 1993) based on the refined sequence alignment with FtsJ/RrmJ (Figure 2). The stereochemistry and pseudoenergetic features of the models passed all tests implemented in ProsaII (Z-scores ≤ -7.7 ; Sippl, 1993). Figure 2A shows the key elements of the predicted AdoMet- and ribose-binding sites of Trm7p, including the predicted catalytic tetrad of two basic and two acidic side chains (K28, D124, K164 and E199, each marked by an asterisk) conserved in many 2'-O-ribose MTases exhibiting the common 'MTase fold' (Bujnicki and Rychlewski, 2001). All three putative MTases from yeast and their orthologues (including *E.coli* FtsJ/RrmJ) exhibit striking conservation of the predicted active site and its neighbourhood, suggesting that their target must be similar.

In the proposed model of interaction with an RNA substrate (Figure 2B), we docked the methylated ribose based on the superposition of the active sites of FtsJ/RrmJ and the yeast proteins with the coordinates of vaccinia mRNA cap-I 2'-O-MTase (Hodel *et al.*, 1998). Although we cannot predict with confidence all specific interactions of the enzyme with the entire RNA molecule, we predict that the solvent-exposed side chains of residues S197 and R194 interact specifically with the phosphate group of the methylated nucleotide. Coordinates and additional structural representations are available (<http://www.crbm.cnrs-mop.fr/~lapeyre/extpages/structure.html>).

Trm7p is able to bind tritiated AdoMet in vitro

To challenge the model presented above, we tested the ability of a recombinant tagged Trm7p to bind AdoMet *in vitro*, as described previously for Spb1p (Pintard *et al.*, 2000). Expression of Trm7'-ZZp (see Materials and methods) was able to complement the growth defect of a *trm7Δ* strain, thus demonstrating that the fusion protein was functional (Figure 3A). The level of expression of Trm7'-ZZp ($M_r = 49\ 700$) was compared with the level of the abundant ZZ-Nop1p by western blot analysis

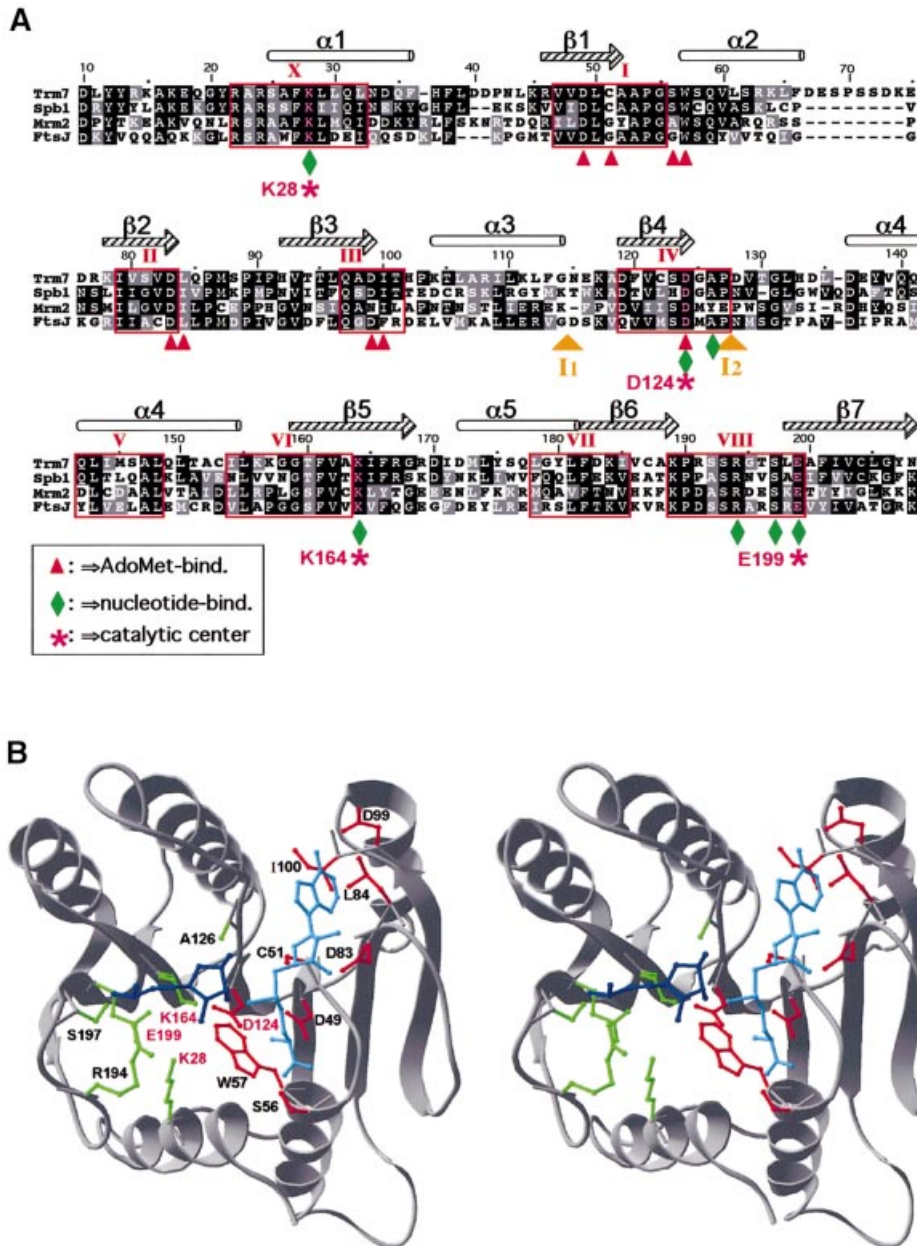


Fig. 2. Three-dimensional structure prediction for Trm7p. (A) Alignment of the AdoMet-binding domain of the three yeast proteins (Trm7p, Spb1p and Mrm2p) with the *E. coli* protein FtsJ/RrmJ. Identical and chemically equivalent residues are highlighted in black and grey, respectively. Secondary structural elements are indicated above the alignment (α -helices as cylinders and β -strands as arrows). Conserved motifs have been labelled according to the nomenclature proposed by Posfai *et al.* (1988). Predicted interactions with AdoMet and the methylated nucleotide are designated by red arrowheads and green diamonds, respectively. The predicted catalytic tetrad K–D–K–E is labelled with purple stars. Two insertions in Mrm2p [I₁ (58 amino acids) and I₂ (21 amino acids)] have been omitted for the sake of clarity. (B) The stereogram of the Trm7p model in cartoon representation. The AdoMet moiety is shown in cyan, and the ribose and the phosphate group of the nucleotide to be methylated are shown in blue. The predicted binding and catalytic residues are shown in the wireframe representation, with colour coding analogous to that in (A).

(Figure 3B). Signals obtained after serial dilutions indicate that Trm7p is ~200 times less abundant in the cell than Nop1p, an observation that is in agreement with the codon adaptation index calculated for the two proteins (0.143 and 0.492, respectively; Sharp and Li, 1987).

Affinity-purified overexpressed recombinant Trm7⁻ZZp was clearly capable of binding tritiated AdoMet *in vitro* (Figure 3C, lane 3). This binding strictly depends on UV irradiation, as is also the case for Spb1p (Pintard, 2000), thus demonstrating that labelling of the proteins

was not due to alkylation by free methyl groups that could arise after degradation of AdoMet. Large amounts of immunoglobulins present in the reaction were not cross-linked to AdoMet under these conditions (Figure 3C, lane 1). Similarly, no signal was detected when bovine serum albumin or recombinant nucleolar Gar1p was used as control (data not shown), thus demonstrating the specificity of the reaction. This result strongly supports the view that Trm7p is a new MTase from *Saccharomyces cerevisiae*.

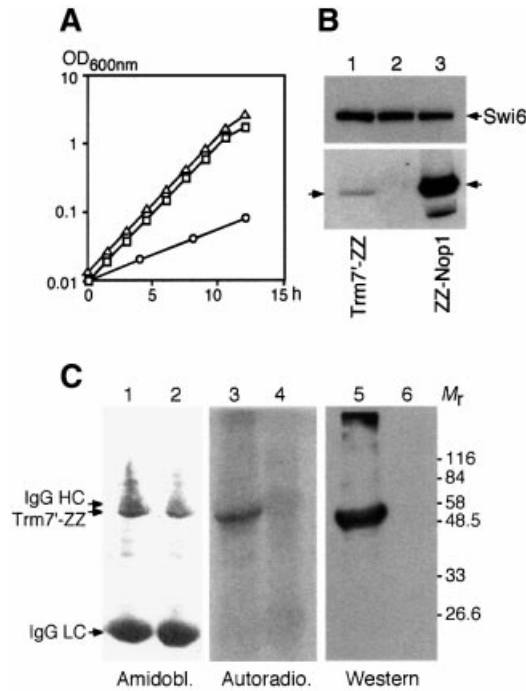


Fig. 3. Trm7p binds tritiated AdoMet *in vitro*. (A) The doubling time was determined for various strains grown in YPD at 30°C. Open squares, wild-type strain (BMA64); open circles, *trm7Δ* strain (YBL4409); open triangles, *trm7Δ* transformed with a centromeric plasmid expressing the *TRM7'-ZZ* gene (YBL4494). (B) Relative expression of Trm7p. Cells expressing various tagged proteins were probed by western blotting using mouse IgG coupled to peroxidase (lower panel). Lane 1, Trm7'-ZZp (arrow on the left, strain YBL4494); lane 2, no tagged protein (BMA64); lane 3, ZZ-Nop1p (arrow on the right points to the major band, and a degradation product is detected below). Similar amounts of protein were loaded in each lane, as demonstrated using anti-Swi6p antibodies (upper panel). (C) Trm7'-ZZp was overexpressed from the *GALI-10* promoter (YBL4502), immunoprecipitated and incubated with 5 μ Ci of [3 H]AdoMet before being UV crosslinked. Radiolabelled complexes were then denatured, separated by 10% SDS-PAGE and transferred to nitrocellulose. The membrane was first stained with amidoblack to localize the heavy and light chains of immunoglobulins used during the immunoprecipitation reaction (lanes 1 and 2). This membrane was then autoradiographed for 4 weeks at -70°C with an intensifying screen (lanes 3 and 4). Finally, the same membrane was analysed by western blotting to reveal Trm7'-ZZp (lanes 5 and 6). Lanes 1, 3 and 5, extracts prepared from the *GAL::TRM7'-ZZ* strain. Lanes 2, 4 and 6, extracts from an untagged strain as a control. M_r , molecular weight markers in thousands.

Deletion of *TRM7* impairs mRNA translation

Sequence similarity existing between Trm7p and Spb1p led us to investigate whether Trm7p could also be a nucleolar protein. However, immunofluorescence studies performed with three different tagged proteins gave signals that were indistinguishable from the background noise. Intense signals were obtained only when *TRM7* was overexpressed from the *GALI-10* promoter, revealing the protein throughout the cytoplasm (data not shown). Unlike Spb1p, Trm7p does not contain a nuclear localization signal, and PSORT software (Nakai and Kanehisa, 1992) predicts a cytoplasmic location for Trm7p (score = 0.650). Although we cannot definitely ascertain the location of the protein, since it was detectable only when overexpressed in the cell, our observations, combined with the PSORT prediction, support the view that Trm7 is a cytoplasmic protein.

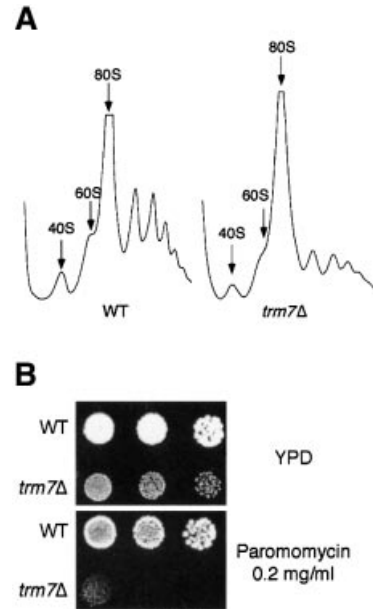


Fig. 4. Translation is impaired in a *trm7Δ* strain. (A) Polysome profile analysis. Cellular extracts were fractionated onto 15–50% linear sucrose gradients. A continuous record of the absorbance at 254 nm for each gradient is presented, with the top of the gradient on the left. The arrows indicate the peaks for the 40S, 60S and 80S subunits. Extracts were prepared from the strains as indicated: WT, wild type (BMA64); *trm7Δ* (YBL4409). (B) The *trm7Δ* strain is sensitive to the translation inhibitor paromomycin. The wild-type strain (WT, BMA64) and the *trm7Δ* strain (YBL4409) were grown in YPD until mid-log phase; then 5 μ l of 5-fold serial dilutions were spotted onto YPD plates containing (or not) 0.2 mg/ml paromomycin as indicated, and strains were grown for 3 days at 30°C.

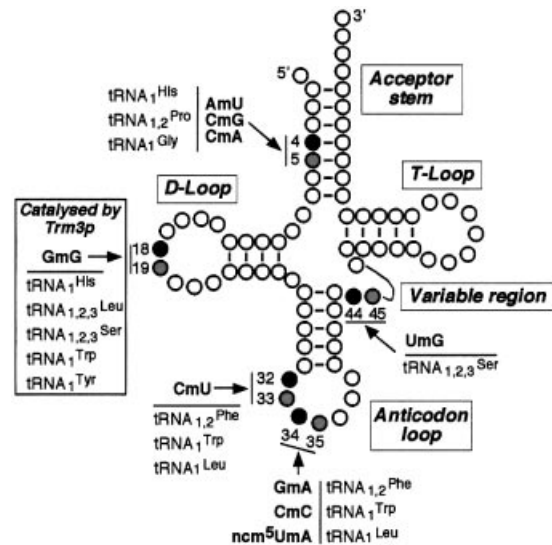


Fig. 5. Position of the five 2'-O-methylnucleotides in the yeast cytoplasmic tRNA and its major domains. Positions in black indicate the site of methylation in tRNA containing 2'-O-methylnucleotide, and positions in grey correspond to its 3'-neighbouring nucleotide. The indicated dinucleotides correspond to those obtained after complete digestion of the tRNA by RNase T2.

To understand better the role of Trm7p, we further analysed the phenotype of the *trm7Δ* strain. Incorporation of labelled methionine revealed that protein synthesis is reduced to 30% in the deleted strain, compared with an

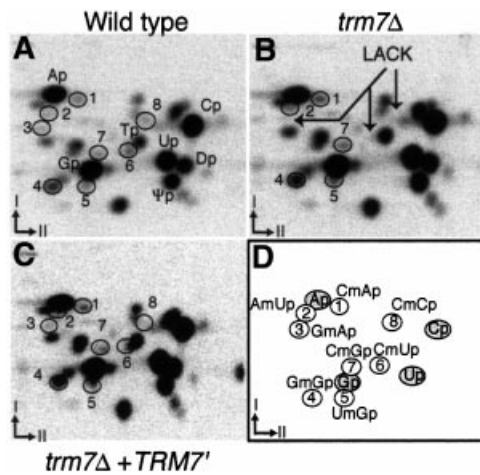


Fig. 6. Position 32 of several tRNAs and position 34 of tRNA^{Phe} and tRNA^{Trp} are not 2'-O-methylated in a *trm7Δ* strain. Autoradiograms of selected two-dimensional TLC of modified mono- and dinucleotides after RNase T2 digestion of various ³²P-labelled tRNA recovered from cells that were grown in the presence of [³²P]orthophosphate. Hydrolysate of total tRNA from (A) wild-type strain (BMA64α), (B) *trm7Δ* strain (YBL4409) and (C) *trm7Δ* strain transformed with a plasmid harbouring the *TRM7'* gene (YBL4363) have been analysed with the N/R chromatographic solvent system. (D) A reference map indicating the location of the nucleotides of interest. Arrows point to the spots that disappear in the disrupted strain.

isogenic wild-type strain. This result was confirmed by the low level of polysomes detected in the mutant, compared with a wild-type strain, when extracts were fractionated on sucrose gradients (Figure 4A). We also noticed that the *trm7Δ* strain is highly sensitive to paromomycin, an antibiotic of the aminoglycoside family that impairs translation by increasing codon misreading in Prokaryota and Eukaryota (Figure 4B) (Chernoff *et al.*, 1994). Taken together, these results suggest that Trm7p is a cytoplasmic protein that plays a role in mRNA translation.

Trm7p is required for tRNA 2'-O-ribose methylation in vivo

The close structural similarity existing between Trm7p and the *E. coli* rRNA MTase FtsJ/RrmJ suggests that Trm7p could be a new yeast 2'-O-RNA MTase. *In vitro*, FtsJ/RrmJ is also able to methylate tRNA (Bügl *et al.*, 2000). This result, taken together with our observation that Trm7p is likely to be a cytoplasmic MTase that plays a role in mRNA translation, prompted us to test whether Trm7p was involved in 2'-O-ribose methylation of a nucleotide within a loop of yeast tRNA. Positions 32 and 34 in the anticodon loop represented good candidates that were tested further (Figure 5). Interestingly, when position 32 of a given tRNA is 2'-O-methylated, the wobble position 34 of the same tRNA is also 2'-O-methylated. To identify the modified nucleotides, we used the property that RNase T2 cleaves all phosphodiester linkages between nucleotides, except those that are 2'-O-methylated. Since the 3'-neighbouring nucleotides of each type of 2'-O-methyl derivative in the different isoacceptor tRNA are different, analysis of the dinucleotides produced by RNase T2 digestion reveals the identity of the modified nucleotide. The result of RNase T2 digestion of total tRNA extracted from various strains grown in the presence of inorganic

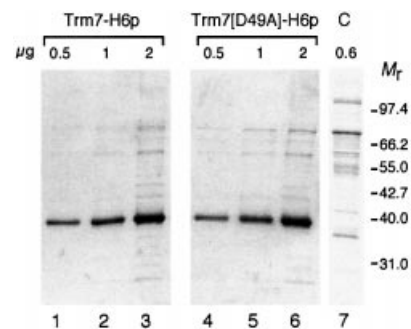


Fig. 7. Purification of the His-tagged wild-type and mutant recombinant Trm7 proteins. Affinity-purified wild-type and D49A mutant Trm7p were analysed by 10% SDS-PAGE. To assess the purity of the proteins, various amounts were loaded (~0.5, 1 and 2 μg, from left to right, as indicated). Lanes 1–3, wild-type Trm7-H6p; lanes 4–6, D49A mutant Trm7-H6p; lane 7, control (C) from an untagged strain. *M_r*, molecular weight markers in thousands.

[³²P]orthophosphate, followed by two-dimensional thin-layer chromatography (TLC), is presented in Figure 6A–C. As expected, each tRNA digest revealed a complex pattern of radiolabelled spots, of which the four canonical 3'-monophosphate nucleosides Ap, Cp, Gp and Up are the most abundant. Spots corresponding to ribothymidine monophosphate (Tp), dihydrouridine monophosphate (Dp) and pseudouridine monophosphate (Ψp), which are present in all naturally occurring tRNA in yeast, are also clearly seen. Other modified nucleotides that are present only in certain tRNA yield spots that are less intense but still easily detected on the autoradiogram. Three spots (3, 6 and 8) of the wild-type strain (Figure 6A) are missing in the *trm7Δ* strain (indicated by arrows in Figure 6B). Spot 3 corresponds to GmAp (positions 34–35 in tRNA^{Phe}), spot 6 corresponds to CmUp (positions 32–33 in tRNA^{Phe,Trp,Leu}) and spot 8 corresponds to CmCp (positions 34–35 in tRNA^{Trp}). Two of these three radioactive spots are clearly visible in the *trm7Δ* strain transformed with the centromeric plasmid harbouring a synthetic *TRM7'* gene (Figure 6C). Of note is the presence in all the chromatograms of the radioactive spots corresponding to CmAp (spot 1, from positions 4–5 in major tRNA^{Gly}), AmUp (spot 2, from positions 4–5 in tRNA^{His}), GmGp (spot 4, from positions 18–19 in several yeast tRNAs), UmGp (spot 5, from positions 44–45 in all tRNA^{Ser}) and CmGp (spot 7, from positions 4–5 in major tRNA^{Pro}). The spot corresponding to the dinucleotide ncm⁵UmAp (positions 34–35 in minor tRNA^{Leu}) was not identified in this experiment, probably because it was in amounts too low to be detected and/or it co-migrates with another radioactive spot on the TLC plates.

Purification of wild-type and mutant recombinant Trm7 proteins

As another means to demonstrate the catalytic activity of Trm7p, we mutated the AdoMet-binding domain of Trm7p to abolish cofactor binding by this enzyme. Based on the homology model of Trm7p and analysis of the FtsJ/RrmJ structure, D49 of Trm7p has been predicted to make an essential water-mediated interaction with AdoMet, the lack of which would interfere with cofactor binding. Therefore, a mutation was introduced into the *TRM7* gene to change this aspartic residue into an alanine

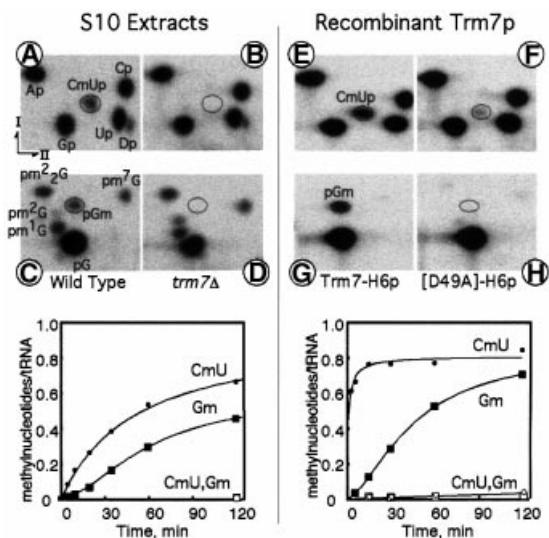


Fig. 8. Affinity-purified Trm7p is able to methylate positions 32 and 34 of tRNA^{Phe} *in vitro*. Upper part: autoradiograms of selected two-dimensional TLC of modified mono- and dinucleotides after RNase T2 (A, B, E and F) or nuclease P1 (C, D, G and H) digestion of synthetic intron-less [α -³²P]GTP-labelled tRNA^{Phe} initially incubated either with S10 cell extracts or with purified recombinant proteins. Left four panels: tRNA incubated for 2 h at 30°C with 2 mg/ml protein from an S10 cell extract prepared from a wild-type strain (BMA64 α) (A and C) or from a *trm7* Δ strain (YBL4409) (B and D). Right four panels: tRNA incubated as above at 30°C with 10 μ g/ml purified Trm7-H6p (E and G) or with D49A mutant Trm7-H6p (F and H). Lower part: time-course formation of Cm32 (detected as CmU) and Gm34 on synthetic intron-less [α -³²P]GTP-labelled tRNA^{Phe} incubated at 30°C either with S10 extracts (left panel) or with recombinant proteins (right panel). Molar ratios of methylated nucleotides versus substrate tRNA plotted as a function of incubation time in minutes (methylnucleotides/tRNA). Left panel: S10 extracts prepared from a wild-type yeast (filled circles, CmU; filled squares, Gm) or from a *trm7* Δ mutant strain that does not give rise to any detectable modification (open circles and open squares, respectively). Right panel: purified recombinant wild-type Trm7-H6p (filled circles, CmU; filled squares, Gm) or with purified mutant Trm7[D49A]-H6p (open circles and open squares, respectively).

(\rightarrow Trm7[D49A]). *In vivo*, a plasmid expressing the recombinant Trm7 wild-type protein tagged with six histidines (Trm7-H6p) was able to complement the growth defect of a *trm7* Δ strain, whereas expression of the mutant Trm7[D49A]-H6p in a *trm7* Δ strain did not restore wild-type growth (data not shown). Then, to assert unambiguously that the MTase activity was borne by Trm7p itself, the two recombinant and mutant proteins were affinity purified (Figure 7) and their tRNA MTase activity was tested *in vitro*. Few minor contaminants were detected by Coomassie Blue staining, the most abundant of which was also present in a fraction prepared from a *trm7* Δ strain expressing no recombinant His-tagged protein (Figure 7, lane 7).

Trm7p is required for tRNA 2'-O-ribose methylation *in vitro*

To test *in vitro* for AdoMet-dependent MTase activity at positions 32 and 34, a synthetic tRNA substrate was prepared. Intron-less yeast tRNA^{Phe} was transcribed *in vitro* using [α -³²P]GTP as a radioactive precursor. This permits, using the same transcript, the detection of the presence of either CmU³²p after RNase T2 digestion or ³²pGm after nuclease P1 digestion. When this substrate

was incubated with a S10 cellular extract prepared from a wild-type strain, enzymatic formation of both Cm32 and Gm34 was detected, although a small but reproducible lag phase in the formation of Gm34 was visible (Figure 8, left part). Formation of Cm32 and Gm34 was completely abolished in an S10 extract from a *trm7* Δ yeast strain (Figure 8B and D). Similar results were obtained with an intron-less yeast tRNA^{Trp} transcript (data not shown). Several other radioactive spots were generated by the S10 extract from the wild-type strain, which were also present in the *trm7* Δ strain extract: Dp from position 17, in addition to the expected CmUp after T2 RNase digestion; and pm²G, pm²₂G, pm¹G and pm⁷G from positions 10, 26, 37 and 46, respectively, in addition to pGm after nuclease P1 digestion (Figure 8A and C). This result demonstrates that only the formation of Cm32 and Gm34 is abolished in the *trm7* Δ strain. Moreover, enzymatic formation of Cm32 and Gm34 was restored when the S10 extract was prepared from a *trm7* Δ strain transformed with a centromeric plasmid expressing the *TRM7* gene (data not shown).

The same synthetic tRNA substrate was then incubated with the recombinant proteins, either the wild-type or the D49A mutant Trm7p, and analysed as above for the presence of modified nucleotides. The wild-type protein was clearly capable of driving the formation of both Cm32 and Gm34, although the rate of Cm32 formation was \sim 10 times faster than the rate of Gm34 formation in intron-less tRNA^{Phe}, and a slight but reproducible lag phase exists for the enzymatic formation of Gm34 (Figure 8E and G, and see the corresponding kinetics below). It is noteworthy that no other spots corresponding to other modified mono- or dinucleotides were detected when using the recombinant protein, thus demonstrating that the affinity-purified Trm7p was not contaminated by other tRNA modification enzymes detectable in our assay. Moreover, mutation D49A abolishes almost completely the formation of Cm32, and no activity was detected for G34 (Figure 8F and H, and see the corresponding kinetics below).

From these results obtained *in vitro* with cellular extracts and recombinant proteins, together with those obtained *in vivo*, after analysing naturally occurring tRNA from wild-type and *trm7* Δ mutant yeast strains (Figure 6), we conclude that *TRM7* codes for a MTase that catalyses the formation of 2'-O-methylnucleotides at positions 32 and 34 of the yeast tRNA^{Phe}, tRNA^{Trp} and, possibly, tRNA^{Leu}.

Discussion

tRNAs contain the highest number of modified nucleotides of all cellular RNAs (Björk, 1995). Several enzymes responsible for the post-transcriptional methylation of tRNAs have now been characterized and named Trm (for tRNA methyltransferase). In *S.cerevisiae*, Trm1p and Trm2p are base MTases that catalyse the site-specific formation of m²G26 and m⁵U54, respectively (Ellis *et al.*, 1986; Nordlund *et al.*, 2000); Trm3p is likely to be the site-specific Gm18 2'-O-ribose MTase (Cavaillé *et al.*, 1999); Trm4p is a multisite-specific m⁵C methylase (Motorin and Grosjean, 1999); Trm5p catalyses the formation of m¹G37 and m¹I37 (Björk *et al.*, 2001); and Trm6 (Gcd10p/Gcd14p) is a two-subunit complex required for the formation of m¹A58 (Anderson *et al.*, 2000).

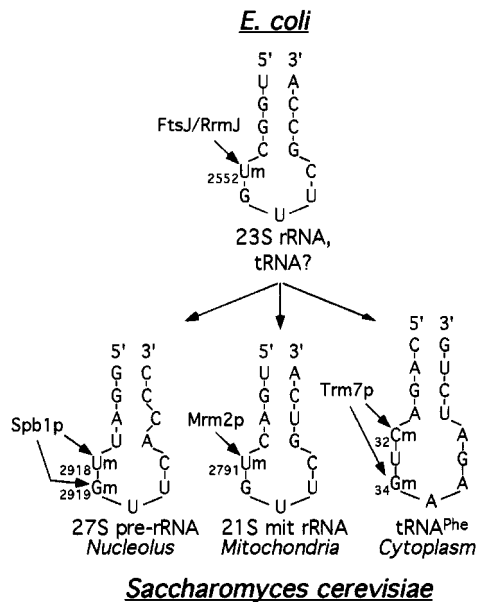


Fig. 9. Schematic representation of the secondary structure of the target sites for each of the three enzymes that belong to the 2'-O-RNA MTase family. Only the 2'-O-ribose methylations discussed in this report are represented. Top: location of U₂₅₅₂ in the hairpin/loop of domain V of the peptidyl transferase centre of the *E. coli* 23S rRNA. In addition, FtsJ/RrmJ can also methylate tRNA *in vitro*; however, the position of the methyl-nucleotides has not yet been determined (Bügl *et al.*, 2000). Bottom, left: location of Um₂₉₁₈ and Gm₂₉₁₉ in the hairpin/loop of the peptidyl transferase centre of the nuclear 27S pre-rRNA. Bottom, middle: location of Um₂₇₉₁ in the hairpin/loop of the 21S mitochondrial rRNA. Bottom, right: location of C32 and G34 in the anticodon loop of tRNA^{Phe}. The modified nucleotides are indicated by arrows, with their known (FtsJ/RrmJ, Trm7p, Mrm2p) or putative (Spb1p) modification enzymes.

In this study, we report the characterization of a new multisite-specific enzyme from yeast that catalyses the formation of 2'-O-methylribose in the tRNA anticodon loop. According to the current nomenclature for tRNA modification enzymes, we propose the name Trm7p for this new enzyme. This proposition is based on the facts that: (i) Trm7p is an AdoMet-binding protein likely to be located in the cytoplasm; (ii) deletion of the *TRM7* gene leads to a low rate of protein synthesis and sensitivity to the translation inhibitor paromomycin; (iii) T2 RNase hydrolysates of total tRNA extracted from the *trm7Δ* strain reveal a lack of dinucleotide CmU (corresponding to positions 32 and 33 of tRNA^{Phe}, tRNA^{Trp} and tRNA^{Leu}), as well as GmA and CmC (corresponding to positions 34 and 35 of tRNA^{Phe} and tRNA^{Trp}, respectively); (iv) extracts prepared from a *trm7Δ* strain can no longer methylate positions 32 and 34 in tRNA^{Phe} and tRNA^{Trp} *in vitro*; (v) the wild-type *TRM7* gene expressed from a centromeric vector in a *trm7Δ* strain restores the enzymatic formation of Cm32 and Gm34 both *in vivo* and *in vitro*; (vi) affinity-purified recombinant Trm7p catalyses *in vitro* the formation of Cm32 and Gm34 in tRNA^{Phe}; and (vii) a single point mutation (D49A) within the AdoMet-binding motif of Trm7p abolishes almost completely the tRNA MTase activity *in vitro* and, *in vivo*, expression of this construction in a *trm7Δ* strain is unable to restore wild-type growth. Taken together, these results demonstrate that Trm7p is the RNA MTase that catalyses the formation

of 2'-O-methylribose at positions 32 and 34 of the anticodon loop in several yeast tRNAs.

Trm7p belongs to a group of three yeast proteins (Trm7p, Spb1p and Mrm2p) that exhibit sequence similarity to the *E. coli* rRNA MTase FtsJ/RrmJ. Bioinformatic analysis allowed the delineation of solvent-exposed features that are common to all these proteins and the categorization of the conserved residues into three different groups (Figure 2A and B). The first group that is conserved in most MTases is involved in the recognition of the AdoMet cofactor. This prediction was experimentally confirmed for position 49, since the mutation D49A was sufficient to abolish almost completely the MTase activity of Trm7p. The second group of residues (K28, D124, K164 and E199) corresponds to the predicted active site conserved between different RNA 2'-O-ribose MTases (Bujnicki and Rychlewski, 2001). Finally, we noticed a group of 14 residues that are perfectly conserved between *E. coli* FtsJ/RrmJ and the three yeast proteins (Figure 2A), suggesting that the four proteins could share other characteristics in addition to being specific for ribose in RNA.

In prokaryotes, a unique enzyme, FtsJ/RrmJ, methylates the peptidyl transferase centre of the large rRNA and possibly also certain tRNAs (Bügl *et al.*, 2000). Its rRNA target has a peculiar structure that is composed of a hairpin and a loop of five nucleotides, with the modified nucleotide being located at the 5' end of the loop (Figure 9, top). Interestingly, in yeast this structure is remarkably conserved in the mitochondrial 21S rRNA, in the 25S rRNA and, to a lesser extent, in the tRNA anticodon loop (Figure 9, bottom). We have demonstrated recently that Mrm2p is a mitochondrial protein that catalyses the formation of Um₂₇₉₁ of the peptidyl transferase centre of the 21S rRNA, at a position equivalent to U₂₅₅₂ of the *E. coli* 23S rRNA (Pintard *et al.*, 2002). Spb1p is a nucleolar protein required for 25S rRNA synthesis. Owing to its structural similarity with FtsJ/RrmJ, we hypothesize that Spb1p is responsible for the methylation of U₂₉₁₈ (and possibly G₂₉₁₉), which is equivalent to U₂₅₅₂ of the *E. coli* 23S rRNA. When compared with the three rRNA loops, the tRNA anticodon loop seems to be slightly different, with a longer loop of seven nucleotides instead of five. However, the target nucleotide to be modified (C32) is also located at the 5' end of the loop, and the seven-nucleotide-long anticodon loop can be reduced to five nucleotides, due to a base-pairing between positions 32 and 38 (Auffinger and Westhof, 2001), thus rendering the tRNA structure even more similar to the rRNA ones. Therefore, we propose that the three yeast proteins Trm7p, Spb1p and Mrm2p have evolved from a common ancestor to function in three different cell compartments, i.e. the cytoplasm, the nucleolus and the mitochondria, where they each recognize a similar hairpin/loop RNA structure (Figure 9). Interestingly, the enzymes of the FtsJ/RrmJ family exhibit no significant similarity to other known yeast 2'-O-ribose MTases, such as Trm3p or Mrm1p/Pet56p. These observations suggest the existence of distinct subfamilies of enzymes that originated at an early point in evolution and then co-evolved with their target RNA, independently of the other classes of 2'-O-ribose MTases.

Table I. Strains used in this study

Strain	Genotype ^a	Plasmid	Main property	Origin
BMA64	MAT α / α		wild type	Baudin <i>et al.</i> (1993)
YBL4409	MAT α <i>trm7</i> Δ :: <i>TRP1</i>		<i>trm7</i> Δ	this work
YBL4363	MAT α <i>trm7</i> Δ :: <i>TRP1</i>	pBL579	+ <i>TRM7</i> [*]	this work
YBL4494	MAT α <i>trm7</i> Δ :: <i>TRP1</i>	pBL598	+ <i>TRM7</i> [*] -ZZ	this work
YBL4502	MAT α / α <i>trm7</i> Δ :: <i>TRP1</i>	pBL601	+ <i>GAL</i> :: <i>TRM7</i> [*] -ZZ	this work
YBL4518	MAT α <i>trm7</i> Δ :: <i>TRP1</i>	YeDP60	<i>trm7</i> Δ	this work
YBL4537	MAT α <i>trm7</i> Δ :: <i>TRP1</i>	pBL606	+ <i>GAL</i> :: <i>TRM7</i> -H6	this work
YBL4538	MAT α <i>trm7</i> Δ :: <i>TRP1</i>	pBL607	+ <i>GAL</i> :: <i>TRM7D49A</i> -H6	this work
ZZ-Nop1p	MAT α <i>nop1</i> Δ :: <i>HIS3</i>	pZZ-Nop1p		Gautier <i>et al.</i> (1997)

^aAll strains were *ade2*, *his3*, *leu2*, *trp1*- Δ , *ura3*, *can1*-100 unless otherwise specified.

Data presented here, together with data published previously, indicate that methylation at positions 32 and 34 exhibits certain similarities but also some significant differences. Amongst the similarities, in yeast the two modifications are catalysed by the same enzyme, Trm7p. Also, both modifications occur at a late stage, after the removal of the intron (reviewed by Grosjean *et al.*, 1997), and they do not depend on other tRNA modifications, since affinity-purified Trm7p modifies efficiently a synthetic intron-less tRNA^{Phe} *in vitro*. As we detected Trm7p in the cytoplasm, it is likely that methylation of the anticodon loop takes place in the cytoplasm on spliced tRNA. Among the differences in the formation of Cm32 and Gm34, first we observed a lag in the formation of Gm34, as compared with Cm32, on tRNA^{Phe} *in vitro* (Figure 8; see also Jiang *et al.*, 1997); secondly, point mutations that affect the three-dimensional structure of the tRNA^{Phe} molecule have little effect on the formation of Cm32 but almost completely prevent the formation of Gm34 (H.G. and F.L., unpublished).

It has been shown that the hypomodified anticodon loop is conformationally more flexible than the modified one present in the mature tRNA (Agris, 1996; Sundaram *et al.*, 2000). We attempted to dock the tRNA^{Phe} structure (Shi and Moore, 2000) to the Trm7 model using the VP39 MTase-mRNA complex (Hodel *et al.*, 1998) as a guide, by superposition of the corresponding protein structures and the C32 and G34 ribose rings onto the 2'-*O*-methylated ribose of mRNA. Docking of C32, whose 2'-*O*-methylated group is buried in the tRNA^{Phe} interior, resulted in extensive steric clashes between tRNA^{Phe} and the Trm7 model (data not shown). However, docking of G34 revealed striking complementarity between the positively charged groove on the Trm7p surface and the tRNA^{Phe} molecule (http://www.crbm.cnrs-mop.fr/~lapeyre/ext_pages/structure.html). This suggests that G34 fits into the active site of Trm7p if tRNA^{Phe} assumes the crystal-like structure, while the conformation of the anticodon loop needs to be altered in order to present C32 to the Trm7p active site. A nucleotide flipping mechanism for tRNA modification has been demonstrated recently in the case of *E. coli* TruB, which catalyses the formation of pseudouridine-55 in the T-loop of tRNA (Hoang and Ferred'Amare, 2001). The flipping mechanism seems quite attractive for C32 methylation by the Trm7p MTase, since it is common in the reaction catalysed by DNA MTases, which are structurally and evolutionarily related to Trm7p (reviewed by Cheng and Roberts, 2001). Based on the

forementioned experimental results and the docking model, we hypothesize that C32 is methylated in the conformationally flexible, hypomodified anticodon hairpin, where its 2'-hydroxyl can be presented to the active site of the enzyme, whereas formation of Gm34 occurs preferably in a rigid, hypermodified loop. It remains to be determined whether the potential of G34 to be methylated is increased only by Cm32 or also by modified nucleotides at other positions of the anticodon loop (m¹G/Wye37, Psi-39 and m⁵C40; Grosjean *et al.*, 1990; Jiang *et al.*, 1997), as suggested by the results obtained *in vitro* with affinity-purified Trm7p.

Materials and methods

Microbiological methods and recombinant DNA work

The strains used in this study (Table I) were constructed from strain BMA64 (Baudin *et al.*, 1993). Disruption of the *TRM7* gene was performed by using a PCR-based strategy with oligonucleotides OBL57 (AAAACAACAGTAGATGACTACAAGGCAAGCTTAAAAAGAAA-GTATTCAAGCTGATGCGGTATTTCTCCT)/OBL58 (ATAATGTAGTACTTATTTAATTGTTTATTAGAGGTAGTAATATTGCAACGGGTGTTGGCGGGTGTC) to amplify a *TRP1* cassette (Baudin *et al.*, 1993). A synthetic *TRM7* gene bearing two unique restriction sites at the initiation and stop codons was constructed as follows. The 5' UTR, the ORF and 3' UTR of *TRM7* were PCR amplified with oligonucleotides OBL59 (GCTGCAGAAAGACACCAAACCTAAGTA)/OBL60 (GGG-GATCCGGCCGACCATCTTGAATACTTCTT), OBL63 (CGGATCCTAGACCCGGGTTGATTGCAATATTACTACTCTC)/OBL64 (CGGTACCGCAGATGTTTAAAGAGACGAG) and OBL61 (CCCCGGCCGTGGTAAGAGCAGCAAAGAT)/OBL62 (GCTCTAGAAGT-GATCTAGTAGTGTTC), respectively, using yeast genomic DNA as a template. The three PCR products were gel purified, digested with *Pst*I and *Eag*I (for the 5' UTR), *Eag*I and *Xba*I (for the ORF), and *Xba*I and *Kpn*I (for the 3' UTR), and inserted between the *Pst*I and *Kpn*I sites of Ycplac22 (Sikorski and Hieter, 1989) to give plasmid pBL579, which encodes a slightly modified Trm7p (denoted Trm7^p), with three additional residues at the N-terminus of the protein (VGR) and four at the C-terminus (LDPG). Tagging Trm7^p with two IgG-binding domains of *Staphylococcus aureus* protein A (Trm7^p-ZZp) was performed by PCR amplification of a ZZ fragment with oligonucleotides OBL144 (TCCCCGGGGAAGCTG-GAGCTCAAAC)/OBL145 (AGGCCCGGGTTGGTTGACTTCCC-CG) and plasmid pBS1173 as a template (Puig *et al.*, 1998). pBL598 was obtained by ligating this fragment digested with *Sma*I into the same site of pBL579. A DNA fragment containing the *GAL1-10* promoter was obtained by PCR using oligonucleotides OBL147 (GCTGCA-GCGACGGCCAGTGAATTCCG)/OBL148 (CCCCGGCCGACCATGGA-TCCGGGTTTTTCTCC) and pDK376 as a template (Kressler *et al.*, 1999). This fragment was digested with *Pst*I and *Eag*I, and used to replace the *TRM7* promoter in pBL598 to yield pBL601. Another recombinant gene encoding a Trm7p derivative tagged with six histidines at the C-terminus (Trm7-H6p) was constructed as follows. Oligonucleotides OBL157 (AAGGATCCATGGGTAA-GAGCAGCAAAGATAAAAAG)/OBL158 (AAGAATCTTTAGTGGTGGTGGTGGTGAAGTACTGATCTAGTGA-GTTTCCCCTCC) were

used to amplify a *Bam*HI–*Eco*RI cassette, which was then introduced into the same sites of YeDP60, a vector shown to achieve a high level expression of cytochrome P-450 under the control of the *GAL1-10* promoter (Urban *et al.*, 1990) and which contains the two selection markers *URA3* and *ADE2*. The resulting plasmid was called pBL606 and introduced into the *trm7Δ* strain YBL4409 to yield strain YBL4537. In order to disrupt the interaction of Trm7p with AdoMet, a mutation was introduced using an ExSite PCR-based kit (Stratagene) to change an aspartate residue into an alanine residue (position 49 of the wild-type protein) with oligonucleotides OBL159 (CTTAAAAAGAGTTGTAGCTTTGTGTGCAGCACACAGG)/OBL160 (CCTGGTGCTGCACACAAAGCTACAACCTTTTTTAAAG). The resulting plasmid, called pBL607, was then transformed into strain YBL4409 to give strain YBL4538.

Protein analysis

Protein extraction, SDS–PAGE and western blotting were carried out as described previously (Pintard *et al.*, 2000). Antibodies were used at the following dilutions: anti-Swi6p, 1/100 000; secondary antibodies coupled to horseradish peroxidase (Sigma), 1/5000 (anti-mouse) or 1/10 000 (anti-rabbit). Immunoprecipitation of Trm7⁺-ZZp was performed using 5 mg of native protein extract prepared from YBL4502 and 20 μl of IgG–Sepharose beads (Pharmacia). Recombinant Trm7-H6p and Trm7[D49A]-H6p were affinity purified using Ni-NTA–Superflow beads (Qiagen). Eluted proteins were dialysed against 100 mM Tris–HCl pH 8, 100 mM Na acetate, 50% glycerol and 1 mM DTT. A 1 l cell culture yields ~0.5 mg of purified protein using 150 μl of Superflow beads. Polysomes and yeast S10 extract from strain BMA64α were prepared as described previously (Jiang *et al.*, 1997; Pintard *et al.*, 2000).

³²P-labelling of tRNA and analysis of modified nucleotides

Cells (0.25 OD_{600 nm}) were labelled with 300 μCi of [³²P]orthophosphate (Amersham, UK) for 38 h at 30°C. Total [³²P]tRNA was phenol extracted and purified by gel electrophoresis as described previously (Grosjean *et al.*, 1990). *In vitro* synthesis of [³²P]tRNA transcripts was as described previously (Jiang *et al.*, 1997). [³²P]tRNA was digested into 3'-phosphate nucleosides by RNase T2 (Sigma). Similarly, synthetic tRNA transcripts radiolabelled with [α-³²P]GMP and modified *in vitro* as described previously (Jiang *et al.*, 1997) with S10 cellular extracts or recombinant purified proteins were digested with RNase T2 or to 5'-phosphate nucleosides with nuclease P1 (Sigma). Hydrolysates were analysed by TLC with solvent system N/R as described previously (Jiang *et al.*, 1997).

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