Identification of yeast tRNA Um_{44} 2′-O-methyltransferase (Trm44) and demonstration of a Trm44 role in sustaining levels of specific $tRNA^{Ser}$ species

LAKMAL KOTELAWALA, ELIZABETH J. GRAYHACK, and ERIC M. PHIZICKY
Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642, USA

ABSTRACT

A characteristic feature of tRNAs is the numerous modifications found throughout their sequences, which are highly conserved and often have important roles. Um_{44} is highly conserved among eukaryotic cytoplasmic tRNAs with a long variable loop and unique to $tRNA^{Ser}$ in yeast. We show here that the yeast ORF *YPL030w* (now named *TRM44*) encodes $tRNA^{Ser}$ Um_{44} 2′-O-methyltransferase. Trm44 was identified by screening a yeast genomic library of affinity purified proteins for activity and verified by showing that a *trm44*-Δ strain lacks 2′-O-methyltransferase activity and has undetectable levels of Um_{44} in its $tRNA^{Ser}$ and by showing that Trm44 purified from *Escherichia coli* 2′-O-methylates U_{44} of $tRNA^{Ser}$ in vitro. Trm44 is conserved among metazoans and fungi, consistent with the conservation of Um_{44} in eukaryotic tRNAs, but surprisingly, Trm44 is not found in plants. Although *trm44*-Δ mutants have no detectable growth defect, *TRM44* is required for survival at 33°C in a *tan1*-Δ mutant strain, which lacks ac^4C_{12} in $tRNA^{Ser}$ and $tRNA^{Leu}$. At nonpermissive temperature, a *trm44*-Δ *tan1*-Δ mutant strain has reduced levels of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$, but not other $tRNA^{Ser}$ or $tRNA^{Leu}$ species. The *trm44*-Δ *tan1*-Δ growth defect is suppressed by addition of multiple copies of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$, directly implicating these $tRNA^{Ser}$ species in this phenotype. The reduction of specific $tRNA^{Ser}$ species in a *trm44*-Δ *tan1*-Δ mutant underscores the importance of tRNA modifications in sustaining tRNA levels and further emphasizes that tRNAs undergo quality control.

Keywords: *S. cerevisiae*; $tRNA^{Ser}$; *TAN1*; quality control

INTRODUCTION

tRNAs play a crucial role in the translation of mRNA into proteins, entering and leaving the ribosome about seven times per second in yeast, and faithfully decoding mRNA through interactions with the anticodon and commensurate addition of the amino acid to the growing peptide chain. This occurs on 300,000 ribosomes using a supply of 3,000,000 tRNAs to decode 60,000 mRNAs in one generation in yeast, and each tRNA is used slightly less than once every second (Waldron and Lacroute 1975; Ares et al. 1999).

A remarkable feature of tRNA is the numerous post-transcriptional base and sugar modifications found throughout the molecule. These modifications include a number of different methylations of distinct bases or of the ribose moiety as well as a number of more complex chemical modifications. In the yeast *Saccharomyces cerevisiae*, the 34 characterized cytoplasmic tRNAs each have an average of 12.7 modifications, and there are a total of 25 different modifications, including 12 distinct methylations of bases or ribose sugars (Sprinzl et al. 1998). Many tRNA modifications are highly conserved within one or more of the archaeal, eubacterial, and eukaryotic kingdoms and often occur at the same position in tRNAs from different organisms, suggesting important structural and functional roles of the modifications.

The roles of these modifications have been a subject of increasing study, as the genes catalyzing these modifications have been identified in the last several years. Thus, a number of modifications in the anticodon region have been shown to be important for translation (Laten et al. 1978; Dihanich et al. 1987; Lecointe et al. 1998; Gerber and Keller 1999; Bjork et al. 2001; Urbonavicius et al. 2001; Pintard et al. 2002; Kalhor and Clarke 2003). Several other modifications elsewhere in the tRNA have important roles, based on growth and translation defects of triple mutants

Reprint requests to: Eric M. Phizicky, Department of Biochemistry and Biophysics, University of Rochester School of Medicine, 601 Elmwood Avenue, Rochester, NY 14642, USA; e-mail: eric_phizicky@urmc.rochester.edu; fax: (585) 271-2683.

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such as *Escherichia coli trmH trmA truB* mutants (lacking Gm_{18}, m^5U_{54}, and Ψ_{55}) (Urbonavicius et al. 2002) and on synthetic lethality of double mutants such as *pus1 pus4* mutants (lacking $\Psi_{26–28,\ 34–36,\ 65,67}$ from Pus1 and Ψ_{55} from Pus4) (Grosshans et al. 2001).

There is also mounting evidence that certain modifications play important roles in tRNA folding or stability. Thus, *trm6*^{ts} (or *trm61*^{ts}) mutants, responsible for m^1A_{58}, have reduced levels of tRNA$_i^{Met}$ at high temperature, due to nuclear degradation of pre-tRNA$_i^{Met}$ by polyadenylation and exonucleolytic degradation, mediated by *TRF4* and *RRP6* (Anderson et al. 1998, 2000; Kadaba et al. 2004; LaCava et al. 2005; Vanacova et al. 2005; Kadaba et al. 2006). Similarly, *trm8*-Δ *trm4*-Δ mutants, which lack m^7G_{46} and $m^5C_{30,40,48,49}$ in their tRNA, are temperature sensitive for growth, and their tRNA$^{Val(AAC)}$ is rapidly degraded and deacylated at high temperature by a pathway that is independent of *TRF4*/*RRP6* (Alexandrov et al. 2006). Such tRNA quality control pathways are not confined to modification defects, since mutations in tRNA$^{Arg(CCG)}$ that lead to misfolding of the anticodon stem–loop or disruption of the acceptor stem (Chakshusmathi et al. 2003) also have reduced tRNA$^{Arg(CCG)}$ levels due to a pathway that is independent of *TRF4* (Copela et al. 2006).

One common tRNA modification is 2′-O-methylation of the ribose sugar. This modification is found at 13 positions in tRNA species from different organisms and at 5 positions in yeast tRNAs (positions 4, 18, 32, 34, and 44). Each of the modifications assigned to a gene product is effected by a 2′-O-methyltransferase rather than by a guide RNA mechanism, as described for several archaeal tRNA modifications (Clouet d'Orval et al. 2001; Renalier et al. 2005) and numerous rRNA and snRNA modifications (Decatur and Fournier 2003; Yu et al. 2005; Piekna-Przybylska et al. 2007). Thus, Trm13 is responsible for formation of Nm_4 in tRNA^{His}, tRNA^{Gly}, and tRNA^{Pro} (Wilkinson et al. 2007), Trm3 for Gm_{18} of numerous tRNAs (Cavaille et al. 1999), and Trm7 for Nm_{32} and Nm_{34} of tRNA^{Leu}, tRNA^{Phe}, and tRNA^{Trp} (Pintard et al. 2002). The enzyme catalyzing 2′-O-methylation at position 44 remains to be identified and is the subject of this report.

There are several reasons why 2′-O-methylation at position 44 is of interest. First, this modification is uniquely found in class II tRNAs, which have a long variable loop and include species of tRNA^{Ser}, tRNA^{Leu}, and tRNA^{Tyr}, and is only observed in tRNA species that have a U_{44} residue (Fig. 1A). Second, the location of Um_{44} at the junction between the anticodon stem and the variable loop suggests an important role of this residue in tRNA structure and/or stability. Indeed, residue 44 is across from residue 26 at the junction of the D-stem and the anticodon stem, and Um_{44} is predicted to form tertiary interactions with the $m^{2,2}G_{26}$ (Sampson and Uhlenbeck 1988). Third, this modification is highly conserved among eukaryotes (Sprinzl et al. 1998). Thus, 21 out of 23 characterized eukaryotic cytoplasmic tRNA^{Ser} species have Um_{44}, and the two exceptions are chicken tRNA^{Ser}, which lacks the long variable loop and has A_{44}, and tRNA$^{Ser(CAG)}$ from *Candida cylindracea*, which is specific for CUG leucine codons in this organism because of its altered genetic code and itself has unusual features in its primary structure (Yokogawa et al. 1992). Um_{44} is also found in 10 of the 22 characterized eukaryotic cytoplasmic tRNA^{Leu} species (although all of these 22 tRNA^{Leu} species have U_{44} and a long variable loop), in all five characterized mitochondrial tRNA^{Leu} species from plants, and

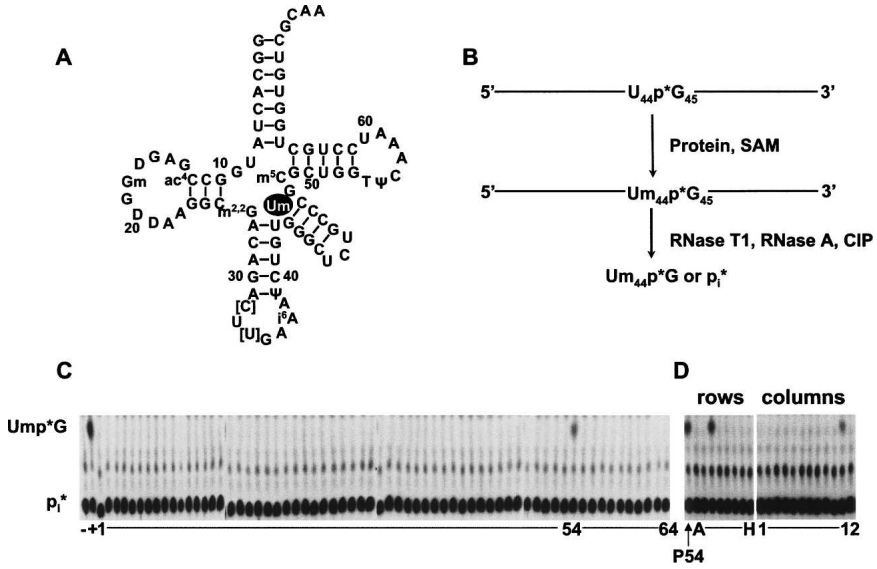

FIGURE 1. Identification of yeast ORF associated with Um_{44} methyltransferase activity of tRNA$^{Ser(UGA)}$. (*A*) Two-dimensional structure of *S. cerevisiae* tRNA$^{Ser(UGA)}$. tRNA is numbered according to convention, in this case with no residue 17, an additional residue 20a, and an 11 residue variable arm between Um_{44} and G_{48}. [C], [U], uncharacterized C and U modifications. Um_{44} is shown in white font within a black oval. (*B*) Assay scheme to detect 2′-O-methylation of tRNA^{Ser} uniquely labeled at U_{44} (U44*Ser). Treatment with RNaseT1, RNaseA and calf intestinal phosphatase (CIP) yields Ump*G if U_{44} is methylated and inorganic phosphate (P_i) if the substrate is unmodified. (*C*) Assay of a genomic collection of GST-ORF pools for Um_{44} methyltransferase activity. GST-ORF fusion proteins were purified and assayed for tRNA^{Ser} Um_{44} 2′-O-methyltransferase using 2 μL of each GST-ORF fusion protein pool, 0.5 mM *S*-adenosyl methionine, and ~1 nM U44*Ser tRNA in methyltransferase buffer at 30°C for 12 h, and then RNA was digested with RNase T1, RNase A, and CIP to produce Ump*G dinucleotide or inorganic phosphate, which were resolved by thin layer chromatography as described in Materials and Methods. (Lane −) no protein; (lane +), yeast crude extract. (*D*) Deconvolution of subpools of Plate 54 to identify the ORF associated with Um_{44} methyltransferase. (P54) GST-ORF proteins purified from pool 54.

in mitochondrial $tRNA^{Tyr}$ from *Tetrahymena pyriformis* (Schnare et al. 1985), but not in the four other organellar $tRNA^{Tyr}$ species with a long variable arm and U_{44}. In yeast, Um_{44} is only found in cytoplasmic $tRNA^{Ser}$ species (Zachau et al. 1966; Etcheverry et al. 1979; Olson et al. 1981).

In this study we identify the yeast $tRNA^{Ser}$ Um_{44} methyltransferase (Trm44), and show that *trm44* mutants that also lack Tan1, required for ac^4C_{12} in yeast $tRNA^{Ser}$ and $tRNA^{Leu}$ (Johansson and Bystrom 2004), have a growth defect, and accumulate reduced levels of specific $tRNA^{Ser}$ species at elevated temperature.

RESULTS

Um_{44} methyltransferase activity copurifies with the product of ORF YPL030w

To identify the $tRNA^{Ser}$ Um_{44} 2′-O-methyltransferase in *S. cerevisiae*, we screened the yeast proteome for this activity using yeast $tRNA^{Ser(UGA)}$ as the substrate (Fig. 1A), specifically labeled at the 3′ phosphate of U_{44} (U44*Ser). Formation of Um_{44} will generate a phosphodiester bond that is resistant to digestion with RNase T1, RNase A, and phosphatase, yielding the dinucleotide $Um_{44}p^*G$, whereas unreacted substrate will yield inorganic phosphate (Pi^*) after this treatment, and these products are easily resolved by thin layer chromatography (Fig. 1B). We screened the yeast proteome for $tRNA^{Ser}$ Um_{44} 2′-O-methyltransferase using pools of purified fusion proteins from a genomic collection of yeast strains expressing yeast ORF fusion proteins, as was done previously (Martzen et al. 1999; Wilkinson et al. 2007). We found 2′-O-methyltransferase activity in pool 54 (Fig. 1C), and deconvolution of this pool shows that activity derives from the strain in row C and column 11 of plate 54 (Fig. 1D), which expresses yeast ORF YPL030w. We show below that this ORF encodes the yeast $tRNA^{Ser}$ Um_{44} 2′-O-methyltransferase, and hence assign the name *TRM44* to this gene.

Trm44 is necessary and sufficient for 2′-O-methylation of U_{44} of $tRNA^{Ser}$ in vitro and in vivo

To determine if Trm44 is required for formation of Um_{44}, we compared Um_{44} methyltransferase activity and $tRNA^{Ser}$ from a *trm44-Δ* deletion strain with that from an otherwise isogenic wild-type strain. *TRM44* is required for $tRNA^{Ser}$ Um_{44} 2′-O-methyltransferase activity in vitro, since crude extracts from a wild-type strain have readily detectable tRNA Um_{44} methyltransferase activity with as little as 0.54 μg extract (Fig. 2A, lanes c–h), whereas crude extracts from a *trm44-Δ* strain did not have detectable activity even with 14.6 μg extract (Fig. 2A, lanes i–m). Moreover, *TRM44* is also required for the modification in vivo, since $tRNA^{Ser(IGA)}$ from a *trm44-Δ* strain specifically lacks Um_{44}. As shown in Figure 2B (upper trace), $tRNA^{Ser(IGA)}$ from the wild-type control strain has all the expected modifications (Ψ, D, m^5C, Um, I, Gm, and ac^4C) as determined by retention times and UV absorbance spectra (Gehrke and Kuo 1989), whereas $tRNA^{Ser(IGA)}$ from a *trm44-Δ* deletion strain has all the same modifications except for Um (Fig. 2B, lower trace). Quantification shows that Um levels in $tRNA^{Ser(IGA)}$ are reduced from 0.84 mol/mole in the wild-type strain to less than 0.01 mol in the *trm44-Δ* strain

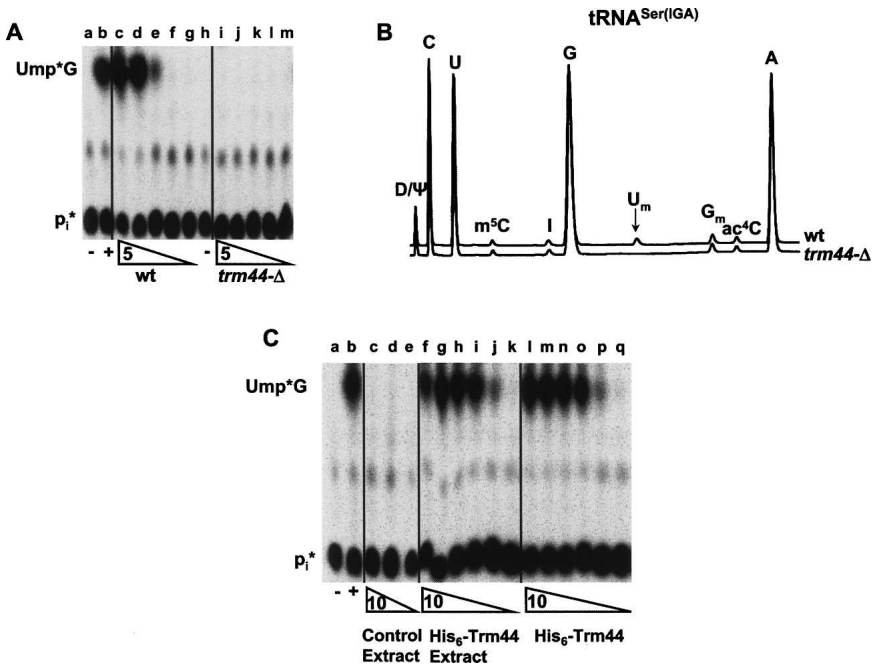

FIGURE 2. *TRM44* is necessary and sufficient for the Um_{44} methyltransferase activity in vitro and in vivo. (*A*) *trm44-Δ* extracts lack $tRNA^{Ser}$ Um_{44} methyltransferase activity. Crude extracts from wild-type and *trm44-Δ* strains were assayed in 10 μL reactions containing 1 nM U44*Ser tRNA, by serial fivefold dilution of wild-type extracts (lanes *c–g*), beginning with 13.4 μg protein or *trm44-Δ* extracts (lanes *i–m*), beginning with 14.6 μg protein. (Lanes *a,h*) no protein; (lane *b*) yeast crude extract. (*B*) $tRNA^{Ser(IGA)}$ isolated from a *trm44-Δ* strain lacks Um. $tRNA^{Ser(IGA)}$ was purified from wild-type and *trm44-Δ* mutant strains, and nucleosides were prepared and resolved on HPLC as described in Materials and Methods. (*C*) Trm44 expressed in *E. coli* has Um_{44} methyltransferase activity. Trm44 was expressed in *E. coli* and purified as described in Materials and Methods and assayed for Um_{44} methyltransferase activity with U44*Ser tRNA substrate and 10-fold serial dilutions of the control extract (lanes *c–e*, beginning with 12 μg protein), Trm44p expressing extract (lanes *f–k*, beginning with 8.6 μg), or purified Trm44p (lanes *l–q*, beginning with 5.2 μg). (Lane *a*) no protein; (lane *b*) yeast crude extract (40 μg).

(Table 1), whereas the levels of all other modifications are similar from both strains. The modest variation in dihydrouridine levels arises from incomplete resolution of the peaks in this system and the difficulty in quantifying dihydrouridine because of the lack of absorption peak (Xing et al. 2004). We also observed lack of Um in nucleosides from $tRNA^{Ser(UGA)}$ purified from the *trm44-Δ* strain (data not shown). Thus we conclude that *TRM44* is required for formation of Um_{44} in $tRNA^{Ser}$ in vitro and in vivo.

Two lines of evidence suggest that Trm44 is sufficient for Um_{44} 2′-O-methyltransferase activity. First, expression of *TRM44* is the limiting factor for activity, since crude extracts made from the strain expressing GST-*TRM44* have 10-fold more activity than the control extract (data not shown). Second, purified yeast Trm44 prepared after expression in *E. coli* has robust $tRNA^{Ser}$ Um_{44} 2′-O-methyltransferase activity. Crude extracts from an *E. coli* strain expressing yeast Trm44 as a His_6-Trm44 fusion protein have a 66-kDa polypeptide that is not observed in the control strain and that corresponds to the expected molecular weight of Trm44, and this polypeptide is substantially enriched after IMAC purification (data not shown). As shown in Figure 2C, extracts from the *E. coli* strain expressing His_6-Trm44 have substantial activity (Fig. 2C, lanes f–k), easily detectable by assay of 0.86 ng protein (Fig. 2C, lane j), whereas the vector control extract (Fig. 2C, lanes c–e) has no detectable activity, even with 14,000-fold more protein (lane c). Moreover, purified His_6-Trm44 has robust activity (Fig. 2C, lanes l–q). Thus, expression of Trm44 is sufficient for $tRNA^{Ser}$ Um_{44} 2′-O-methyltransferase activity in *E. coli*. Furthermore, the 20% yield of activity after purification suggests that no dissociable cofactors are required for activity under these conditions, other than *S*-adenosylmethionine, which is included in the reaction mixture. *S*-adenosylmethionine (SAM) is required for the methyltransferase reaction, based on the complete absence of detectable activity (less than 0.3%) observed with 800 nM His_6-Trm44 protein and 800 nM singly labeled $tRNA^{Ser}$, compared with the conversion of 35% of the substrate to product when SAM is added under the same conditions.

TABLE 1. Quantification of nucleosides in $tRNA^{Ser(IGA)}$ from a wild-type and a *trm44-Δ* strain

Modified nucleoside	Expected/mole[a]	$tRNA^{Ser(IGA)}$, wild type[b]	$tRNA^{Ser(IGA)}$, *trm44-Δ*[b]
Um	1	0.84	<0.01
m^5C	1	0.86	0.86
ac^4C	1	0.93	0.92
Gm	1	0.96	0.95
I	1	0.93	0.98
ψ	3	2.94	2.90
D	3	3.26	2.50

[a]Sprinzl et al. (1998).
[b]Calculated from the areas under peaks observed in HPLC.

The Trm44 protein family is highly conserved among metazoans and fungi but not plants

To assess the occurrence of Trm44 in other species we did a BLAST search of the NCBI translated nucleotide database and the nonredundant protein database. Trm44 protein is highly similar to predicted proteins from other fungi, with E values ranging from 0.0 for close relatives to e-37 for *Ustilago maydis* and somewhat similar to predicted proteins from a number of metazoans, including *Anopheles gambiae* (e^{-34}), *Drosophila melanogaster* (e^{-33}), *Xenopus laevis* (e^{-28}), *Homo sapiens* (e^{-28}), *Strongylocentrotus purpuratatus* (e^{-26}), *Caenorhabditis elegans* (e^{-20}), *Danio rerio* (e^{-19}), and *Schistosoma japonicum* (e^{-8}). None of these proteins has been characterized, although most are named as "putative methyltransferase." The widespread occurrence of members of the Trm44 protein family among eukaryotes is consistent with the widespread occurrence of Um_{44} in eukaryotes. Surprisingly, however, plants do not appear to have a Trm44 homolog, although $tRNA^{Ser}$ and $tRNA^{Leu}$ species in plants have Um_{44}. Thus, plants may possess another mechanism for Um_{44} modification of tRNAs.

Most of the homology in the Trm44 protein family is restricted to an ~200 amino acid region toward the C terminus of the protein, which is called DUF1613 by Pfam. An alignment from BLAST analysis is shown in Figure 3, which reveals the presence of a conserved methyltransferase domain, featuring the characteristic GXGXG amino acid sequence found in this superfamily (Kagan and Clarke 1994).

Cells lacking both Trm44 and the tRNA modifying enzyme Tan1 have an aggravated growth defect

Although Um_{44} is highly conserved among eukaryote $tRNA^{Ser}$ species, *trm44-Δ* mutant strains do not have any observable growth defect at a range of temperatures from 18°C to 37°C in minimal or rich media containing either glucose or glycerol (data not shown). To further probe the role of Trm44, we created and tested the growth phenotypes of a set of double mutant strains, each bearing a *trm44-Δ* deletion as well as a deletion in a second tRNA modification gene that acts on $tRNA^{Ser}$ species, the only known substrate of Trm44 (Fig. 1A). We used a similar approach previously to assign a role for *trm8* mutants (Alexandrov et al. 2006). We thus targeted *TAN1*, encoding tRNA cytidine N^4 acetyltransferase (Johansson and Bystrom 2004), *DUS1*, *DUS2*, and *DUS4*, encoding dihydrouridine synthases for D_{16}, D_{20}, and D_{20a} (Xing et al. 2002, 2004), *TRM3*, encoding Gm_{18} methyltransferase (Cavaille et al. 1999), *TRM1*, encoding $m^{2,2}G_{26}$ dimethyltransferase (Ellis et al. 1986), *MOD5*, encoding i^6A isopentenyltransferase (Laten et al. 1978; Dihanich et al. 1987), *TRM4*, encoding m^5C_{48} methyltransferase (Wu et al. 1998), and *TRM2*, encoding m^5U_{54} methyltransferase (Hopper et al.

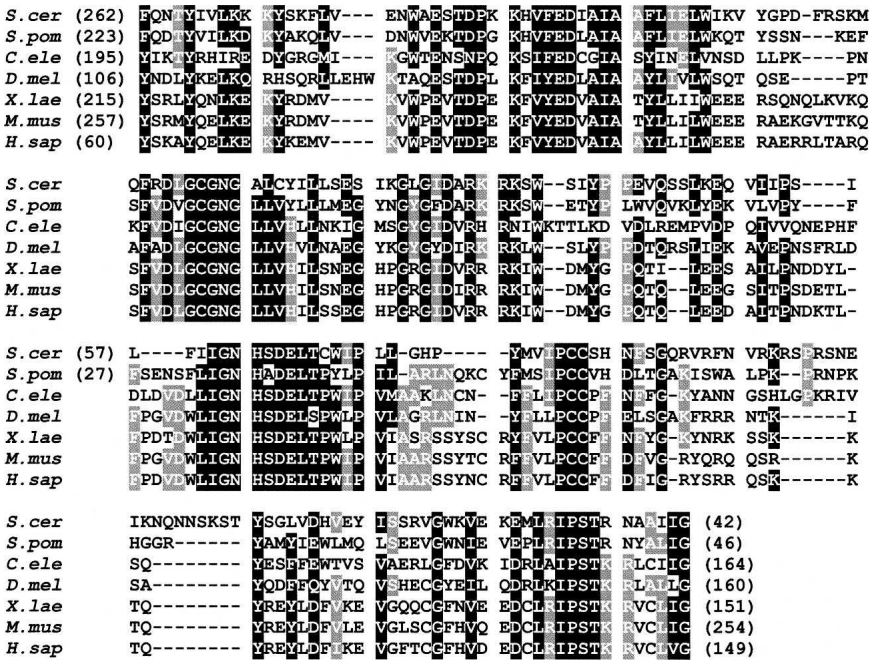

FIGURE 3. Alignment of Trm44 homologs. Trm44 homologs were identified by BLAST search (Altschul et al. 1997), and several proteins representing yeast to humans were aligned using Multalin (Corpet 1988). Sequences with little or no homology are omitted from proteins for clarity, as indicated by numbers in parentheses. Residues shaded in dark shading have >70% consensus and light shading have >50% consensus.

1982; Nordlund et al. 2000). These genes are all known to be nonessential, and all but *MOD5* act outside the anticodon region. In addition, we mutated the yeast La homolog *LHP1* (Lin-Marq and Clarkson 1995), which is known to affect 3′-end formation of tRNA and tRNA stability (Yoo and Wolin 1997; Chakshusmathi et al. 2003; Copela et al. 2006; Huang et al. 2006), and the 2′-O-methyltransferase *TRM7* (Pintard et al. 2002) as a negative control.

As expected from previous published results, we found no obvious growth defects of single mutant strains lacking *TRM2*, *TRM3*, *TRM4*, *MOD5*, *LHP1*, *DUS1*, *DUS2*, and *DUS4* (Fig. 4A, panels a,c,e,g), and we found that *trm7*-Δ mutant strains were quite sick under a variety of conditions (Fig. 4A, row 5; Pintard et al. 2002). In addition, we find that *trm1*-Δ mutants and *tan1*-Δ mutants each grow poorly on YP media containing glucose (YPD) at 37°C (Fig. 4A, rows 1,8), which has not been previously reported.

We find that *TRM44* is essential for growth of *tan1*-Δ mutant strains in YPD media or in YP+ Glycerol media at 35°C (Fig. 4A, row 8) or 33°C (data not shown) and in synthetic media containing glucose (SD) or glycerol at 33°C or 35°C (data not shown). The synthetic growth phenotype of *trm44*-Δ *tan1*-Δ double mutant strains is complemented by introduction of either missing gene, expressed under control of its own promoter on a single-copy (*CEN*) plasmid (P_{TAN1}-*TAN1* or P_{TRM44}-*TRM44*) but not by an empty vector, confirming that the phenotype is due to the *TRM44* and *TAN1* genes (Fig. 4B, panels b,c, rows 7–9). Notably, *trm44*-Δ mutants do not display a synthetic growth defect with any of the other tested deletions, including the controls *LHP1* and *TRM7*.

Lack of *TRM44* and *TAN1* leads to reduced levels of tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$ at restrictive temperature

The conditional growth defect of *trm44*-Δ *tan1*-Δ double mutant strains is likely due to effects on tRNA^{Ser} function, since in yeast only tRNA^{Ser} is known to have Um_{44}, and the ac^4C_{12} product of Tan1 is also found in tRNA^{Ser} as well as in tRNA^{Leu}. tRNA^{Ser} function might be affected either by reduced activity of one or more tRNA^{Ser} species or by reduced levels of these species at restrictive temperature. Our results indicate that levels of tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$ are dramatically reduced in *trm44*-Δ *tan1*-Δ mutant strains grown in restrictive conditions (YP media containing glycerol at 36.5°C). tRNA$^{Ser(CGA)}$ levels are at 60% of wild-type levels before the temperature shift and are reduced to 34% 1.3 h after temperature shift, 22% after 3.9 h, and 13% after 6.7 h (Fig. 5). By contrast, tRNA$^{Ser(CGA)}$ levels are more modestly reduced in *tan1*-Δ single mutants and are nearly constant in wild-type cells and in *trm44*-Δ mutants. A similar reduction in tRNA levels is observed with tRNA$^{Ser(UGA)}$, although this is not quite as severe as that with tRNA$^{Ser(CGA)}$.

Strikingly, there is no reduction in the other two tRNA^{Ser} isoacceptors, tRNA$^{Ser(IGA)}$ and tRNA$^{Ser(GCU)}$ (Fig. 5), although tRNA$^{Ser(IGA)}$ is known to have the same modifications (Zachau et al. 1966), and tRNA$^{Ser(GCU)}$ is likely to have both modifications but is not characterized. Similarly, there is little observed decrease in any of the four different tRNA^{Leu} species (other than a modest reduction in tRNA$^{Leu(GAG)}$ that is observed in *tan1*-Δ mutant strains), which are the only other tRNA species known to be modified by Tan1 (Sprinzl et al. 1998), and there is no observed decrease in each of several control tRNAs (Fig. 5).

The growth defect of *trm44*-Δ *tan1*-Δ double mutants is due to loss of tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$

Because Northern analysis of *trm44*-Δ *tan1*-Δ double mutants reveals specific loss of tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$ species, but not other tRNAs, at restrictive temperature,

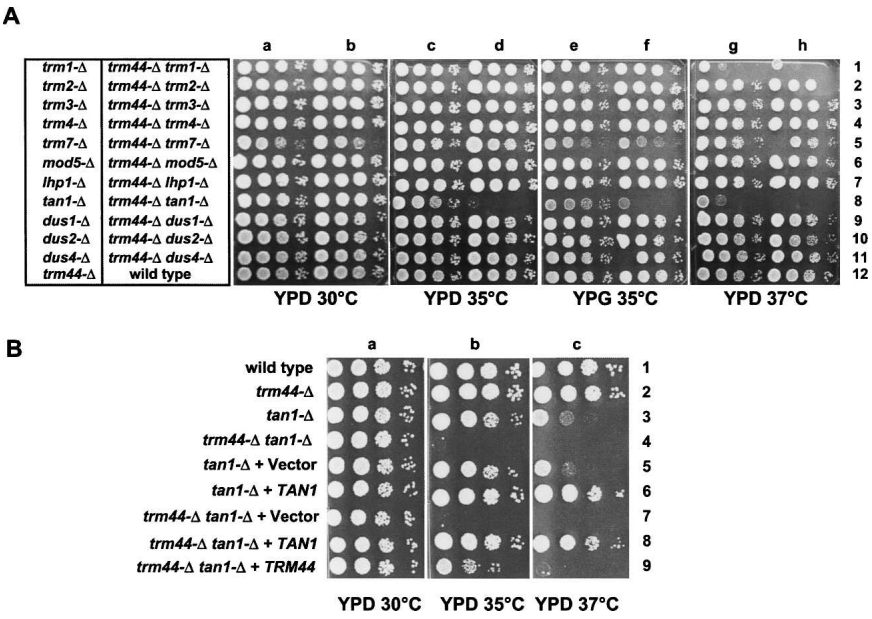

FIGURE 4. *trm44*-Δ *tan1*-Δ double mutants have a synthetic growth phenotype. (*A*) Analysis of mutant strains for growth phenotypes. Homozygous diploid strains bearing single and double deletions (in conjunction with a *trm44*-Δ mutation) of genes encoding tRNA modifying enzymes were constructed as described in Materials and Methods, grown in YP+ Dextrose media overnight at 25°C, diluted to OD_{600} = 1, serially diluted (10-fold), spotted on plates containing YP+ Dextrose and YP+ Glycerol, and incubated at indicated temperatures for 2 d. (*B*) Complementation of the growth defect of *trm44*-Δ *tan1*-Δ mutant strains. *trm44*-Δ *tan1*-Δ mutant strains were transformed with *CEN URA3* plasmids containing *TAN1* or *TRM44* or an empty vector, and transformants were grown in SD-uracil media at 25°C overnight, diluted to OD_{600} = 1, 10-fold serially diluted, and spotted on YPD plates and incubated for 2 d at indicated temperatures.

it seemed likely that the loss of tRNA$^{Ser(CGA)}$ and/or tRNA$^{Ser(UGA)}$ was responsible for the growth defect of *trm44*-Δ *tan1*-Δ mutants. In agreement with this, we find that introduction of multicopy plasmids carrying tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$ can completely suppress the growth defect of *trm44*-Δ *tan1*-Δ mutants (Fig. 6, row 9), whereas no suppression is observed after introduction of empty control plasmids or plasmids containing other tRNA species. Indeed, expression of tRNA$^{Ser(CGA)}$ alone is sufficient for nearly complete suppression of the growth defect of this strain at 37°C (Fig. 6, row 7), whereas expression of tRNA$^{Ser(UGA)}$ can only suppress the growth defect at 35°C (Fig. 6, row 8). This result provides strong evidence that the growth defect of *trm44*-Δ *tan1*-Δ double mutant strains at high temperatures is primarily due to loss of tRNA$^{Ser(CGA)}$ and secondarily to the loss of tRNA$^{Ser(UGA)}$.

DISCUSSION

In this work we have identified the *S. cerevisiae* gene encoding tRNA^{Ser} Um$_{44}$ methyltransferase (Trm44) as the product of ORF YPL030w and demonstrated a role for this protein in helping to maintain levels of specific tRNA^{Ser} species. We identified Trm44 as the Um$_{44}$ 2′-O-methyltransferase by assay of a genomic collection of purified yeast GST-ORF fusion proteins, using tRNA$^{Ser(UGA)}$ specifically labeled at U$_{44}$ as the substrate and *S*-adenosylmethionine as the methyl donor. Trm44 is necessary for Um$_{44}$ formation in vivo because Um$_{44}$ is absent in tRNA$^{Ser(IGA)}$ and tRNA$^{Ser(UGA)}$ from a *trm44*-Δ strain, and Trm44 protein is sufficient for activity because yeast Trm44 protein purified from *E. coli* catalyzes 2′-O-methyltransferase activity. We have also shown that *trm44*-Δ *tan1*-Δ mutant strains (which lack both Um$_{44}$ and ac^{4}C$_{12}$) are temperature sensitive and provided evidence that the loss of viability occurs because of loss of tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$, since these species are specifically depleted at high temperature and since introduction of more copies of these tRNA genes rescues the temperature-sensitive defect of the strain. Moreover, since the growth defect at 37°C is due to the *tan1*-Δ mutation, this result suggests that the defect in *tan1*-Δ mutants is due to the same two tRNA species.

The two tRNA^{Ser} species that are specifically reduced in *trm44*-Δ *tan1*-Δ mutants are remarkably similar to one another. Thus, tRNA$^{Ser(CGA)}$ and tRNA$^{Ser(UGA)}$ have 82 identical residues among their 85 bases, whereas tRNA$^{Ser(CGA)}$ has 13 differences with tRNA$^{Ser(IGA)}$ and 26 differences with tRNA$^{Ser(GCU)}$. Although this similarity explains why tRNA$^{(Ser)CGA}$ and tRNA$^{Ser(UGA)}$ behave similarly, it is not immediately obvious why tRNA$^{Ser(CGA)}$ is more sensitive than tRNA$^{Ser(UGA)}$ as measured by both the reduction in tRNA levels and by suppression of the temperature sensitivity of *trm44*-Δ *tan1*-Δ mutants with introduction of additional copies of tRNA genes. tRNA$^{Ser(CGA)}$ has a G$_{28}$–C$_{42}$ base pair in the anticodon stem instead of a C–G pair in tRNA$^{Ser(UGA)}$ and C$_{34}$ rather than U$_{34}$ at the anticodon, and these differences are predicted to preferentially stabilize the anticodon stem–loop from tRNA$^{Ser(CGA)}$ by only a very modest 0.2 kcal/mol (Mathews et al. 1999). The discrepancy in the loss rates of these tRNA^{Ser} species may be related to differences in ongoing transcription or to subtle differences in cellular recognition of these undermodified tRNAs.

tRNA$^{Ser(CGA)}$ appears to be unusually sensitive to alterations in its modifications. Bystrom and coworkers (Johansson and Bystrom 2002, 2004) have shown that a strain with a mutation in the variable arm of tRNA$^{Ser(CGA)}$ has a synthetic growth defect when combined with deletion

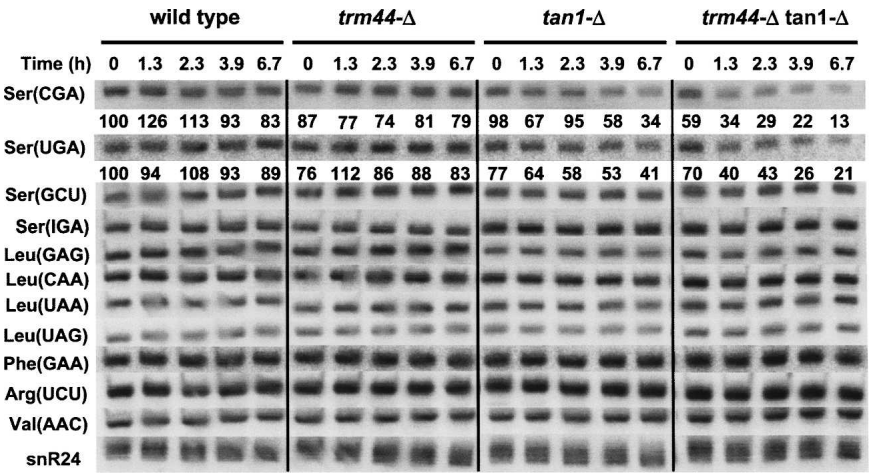

FIGURE 5. Levels of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ are reduced in a *trm44*-Δ *tan1*-Δ mutant strain at 36.5°C in YP+ Glycerol. Wild-type, *trm44*-Δ, *tan1*-Δ, and *trm44*-Δ *tan1*-Δ strains were grown in YP+ Glycerol media at 28°C and shifted to 36.5°C for indicated times, and RNA was analyzed by Northern blotting as described in Materials and Methods. Quantification of the levels of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ is shown *under* each corresponding panel, as normalized first to snR24 (snRNA), and then to levels of the corresponding tRNA species in wild-type cells at permissive temperature.

of any of several genes encoding modification enzymes acting on this tRNA, including *TRM2*, encoding m^5U_{54} methyltransferase (Hopper et al. 1982; Nordlund et al. 2000), *TRM1* ($m^{2,2}G_{26}$) (Ellis et al. 1986), *TRM3* (Gm_{18}) (Cavaille et al. 1999), *PUS4* (Ψ_{55}) (Becker et al. 1997), or *TAN1* (ac^4C_{12}) (Johansson and Bystrom 2004), as well as *LHP1*, which affects 3′ processing of tRNAs and has a chaperone function (Yoo and Wolin 1997; Chakshusmathi et al. 2003). Moreover, each of these combinations of $tRNA^{Ser(CGA)}$ mutations and modification genes or *LHP1* mutations affects levels of $tRNA^{Ser(CGA)}$ (Johansson and Bystrom 2002). This unusual sensitivity of $tRNA^{Ser(CGA)}$ may in part be due to the fact that it is a single copy essential tRNA gene (Etcheverry et al. 1982).

We note that *tan1*-Δ mutants also have reduced levels of both $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ at 36.5°C, albeit not as severely reduced as in *trm44*-Δ *tan1*-Δ double mutants. A likely explanation for this observation is that the instability of these $tRNA^{Ser}$ species in *trm44*-Δ *tan1*-Δ mutants is due more to the lack of Tan1 than to the lack of Trm44, consistent with the observed temperature sensitivity of *tan1*-Δ mutants at 37°C but not at the lower temperatures at which *trm44*-Δ *tan1*-Δ mutants are defective for growth (Fig. 4).

The mechanism by which 2′-O-methylation of U_{44} contributes to stability of $tRNA^{Ser(CGA)}$ is unclear. One possibility is that the effects are due to thermal stability conferred by Um_{44}. It is known that a 2′-O-methyl group on uridine can stabilize the C3′-endo form of RNA by ~0.8 kcal mol^{-1}, attributed to decreased repulsion between the 2-carbonyl group of uridine, the 2′-O-methyl group, and the 3′-phosphate group relative to the C2′-endo form (Kawai et al. 1992). It is also known that the 2′-O-methyl group can confer increased stability, attributed to stacking, of poly-U polymers (Zmudzka and Shugar 1970) and of nucleotide dimers of form 5′NmpN3′, when the 5′ nucleoside is cytidine, uridine, or guanosine but not adenosine (Drake et al. 1974). It is interesting to note in this regard that the amount of 2′-O-methylated ribose moieties in tRNA

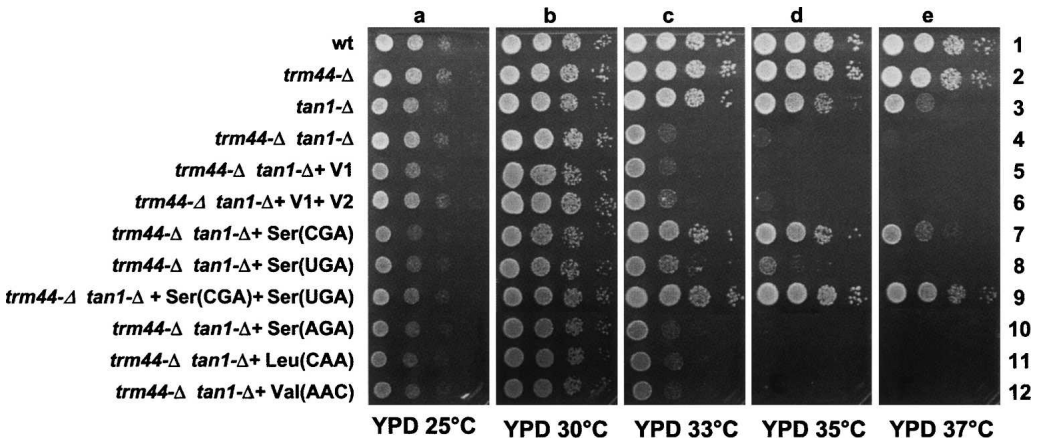

FIGURE 6. The synthetic growth phenotype of a *trm44*-Δ *tan1*-Δ mutant strain is suppressed by $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$. A *trm44*-Δ *tan1*-Δ mutant strain was transformed with a multicopy plasmid(s), containing $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ or a vector control, and transformants were grown in synthetic media, diluted to OD_{600} = 1, spotted on YPD plates, and incubated at indicated temperatures for 2 d.

increases threefold when cultures of *Bacillus stearothermophillus* are shifted from 50°C to 70°C (Agris et al. 1973). A second possibility is that the role of 2′-O-methylation at U_{44} is to stabilize a particular conformation for recognition purposes. Indeed, there is evidence that fixing the base at the wobble position of the tRNA anticodon (N_{34}) at C3′-endo form or C2′-endo form can lead to proper recognition of codons (Yokoyama et al. 1985).

The finding that members of the Trm44 family are not found in plants is somewhat surprising. The Trm44 protein family is distributed widely among fungi and among metazoans, including the arthropods, chordates, echinoderms, nematodes, and platyhelminthes. This distribution is consistent with the occurrence of Um_{44} in $tRNA^{Ser}$ in all of the 14 characterized cytoplasmic $tRNA^{Ser}$ species from metazoan and fungal species. It is also likely consistent with the occurrence of Um_{44} in four $tRNA^{Leu}$ species from metazoans and fungi, since most modifications at the same position in different tRNAs are formed by the same proteins (Hopper and Phizicky 2003). However, the lack of Trm44 family members in plants is not consistent with the presence of Um_{44} in each of seven cytoplasmic $tRNA^{Ser}$ species from three different orders within the class magnoliopsida (flowering plants), represented by *Lupinus luteus*, *Nicotiana rustica*, and *Spinacia oleracea*, or with the six $tRNA^{Leu}$ species containing Um_{44} found in flowering plants. The complete absence of a recognizable Trm44 ortholog in plants might have two possible causes: First, the Trm44 protein family might be somewhat diverged in plants, consistent with the observation that although the Trm44 family is very highly conserved in fungi, it is much less conserved in metazoans. Second, 2′-O-methylation in plants might be catalyzed through appropriate Box C/D snoRNA guide molecules and the Nop1/Fibrillarin snoRNP complex (Galardi et al. 2002), much as 2′-O-methylation is catalyzed either enzymatically or by Box C/D snRNPs at residue N_{34} (Clouet d'Orval et al. 2001; Pintard et al. 2002) and at residue C_{56} (Renalier et al. 2005) in different groups of species.

The reduced levels of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ observed in *trm44-Δ tan1-Δ* double mutants at restrictive temperature underscore the importance of modifications remote from the anticodon for tRNA stability in the cell and the importance of synergistic interactions among modifications. Lack of these tRNA modifications can lead to tRNA degradation by at least two pathways. First, cells lacking m^1A_{58} have reduced $tRNA_i^{Met}$ because pre-$tRNA_i^{Met}$ is degraded by polyadenylation and exonucleolytic degradation, using the TRAMP complex containing Trf4, Air2, and Mtr4, as well as Rrp6 and the nuclear exosome (Kadaba et al. 2004, 2006; LaCava et al. 2005; Vanacova et al. 2005). Second, cells lacking m^7G_{46} and m^5C_{49} (*trm8-Δ trm4-Δ* mutants) have mature $tRNA^{Val(AAC)}$ that is rapidly degraded at high temperature by a pathway independent of the Trf4/Rrp6 degradation pathway (Alexandrov et al. 2006). The observed loss of $tRNA^{Ser(CGA)}$ and $tRNA^{Ser(UGA)}$ in *trm44-Δ tan1-Δ* mutants lacking Um_{44} and ac^4C_{12} emphasizes the crucial importance of a number of modifications for tRNA stability, reinforcing the need to understand the mechanism and regulation of these tRNA quality control pathways.

MATERIALS AND METHODS

Strains and plasmids

Yeast strains deleted for specific genes and their isogenic parents (BY4741 and BY4742) were obtained from the yeast knockout collection (Open Biosystems). The *kanMX* cassette of MAT**a** and MAT**α** strains deleted for ORF YPL030w (*trm44-Δ::kanMX*) were each replaced with the nourseothricin resistance cassette by transformation with linearized DNA derived from plasmid p4339 by EcoRI digestion (Tong et al. 2001) to generate yeast strains LY1050 (MAT**a** *trm44Δ::natMX*) and LY1023 (MAT**α** *trm44Δ::natMX*). The *trm44-Δ::kanMX*, *trm1-Δ::kanMX, trm2-Δ::kanMX*, *trm3-Δ::kanMX*, *trm4-Δ::kanMX*, *trm7-Δ::kanMX*, *dus1-Δ::kanMX*, *dus2-Δ::kanMX*, *dus4-Δ::kanMX*, *tan1-Δ::kanMX*, *mod5-Δ::kanMX*, and *lhp1-Δ::kanMX* alleles were PCR amplified from genomic DNA prepared from the corresponding knockout strains using upstream and downstream primers and transformed into either the *trm44Δ::natMX* or wild-type haploid strains to generate the corresponding *trm44Δ::natMX geneX-Δ::kanMX* double mutant or *geneX-Δ::kanMX* single mutant strains. All strains were confirmed by PCR with flanking primers and then mated to generate the corresponding homozygous diploid knockout strains and their isogenic parents (BY4741/BY4742). Strains are listed in Table 2.

For complementation, the *TRM44* and *TAN1* genes were PCR amplified together with ~400 bp of upstream and downstream flanking sequence using primers TRM44 XbaI 5′ and TRM44 EcoR1 3′ or TAN1 HindIII Lac 5′ and TAN1 EcoR1 Lac 3′ and then digested accordingly and cloned into yCPlac33 *URA3* (Gietz and Sugino 1988) to create plasmids pLK300 (*CEN TRM44*) and pLK313 (*CEN TAN1*), which were sequence verified. Multicopy plasmids containing $tRNA^{Ser(UGA)}$ (pLK328) and $tRNA^{Ser(CGA)}$ (pLK322), were constructed in a similar manner using primers Ser UGA HindIII 5′ and Ser UGA EcoRI 3′ to amplify the tS(UGA)E locus and ~400 bp of flanking sequences and primer pairs tS(CGA)BamHI 5′ and tS(CGA)EcoRI 3′ to amplify tS(CGA)C locus followed by digestion and ligation into the 2 μ *URA3* vector yEPlac195 (Gietz and Sugino 1988). Control multicopy plasmids were also created by similar cloning of tS(AGA)D3 and tL(CAA)A loci using primer pairs tS(AGA) HindIII 5′ and tS(AGA)EcoRI 3′ and tL(CAA) BamHI 5′ and tL(CAA) EcoRI 3′, respectively. The sequences of the primers are listed in Table 3. Construction of the multicopy plasmid containing $tRNA^{Val(AAC)}$ was previously described (Alexandrov et al. 2006).

S. cerevisiae TRM44 was cloned into the *E. coli* expression vector AVA421 (Alexandrov et al. 2004) by PCR amplification of *TRM44* using primers YPL030W_Fwd (5′-GGGTCCTGGTTCGATGACT GGCGACGGTAGTGC-3′) and YPL030W_Rev (5′-CTTGTTCG TGCTGTTTATTAATGGTTTCTTGGGTTTCTTTTC-3′) followed by ligation-independent cloning to generate plasmid pLKD306 for expression of His_6-Trm44 protein in *E. coli*.

TABLE 2. Strains used in this study

Strain name	Genotype	Parents
LY1481	MATa/MATα, *trm1*-Δ:: *kanMX/trm1*-Δ:: *kanMX*	LY1435/LY1440
LY1547	MATa/MATα, *trm2*-Δ::*kanMX/trm2*-Δ::*kanMX*	LY1254/LY1529
LY1549	MATa/MATα, *trm3*-Δ::*kanMX/trm3*-Δ::*kanMX*	LY1256/LY1531
LY1552	MATa/MATα, *trm4*-Δ::*kanMX/trm4*-Δ::*kanMX*	LY1258/LY1534
LY1485	MATa/MATα, *trm7*-Δ::*kanMX/trm7*-Δ::*kanMX*	LY1459/LY1462
LY1486	MATa/MATα, *trm44*-Δ::*kanMX/trm44*-Δ::*kanMX*	LY1390/LY1402
LY1487	MATa/MATα, *mod5*-Δ::*kanMX/mod5*-Δ::*kanMX*	LY1393/LY1405
LY1554	MATa/MATα, *lhp1*-Δ::*kanMX/lhp1*-Δ::*kanMX*	LY1538/LY1298
LY1489	MATa/MATα, *tan1*-Δ::*kanMX/tan1*-Δ::*kanMX*	LY1387/LY1400
LY1555	MATa/MATα, *dus1*-Δ::*kanMX/dus1*-Δ::*kanMX*	LY1539/LY1289
LY1491	MATa/MATα, *dus2*-Δ::*kanMX/dus2*-Δ::*kanMX*	LY1265/LY1291
LY1492	MATa/MATα, *dus4*-Δ::*kanMX/dus4*-Δ::*kanMX*	LY1267/LY1294
LY1493	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, trm1*-Δ::*kanMX/trm1*-Δ::*kanMX*	LY1447/LY1451
LY1494	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, trm2*-Δ::*kanMX/trm2*-Δ::*kanMX*	LY1176/LY1178
LY1495	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, trm3*-Δ::*kanMX/trm3*-Δ::*kanMX*	LY1180/LY1182
LY1496	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, trm4*-Δ::*kanMX/trm4*-Δ::*kanMX*	LY1196/LY1198
LY1497	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, trm7*-Δ::*kanMX/trm7*-Δ::*kanMX*	LY1465/LY1470
LY1498	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, mod5*-Δ::*kanMX/mod5*-Δ::*kanMX*	LY1204/LY1207
LY1499	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, lhp1*-Δ::*kanMX/lhp1*-Δ::*kanMX*	LY1184/LY1186
LY1500	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, tan1*-Δ::*kanMX/tan1*-Δ::*kanMX*	LY1172/LY1174
LY1501	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, dus1*-Δ::*kanMX/dus1*-Δ::*kanMX*	LY1188/LY1190
LY1557	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, dus2*-Δ::*kanMX/dus2*-Δ::*kanMX*	LY1301/LY1541
LY1503	MATa/MATα, *trm44*-Δ::*natMX/trm44*-Δ::*natMX, dus4*-Δ::*kanMX/dus4*-Δ::*kanMX*	LY1192/LY1194
LY1504	MATa/MATα, wild type	BY4741/BY4742

Growth and preparation of extracts and purified proteins

The purification of GST-ORF fusion protein pools and subpools was previously described (Martzen et al. 1999; Phizicky et al. 2002).

Wild-type and *trm44-Δ* yeast strains were grown in YP media containing glucose to midlog phase, and 300 OD of cells were resuspended in 0.8 mL extraction buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM DTT, 10% glycerol, 1 M NaCl, and protease inhibitors (1 μg/mL pepstatin, 2 μg/mL leupeptin, and 1 mM pefabloc), lysed with glass beads (10 times for 20 sec in a bead beater), and centrifuged 10 min to remove debris and generate crude extract (20 mg/mL for wild type and 22 mg/mL for *trm44-Δ* mutants), as described (Jackman et al. 2003).

His_6-Trm44 was purified from *E. coli* essentially as described (Steiger et al. 2001). BL21DE3pLysS cells transformed with pLKD306 were grown in 500 mL LB+Amp at 30°C to OD_{600} = 0.5 and induced with IPTG for 3 h, and harvested cells were resuspended in 10 mL buffer containing 20 mM HEPES (pH 7.5), 5% glycerol, 1 M NaCl, 2 mM β−mercaptoethanol, and protease inhibitors, and lysed by sonication, followed by centrifugation to produce crude extract (4.3 mg/mL). Then His_6 tagged protein was

TABLE 3. DNA oligonucleotides used for cloning genes

Gene	Primer	Sequence
tS(UGA)E	Ser UGA HindIII 5′	5′-GAAGCCAAGCTTGGGACACTATCTGATGTATTTTTTATTGTTGAAATTTCTG
tS(UGA)E	Ser UGA Eco I 3′	5′-GGGAATTCGGCGATCACACTATATTTTCTAGAAATAACTTAAACCATAGTG
tS(AGA)D3	tS(AGA)HindIII 5′	5′-GAAGCCAAGCTTGGGGAGACTTCGCTTTTGATCAAAGTGATAACATTATG
tS(AGA)D3	tS(AGA)EcoRI 3′	5′-GGAATTCGGCGTTAGTGCTCTAAGTTGTAAACTTTCTCTTG
tS(CGA)C	tS(CGA)BamHI 5′	5′-GCCGGATCCGCGTTCACTTTCAACCTCAGTGTTGGATTCGTTTCTTTG
tS(CGA)C	tS(CGA)EcoRI 3′	5′-GGAATTCGGCGAGCTGCTCATTGGACTTTGCTGCGAGCTTAGACGACTTG
tL(CAA)A	tL(CAA)BamHI 5′	5′-GCCGGATCCGCGCTGGATTAGTAGTATAGCAAAAGTAACACTTGTCCACCG
tL(CAA)A	tL(CAA)EcoRI 3′	5′-GGAATTCGGCGAGCTTTGCTATTTTGCCTTGAAACAGCATATCTCATGGCGAGCG
TAN1	TAN1 HindIII Lac 5′	5′-GGCCTAAGCTTGGGTTGAGCAAGATTCAGCACTTCGTCTCATCTG
TAN1	TAN1 EcoRI Lac 3′	5′-GACGGAATTCCCAGTATTACAAGTACAGAAGGCATGGCAAATTG
TRM44	TRM44 XbaI 5′	5′-GCTCTAGAGCTACGGGCTTACGAGTCTACGTGTATGG
TRM44	TRM44_EcoRI 3′	5′-GGAATTCCAGGTCTTTTCACAATCCAATCCTTATCATG

TABLE 4. Oligonucleotides used to analyze RNA expression in Northern blots

Probe	Target	Sequence
Mature-Ser CGA-2	Mature $tRNA^{Ser(CGA)}$	5′-CAGAGCCCAAGAGATTTCGAGTCT-3′
Ser1-UGA-2	$tRNA^{Ser(UGA)}$	5′-GCAGAGCCCAACAGATTTCAAGTCTG-3′
Ser-GCU1	$tRNA^{Ser(GCU)}$	5′-AAAACCCAATGCCTTAGCAGGG-3′
Ser-IGA	$tRNA^{Ser(IGA)}$	5′-GCAAAGCCCAAAAGATTTCCAA-3′
Leu-GAG1	$tRNA^{Leu(GAG)}$	5′-CGAAGAGATCAGGACCTCGACC-3′
Leu CAAP1	$tRNA^{Leu(CAA)}$	5′-AGATTCGAACTCTTGCATCTT-3′
Leu UAA pr1	$tRNA^{Leu(UAA)}$	5′-AACCGTCCAACAGATCTTAAGT-3′
Leu UAG pr1	$tRNA^{Leu(UAG)}$	5′-CCGAAGATATCAGAGCCTAAAT-3′
tRNAPheloop	$tRNA^{Phe(GAA)}$	5′-TGTGGATCGAACACAGGACC-3′
G9ArgSa	$tRNA^{Arg(ACG)}$	5′-ACCATTGGGCCACGAGGAA-3′
Val(CCA)	$tRNA^{Val(CCA)}$	5′-TGGTGATTTCGCCCAGGA-3′
snR24-39-63	snR24	5′-GTATGTCTCATTCGGAACTCAAAG-3′

purified by immobilized metal-ion chromatography (IMAC) using TALON resin (Clontech), as described (Steiger et al. 2001), followed by dialysis of eluted protein into buffer containing 20 mM HEPES (pH 7.5), 55 mM NaCl, 4 mM $MgCl_2$, 1 mM DTT, and 50% glycerol. The purified protein (2.6 mg/mL) was estimated to be∼40% pure on SDS-PAGE by Coomassie staining (not shown).

Preparation of labeled $tRNA^{Ser}$ substrate and assay of U_{44} methyltransferase activity

To prepare $tRNA^{Ser(UGA)}$ substrate singly labeled at U_{44} (U44*Ser), $tRNA^{Ser(UGA)}$ was cloned in two fragments into a pUC13-derived vector, as a 5′ half molecule comprising bases 1–44 (plasmid LKD260) and a 3′ half molecule comprising bases 45–85 to generate (LKD261). The 5′ half molecule was assembled from synthetic oligonucleotides to contain, in order, 5′ PstI site, T7 promoter, $tRNA^{Ser(UGA)\ 1–44}$, HpaI site, and BamH1, and the resulting plasmid was cut with HpaI for transcription. The 3′ half molecule was constructed in the same way except that the HpaI site was replaced by BstNI for BstNI-runoff transcripts. Transcripts made using T7 RNA polymerase were gel purified, and then the 3′ half molecule was treated with phosphatase and T4 PNK to label its 5′-phosphate, followed by ligation to the 5′ half molecule using T4 DNA ligase (USB) and a bridging DNA oligonucleotide (5′-GGCAGAGCCCAACAGATTTCAAGTC-3′), as previously described (Yu 1999; Jackman et al. 2003), The final ligated $tRNA^{Ser(UGA)}$ product was analyzed with RNase A and P1 nuclease to show that the junction sequence was UpG, as predicted.

The methyltransferase assay was performed with U44*Ser in 10-μL reaction mixtures containing 50 mM HEPES (pH 7.5), 50 mM ammonium acetate, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM SAM, ∼1 nM U44*Ser, and a protein source (either crude extract or purified protein). Reactions were initiated by addition of protein and incubated for 2–12 h at 30°C. Then tRNA was supplemented with carrier RNA (20 μg), phenol extracted and ethanol precipitated, resuspended in 4 μL buffer containing 20 mM sodium acetate (pH 5.2), 1 mM EDTA, RNaseT1 (0.5 units), and RNaseA (1 μg), incubated for 30 min at 50°C, and treated with alkaline phosphatase (1 unit) for 30 min at 37°C in 1× buffer, and then the final product was applied to PEI cellulose TLC plates (EM Science) and resolved in solvent containing 0.5 M LiCl.

Preparation of low molecular weight RNA from yeast

Small molecular weight RNA was extracted from 300 OD of ***MAT*a** haploid wild-type and *trm44*-Δ mutant trains, using hot phenol, followed by two ethanol preicpitations, and resuspension in 1 mL H_2O, yielding 3–5 mg RNA. The concentration of RNA was calculated by assuming A^{260} of 40 μg/mL RNA = 1.

Purification of $tRNA^{Ser(UGA)}$ and $tRNA^{Ser(IGA)}$ from yeast

$tRNA^{Ser(UGA)}$ and $tRNA^{Ser(IGA)}$ were purified from small molecular weight RNA extracted from wild-type and *trm44*-Δ strains using 5′ biotinylated oligonucleotides (Integrated DNA Technologies) complementary to the residues between e23–73 for $tRNA^{Ser(UGA)}$ (5′Bio tRNASer1, 5′Bio-CGACACCAG CAGGATTTGAACCAGCGCGGG) and 1–32 for $tRNA^{Ser(IGA)}$ (5′Bio Ser3 5′ bio/ATCTTTCGCCTTAACCACTCGGCCAAGT TGCC) and streptavidin (SA) magnetic beads (Roche), as previously described (Jackman et al. 2003), using 2 mg of beads bound with oligomer and 1 or 3 mg of low-molecular-weight RNA to purify $tRNA^{Ser(IGA)}$ (10 μg) and $tRNA^{Ser(UGA)}$ (4 μg).

HPLC analysis of nucleosides

Purified tRNAs (1 μg) were analyzed for modifications by treatment of tRNA with Nuclease P1 (Calbiochem) followed by calf intestinal alkaline phosphatase, followed by resolution of nucleosides on a reverse phase C18 column (supelcosil LC-18-T, 25 cm × 4.6 μm; Supelco, Inc.) using an HPLC (Waters Alliance Model 2690, equipped with Waters 996 phosphodiode array detector) as described previously (Gehrke and Kuo 1989), and individual spectra of nucleosides were used to confirm the assignments and quantify nucleosides as described (Jackman et al. 2003).

Analysis of tRNA levels

Wild-type, *trm44*-Δ, *tan1*-Δ, and *trm44*-Δ *tan1*-Δ homozygous diploid yeast strains were grown in YP+ glycerol media at 25°C and switched to 36.5°C, cells were harvested at specific time points, and 2 μg of small molecular weight RNA from each sample was used for Northern analysis, as previously described (Alexandrov et al. 2006), with 5′ ^{32}P-labeled oligonucleotide probes listed in Table 4.

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Identification of yeast tRNA Um_{44} 2′-O-methyltransferase (Trm44) and demonstration of a Trm44 role in sustaining levels of specific $tRNA^{Ser}$ species

Lakmal Kotelawala, Elizabeth J. Grayhack and Eric M. Phizicky

RNA 2008 14: 158-169