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Identification of the *TRM2* gene encoding the tRNA(m^5U_{54})methyltransferase of *Saccharomyces cerevisiae*

MONICA E. NORDLUND,[1] J.O. MARCUS JOHANSSON,[1] ULRICH VON PAWEL-RAMMINGEN,[1,2] and ANDERS S. BYSTRÖM[1]

[1]Department of Microbiology, Umeå University, 901 87 Umeå, Sweden

ABSTRACT

The presence of 5-methyluridine (m^5U) at position 54 is a ubiquitous feature of most bacterial and eukaryotic elongator tRNAs. In this study, we have identified and characterized the *TRM2* gene that encodes the tRNA(m^5U_{54})methyltransferase, responsible for the formation of this modified nucleoside in *Saccharomyces cerevisiae*. Transfer RNA isolated from *TRM2*-disrupted yeast strains does not contain the m^5U_{54} nucleoside. Moreover, a glutathione S-transferase (GST) tagged recombinant, Trm2p, expressed in *Escherichia coli* displayed tRNA(m^5U_{54})methyltransferase activity using as substrate tRNA isolated from a *trm2* mutant strain, but not tRNA isolated from a *TRM2* wild-type strain. In contrast to what is found for the tRNA(m^5U_{54})methyltransferase encoding gene *trmA*$^+$ in *E. coli*, the *TRM2* gene is not essential for cell viability and a deletion strain shows no obvious phenotype. Surprisingly, we found that the *TRM2* gene was previously identified as the *RNC1*/*NUD1* gene, believed to encode the yNucR endo-exonuclease. The expression and activity of the yNucR endo-exonuclease is dependent on the *RAD52* gene, and does not respond to increased gene dosage of the *RNC1*/*NUD1* gene. In contrast, we find that the expression of a *trm2-LacZ* fusion and the activity of the tRNA(m^5U_{54})methyltransferase is not regulated by the *RAD52* gene and does respond on increased gene dosage of the *TRM2* (*RNC1*/*NUD1*) gene. Furthermore, there was no nuclease activity associated with a GST-Trm2 recombinant protein. The purified yNucR endo-exonuclease has been reported to have an NH_2-D-E-K-N-L motif, which is not found in the Trm2p. Therefore, we suggest that the yNucR endo-exonuclease is encoded by a gene other than *TRM2*.

Keywords: 5-methyluridine; gene expression; modification; *NUC2*; *NUD1*; *RNC1*; tRNA biosynthesis; yNucR endo-exonuclease

INTRODUCTION

During the translational process, tRNA molecules mediate decoding of the mRNAs' genetic information. Transfer RNA is characterized by specific base or ribose modifications, which allow the tRNA to function with high efficiency and flexibility. Modified nucleosides are found in all phylogenetic domains and also in identical positions of the tRNA, which suggests a conserved function of some tRNA modifications (Björk, 1986; Cermakian & Cedergren, 1998). Although most mutants defective in tRNA modifications have been isolated in bacteria (Björk, 1995), several have also been characterized in eukaryotes. In *Saccharomyces cerevisiae*, the following tRNA modification genes have been identified; *TRM1*, encoding the N2,N2-dimethylguanosinetransferase (Ellis et al., 1986); *TRM3*, encoding the putative tRNA Gm18 2′-*O*-ribose methyltransferase (Cavaillé et al., 1999); *TRM4*, encoding the tRNA:m^5C methyltransferase (Motorin & Grosjean, 1999); *MOD5*, encoding the N6-isopentenyladenosinetransferase (Dihanich et al., 1987); *RIT1*, encoding the 2′-*O*-ribosyl phosphate transferase (Åström & Byström, 1994); *PUS1*, *PUS3*/*DEG1*, *PUS4*, encoding pseudouridine synthases (Simos et al., 1996; Becker et al., 1997; Lecointe et al., 1998); and *TAD2/TAD3*, encoding the adenosine deaminase that forms I_{34} in tRNA (Gerber & Keller, 1999). In addition, *gcd10* and *gcd14* mutants have been found to be deficient in 1-methyladenosine (Anderson et al., 1998; Calvo et al., 1999). Finally, a *trm2* mutant lacking the tRNA modification m^5U has been isolated, but the corresponding structural gene, *TRM2*, has not been identified (Hopper et al., 1982). The m^5U modification is present at position 54 in the TψC loop in tRNAs of

Reprint requests to: Anders S. Byström,Department of Microbiology, Umeå University, 901 87 Umeå, Sweden; e-mail: Anders.Bystrom@micro.umu.se.

[2]Present address: Department of Cell and Molecular Biology, Lund University, P.O. Box 94, 221 00 Lund, Sweden.

most eubacteria and in eukaryotic elongator tRNAs. It is one of the most abundant tRNA modifications. Kersten et al. (1981) reported that m^5U_{54}-deficient $tRNA^{Lys}$ was more efficient in an in vitro translation system when compared to the modified tRNA. It was suggested that this effect was due to reduced binding to the ribosomal A-site and an improved translocation process. To clarify whether the methylation of U at position 54 is an important determinant in translation, we set out to isolate the *TRM2* gene and create a *trm2* null allele to study its function in vivo by introducing it into appropriate yeast strains.

In this paper we report the cloning of the *TRM2* gene encoding the tRNA(m^5U_{54})methyltransferase of *S. cerevisiae*. We found that the *TRM2* gene was not essential and a deletion strain showed no obvious growth phenotype, which was in accordance with the phenotype of the *trm2* mutant isolated by Hopper et al. (1982). Others have previously suggested that the *TRM2* gene encoded the yNucR endo-exonuclease (Chow et al., 1992; Sadekova & Chow, 1996; Asefa et al., 1998), which has been referred to as the *NUC2*, *RNC1*, or *NUD1* gene (Chow & Kunz, 1991; Chow et al., 1992; Van Vliet-Reedijk & Planta, 1993). The terms *NUC2* and *RNC1* represent alternative names for the same cloned gene. The *NUC2*/*RNC1* gene was shown to be a cloning artefact that was due to a deletion between *RHO4* and *NUD1* genes, thereby creating a *RHO4-NUD1* gene fusion (Van Vliet-Reedijk & Planta, 1993). Although *NUD1* represents the open reading frame (ORF) separated from *RHO4* and has been used in recent work to denote the gene encoding the yNucR endo-exonuclease (Sadekova & Chow, 1996; Asefa et al., 1998), *RNC1* is used in the gene registry because *NUD1* is the gene symbol for another unrelated gene. To avoid confusion when referring to the yNucR endo-exonuclease we will use the gene symbol *TRM2* (*RNC1/NUD1*). Our data suggest that *TRM2* does not encode the yNucR endo-exonuclease and that it is encoded by another, yet unidentified gene.

RESULTS

Identification of the *S. cerevisiae* tRNA(m^5U_{54})methyltransferase (*TRM2*) gene

A candidate for the *TRM2* gene was identified in a BLAST peptide homology search using the *Escherichia coli* tRNA(m^5U_{54})methyltransferase gene *trmA*$^+$ as a query. The search revealed strong homology between the carboxy terminal end of the TrmA protein and the corresponding domain of the previously identified protein Nud1p, (Fig. 3). *RNC1*/*NUD1* is located on chromosome XI and was suggested to encode a protein with endo-exonuclease activity (Chow et al., 1992; Van Vliet-Reedijk & Planta, 1993; Sadekova & Chow, 1996; Asefa et al., 1998). Because there have been contradictory reports on the genomic organization in the region containing the *RNC1*/*NUD1* gene (Chow et al., 1992; Van Vliet-Reedijk & Planta, 1993), we investigated it using Southern blot and polymerase chain reaction (PCR) analysis (data not shown). Our analysis showed that the genomic organization in strains FY23 and FY86 is the same as proposed by Van Vliet-Reedijk and Planta (1993), with *RHO4* and *NUD1* as two independent reading frames.

The *TRM2* (*RNC1/NUD1*) gene complemented the m^5U deficiency of a *S. cerevisiae trm2* and the *E. coli trmA5* mutation

Plasmid pGKU144 contains a 27-kb DNA fragment from the right arm of chromosome XI and contains the *TRM2* (*RNC1/NUD1*) gene (Van Vliet-Reedijk & Planta, 1993). A 3,555 bp *Eco*RI-*Xba*I fragment containing the *TRM2* and *RHO4* genes was subcloned from plasmid pGKU144 into plasmid pRS316. The resulting plasmid, pRS316-*RHO4-TRM2*, was used to complement the tRNA(m^5U_{54})methyltransferase-deficient *S. cerevisiae trm2* mutant strain 4010-2 (Hopper et al., 1982). Total tRNA from strain 4010-2 transformed with either plasmid pRS316 or pRS316-*RHO4-TRM2* was prepared and analyzed on HPLC. The m^5U was absent in tRNA prepared from the *trm2* mutant carrying plasmid pRS316, but the same strain transformed with pRS316-*RHO4-TRM2* showed a nucleoside with a retention time corresponding to m^5U (Fig. 1, Table 1). Moreover, this nucleoside has a spectrum identical to m^5U (data not shown). A 2,001-bp *Hin*dIII-*Xba*I fragment containing only the *TRM2* gene was subcloned into plasmid pRS316. The resulting plasmid, pRS316-*TRM2*, also complemented the *trm2* mutation (Table 1), showing it was the *TRM2* (*RNC1/NUD1*) gene and not the *RHO4* gene that was responsible for the complementing activity. Furthermore, no m^5U nucleoside was observed in the HPLC elution profiles when tRNA was analyzed from strain 4010-2 containing plasmid pRS316-*RHO4-trm2::LEU2*, which has an entire deletion of the *TRM2* gene, or plasmid pRS316-*RHO4-trm2::HIS3*, which contains an insertion of *HIS3* at the *Bam*HI site at the carboxy terminal of the *TRM2* gene (Table 1). Thus, we conclude that the *TRM2* (*RNC1/NUD1*) gene complements the m^5U deficiency of the *trm2* mutant 4010-2.

In *E. coli*, the *trmA*$^+$ gene encodes the tRNA(m^5U_{54}) methyltransferase and the mutant allele, *trmA5*, present in strain GB1-5-39 results in production of m^5U-deficient tRNA (Björk & Neidhardt, 1975). We used plasmid pRS316-*RHO4-TRM2* to complement the m^5U-deficient strain GB1-5-39. As controls we used plasmid pRS316 and the prokaryotic *trmA*$^+$ gene on plasmid pRS316. Strain GB1-5-39 was transformed with plasmids pRS316, pRS316-*trmA*$^+$, or pRS316-*RHO4-TRM2*, and total tRNA was prepared and analyzed on HPLC. As expected, no m^5U nucleoside could be detected in tRNA

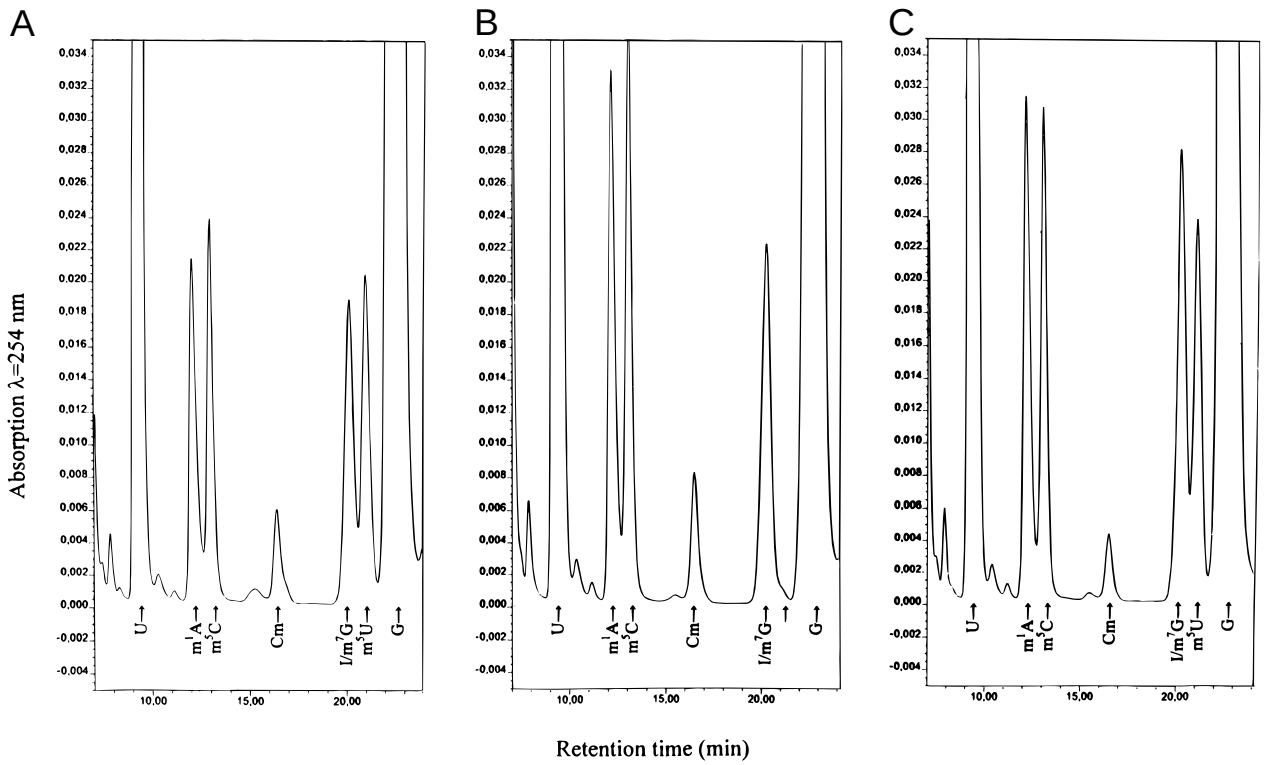

FIGURE 1. RP-HPLC elution profiles of total tRNA preparations from (**A**) strain L1937 *TRM2* carrying pRS316; (**B**) *trm2* mutant strain 4010-2 lacking m^5U (Hopper et al., 1982) carrying pRS316; and (**C**) strain 4010-2 carrying pRS316-*RHO4-TRM2*. Only peaks with retention times between 8 and 24 min are shown. Nucleosides were identified by their relative retention times and UV spectra. Peaks corresponding to m^5U are indicated by m^5U marked arrows; in profiles lacking m^5U the absence is indicated by arrow.

analyzed from strain GB1-5-39 transformed with pRS316. In contrast, analysis of tRNA from the strain transformed with pRS316-*RHO4-TRM2* revealed a nucleoside with the retention time and UV spectrum identical to that of m^5U, and this nucleoside was present at approximately 10% of the amount of tRNA derived from pRS316-*trmA*$^+$-transformed GB1-5-39 (Table 1). Although, the relative amount of m^5U nucleoside present in strain GB1-5-39 transformed with pRS316-*RHO4-TRM2* was low, these results showed that *TRM2* (*RNC1/NUD1*) is the structural gene for the *S. cerevisiae* yeast tRNA(m^5U_{54})methyltransferase enzyme and demonstrates the conservation of this activity between species. We have therefore decided to rename gene *RNC1/NUD1* to *TRM2* as suggested by Hopper et al. (1982).

Sequence analysis of the *TRM2* gene

The m^5U_{54} tRNA methylation reaction was shown to be similar to that of other enzymes that catalyze the transfer of hydroxyl or methyl groups to pyrimidine substrates (Bellisario et al., 1976; Wu & Santi, 1987; Osterman et al., 1988). In all reactions a cysteine residue has been implicated as the catalytic nucleophile and for the *E. coli* TrmA protein, Cys324 was identified as the catalytic cysteine (Kealey & Santi, 1991). Despite the homologies between the C-terminal portions of the Trm2 and TrmA proteins, we could not identify a potential catalytic cysteine in the proposed protein-encoding region of the *TRM2* gene. However, in another reading frame we detected a region that showed homology to the catalytic cysteine region of the TrmA protein. Therefore we decided to sequence the *TRM2* gene again. The sequence analysis confirmed the previously published DNA sequence (Van Vliet-Reedijk & Planta, 1993), except for an additional adenine residue inserted in the *TRM2* sequence at position 1534 (calculated from the start codon AUG3; Fig. 2). This insertion results in the substitution of isoleucine 511 to an aspargine and extends the reading frame by 22 amino acids. The longer ORF included the potential catalytic cysteine residue in position 521 (Fig. 3). The amino acid sequence L/I/V-V/L-E/D-V/I-G-C/G-G-P/T-G has been suggested to be a consensus sequence for motif I of the *S*-adenosylmethionine (AdoMet) binding site (Kagan & Clarke, 1994). As part of the AdoMet binding site located an average of 13 amino acids from motif I, there is another consensus motif post-I, h-h-X-h-D/E, in which h corresponds to a hydrophobic and X to any amino acid (Niewmierzycka & Clarke, 1999). The se-

TABLE 1. Quantification of the m^5U nucleoside assayed by HPLC analysis of tRNA from *S. cerevisiae* and *E. coli* strains, containing indicated plasmid derivatives.

Strains	Plasmid construct	m^5U/t^6A
UMY1901 (*TRM2*)	none	2.0 ± 0.2
UMY1902 (*trm2::LEU2*)	none	<0.01
UMY1903 (*TRM2*)	none	1.6 ± 0.1
UMY1904 (*trm2::LEU2*)	none	<0.01
4010-2 (*trm2*)	pRS316	<0.01
	pRS316-*RHO4-TRM2*	1.4 ± 0.3
	pRS316-*TRM2*	1.4 ± 0.1
	pRS316-*RHO4-trm2::LEU2*	<0.01
	pRS316-*RHO4-trm2::HIS3*	<0.01
	pRS316-*RHO4-TRM2*-UUG1	1.3 ± 0.3
	pRS316-*RHO4-TRM2*-UUG2	1.4 ± 0.2
	pRS316-*RHO4-TRM2*-UUG3	1.4 ± 0.2
	pRS316-*RHO4-TRM2*-AUG2/-1fr	1.4 ± 0.2
	pRS316-*RHO4-TRM2*-AUG3/-1fr	0.19 ± 0.08
	pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG4	0.19 ± 0.01
	pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG5	0.26 ± 0.11
	pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG4-UUG5	<0.01
GB1-5-39 (*trmA5*)	pRS316	<0.01
	pRS316-*trmA*$^+$	10.6
	pRS316-*RHO4-TRM2*	0.98

The *S. cerevisiae trm2* and *E. coli trmA5* mutations results in m^5U deficient tRNA. The peak corresponding to m^5U was correlated to the t^6A peak and the number given represents the absorption ratio m^5U/t^6A. The absence of the m^5U nucleoside is indicated by <0.01, which represents the detection limit. The quantification of the m^5U nucleoside in tRNA prepared from yeast is based on three to five independent experiments and the standard deviation is indicated. Only one representative value is shown when tRNA was prepared from *E. coli* strains.

quence analysis of the Trm2p revealed a potential AdoMet binding site, having motif I and spaced by 12 amino acids from a post-I motif. Motif I is located between amino acids 422 and 430, with the sequence L-V-D-A-Y-C-G-S-G and the post-I motif V-I-G-V-E at amino acids 443 through 447 (Fig. 3). Motif I is homologous to the suggested AdoMet binding site of the TrmA protein, that is, L-L-E-L-Y-C-G-N-G (Gustafsson et al., 1991). Residues that differ from the consensus sequence are underlined and found in the same position. Despite the different sizes of the Trm2 and TrmA proteins, the distance between the potential AdoMet binding site and the catalytic cysteine residue is similar in both proteins, that is, 94 amino acids in Trm2p and 104 in TrmAp.

Transcriptional and translational start sites

Sequence analysis of the *TRM2* gene revealed an ORF encoding a peptide of 73 kDa. However, we found a number of in-frame AUG codons that could potentially act as translational start codons in vivo. To determine which AUG start codon was used, we first investigated the transcriptional start sites of the *TRM2* gene. To accomplish this, we performed primer extension analysis of total RNA using two different oligonucleotides as primers (Göransson et al., 1989). Three major primer extension products, potentially representing transcriptional start sites, were observed and are identified by asterisks in Figure 2. All transcriptional start sites are located 3′ of the first AUG codon of the longest ORF (Fig. 2). Two of the major presumptive transcriptional start sites are in close proximity to the second AUG codon; one 17 nt upstream and one 2 nt downstream. A third major transcriptional start is located 31 nt downstream of the second AUG codon and is succeeded by three additional in-frame AUG codons (Fig. 2). To identify the physiological start codon of the Trm2 protein, we independently changed the first, the second, and the third AUG of the ORF to UUG codons on plasmid pRS316-*RHO4-TRM2*, generating plasmids pRS316-*RHO4-TRM2*-UUG1, pRS316-*RHO4-TRM2*-UUG2, and pRS316-*RHO4-TRM2*-UUG3. The mutated plasmid constructs were transformed into the m^5U-deficient strain, 4010-2, and total tRNA was analyzed by HPLC. All three constructs complemented the m^5U-deficient strain 4010-2, suggesting that none of these three AUG codons acted as translational start codons (Table 1). However, the majority of the translational initiation events in eukaryotes are mediated by scanning the mRNA from the 5′ end and the first AUG acts as start a codon (Kozak, 1989). Thus, we considered a model in which either AUG2 or AUG3 still could be the correct start codon and the introduction of UUG would allow scanning to continue to a downstream AUG codon to produce a functional Trm2p. Because no transcriptional

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                                        I
-329 AAGTACTAAT TCGATATTAA TCTAGCCTAA TTACATTAAA TACTTAATAA

-279 AAGCTTCTGT GACATAAAAG TACAAATCTG TCATTTTATT TTAGAGGAAT

                                        I
-229 AGTTTAGGAC AAAGTCATTA TGTACGAACA GTTTGAATTT TCTTTTTTTT

-179 TCTTCGAAAA TTCAGACAAT AAGGTTAAAT ATAAAGCTCA TCTCATCTCA

                         *                      II  *
-129 TCGATAAAAC GCTGGAGTAT TATCACATGC ATGCGTTGCT TTTGGACCGT

                     *
-79  ACAGAAGTCT ATATTTAAAG CTAGGTTTTT CGCTTGCAGA AACTTTGTCA

                                          III                    IV
-29  AGAAGCATAA TTATAAACTA ATCAGCACCA TGACTGGAAG TACTGAAATG

                       V
 22  GTACCACCAA CAATGAAACA ----------------//----------------
1522 TAGCCGCATA TAATCCAGCC AAGATTATTT ACATATCGTG TAATGTCCAT
1572 TCCCAGGCAC GTGATGTCGA GTACTTCCTC AAAGAAACAG AAAACGGTTC
1622 CGCCCACCAG ATTGAAAGCA TAAGAGGATT TGATTTCTTT CCACAAACGC
1672 ACCACGTTGA GAGTGTGTGT ATAATGAAGA GAATCTAATT GCACATATAA
1722 AATATCTATC ACTATGTAT
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FIGURE 2. Nucleotide sequence of the 5′ and 3′ regions of the *TRM2* gene. The termination codon and the first five in-frame AUG codons are printed in bold and numbered in Roman numerals. The numbering of the sequence is correlated to the A residue of the major physiological start AUG (+1) for the tRNA(m^5U_{54})methyltransferase, which is the third AUG codon. Asterisks above the DNA sequence mark potential transcriptional start sites. The extra adenine residue at position 1534, which leads to a frame shift and extends the Trm2p with 22 amino acids compared with the sequence of Van Vliet-Reedijk & Planta (1993), is shown in bold. The DNA sequence of the *TRM2* gene appears in the EMBL database under the accession number AJ250970.

start site was identified 5′ of the AUG1 codon (Fig. 2), we did not consider this codon as a potential translation start site of the Trm2 protein. To test this model, we introduced single-nucleotide insertions behind the second and third in-frame AUG codons (pRS316-*RHO4-TRM2*-AUG2/-1fr and pRS316-*RHO4-TRM2*-AUG3/-1fr). If one of these two codons would represent the physiological start codon, the ribosome would initiate translation but terminate after eleven or four amino acids, respectively, due to stop codons in the −1 frame. The m^5U nucleoside was present in tRNA when plasmid pRS316-*RHO4-TRM2*-AUG2/-1fr was transformed into strain 4010-2 (Table 1). However, when total tRNA was analyzed from strain 4010-2 carrying plasmid

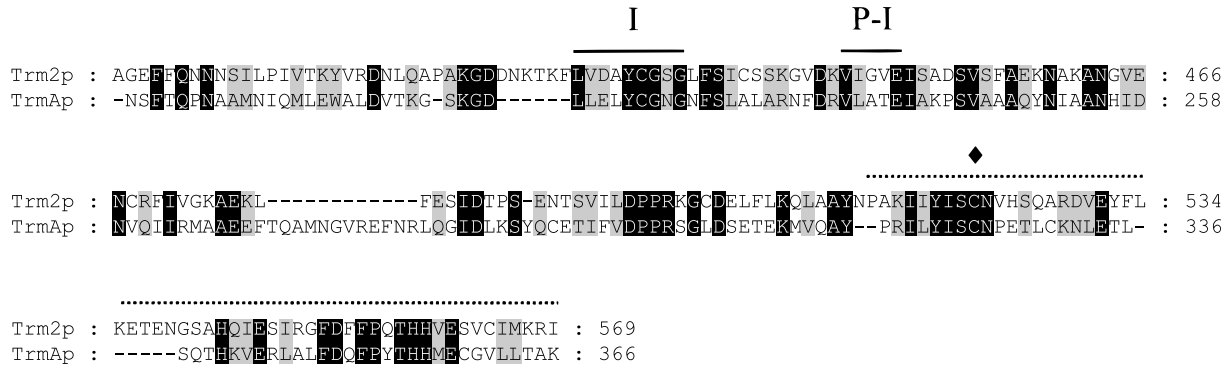

FIGURE 3. C-terminal homology between amino acids 386 and 569 of the *S. cerevisiae* Trm2 protein and amino acids 186 and 366 of the *E. coli* TrmA protein. The potential motif I (I) and post I (P-I) of the AdoMet binding site are overlined. The catalytic cysteine residue is marked with a diamond. Amino acids of the corrected C-terminus are indicated with a dashed line over the sequence. Identical and similar residues are shown in black and grey, respectively. In the BLAST2 sequence search (Tatusova & Madden, 1999), 32% identity and 51% similarity was obtained between amino acids 411 and 561 and 210 and 358 of the Trm2 and TrmA proteins, respectively, and the remaining alignment was refined manually.

pRS316-*RHO4-TRM2*-AUG3/-1fr, the m^5U nucleoside was only present in ~15% of the wild-type amounts (Table 1). These results showed that the third AUG codon acts as a start codon (see Fig. 2). The residual tRNA(m^5U_{54})methyltransferase activity observed from construct pRS316-*RHO4-TRM2*-AUG3/-1fr may be caused by a translational termination of the ribosome at the stop codon in the −1 frame and a reinitiation of translation at the neighboring fourth or fifth in-frame AUG codon. Alternatively, a translational initiation could occur at AUG4 or AUG5 using a minor transcript. To test the significance of AUG4 and AUG5, we constructed double mutants containing the −1 frameshift after the third AUG codon and a UUG codon instead of the fourth or the fifth AUG codon, and a triple mutant (pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG4, pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG5, and pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG4-UUG5). HPLC analysis of total tRNA from strain 4010-2 containing either pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG4 or pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG5 revealed ~15% of wild-type amounts of m^5U nucleoside in the tRNA. However, when transformed with the triple mutant plasmid pRS316-*RHO4-TRM2*-AUG3/-1fr-UUG4-UUG5, no m^5U was detected in the analysis (Table 1). We conclude that the third in-frame AUG acts as a major translational start site of the *TRM2* mRNA and that AUG4 and AUG5 are responsible for the remaining 15% of m^5U observed in the AUG3/-1fr mutant. The ORF starting at AUG3 would generate a peptide with the molecular weight of 64.2 kDa. However, we cannot exclude that longer and shorter minor forms of the Trm2 protein are also produced from the second, fourth, or fifth AUG codon (Fig. 2).

TRM2 was not essential

Hopper et al. (1982) studied the physiological consequences of the m^5U_{54} deficiency in *S. cerevisiae*. They could not detect any difference in growth rates between *trm2* mutant and *TRM2* wild-type cells and the wild type did not show a competitive advantage over *trm2* cells when cocultured in rich media for 35 generations (Hopper et al., 1982). However, an *E. coli* mutant deficient for m^5U_{54} in its tRNA exhibits a 4% growth reduction and is outgrown when cocultured with wild-type cells in minimal medium (Björk & Neidhardt, 1975; Björk, 1986). Furthermore, the *E. coli trmA*$^+$ gene was shown to be essential for cell viability, although the known catalytic activity of the tRNA(m^5U_{54})methyltransferase was not (Persson et al., 1992).

To determine the null phenotype of the *trm2* gene, we used a *trm2::LEU2* plasmid disruption construct in which almost the entire ORF of the *TRM2* gene is replaced by a functional *LEU2* gene (see Materials and methods). In a diploid formed between the haploid strains FY23 and FY86, we replaced one chromosomal copy of the *TRM2* gene using this construct. Tetrad analysis of the resulting heterozygous *TRM2/trm2::LEU2* diploid strain showed that in all 20 tetrads examined, four viable spores were obtained and that the *LEU2* marker segregated 2:2. Total tRNA of a tetrad was prepared (strains UMY1901–UMY1904) and analyzed by HPLC. We found that the *LEU2* marker correlated with the m^5U deficiency (Table 1). Because we obtained four viable spores in all tetrads, we concluded that the *TRM2* gene is not essential for cell viability.

We investigated the strains derived from this tetrad for growth at different temperatures (17, 26, 30, and 37 °C), on minimal and rich plates (Rose et al., 1990) and for mitochondrial dysfunction on nonfermentable carbon sources, that is, YEP plates with 2% glycerol and 3% ethanol (Yaffe, 1991). We did not observe any growth difference in either case between *TRM2* wild-type and *trm2* mutant cells (data not shown).

Role of the m^5U_{54} modification in initiator and elongator tRNA identity

Eukaryotic initiator tRNAs have a conserved adenosine in position 54 of the TψC loop, whereas eukaryotic elongators have m^5U. Previously we showed that a $tRNA_i^{Met}$ containing C_{60}-U/m^5U_{54} lost the ability to initiate, but gained ability to elongate during protein synthesis. This suggests that when U or m^5U is placed in position 54 of an initiator tRNA, it acts as an antideterminant in initiation of protein synthesis (von Pawel-Rammingen et al., 1992; Åström et al., 1993). Introduction of a C_{60}-U/m^5U_{54} into $tRNA_i^{Met}$ would make the mutant $tRNA_i^{Met}$ a substrate for the tRNA(m^5U_{54}) methyltransferase (von Pawel-Rammingen et al., 1992; Kealey et al., 1994). Furthermore, the initiator-specific adenosine introduced at position 54 of elongator methionine tRNA ($tRNA_i^{Met}$) lowered the activity of this tRNA in the elongation of translation, suggesting that a U or a m^5U at position 54 is important for optimal elongator tRNA function (Åström et al., 1993). To determine whether the methylation of U_{54} is important in initiator/elongator tRNA discrimination, we first introduced a *trm2* null allele into a yeast strain in which the effect of one single mutated initiator tRNA gene can be investigated (von Pawel-Rammingen et al., 1992). An initiator $tRNA_i^{Met}$ with the C_{60}-U_{54} mutation could not act as an initiator tRNA, either in the *TRM2* or the *trm2* background (data not shown). Thus, we conclude that the methyl group of m^5U_{54} as such is not an antideterminant in initiation of translation.

A strain with four of the five *EMT* genes disrupted has a reduced growth rate, that is, 71% compared to that of wild type (Åström et al., 1993). We investigated the effect on growth of introducing a *trm2::LEU2* allele into this strain and comparing it with its isogenic *TRM2* derivative. We could not detect any difference in growth between these two strains. Thus, strains with reduced

translational capacity due to low gene dosage of *EMT* genes do not exhibit an increased growth defect when lacking the m^5U_{54} modification (data not shown).

A heterologously expressed Trm2p has tRNA(m^5U_{54})methyltransferase activity

To establish tRNA(m^5U_{54})methyltransferase activity for the Trm2 peptide, we cloned the *TRM2* gene into the expression plasmid pGEX-4T-2 and expressed a GST-tagged version of the protein, which subsequently was purified to near homogeneity (Fig. 4; see Material and methods). We investigated the tagged protein for in vitro tRNA methyltransferase activity using various amounts of total tRNA prepared from strains UMY1901 (*TRM2*) or UMY1902 (*trm2::LEU2*) as substrates. We found that it efficiently methylated tRNA, lacking the m^5U modification, but it was not active on preparations of fully modified tRNAs from the *TRM2* strain (Fig. 5). The maximum incorporation of methyl groups into tRNA prepared from strain UMY1902 (*trm2::LEU2*) was 31 pmol/μg. As nearly all tRNAs contain m^5U_{54}, initiators excluded, we estimated that 78% of total tRNA chains were methylated by the GST-Trm2 protein in the in vitro assay.

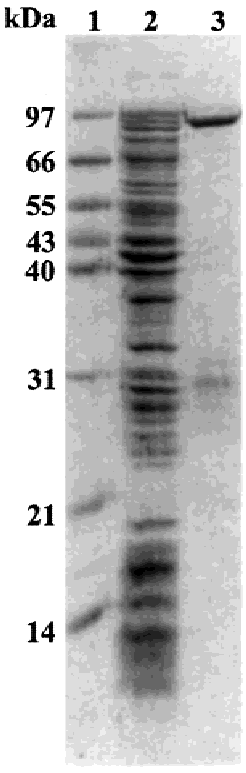

FIGURE 4. Electrophoresis of affinity purified GST-Trm2 protein on a 10% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1: molecular weight standard. Lane 2: *E. coli* crude extract with overexpressed GST-Trm2 protein. Lane 3: Affinity purified GST-Trm2 protein eluted from a glutathione sepharose column with glutathione.

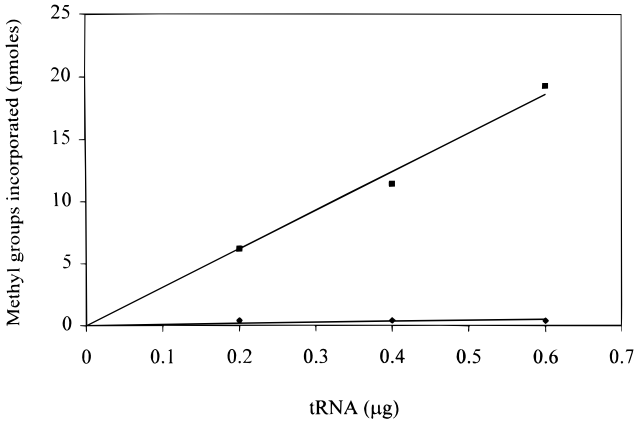

FIGURE 5. The extent of methylation as a function of the amount of tRNA. Substrates were total tRNA preparations from strains UMY1901 (*TRM2*) (♦) and UMY1902 (*trm2::LEU2*) (■). The enzyme used was affinity purified recombinant GST-Trm2p. Conditions for the methylation reaction and sample treatment are described in Materials and methods.

Although m^5U has so far only been found in position 54 of tRNA (Sprinzl et al., 1999), we wanted to investigate if the GST-Trm2 protein catalyzed incorporation of methyl groups in this position. Methylation reactions containing UMY1902 (*trm2::LEU2*) total tRNA and crude protein extracts of GB1-5-39 (*trmA5*) or BL21/pMTRX1 were performed. The *trmA5* allele of GB1-5-39 encodes an inactive *E. coli* tRNA(m^5U_{54})methyltransferase unable to modify U_{54} to m^5U_{54} (Björk & Neidhardt, 1975). Plasmid pMTRX1 contains a wild-type *TrmA* gene regulated by the *tac* promoter (Gu & Santi, 1990). UMY1902 (*trm2::LEU2*) tRNA incorporated methyl groups with the BL21/pMTRX1 protein extract containing the active tRNA(m^5U_{54})methyltransferase but not with the inactive tRNA(m^5U_{54})methyltransferase of the GB1-5-39 (*trmA5*) extract (Fig. 6). Purified GST-Trm2 protein was added to part of the above reactions and the incubation was prolonged. In this case only a minor incorporation was obtained in the reaction preincubated with BL21/pMTRX1 protein extract, whereas a significant incorporation was obtained in the reaction preincubated with GB1-5-39 (*trmA5*) extract (Fig. 6). In a similar experiment UMY1901 (*TRM2*) tRNA did not accept methyl groups using the BL21/pMTRX1 protein extract (data not shown). Thus, an active *E. coli* tRNA(m^5U_{54})methyltransferase with known specificity will incorporate methyl groups into UMY1902 (*trm2::LEU2*) tRNA but not UMY1901 (*TRM2*) tRNA. Furthermore, the GST-Trm2 protein only significantly incorporates methyl groups in UMY1902 (*trm2::LEU2*) tRNA that have not been preincubated with active *E. coli* tRNA(m^5U_{54})methyltransferase. To confirm the nature of the product, UMY1901 (*TRM2*) tRNA, UMY1902 (*trm2::LEU2*) tRNA, and tRNA of an in vitro methylation reaction using GST-Trm2p, UMY1902 (*trm2::LEU2*) tRNA and nonradioactive S-adenosyl-L-methionine was

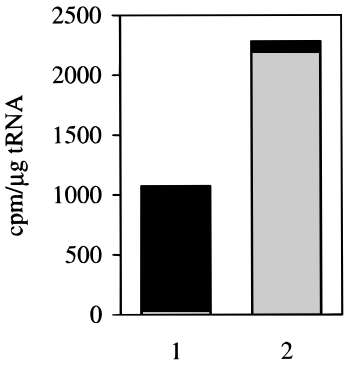

FIGURE 6. The GST-Trm2p methylates position U_{54} of tRNA. UMY1902 (*trm2::LEU2*) tRNA was incubated for 2 h with GB1-5-39 (*trmA5*) (lane 1) and BL21/pMTRX1 (lane 2) crude protein extracts. Samples were withdrawn and incorporation of ^{14}C-methyl groups was determined (grey bars). Purified GST-Trm2p was added to remaining samples and incubation continued for another 2 h and incorporation of ^{14}C-methyl groups was determined (black bars). Values shown are an average of two measurements and corrected for unspecific incorporation of the GB1-5-39 (*trmA5*) and BL21/pMTRX1 crude protein extracts. Conditions for the methylation reaction and sample treatment are described in Materials and methods.

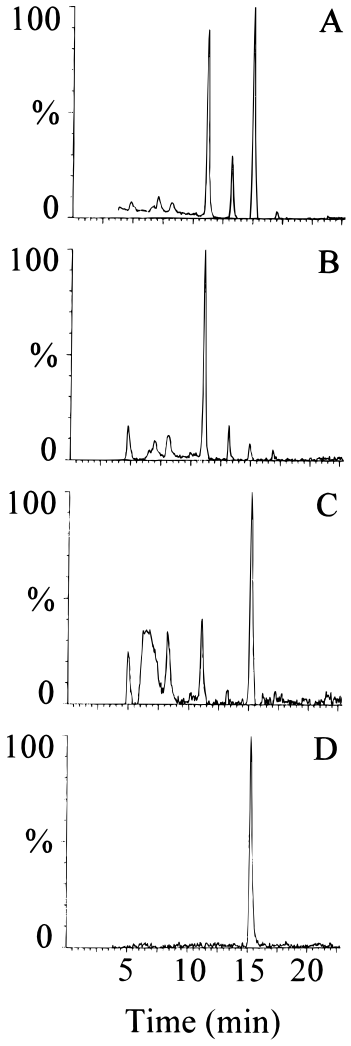

FIGURE 7. HPLC-coupled MS analysis of m^5U in tRNA. Identification of m^5U in tRNA using selected ion profile for the protonated nucleoside, *m/z* 259. Relative levels of protonated nucleosides *m/z* 259 is plotted as a function of elution time. **A**: tRNA from strain UMY1901 (*TRM2*). **B**: tRNA from strain UMY1902 (*trm2::LEU2*). **C**: tRNA from strain UMY1902 (*trm2::LEU2*) in vitro methylated with GST-Trm2p. **D**: Five nanomoles each of nucleosides guanosine (G), uridine (U), cytosine (C), pseudouridine (ψ) and 5-methyluridine (m^5U).

subjected to combined HPLC–mass spectrometry (MS). To detect the presence of m^5U in tRNA, MS analysis in the form of selected-ion recordings was carried out and the ion used for detection of m^5U was *m/z* 259. The analyses revealed that tRNA from strain UMY1902 (*trm2::LEU2*) was missing one peak in the HPLC and MS profiles compared to UMY1901 (*TRM2*) (Fig. 7 and data not shown). This peak present in UMY1901 (*TRM2*) tRNA has the retention time and spectra of m^5U using HPLC analysis and a *m/z* of 259 as analyzed by MS, which corresponds to a protonated m^5U nucleoside (Fig. 7). UMY1902 (*trm2::LEU2*) tRNA in vitro methylated with GST-Trm2p restored the missing peak both in the HPLC and MS analysis and it corresponds to the peak obtained with m^5U (Fig. 7). In conclusion, the GST-Trm2p methylated U at position 54 and the product has the retention time, spectra, and molecular weight of the m^5U nucleoside.

The finding that *TRM2* is allelic to the potential endo-exonuclease gene *RNC1*/*NUD1* was unexpected; therefore we investigated if the GST-Trm2 protein fusion had deoxyribonuclease activity. The deoxyribonuclease assays were performed essentially according to Chow and Resnick (1987) using superhelical YEp351 plasmid or ss-M13mp19 as substrates. In a total reaction volume of 300 μL, we added 6 μg of plasmid and 5–50 μg of purified GST-Trm2 protein. The reaction was incubated at 37 °C and samples of 50 μL were taken out at time intervals and analyzed on agarose gels (data not shown). In some preparations we found no degradation of the plasmid DNA whereas in others, the efficiency of the degradation varied dramatically. When we used the same amount of purified GST protein lacking the Trm2 moiety, the same result was obtained, that is, from no degradation to varying efficiency of degradation. From these experiments, we concluded that the GST-Trm2p did not have nuclease activity and when nuclease activity was observed it was due to contaminating proteins from *E. coli*.

A *trm2-LacZ* fusion and the tRNA(m^5U_{54}) methyltransferase activity showed gene dosage response but no *RAD52* dependence of in exponentially growing cells

Others have previously described the *TRM2* gene to be the *RNC1/NUD1* gene (Chow et al., 1992; Van Vliet-Reedijk & Planta, 1993; Sadekova & Chow, 1996; Asefa

et al., 1998). The *RNC1/NUD1* gene was suggested to encode a protein with endo-exonuclease activity, yNucR, that was identified using antibodies raised against a *Neurospora crassa* endo-exonuclease. In an exponentially growing *rad52* mutant, only about 20% yNucR endo-exonuclease activity was precipitated by the antibodies compared to a *RAD52* strain and the difference was suggested to be at the expression level (Chow & Resnick, 1988). We decided to investigate a possible dependence of the allelic state of the *RAD52* gene on *TRM2* gene expression and the activity of its product, the tRNA(m^5U_{54})methyltransferase. The expression of the *TRM2* gene was measured in *rad52* mutant and *RAD52* wild-type strains by assaying β-galactosidase activities of a *trm2-lacZ* fusion plasmid integrated at the *TRM2* locus (see Materials and methods). Furthermore, we used the same extract to measure the specific activity of the tRNA(m^5U_{54})methyltransferase. In cells growing exponentially in synthetic complete media (SC) lacking uracil, we found no difference between the isogenic *rad52* mutant or *RAD52* wild-type cells with respect to *trm2-lacZ* expression or the specific activity of the tRNA(m^5U_{54})methyltransferase (Table 2). These data are not consistent with the data for the yNucR endo-exonuclease (Chow & Resnick, 1988), where the reported amount of DNase activity precipitated with the antibodies was dependent on the *rad52* genotype. Furthermore, we examined the level of the m^5U nucleoside in tRNA from a *rad52* mutant strain using HPLC analysis and did not observe any effect on the modification level (data not shown).

The amount of yNucR endo-exonuclease activity precipitated by the antibodies only increased twofold in a yeast strain having a cloned *TRM2* (*RNC1/NUD1*) gene on a YEp high copy vector, whereas the *TRM2* (*RNC1/NUD1*) mRNA increased tenfold or more (Sadekova & Chow, 1996). We introduced a YEp-*trm2-LacZ* or YEp-*TRM2* plasmid into strain L1937 and measured the β-galactosidase and the tRNA(m^5U_{54})methyltransferase specific activity in exponentially growing cells. For these two transformed strains, we observed a 19-fold and 10-fold increase, respectively (Table 2). In summary, the yNucR endo-exonuclease suggested to be encoded by the *TRM2* (*RNC1/NUD1*) gene is not regulated as the *trm2-lacZ* fusion or the tRNA(m^5U_{54}) methyltransferase specific activity with respect to the status of the *RAD52* allele or increased expression of mRNA from the *TRM2* (*RNC1/NUD1*) gene.

Involvement of the Nud1p/Trm2p in DNA metabolism

Because of the dependence of the *RAD52* gene product (Chow & Resnick, 1988), the elevated levels of immunoprecipitable nuclease in meiosis (Resnick et al., 1984), and the observed cross-reactivity of the *N. crassa* antibodies to the *E. coli recC* gene product (Fraser et al., 1990), the yNucR endo-exonuclease was proposed to be important in DNA recombination and/or DNA repair (Chow & Kunz, 1991; Moore et al., 1993; Sadekova & Chow, 1996; Asefa et al., 1998). *RAD52* and other members of the *RAD52* epistasis group are required for X-ray damage repair and various types of intra- and interchromosomal mitotic recombination repair. *RAD52* is also required for repair of double-stranded breaks during induction of HO-endonuclease and is essential during sporulation (Friedberg et al., 1995).

To investigate the potential role of the Trm2p in recombination or DNA repair, we performed a set of assays in isogenic *TRM2* and *trm2::LEU2* strains to measure the spontaneous mutation frequencies and the recombination efficiency. We measured the increase in canavanine resistance, Can^R, reversion or suppression of the *hom3-10* allele having a T insertion in the structural gene and the *lys2-Bgl* allele having a

TABLE 2. *RAD52* and gene dosage dependence of a *trm2-LacZ* fusion and the tRNA(m^5U_{54})methyltransferase activity in exponentially growing cells.

Strain	Units of β-galactosidase	Specific activity of tRNA(m^5U_{54})methyltransferase
UMY2229 *RHO4::trm2-LacZ* (*RAD52*)	6.1 ± 0.3	6.1 ± 1.1
UMY2235 *RHO4::trm2-LacZ* (*rad52*)	5.9 ± 0.1	6.1 ± 1.0
L1937 YEp352-*TRM2* (*RAD52*)	n.a.[a]	63 ± 19
L1937 YEp358-*trm2-LacZ* (*RAD52*)	116 ± 19	n.d.[b]

The cells were exponentially grown in synthetic complete (SC) medium lacking uracil and harvested at approximately $OD_{600} = 0.5$. The values for UMY2229 and UMY2235 are based on five independent experiments, whereas, the values on L1937 YEp352-*TRM2* and L1937 YEp358-*trm2-LacZ* are based on four independent transformants. The standard deviation is indicated. The tRNA(m^5U_{54})methyltransferase-specific activities are given in microunits per milligram of protein. One enzymatic unit transfers 1 μmol of methyl group to tRNA per minute at 37 °C. Strains L1937, UMY2229, and UMY2235 are isogenic except for the *rad52* and *RHO4::trm2-LacZ* alleles.

[a] n.a.: not applicable.

[b] n.d.: not determined.

+4 frameshift mutation (Marsischky et al., 1996). No difference in mutation frequencies was observed for any of these alleles in *TRM2* and *trm2::LEU2* strains (data not shown). We measured the mitotic recombination frequencies between two tandem direct repeats of truncated alleles of the *ade2* gene separated by an *URA3* gene (Fiorentini et al., 1997) and the nonallelic *ade2* sequences located on homologous chromosomes in diploids (Bai & Symington, 1996). In neither strain did a *trm2::LEU2* allele influence recombination frequencies (data not shown). We also investigated isogenic *TRM2* and *trm2::LEU2* strains for their tolerance to X-ray, ultra violet (UV) light, and MMS (methylmethan-sulfonate), and observed no difference in sensitivity.

The levels of immunoprecipitated yNucR endo-exonuclease, increased within the first hour of meiosis in a *RAD52* diploid strain but not in *rad52* homozygous diploids (Resnick et al., 1984; Chow & Resnick, 1988). The *RAD52* is an essential gene for sporulation and affects the activity, most likely at the expression level, of the yNucR endo-exonuclease (Resnick et al., 1984; Chow & Resnick, 1988; Chow et al., 1992). Because *TRM2* was identical to *RNC1/NUD1*, we argued that meiosis might be deleterious in a *trm2* homozygous diploid. Therefore, we investigated the ability of a *trm2::LEU2* homozygous diploid, formed between UMY1902 and UMY1904, to go through meiosis. No difference in spore survival was observed when compared with the cross-generating strains UMY1901 to UMY1904 (data not shown). Furthermore, during the first half hour of sporulation, the *TRM2* (*RNC1/NUD1*) transcript decreases threefold (Chu et al., 1998). Thus, we concluded that the Trm2p is not essential for meiosis and the reported increase in yNucR endo-exonuclease activity (Resnick et al., 1984; Chow & Resnick, 1988) did not correlate with an increase of *TRM2* (*RNC1/NUD1*) mRNA.

The *HO* gene codes for a site-specific endonuclease that will generate a double-strand break at the mating type (*MAT*) locus. Strains defective in the *RAD52* gene are not viable in the presence of the *HO* gene (Malone & Esposito, 1980). Recently, Asefa et al. (1998) demonstrated that prolonged induction of HO endonuclease in a *trm2 (rnc1/nud1)* null strain showed 9% viability when compared with isogenic *TRM2* (*RNC1/NUD1*) strain. Furthermore, a strain with both the *rad52::URA3* and the *trm2 (rnc1/nud1)* null allele shows the same viability as the strain with the *trm2 (rnc1/nud1)* null allele, suggesting that the *trm2 (rnc1/nud1)* mutation is suppressing the deleterious effects of the *rad52* mutation. As we have been unable to link the *TRM2* gene product with a nuclease activity, we decided to repeat this experiment in the SK1 strain background, which is the genetic background used earlier to define the yNucR endo-exonuclease (Resnick et al., 1984; Chow & Resnick, 1987; 1988; Chow & Kunz, 1991; Chow et al., 1992; Moore et al., 1993; Sadekova & Chow, 1996; Asefa et al., 1998). Isogenic derivatives of *rad52::hisG*, *trm2::LEU2*, and the combination thereof were constructed in the SK1 derivative, S2889, and transformed with the pGAL-HO plasmid, pSB283. However, no effect of the *trm2::LEU2* allele, either alone or in combination with the *rad52::hisG* allele under prolonged *HO* induction, was observed (Table 3). We also investigated the effect of prolonged *HO* induction in S288C background in isogenic *TRM2* and *trm2::LEU2* derivatives and observed no difference in survival (data not shown).

DISCUSSION

The nucleoside m^5U at position 54 in most bacterial and eukaryotic tRNAs is one of the most abundant nucleoside modifications. Still, it has been difficult to establish a biological role for this modification. In this article we describe the identification and characterization of the structural gene *TRM2*, which encodes the tRNA(m^5U_{54})methyltransferase in *S. cerevisiae*. In contrast to what was found earlier for the tRNA(m^5U) methyltransferase-encoding gene $TrmA^+$ in *E. coli* (Persson et al., 1992), a deletion was not lethal and showed no obvious phenotype. The major homology between the TrmA and the Trm2 proteins is located in the terminal carboxyl region (Fig. 3). This may indicate that the terminal amino part of the TrmA protein provides the essential function in *E. coli*, which is not present in the Trm2p. The *TRM2* gene complemented the m^5U deficiency of a *trm2* mutant strain of *S. cerevisiae*, as well as a *trmA5 E. coli* strain. In addition, total tRNA isolated from a *trm2* mutant strain acts as a substrate in a methylation reaction using purified GST-Trm2p produced in *E. coli* and the enzyme modifies U at position 54 to m^5U.

Alternative translational start sites of the Trm2p

When analyzing the transcriptional and translational start sites of *TRM2*, we found that the major physiological

TABLE 3. The effect of HO-endonuclease expression on cell survival.

Strain	Surviving fraction
S2889	0.96 ± 0.05
UMY2353 (*trm2::LEU2*)	0.97 ± 0.07
UMY2361 (*rad52::hisG*)	0.006 ± 0.001
UMY2362 (*trm2::LEU2 rad52::hisG*)	0.008 ± 0.006

Cells were incubated for at least 72 h at 30 °C. Strain S2889 is an SK1 derivative and strains UMY2353, UMY2361, and UMY2362 are isogenic to this strain. The values are based on three independent transformants and the standard deviation is indicated.

AUG start codon was the third in-frame AUG codon. However, one potential transcriptional start site (weakest of three major transcripts) was located 5′ of the second in-frame AUG codon and there were some minor transcripts that ended upstream of AUG4 and AUG5. Our analysis could not completely exclude that these AUGs also could act as start codons, in addition to the third AUG codon, and perhaps contribute with a small fraction of differently sized Trm2p. Interestingly, some genes in *S. cerevisiae* express two different proteins by using alternative start codons, leading to two different proteins with divergent N-terminal sequences (Hinnebusch & Liebman, 1991). Two of these genes are *TRM1* and *MOD5*, both of which encode tRNA modification enzymes (Rose et al., 1992; Tolerico et al., 1999) and the amino terminal extended protein is imported into the mitochondria. This could also be the case for the Trm2p, as mitochondrial tRNAs contain m^5U. However, the deletion of the *TRM2* gene did not affect growth of the yeast cells on nonfermentable carbon sources, indicating that a functional *TRM2* gene is not essential for normal mitochondrial function. The m^5U modification is found in unspliced tRNAs, suggesting that the action of the tRNA(m^5U_{54})methyltransferase is within the nucleus (Hopper et al., 1982). The presence of a candidate motif for a bipartite nuclear localization signal (Makkerh et al., 1996) at amino acid positions 19–35 with the sequence Lys-Arg-Leu-Ser-Ser-Pro-Leu-Thr-Asp-Ser-Gly-Asn-Arg-Arg-Thr-Lys-Lys is likely how the Trm2p is targeted to this compartment.

Does the Trm2 protein have nuclease activity?

We have identified the *TRM2* gene to be the structural gene for the tRNA(m^5U_{54})methyltransferase of *S. cerevisiae*. The *TRM2* gene was previously cloned and suggested to encode an endo-exonuclease, Rnc1p/Nud1p. This endo-exonuclease activity was identified in *S. cerevisiae* using antibodies made against a 31-kDa enzyme purified from *N. crassa* with the same activities (Chow & Fraser, 1983). The antibodies were also used to purify a endo-exonuclease with these activities from *S. cerevisiae*. After an 850-fold purification, a major band at 72 kDa was obtained by SDS gel electrophoresis (Chow & Resnick, 1987; Chow et al., 1992). This band was extracted from the gel, renatured, and found to retain its nuclease activity. The yeast enzyme was named yNucR. From the purest fraction of the *S. cerevisiae* yNucR enzyme, the amino terminal was determined to be NH_2-D-E-K-N-L (Chow & Resnick, 1987; Chow et al., 1992). Based on the sequence of *NUD1* (Van Vliet-Reedijk & Planta, 1993) and the corrected *TRM2/NUD1* sequence shown here, there is no amino acid motif -D-E-K-N-L present in Trm2p. Thus, if the *TRM2* gene should encode the yNucR endo-exonuclease, it must be a minor constituent of the purified yNucR endo-exonuclease fraction or not be encoded by the *TRM2* (*RNC1/NUD1*) gene. Furthermore, we also considered that the yNucR endo-exonuclease might be encoded by an alternative ORF within the *TRM2* DNA fragment. However, the second longest ORF would encode a protein of 5.7 kDa, excluding this possibility.

We found that an affinity-purified GST-Trm2 protein fusion expressed in *E. coli* had tRNA(m^5U_{54})methyltransferase activity. However, we could not detect any significant nuclease activity from the GST-Trm2 protein when compared to the same amount of purified GST protein (data not shown). However, the difference in our results and the results of Chow and Resnick (1987) could be explained by three points: First, a native Trm2 protein is needed, that is, the GST tag will interfere with the nuclease activity. Second, the Trm2 protein could be posttranslationally modified in yeast to obtain nuclease activity. Third, in all experiments defining the *S. cerevisiae* yNucR endo-exonuclease, antibodies against a purified endo-exonuclease from *N. crassa* was used to precipitate the nuclease activity (Resnick et al., 1984; Chow & Resnick, 1987; 1988; Chow & Kunz, 1991; Chow et al., 1992; Moore et al., 1993; Sadekova & Chow, 1996). Thus, as the antibodies do recognize the *S. cerevisiae* Trm2p (Rnc1p/Nud1p) (Asefa et al., 1998), the yNucR endo-exonuclease could be coprecipitated and copurified with the Trm2p. Alternatively, the antibodies recognize both the Trm2p and the yNucR nuclease and they happened to be in the same size range. One of the two assumptions in the third point must be correct, as the major peptide in the yNucR endo-exonuclease purification using the antibodies has a D-E-K-N-L amino terminal end (Chow & Resnick, 1987; Chow et al., 1992) not present in the Trm2p.

In exponentially growing cells, the expression and activity of the yNucR endo-exonuclease has been reported to be dependent on the allelic state of the *RAD52* gene (Chow & Resnick, 1987; 1988). We find no such dependence when measuring the β-galactosidase activities of a *trm2-LacZ* fusion integrated at the *TRM2* locus or the endogenous tRNA(m^5U)methyltransferase-specific activity. Furthermore, the levels of the m^5U modification in tRNA are not affected in a *rad52* mutant strain. Moreover, it has been shown that the *N. crassa* antibody-precipitated nuclease activity from a yeast strain having a *TRM2* (*RNC1/NUD1*) gene on a high copy YEp vector only increases twofold, whereas the *TRM2* (*RNC1/NUD1*) mRNA increased tenfold or more (Sadekova & Chow, 1996). In contrast, we find that in yeast strains transformed with YEp-*trm2-LacZ* fusion or YEp-*TRM2*, the β-galactosidase activity and the tRNA(m^5U)methyltransferase-specific activity increased 19-fold and 10-fold, respectively. Thus, our *trm2-LacZ* expression data and measurements of tRNA(m^5U) methyltransferase-specific activity does not correlate with the earlier data on yNucR expression and nucle-

ase activity in response of the allelic state of the *RAD52* allele and gene dosage of the *TRM2* (*RNC1/NUD1*) gene. To relate our Trm2p expression and activity data with that of the yNucR endo-exonuclease, one has to consider very complicated explanations. In response to increased gene dosage, only a fraction of the Trm2p peptide will be posttranslationally modified or has to interact with a limiting factor in the cell to obtain nuclease activity. With respect to the allelic state of the *RAD52*, we have to postulate that strains used earlier to identify the yNucR endo-exonuclease contained a modifier of the Trm2p expression and activity and it was not present in the strains used by us. Furthermore, the activity of this modifier should be dependent on the status of the *RAD52* allele.

We have identified the gene encoding the tRNA(m^5U_{54})methyltransferase of *S. cerevisiae* and denoted it, *TRM2*, according to the nomenclature of Hopper et al. (1982). This gene is identical with the *RNC1/NUD1* gene, suggested to encode a endo-exonuclease designated yNucR (Chow et al., 1992; Sadekova & Chow, 1996; Asefa et al., 1998). However, we have been unable to show a nuclease activity associated with the *TRM2* gene product or correlate its expression and the activity of the encoded tRNA(m^5U_{54})methyltransferase to that of the yNucR endo-exonuclease. We have also been unable to repeat experiments concerning the effect of a *trm2 (rnc1/nud1)* null allele on cells after prolonged *HO* nuclease induction. To relate our data on the Trm2p to the earlier data on the yNucR endo-exonuclease, we have to make very complicated and laborious assumptions and explanations that we find highly unlikely. Furthermore, the purified yNucR endo-exonuclease has been reported to have an NH_2-D-E-K-N-L motif, which is not found in the Trm2p. Thus, we suggest that the *TRM2* gene, per se, does not encode the yNucR endo-exonuclease and that it is encoded by another yet unidentified gene.

MATERIALS AND METHODS

Materials

All restriction enzymes and DNA modification enzymes were purchased from Roche Molecular Biochemicals and New England Biolabs. *Pwo* and *Taq* DNA polymerase for PCR amplifications was purchased from Roche Molecular Biochemicals. Hybridizations were carried out on Zeta-Probe membranes from Bio-Rad. Southern blots were visualized using a PhosphorImager (Molecular Dynamics). Sequencing gels were autoradiographed on Amersham MP hyper film. The 5-flouro-orotic acid was purchased from SCM Speciality Chemicals. Adenosine 5′[γ^{32}P]-triphosphate (3,000 Ci/mmol), Adenosine 5′[α^{35} S]-triphosphate (600 Ci/mmol) and S-adenosyl-L-(methyl-14-C)methionine (54 mCi/mmol) were purchased from Amersham. S-adenosyl-L-methionine was purchased from Sigma.

Strains and genetic procedures

Genotypes and sources of *S. cerevisiae* strains used in this study are listed in Table 4. Yeast media and genetic procedures have been previously described (Rose et al., 1990; Gietz et al., 1992). Disruption of the chromosomal *TRM2* gene locus by a *LEU2* selectable marker was performed using a two-step gene replacement method described by Scherer and Davis (1979). All gene disruptions were confirmed by Southern blot hybridization or PCR and HPLC analysis of total tRNA as described below. To target the *trm2::LEU2* disruption to the correct chromosomal locus, a unique *Mlu*NI site was utilized to linearize plasmid pRS306-*RHO4-trm2::LEU2* (Orr-Weaver et al., 1981). One *TRM2* allele was replaced in a diploid derived from a cross between FY23 (*MATa trp1*Δ*63 ura3-52 leu2*Δ*1*) and FY86 (*MAT*α *his3*Δ*200 ura3-52 leu2*Δ*1*). The heterozygous *TRM2/trm2::LEU2* strain was sporulated and subjected to tetrad analysis. Strains UMY1901–UMY1904 are derived from ascospores of one tetrad in which UMY1901 and UMY1903 represent *TRM2* wild-type cells, whereas UMY1902 and UMY1904 represent strains in which *TRM2* was disrupted by a functional *LEU2* gene. To construct strain UMY2229, plasmid YIp358-*RHO4-trm2* containing an in-frame *trm2-LacZ* fusion was linearized with *Mlu*NI and targeted to the *RHO4* gene, leaving the chromosomal *RHO4* and *TRM2* genes intact. Strain UMY2235 was generated in a cross between strains UMY2229 and UMY2230. Strain UMY2230 was constructed using a *rad52::hisG-URA3-hisG* construct (Curcio & Garfinkel, 1994). Strains UMY2353 (*trm2::LEU2*) and UMY2361 (*rad52::hisG*) were generated from SK1 derivative, S2889, using plasmid pRS306-*RHO4-trm2::LEU2* and the *rad52::hisG-URA3-hisG* construct as described. Strain UMY2362 (*trm2::LEU2 rad52::hisG*) was generated from strain UMY2353 (*trm2::LEU2*) using the *rad52::hisG-URA3-hisG* construct.

DNA methods and plasmid constructions

Isolation of plasmid DNA, restriction enzyme analysis, and agarose gel electrophoresis were performed following standard procedures (Sambrook et al., 1989). The 2-kb *Hin*dIII-*Xba*I fragment containing the *TRM2* (*RNC1/NUD1*) gene was sequenced using the chain termination method described by Sanger et al. (1977). Preparation of chromosomal DNA and Southern blot analysis were described by Boeke et al. (1985) and Byström and Fink (1989), respectively. A 5′-ATT TTA GAG GAA TAG TTT AG-3′ oligonucleotide was used as a hybridization probe in Southern blot analysis against *TRM2* or *trm2*.

The *RHO4* and *TRM2* (*RNC1/NUD1*) genes were isolated as part of a 3,555-bp *Eco*RI-*Xba*I fragment from plasmid pGKU144 (Van Vliet-Reedijk & Planta, 1993) and subcloned into the corresponding sites of plasmids pRS316 and pRS306 (Sikorski & Hieter, 1989), generating plasmids pRS316-*RHO4-TRM2* and pRS306-*RHO4-TRM2*. Plasmid pRS316-*TRM2* was constructed by digesting plasmid pRS316-*RHO4-TRM2* with *Hin*dIII and religating, thereby eliminating a 778-bp fragment that contains the *RHO4* gene. A 2-kb *Hin*dIII-*Xba*I fragment containing the *TRM2* gene was cloned into the corresponding sites of YEp352 (Hill et al., 1986), generating plasmid YEp352-*TRM2*. A 1.1-kb *Eco*R1-*Hin*dIII fragment con-

TABLE 4. Yeast and *E. coli* strains used in this study.

	Genotype	Source
Yeast strains		
FY23	*MATa ura3-52 trp1Δ63 leu2Δ1*	F. Winston
FY86	*MATα ura3-52 his3Δ200 leu2Δ1*	F. Winston
GRF88	MATα his4-39	G. Fink
L1937	*MATa leu2-3/112 ura3-52 trp1Δ1*	G. Fink
L1938	*MATα leu2-3/112 ura3-52 trp1Δ1*	G. Fink
4010-2	*MATα SUP4 gal1-ochre trm2 gal7 can1-100 lys2-1 ade2-1 ura3 met*	Hopper et al., 1982
S2889	*MATa lys2 ura3 leu2::hisG ho::LYS2 trp1-H3*	S. Roeder
UMY1901	*MATa ura3-52 his3Δ200 leu2Δ1*	This study
UMY1902	*MATa ura3-52 trp1Δ63 trm2::LEU2*	This study
UMY1903	*MATα ura3-52 trp1Δ63 leu2Δ1*	This study
UMY1904	*MATα ura3-52 his3Δ200 trm2::LEU2*	This study
UMY2229	as L1937 but also *RHO4::trm2-LacZ*	This study
UMY2230	as L1938 but also *rad52::hisG*	This study
UMY2235	as UMY2229 but also *rad52::hisG*	This study
UMY2353	as S2889 but also *trm2::LEU2*	This study
UMY2361	as S2889 but also *rad52::hisG*	This study
UMY2362	as S2889 but also *trm2::LEU2 rad52::hisG*	This study
E. coli strains		
BL21	*F⁻ ompT hsdS* (r_B^-,m_B^-) *gal dcm*	Studier & Moffatt, 1986
CJ236	*dut1 ung1 thi relA*/pCJ105 (Cm^R)	Kunkel, 1985
DH5α	F⁻ *endA1 hsdR17* (r_K^-,m_K^+) *supE44 thi-1 d⁻ recA gyrA96 relA1* Δ(*argF-lacZYA*) U169 φ80d*lacZ* ΔM15	Betheseda Research Laboratories
GB1-5-39	*trmA5 argH lac*	Björk & Neidhardt, 1975

taining the prokaryotic *trmA*$^+$ gene was cloned into the corresponding sites of plasmid pRS316 (obtained from J. Urbonavicius, Umeå University). To construct a disruption allele of the *TRM2* gene, two *Xho*I sites were introduced with oligonucleotide-directed mutagenesis at positions 1610 and 3440 of the 3,555-bp fragment on the plasmid pRS306-*RHO4-TRM2* using oligonucleotides, 5′-AAA AAA GAA AAC TCG AGC TGT TCG TAC AT-3′ and 5′-CTC TTA TGC TCT CGA GCT GGT GGG CGG AA-3′, respectively (Kunkel, 1985). The *TRM2* sequence between the *Xho*I sites was replaced by a 2.5-kb *Sal*I fragment containing a functional *LEU2* gene, resulting in plasmids pRS306-*RHO4-trm2::LEU2* and pRS316-*RHO4-trm2::LEU2*. To construct plasmid pRS316-*RHO4 trm2::HIS3*, a 1,760-bp *Bam*HI fragment containing the *HIS3* gene was inserted into a unique *Bam*HI site of the *TRM2* gene.

To convert the potential AUG translational start codons of the *TRM2* gene to UUG codons, oligonucleotide directed mutagenesis was performed as described by Kunkel (1985). The following oligonucleotides were used: 5′-TCA AAC TGT TCG TAC AAA ATG ACT TTG TCC T-3′ (AUG1–UUG1), 5′-TCC AAA AGC AAC GCA AGC ATG TGA TAA TAC T-3′ (AUG2–UUG2), and 5′-AAA CTA ATC AGC ACC TTG ACT GGA AGT ACT GAA-3′ (AUG3–UUG3). Frameshifts (−1) were introduced using oligonucleotides: 5′-GTC CAA AAG CAA CGA CAT GCA TGT GAT AAT A-3′ (1 nt insertion after AUG2), 5′-TTC AGT ACT TCC AGT ACA TGG TGC TGA TTA G-3′ (1 nt insertion after AUG3), and 5′-GTG GTA CCA ATT CAG TAC TTC CAG TAC ATG GTG CTG-3′ (1 nt insertion after AUG3 and conversion of AUG4 to UUG4). To change AUG5 to UUG5, we made a PCR-based mutagenesis of plasmid pRS316-*TRM2* using oligonucleotides 5′-GAA ATG GTA CCA CCA ACA TTG AAA CAT ACC GTT GAC-3′ and 5′-GTC AAC GGT ATG TTT CAA TGT TGG TGG TAC CAT TTC-3′, generating plasmid pRS316-*TRM2*-UUG5. A 351-bp *Kpn*I fragment was exchanged from plasmid pRS316-*TRM2*-UUG5 with a 1,883-bp *Kpn*I fragment from plasmid pRS316-*RHO4-TRM2* AUG3/-fr or pRS316-*RHO4-TRM2* AUG3/-fr-UUG4 to generate plasmids pRS316-*RHO4-TRM2* AUG3/-fr-UUG5 and pRS316-*RHO4-TRM2* AUG3/-fr-UUG4-UUG5 (*Kpn*I sites are in the multiple cloning cassette of pRS316 and between AUG4 and AUG5 of the *TRM2* gene).

For β-galactosidase assays a 1,834-bp *Eco*RI-*Kpn*I *trm2* fragment was cloned from pRS316-*RHO4-TRM2* into the corresponding sites of YIp358 or YEp358 (Myers et al., 1986), creating an in-frame *trm2-lacZ* fusion, encoding eight amino acids of the Trm2p.

For protein expression and purification, the *TRM2* gene was subcloned into the expression plasmid pGEX-4T-2 (Pharmacia Biotech) as follows: A *Bgl*II site was introduced at the physiological ATG start site of the *TRM2* gene by oligo-directed mutagenesis according to Kunkel (1985) using oligo 5′-TTC AGT ACT TCC AGA TCT GGT GCT GAT TAG T-3′. A 1,890-bp *Bgl*II-*Pvu*II fragment from plasmid pRS316-*RHO4-TRM2* was subcloned into the *Bam*HI-*Sma*I site of pGEX-4T-2. In this plasmid, transcription is under the *tac* promoter and the product made is a Trm2 protein with the GST tag and thrombin cleavage site located at the amino terminal end.

RNA methods

Total tRNA for HPLC was prepared as follows: *S. cerevisiae* strains were inoculated in 100 mL of YEPD or selective media and grown to approximately 1.0 OD_{600} unit. The cells were

harvested, washed in sterile water, and stored at −20 °C. The pellet was resuspended in 2 mL extraction buffer (0.5 M NaCl; 0.2 M Tris-HCl, pH 7.6; 10 mM EDTA), mixed with 2 mL PCIA (25:24:1 phenol:chloroform:isoamylalcohol, equilibrated with extraction buffer) and 3 mL sterile RNase-free glass beads (0.45–0.55 mm, Sigma Chemicals). The mixture was vortexed for 5 min to break the yeast cells and centrifuged at 6,500 × *g* for 5 min. The aqueous phase was removed and re-extracted with an equal volume of PCIA. After the second centrifugation, the aqueous phase was loaded onto a QIAGEN® plasmid Midi prep column that had been equilibrated with tRNA equilibration buffer (0.5 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100). The column was washed with 15 mL tRNA washing buffer (0.6 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol), and finally eluted with 3 mL of tRNA elution buffer (0.7 M NaCl; 50 mM Tris-HCl, pH 7.4; 15% ethanol). Total tRNA was precipitated with 7.5 mL ethanol, centrifuged at 12,000 × *g* for 30 min, dried, and resuspended in 100 μL TE. The qualities of the tRNA preparations were controlled on a 2% agarose gel. Large scale purification of crude RNA from strain UMY1901 *TRM2* and UMY1902 *trm2::LEU2*, using 50 g of cells from each strain, was prepared as described above and fractionation of tRNA was according to Avital and Elson (1969).

Prokaryotic tRNA from strain GB1-5-39 containing plasmids pRS316, pRS316-*trmA*$^+$, or pRS316-*RHO4-TRM2* was prepared as follows: Cells were grown in 100 mL LB medium (supplemented with 50 μg/mL of carbenicillin) to approximately 1.0 OD_{600} unit. The cells were harvested, washed in 0.9% NaCl, and resuspended in extraction buffer (10 mM Tris-HCl, pH 8.0; 10 mM MgAc, pH 8.0) containing DNase (25 μg/mL) and lysozyme (250 μg/mL). The suspension was frozen and thawed repeatedly (up to three times), phenol extracted twice (phenol equilibrated with extraction buffer), and finally the aqueous phase was ethanol precipitated. The pellet was resuspended in 1 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and subjected to the QIAGEN® purification protocol, as described above.

Potential transcriptional start points of *TRM2* were established by primer extension analyses (Göransson et al., 1989) using two different oligonucleotides: 5′-GTT GTC AAC GGT ATG TTT CAT TG-3′ and 5′-TCT AGG ACA CCC ATC GG-3′ hybridizing to positions 32–54 and 150–160 of the *TRM2* gene. Total RNA for primer extension analyses was prepared according to Carlson and Botstein (1982) from strain GRF88 exponentially grown in YEPD.

Analysis of the m^5U content by HPLC and HPLC-coupled MS

For HPLC analysis, 50 μg tRNA were digested with nuclease P1 and bacterial alkaline phosphatase (Gehrke et al., 1982) and the hydrolysate was separated on a RP-HPLC column according to Gehrke and Kuo (1990). The nucleoside mixture from tRNA was analyzed on a Waters™ System liquid chromatograph with a Waters™ 996 diode array UV detector (Waters™ Corporation, Milford, Massachusetts). Separation of nucleosides by HPLC was achieved using a Supelcosil LC-18-S reverse-phase column (4.6 × 250 mm) and a Supelguard LC-18-S, 2.1 × 20 mm guard column (Supelco, Bellefonte, Pennsylvania) thermostatted to 26 °C, at a flow rate of 1 mL/min. For MS analysis the flow rate was 2 mL/min and the UV detector was directly interfaced to a VG platform mass spectrometer equipped with an electrospray ionization source (Fisons Instruments, Altrincham, United Kingdom). In MS analysis nucleosides were eluted using the gradient of Buck et al. (1983) altered to accommodate a lower ammonium acetate concentration (5 mM) which is more compatible with electrospray ionization. UV data were recorded continuously, and mass spectra were recorded every 1.0 s during the 60 min chromatography. The procedures and interpretation of data for qualitative LC-MS analysis of nucleosides in RNA hydrolysates have been previously described in detail (Pomerantz & McCloskey, 1990).

Preparation of protein extracts and purification of the Trm2 protein

Total protein extracts of *S. cerevisiae* were done as previously described (Rose et al., 1990) and dialyzed against 10 mM Tris-HCl, pH 8.0, and 0.1 mM DTT. To purify the Trm2 protein the bacterial strain BL21 (Studier & Moffatt, 1986) was transformed with pGEX-4T-2-*TRM2*. A total volume of 1 L of LB medium containing 50 μg/mL of carbenicillin was inoculated, grown to OD_{600} = 1.0 at 37 °C, chilled on ice to 15 °C before IPTG was added to a final concentration of 1 mM. After 4 h induction at 15 °C the cells were harvested. The pellet was dissolved in 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 1.5 M NaCl, 5% NP-40, 10% glycerol and protease inhibitors at a final concentration of 2 μg/mL Chymostatin, 0.3 μg/mL leupeptin, 1.4 μg/mL pepstatin A, 0.2 mg/mL PMSF, and 0.3 mg/mL benzamidin. *E. coli* cells were broken using a French Press and cell debris was removed by centrifugation at 48,000 × *g* for 10 min. The GST-Trm2p was purified using Glutathione Sepharose® 4B (Pharmacia Biotech) according to Smith and Johnson (1988). Crude protein extracts of *E. coli* strains GB1-5-39 (*trmA5*) and BL21/pMTRX1 were prepared according to Gu & Santi (1990).

In vitro methyltransferase assay

In vitro tRNA(m^5U_{54})methyltransferase assays were performed with total *S. cerevisiae* and *E. coli* protein extracts or with the GST-Trm2 fusion protein purified from *E. coli*. Cultures used were *S. cerevisiae* strains UMY2229, UMY2235, and L1937/YEp352-*TRM2* or *E. coli* strains GB1-5-39, BL21/pMTRX1, and BL21/pGEX-4T-2-*TRM2*. A total of 50 μg m^5U_{54} deficient or m^5U_{54} containing crude tRNA, were mixed with various amounts of total yeast protein extracts or purified GST-Trm2 protein in 50 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 20 mM $(NH_4)_2Cl_2$; 0.1 mM DTT; and 38 μM S-adenosyl-L-(methyl-^{14}C)methionine (54 mCi/mmol) in a total volume of 100 μL. The reaction mixture was incubated at 37 °C for 30 min. Reactions were terminated by adding 3 mL of 0.5 M perchloric acid containing 0.01 M lanthanium nitrate. Samples were collected on GF/C filters, washed repeatedly with 5% TCA and once with acetone, dried, and counted in a scintillation counter.

When measuring the extent of methylation as a function of the amount of tRNA prepared from UMY1901 (*TRM2*) or UMY1902 (*trm2::LEU2*), the same buffer conditions and sample preparation as above was used. The reaction was carried out in a total volume of 300 μL with 1.2, 2.4, and 3.6 μg of

tRNA and 5μg of GST-Trm2 protein. Samples of 50 μL were withdrawn over a 5-h period. The maximum incorporation of methyl groups, plateau value, was used in the calculations, and to estimate the fraction of the tRNA pool that incorporated methyl groups we used a molecular weight of 2.5×10^4 g/mol for a tRNA.

To determine if the Trm2p methylates U at position 54 of tRNA, an experiment using UMY1901 (*TRM2*), UMY1902 (*trm2::LEU2*) total tRNA, purified GST-Trm2, and crude protein extracts of GB1-5-39 (*trmA5*) and BL21/pMTRX1 was performed. The *trmA5* allele of GB1-5-39 encodes an inactive *E. coli* tRNA(m^5U_{54})methyltransferase unable to modify U_{54} to m^5U_{54} (Björk & Neidhardt, 1975). Plasmid pMTRX1 contains a wild-type *TrmA* gene regulated by the *tac* promoter (Gu & Santi, 1990). Buffer conditions used for the methylation reaction and treatments of samples were as above. The reaction was carried out in a total volume of 300 μL with 12 μg of UMY1902 (*trm2::LEU2*) tRNA and 150 μg of GB1-5-39 (*trmA5*) or BL21/pMTRX1 crude protein. The tRNA was incubated for 2 h and samples representing 4 μg of tRNA were withdrawn and incorporation of ^{14}C-methyl groups was determined. To the remaining reactions 5 μg of purified GST-Trm2 protein were added and the incubation was continued for another 2 h. Samples representing 4 μg of tRNA were withdrawn and incorporation of ^{14}C-methyl groups was determined. The UMY1901 (*TRM2*) tRNA was incubated with the protein extract BL21/pMTRX1 using the same conditions. In HPLC-coupled MS analysis of m^5U content in in vitro methylated UMY1902 (*trm2::LEU2*) total tRNA, nonradioactive S-adenosyl-L-methionine was used. Reaction conditions were as above.

β-galactosidase assays

β-galactosidase assays were measured on protein extracts as previously described (Rose et al., 1990). The activities are expressed as nanomoles of o-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram protein. Protein concentrations were measured by the Bradford dye binding method (BioRad) with bovine serum albumin as the standard.

Induction of HO endonuclease

Overnight cultures of strains S2889, UMY2353, UMY2361, and UMY2362 carrying plasmid pSB283 were grown at 30 °C in SC medium lacking uracil and with 2% glucose as a carbon source. Joshua Trueheart provided plasmid pSB283 that is pGAL-HO (Herskowitz & Jensen, 1991), with a *LEU2* gene located on a *Sal*I-*Xho*I fragment cloned into the *Sal*I site. Cells were collected by centrifugation, washed, and resuspended in sterile water. The cell suspension was diluted and plated on SC medium lacking uracil with 2% glucose or galactose as carbon sources and incubated at 30 °C for at least 72 h.

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