tRNA m⁷G methyltransferase Trm8p/Trm82p: Evidence linking activity to a growth phenotype and implicating Trm82p in maintaining levels of active Trm8p

ANDREI ALEXANDROV, ELIZABETH J. GRAYHACK, and ERIC M. PHIZICKY

Department of Biochemistry and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642, USA

ABSTRACT

We show that *Saccharomyces cerevisiae* strains lacking Trm8p/Trm82p tRNA m⁷G methyltransferase are temperature-sensitive in synthetic media containing glycerol. Bacterial *TRM8* orthologs complement the growth defect of *trm8-Δ*, *trm82-Δ*, and *trm8-Δ trm82-Δ* double mutants, suggesting that bacteria employ a single subunit for Trm8p/Trm82p function. The growth phenotype of *trm8* mutants correlates with lack of tRNA m⁷G methyltransferase activity in vitro and in vivo, based on analysis of 10 mutant alleles of *trm8* and bacterial orthologs, and suggests that m⁷G modification is the cellular function important for growth. Initial examination of the roles of the yeast subunits shows that Trm8p has most of the functions required to effect m⁷G modification, and that a major role of Trm82p is to maintain cellular levels of Trm8p. Trm8p efficiently cross-links to pre-tRNA^{Phe} in vitro in the presence or absence of Trm82p, in addition to its known residual tRNA m⁷G modification activity and its SAM-binding domain. Surprisingly, the levels of Trm8p, but not its mRNA, are severely reduced in a *trm82-Δ* strain. Although Trm8p can be produced in the absence of Trm82p by deliberate overproduction, the resulting protein is inactive, suggesting that a second role of Trm82p is to stabilize Trm8p in an active conformation.

Keywords: tRNA modification; *S. cerevisiae*; S-adenosylmethionine; methyltransferases; Trm8; Trm82; YggH; TM0925; 7-methylguanosine; m⁷G

INTRODUCTION

A continuing puzzle in RNA biology is the precise role of tRNA modifications. Although more than 79 different modifications have been identified in tRNA (Limbach et al. 1994; Bjork 1995), and 25 of these modifications are found in yeast (Sprinzl et al. 1998), a cellular role for many of them has not yet been defined. The recent identification of a substantial number of genes responsible for tRNA modification (Hopper and Phizicky 2003) has allowed the opportunity to study their roles by examining mutant phenotypes.

Surprisingly, deletion of the majority of enzymes responsible for tRNA modification revealed few obvious defects. Only three tRNA modifying enzymes in yeast are known to be essential, including m¹A₅₈ methyltransferase Gcd10p/

Gcd14p (Anderson et al. 1998), I₃₄ adenosine deaminase Tad2p/Tad3p (Gerber and Keller 1999), and tRNA^{His} G₋₁ guanylyltransferase Thg1p (Gu et al. 2003). Strains lacking each of six other modification enzymes affecting residues in the anticodon region have distinct growth or translation phenotypes, including 2'-O-Me_{32,34} methyltransferase Trm7p (Pintard et al. 2002), m¹G₃₇/m¹I₃₇ methyltransferase Trm5p (Bjork et al. 2001), Ψ_{38,39} pseudouridylase Pus3p (Lecointe et al. 1998), mcm⁵U/mcm⁵s²U₃₄ carboxyl methyltransferase Trm9p (Kalhor and Clarke 2003), m⁵C_{34,40,48,49} methyltransferase Trm4p (Wu et al. 1998), and i⁶A₃₇ isopentenyl transferase Mod5p (Laten et al. 1978; Janner et al. 1980; Dihanich et al. 1987).

The other 16 known modification enzymes in yeast each modify residues remote from the anticodon region, and mutants lacking these enzymes have only subtle phenotypes. Each of these mutants has little obvious growth or translation defect, and the observation of distinct phenotypes has required more sensitive approaches such as synthetic interaction screens (Grosshans et al. 2001; Johansson and Bystrom 2002; Urbonavicius et al. 2002), or, as shown in *Escherichia coli*, growth competition experiments (Gutg-

Reprint requests to: Eric Phizicky, Department of Biochemistry and Biophysics, University of Rochester School of Medicine, 601 Elmwood Ave., Box 712, Rochester, NY 14642, USA; e-mail eric_phizicky@urmc.rochester.edu.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2030705>.

sell et al. 2000). The lack of obvious phenotype of strains lacking these modification enzymes contrasts sharply with the strong evolutionary conservation of a number of these enzymes and their modifications. Even more unexpectedly, some of the subtle phenotypes associated with defects in tRNA modification, including those of yeast m^5U_{54} methyltransferase Trm2p (Johansson and Bystrom 2002) and *E. coli* Ψ_{55} pseudouridylylase TruB (Gutgsell et al. 2000), were complemented with catalytically inactive mutants, suggesting that lack of the modification of tRNA is not the cause of the defect, and that tRNA-modifying enzymes may have other distinct cellular roles.

We focus here on m^7G (7-methylguanosine) modification of tRNA. This modification is highly conserved, based on its presence in >40% of all sequenced bacterial and eukaryotic tRNA species. In all but three cases, m^7G is found at position 46 in the extra loop, a site known from the crystal structures of tRNA^{Phe} (Kim et al. 1974; Robertus et al. 1974) and tRNA^{Met} (Basavappa and Sigler 1991) to form tertiary interactions with bases at positions 13 and 22. Additionally, the m^7G and m^1A modification are the only two yeast modifications that confer a positive charge to the base.

We previously used a biochemical genomics approach to identify two proteins, Trm8p and Trm82p, that copurify with tRNA m^7G methyltransferase activity. We showed that these two proteins are both necessary for m^7G_{46} modification of tRNA in vivo and in vitro, form a complex in vivo, and are sufficient for activity in vitro (Alexandrov et al. 2002). Neither Trm8p nor Trm82p is significantly related to yeast Abd1p, which catalyzes m^7G formation during capping of mRNAs (Mao et al. 1995), other than within the S-adenosylmethionine (SAM)-binding domain of Trm8p. Trm8p is highly conserved in eukaryotes and bacteria, extending from humans to *Mycoplasma genitalium*, with less than 500 ORFs (Bahr et al. 1999), and Trm82p orthologs are present in the majority of sequenced eukaryotes. Nevertheless, deletion of *TRM8* or *TRM82* results in no obvious growth defect under standard laboratory conditions (Alexandrov et al. 2002), raising the question of the role of these proteins in these organisms.

Surprisingly, whereas Trm8p/Trm82p complex appears to be conserved in eukaryotes, it may be a single Trm8p subunit in bacteria, since bacteria appear to lack Trm82p (Michaud et al. 2000). We showed previously that the two-protein mechanism of tRNA m^7G formation is conserved in higher eukaryotes, since expression of both human orthologous proteins (METTL1 and WDR4) in yeast was required to restore m^7G methyltransferase activity in extracts (Alexandrov et al. 2002). However, it appears that a single bacterial protein may be sufficient for activity, consistent with the implication of a single purified 25-kDa *Salmonella typhimurium* polypeptide in tRNA m^7G methylation (Colonna et al. 1983), and the demonstration of tRNA m^7G -modifying activity for *E. coli* YggH (De Bie et al. 2003), and

Aquifex aeolicus aq065 protein purified from *E. coli* (Okamoto et al. 2004).

As a first step toward determining the physiological roles of *TRM8*/*TRM82* genes and the role of m^7G modification of tRNA, we have found and characterized a phenotype associated with loss of either or both of the *TRM8* and *TRM82* genes. We show that this growth phenotype is strongly correlated with m^7G methyltransferase activity in vitro and in vivo. We also provide in vivo evidence that the bacterial Trm8 ortholog does not require a second subunit to function in yeast, raising the issue of the function of each of the two yeast subunits.

We provide evidence that a crucial function of yeast Trm82p is to maintain the levels of active Trm8p in vivo, since deletion of Trm82p results in a severe reduction in the level of Trm8p protein, and Trm8p prepared from yeast in the absence of Trm82p is not detectably active. In contrast, Trm8p appears to possess the requisite functions of the catalytic subunit: it has a SAM-binding domain, has residual catalytic activity when purified from *E. coli*, and cross-links to pre-tRNA^{Phe} in vitro.

RESULTS

TRM8 and *TRM82* are both required for growth at 38°C on glycerol-containing synthetic media

We found a distinct growth defect that results from the loss of *TRM8* or *TRM82* genes. As demonstrated in Figure 1 by serial 10-fold dilutions of yeast cells, homozygous diploid strains lacking either Trm8p (relevant genotype: *trm8-Δ/trm8-Δ*) or Trm82p (relevant genotype: *trm82-Δ/trm82-Δ*) grow extremely poorly relative to wild-type strains on synthetic minimal media (Sherman 1991) containing 2% glycerol at 38°C. No obvious growth differences were observed between mutants and the wild-type strain on this media at 30°C or at 36°C, or on two other media at either 30°C or

FIGURE 1. Growth defect of *trm8-Δ/trm8-Δ* and *trm82-Δ/trm82-Δ* strains on glycerol-containing minimal media. Homozygous diploid *trm8-Δ/trm8-Δ*, *trm82-Δ/trm82-Δ*, or wild-type strains, containing a *CEN URA3* plasmid bearing *TRM8* or *TRM82* under control of its own promoter as indicated, were grown overnight, and 10-fold serial dilutions were plated on synthetic media lacking uracil. (A) Cells plated on glucose-containing media, incubated for 2 d at 30°C. (B) Cells plated on glycerol-containing media, incubated for 7 d at 38°C.

38°C: synthetic minimal media containing 2% dextrose, and yeast extract-peptone media containing 2% glycerol (data not shown). Since growth of the mutants was also not compromised on acetate or lactate media (data not shown), this phenotype does not originate from a general loss of mitochondrial function. This growth defect is due to loss of Trm8p or Trm82p, because it is restored by a single-copy plasmid containing the corresponding *TRM8* or *TRM82* under control of its own promoter, but not by the vector control (Fig. 1).

The similar conditions, magnitude, and temperature de-

pendence of the growth defects of *trm8*-Δ and *trm82*-Δ strains, and the absence of an additional effect in the *trm8*-Δ *trm82*-Δ double deletion strain (see below) suggest the mutual involvement of *TRM8* and *TRM82* in the same biological process, implying that the growth phenotype is due to loss of functional Trm8p/Trm82p complex.

We used this phenotype to address two separate issues of Trm8p/Trm82p-catalyzed m⁷G formation. First, we provide evidence confirming that bacterial orthologs of Trm8p can affect m⁷G modification alone in vivo. Second, we provide evidence that the catalytic activity of Trm8p/Trm82p m⁷G methyltransferase is central to its function in vivo. The results of these experiments are shown together in Figures 2 and 3, and Table 1.

Bacterial Trm8p orthologs complement the *trm8*-Δ *trm82*-Δ growth phenotype and restore m⁷G-methyltransferase activity in vitro and in vivo

Reports from other investigators indicate that purified bacterial Trm8p orthologs YggH from *E. coli* (De Bie et al. 2003) and aq065 from *Aquifex aeolicus* (Okamoto et al. 2004) have tRNA m⁷G methyltransferase activity. To determine whether and to what extent bacterial proteins could function in yeast, we tested *E. coli* protein YggH and *Thermotoga maritima* protein TM0925 for complementation of the growth defect of *trm8*-Δ and *trm82*-Δ strains, and for m⁷G-methyltransferase activity. As demonstrated by serial dilutions (Fig. 2A), YggH and TM0925 each successfully complemented the yeast *trm8*-Δ phenotype nearly as well as observed with yeast Trm8p (lanes 3a,4a,1a). Strikingly, expression of either of the prokaryotic orthologs also complemented the phenotypes of *trm82*-Δ mutants and of *trm8*-Δ *trm82*-Δ double mutants, whereas yeast *TRM8* or *TRM82* each complemented only their own deletions strains (Fig. 2A, lanes 1a,2). Moreover, expression of either YggH or TM0925 fully restored m⁷G-methyltransferase activity in extracts from a *trm8*-Δ *trm82*-Δ double mutant strain, as measured using yeast pre-tRNA^{Phe} as substrate (Fig. 3A, lanes 3a,4a). Additionally, expression of either YggH or TM0925 fully restored the in vivo levels

FIGURE 2. Complementation of the growth defect by variants of *TRM8* and of two of its bacterial orthologs. Homozygous diploid strains with genetic background indicated at the top of each titration panel, and containing plasmids expressing GST-fusion proteins under P_{CUP1} control as indicated at the left, were grown overnight and plated after serial 10-fold dilutions, on synthetic media lacking leucine and uracil, and containing carbon sources as indicated. (A) Cells plated on media containing glycerol, and incubated at 38°C for 7 d. (B) Cells plated on media containing glucose, and incubated at 30°C for 3 d. Rows 1a–1e, plasmids expressing variants of yeast *TRM8*, as described in the text, as GST-fusion proteins; 2, plasmid expressing *TRM82*; 3a–3c, plasmids expressing *E. coli* YggH variants; 4a–4c, plasmids expressing *T. maritima* TM0925 variants; 5, vector control; 6, wild-type strain control, with vector control.

FIGURE 3. m⁷G-methyltransferase activity is altered in cells expressing Trm8p, YggH, and TM0925 mutant variants. (A) Analysis of m⁷G methyltransferase activity of variants. Activity was assayed with [α -³²P]GTP-labeled yeast pre-tRNA^{Phe} substrate and fivefold dilutions of crude extracts beginning with 6 μ g of protein, and modified nucleotides were analyzed as described in Materials and Methods. Open arrows, m⁷G methyltransferase activity; black arrows, m²G methyltransferase activity, as internal control. (B) SDS-PAGE analysis of expressed GST fusion proteins. Expressed GST-fusion proteins were purified by glutathione sepharose chromatography from extracts of yeast strains shown in Figures 2 and 3A, analyzed by 4%–15% SDS-PAGE, and stained with Coomassie. Arrows indicate protein bands of the expected size. (C) Alignment of Motif I and Post I for putative SAM-binding domains of Trm8p-orthologous proteins. Sites of point mutations used in this study (G103, G105, and G124) are indicated with triangles.

of m⁷G in tRNA^{Phe} in a *trm8*- Δ *trm82*- Δ strain to those of the wild-type strain (Table 1, cf. 3a, 4a, and 6). Thus, bacterial orthologs of Trm8p can rescue the growth defect of yeast strains lacking both proteins of the Trm8p/Trm82p complex and, unlike their eukaryotic counterparts, do not require either a bacterial partner or Trm82p to catalyze formation of m⁷G in tRNA either in vitro in extracts or in vivo.

The growth defect of *trm8* and *trm82* mutants is associated with lack of tRNA m⁷G methyltransferase activity in vitro and in vivo

A growth phenotype such as that described above can occur if the methyltransferase activity of Trm8p/Trm82p is important for function, or if some other unknown activity of the complex is important, as was observed with phenotypes

of *trm2* yeast strains and *truB* *E. coli* strains (Gutgsell et al. 2000; Johansson and Bystrom 2002). To determine whether tRNA m⁷G modification activity correlates with phenotype, we altered the S-adenosylmethionine (SAM)-binding domain in Trm8p and in its bacterial orthologs, and assayed complementation, biochemical activity, and tRNA m⁷G content in vivo. As shown in Figure 3C, Trm8p orthologs, like a number of other methyltransferases, contain a Motif I and a Post I motif, regions which have been implicated in SAM binding in other proteins (Cheng et al. 1993; Labahn et al. 1994; Hodel et al. 1996; Djordjevic and Stock 1998; Niewmierzycza and Clarke 1999). Trm8p and variants were altered at three conserved glycines within these motifs, to make a G103A mutation (designated G/A), a G103A G105A double mutation (GG/AA), and a G103A G105A G124E triple mutation (GGG/AAE). Corresponding G/A and GG/AA mutations in the SAM-binding domain of yeast m⁷G cap methyltransferase Abdlp (Mao et al. 1996) and m¹A methyltransferase Gcd14p (Anderson et al. 2000) impaired their catalytic function. We also tested a *trm8* (1–39 Δ) mutant because this region is different in eukaryotes and bacteria. All mutants were expressed under P_{CUP1} control as N-terminal GST fusions.

As shown in Figure 2A, both *trm8* (G/A), and *trm8* (1–39 Δ) complemented the growth defect of *trm8*- Δ mutants as well as did wild-type *TRM8* (lanes 1a,1b,1e), whereas *trm8* (GG/AA) and *trm8* (GGG/AAE) (lanes 1c,1d) failed to complement. Similarly, tm0925 (G/A) and tm0925 (GG/AA) complemented the growth defect of a *trm8*- Δ strain (lanes 4b,4c), whereas yggH (G/A) and yggH (GG/AA) mutants did not complement at all (lanes 3b,3c).

As determined by assay of yeast extracts in the presence of excess SAM, m⁷G methyltransferase activity correlates with complementation (Fig. 3A). Activity was not detected in all extracts derived from mutants in which complementation was not observed, including *trm8* (GG/AA), *trm8* (GGG/AAE), yggH (G/A), and yggH (GG/AA) (Fig. 3A, lanes 1c,1d,3b,3c). In contrast, m⁷G methyltransferase activity was detected, albeit at various levels, in all cases where complementation was observed: at high levels in extracts from strains expressing *TRM8*, *trm8* (1–39 Δ), YggH, tm0925, and tm0925 (G/A) (Fig. 3A, lanes 1a,1e,3a,4a,4b), and at lower levels in extracts from strains expressing *trm8*

TABLE 1. Complementation of growth defect correlates with m⁷G formation in tRNA^{Phe} in vitro and in vivo

	Strain	Plasmid	Growth defect complementation ^a	m ⁷ G activity in vitro ^b	% of m ⁷ G in vivo ^c
1a	<i>trm8-Δ</i>	<i>TRM8</i>	Yes	+++	110
1b		<i>trm8</i> (G/A)	Yes	+	5.1
1c		<i>trm8</i> (GG/AA)	No	–	not detected ^d
1d		<i>trm8</i> (GGG/AAG)	No	–	not detected
1e		<i>trm8</i> (1–39Δ)	Yes	++	39
3a	<i>trm8-Δ</i> <i>trm82-Δ</i>	YggH	Yes	+++	119
3b		yggH (G/A)	No	–	not detected
3c		yggH (GG/AA)	No	–	not detected
4a		TM0925	Yes	+++	112
4b		tm0925 (G/A)	Yes	+++	28
4c		tm0925 (GG/AA)	Yes	+	5.5
5		vect.	No	–	not detected
6	Wild type	vect.	Yes	+++	100

^aAs shown in Figure 2.^bAs shown in Figure 3A.^cAs determined by HPLC analysis of m⁷G content of tRNA^{Phe} purified from the corresponding strain. 100% corresponds to m⁷G content of tRNA^{Phe} purified from the wild-type strain (in this experiment: 0.80 m⁷G per tRNA).^dDetection limit of m⁷G was 2% of the wild-type amount.

(G/A) and tm0925 (GG/AA) (lanes 1b,4c). Internal controls show that the m²G methyltransferase activity was virtually the same in the extracts (Fig. 3A), and that varying levels of m⁷G methyltransferase activity were not due to variations in the amount of protein, as measured by yield of Trm8p orthologs or variants after purification of the expressed protein (Fig. 3B).

Examination of tRNA^{Phe} modification levels in vivo demonstrates directly that m⁷G content correlates with complementation of the growth phenotype of *trm8* mutants. To evaluate the extent of tRNA^{Phe} m⁷G methylation, we purified the tRNA from each of these strains using biotinylated DNA and streptavidin beads, and analyzed the nucleoside content using HPLC. As shown in Table 1, all strains that did not complement a *trm8-Δ* mutant had no detectable m⁷G in their tRNA^{Phe}, whereas strains with wild-type or mutant alleles that complemented the growth defect also had detectable amounts of m⁷G in tRNA^{Phe}. As with the assay of extracts, there was variability in the amount of m⁷G detected in vivo; however, there was a very high correlation of m⁷G modification levels of tRNA^{Phe} found in vivo (Table 1) with tRNA^{Phe} m⁷G modification activity observed in extracts in vitro (Fig. 3A; Table 1).

Trm82p controls the cellular levels of Trm8p protein in addition to its requirement for activity

The finding that a two-protein yeast m⁷G methyltransferase can be functionally substituted by a single subunit bacterial enzyme prompted us to examine the roles of yeast Trm8p and Trm82p subunits. Surprisingly, we found that the level of chromosomally TAP-tagged Trm8p was reduced at least

16-fold in crude yeast SDS lysates from a *trm82-Δ* strain, relative to that observed in extracts from a *TRM82⁺* strain (Fig. 4A, cf. lanes d–f and a–c). Furthermore, the Trm8p levels were fully restored upon introduction of a single-copy plasmid expressing Trm82p into this *trm82-Δ* strain (lanes g–i). As demonstrated by Northern blot analysis with a *TRM8*-specific probe (Fig. 4C), the *TRM8* mRNA level is virtually the same in a *trm82-Δ* strain as in a *TRM82⁺* strain (Fig. 4A, cf. lanes d–f and lanes a–c) and, as expected, is absent in mRNA from a specificity control *trm8-Δ* strain (lanes g–i). This result suggests that Trm82p does not affect the amount of *TRM8* mRNA but, instead, is involved in the regulation or maintenance of Trm8p at the protein level.

We note that deliberate overproduction of Trm8p in a *trm82-Δ* strain can bypass the control of Trm8 protein levels exerted by Trm82p, since Trm8p can be overexpressed and purified from a *trm82-Δ* strain in a yield comparable to that from wild-type cells (Fig. 5A); however, Trm8p produced from a *trm82-Δ* strain is essentially inactive (Fig. 5B). Presumably the tRNA m⁷G methyltransferase activity observed with purified Trm8p prepared from *TRM82⁺* cells is due to endogenous Trm82p that copurifies with Trm8p as part of the Trm8p/Trm82p complex observed previously (Alexandrov et al. 2002). The Trm82p is not visible in the Coomassie-stained gel shown in Figure 5A, because only Trm8p is overproduced in these strains.

Trm8p carries partial catalytic and tRNA-binding determinants

To further examine the function of the subunits, we examined tRNA binding. The presence of a consensus SAM-

FIGURE 4. Trm8p protein, but not mRNA, is severely reduced in a *trm82-Δ* strain. (A) Comparison of Trm8p expression in a *trm82-Δ* and a *TRM82⁺* strain. Otherwise isogenic haploid strains containing wild-type *TRM8* or chromosomally epitope-tagged *TRM8* (tagged with His⁶-HA-3C^{protease site}-ZZ^{Protein A}, abbreviated ZZ), and *TRM82⁺* or *trm82-Δ* as indicated, were grown, harvested, and lysed with SDS and glass beads as described in Materials and Methods. Lysates were serially diluted fourfold, starting with 2.7 OD₆₀₀ units of cells, resolved by SDS-PAGE, and analyzed for Trm8-ZZ expression by Western blot analysis, as described in Materials and Methods. Lanes a–c, *TRM8-ZZ TRM82⁺* strain; d–f, *TRM8-ZZ trm82-Δ* strain; g–i, *TRM8-ZZ trm82-Δ* strain transformed with plasmid containing *CEN LEU2 P_{TRM82}TRM82*; j–l, *TRM8 TRM82⁺* strain, with no epitope tag. (B) SDS-PAGE analysis of lysates described in (A) and stained with Coomassie. (C) Comparison of *TRM8* mRNA in a *trm82-Δ* and a *TRM82⁺* strain. RNA from otherwise isogenic wild-type (lanes a–c), *trm82-Δ* (d–f), and *trm8-Δ* (g–i) haploid strains was resolved on a 1% agarose gel, transferred to membrane, and hybridized with ³²P-labeled *TRM8* or *ACT1*-specific probes, as described in Materials and Methods. Successive lanes contain 15, 7.5, and 3.8 μg RNA.

binding domain in Trm8p, but not in Trm82p, implicates Trm8p in the methyl transfer, but the source of the RNA binding was unknown. To address this, we used in vitro UV cross-linking to identify subunit(s) in contact with the RNA, using pre-tRNA^{Phe} transcribed with [α -³²P]UTP (Fig. 6). As can be seen in Figure 6B, Trm8p efficiently cross-links to pre-tRNA^{Phe} in a UV-dependent manner, and cross-links as efficiently when part of the Trm8p/Trm82p complex (lanes g,o) or when alone (lanes f,n). In contrast, Trm82p does not detectably cross-link to pre-tRNA, whether as part of the Trm8p/Trm82p complex or when present alone (lanes e,m,g,o), and no cross-linking is observed in a control purification (lanes h,p). Thus, Trm8p likely has both the SAM and the tRNA binding sites.

DISCUSSION

We obtained evidence that lack of functional Trm8p or Trm82p in yeast is associated with a distinct phenotype: temperature-sensitive growth on glycerol-containing synthetic media. This phenotype underscores the importance of these proteins in survival, as implied by the widespread

evolutionary conservation of putative orthologs of Trm8p and Trm82p (Bahr et al. 1999; Michaud et al. 2000; Alexandrov et al. 2002). The finding of nearly identical phenotypes of *trm8-Δ* and *trm82-Δ* mutants extends our previous genetic and biochemical data implicating *TRM8* and *TRM82* in the same process, as indicated by their mutual participation in tRNA m⁷G modification in vitro and in vivo, and by their presence in the same complex (Alexandrov et al. 2002).

We also obtained evidence that the growth defect of *trm8-Δ* and *trm82-Δ* mutants is associated with lack of tRNA m⁷G modification activity. Establishment of this connection was important because of two other cases in which phenotypes associated with tRNA-modifying enzymes were complemented by catalytically inactive enzyme mutants (Gutgsell et al. 2000; Johansson and Bystrom 2002), suggesting a second function for these tRNA-modifying proteins. For each of the 10 tested Trm8p orthologs and variants, we found that complementation of the growth defect correlates with tRNA m⁷G methyltransferase activity in extracts and with m⁷G levels in isolated tRNA^{Phe} (Figs. 2, 3; Table 1). However, we showed above that complementation

FIGURE 5. Trm8p overexpressed in a *trm82-Δ* strain has much reduced activity. (A) GST-Trm8p or GST alone (mock) were overexpressed under P_{CUP1} control in a *trm82-Δ* or a *TRM82⁺* strain as indicated, and expressed proteins were purified from extracts by chromatography on glutathione sepharose resin, followed by release of the Trm8p moiety by cleavage with GST-3C protease, and then analyzed by 4%–15% SDS PAGE and Coomassie staining. (B) Analysis of m⁷G methyltransferase activity of purified Trm8p. Trm8p purified as described in A from a *trm82-Δ* (lanes d–f) or a *TRM82⁺* strain (j–l), or mock-purified controls (lanes g–i, m–o), was analyzed for methyltransferase activity, using serial fivefold dilutions of preparations. Controls: a, yeast m²G tRNA methyltransferase, b, yeast Trm8p/Trm82p m⁷G methyltransferase, c, buffer only.

is observed in two cases in which there is as little as 5% m⁷G modification of tRNA^{Phe} (Table 1, lines 1b,4c). We propose three different explanations for how such low m⁷G levels could be sufficient for complementation. First, the cell may only require 5% of the normal amount of m⁷G-containing tRNA for growth under these conditions. Second, the low observed m⁷G levels may result from evaluation of m⁷G under permissive conditions instead of nonpermissive conditions. We note that the narrow temperature window of this phenotype did not permit evaluation of m⁷G under nonpermissive conditions. Third, the m⁷G values for tRNA^{Phe} may not be the crucial determinant for growth; since m⁷G is found in 11 tRNA species in yeast, the m⁷G content of any one of these species could be the crucial limiting factor.

Although the evidence cited above establishes a correlation of m⁷G methyltransferase activity with a growth phenotype, it is formally possible that the physiologically important effect is linked to m⁷G formation in another substrate. We note that yeast Pus1 protein is known to modify tRNA as well as U2 snRNA substrates (Massenet et al. 1999), that mPus1 can also act on Steroid Receptor RNA Activator (Zhao et al. 2004), and that Pus7p can act on tRNA and U2 snRNA (Ma et al. 2003).

We have shown that bacterial Trm8p orthologs YggH of *E. coli* and TM0925 of *T. maritima* can act alone to complement the growth defect of *trm8-Δ trm82-Δ* double mutants (Fig. 2, lanes 3a,4a), and have the requisite m⁷G methyltransferase activity (Fig. 3; Table 1). This result is consistent with the lack of a Trm82p ortholog detected in any sequenced prokaryotic organism (Michaud et al. 2000), ex-

tends previous in vitro results demonstrating m⁷G methyltransferase activity of purified *E. coli* protein YggH (De Bie et al. 2003) and *Aquifex aeolicus* protein aq065 (Okamoto et al. 2004), and is in contrast with the established two-protein mechanism of m⁷G formation in yeast and likely in humans (Alexandrov et al. 2002). We note that another single-subunit bacterial modifying enzyme, *Thermus thermophilus* m¹A₅₈ methyltransferase (Droogmans et al. 2003), is also represented by a two-subunit protein complex (Gcd10p/Gcd14p) in yeast (Anderson et al. 2000).

Our results suggest a different partitioning of the roles of the subunits in Trm8p/Trm82p than in the other two known yeast tRNA-modifying complexes. The observation that Trm8p can be cross-linked to tRNA in the absence or presence of Trm82p suggests that the Trm8p subunit, which is known to have a SAM-binding domain, also has tRNA-binding activity. In contrast, in the Gcd10p/Gcd14p m¹A₅₈ methyltransferase complex, Gcd14p has a SAM-binding domain, whereas the Gcd10p subunit binds tRNA (Anderson et al. 1998), and in the Tad2p/Tad3p adenosine deaminase complex, association of both subunits is required for its cross-linking to tRNA^{Ala} substrate (Gerber and Keller 1999). The presence of cofactor and likely substrate binding domains in the Trm8p subunit, as well as its residual m⁷G methyltransferase activity (Alexandrov et al. 2002), suggest that Trm8p has most of the required functions for m⁷G modification, but sheds little light on the role of Trm82p.

Our experiments demonstrate that lack of Trm82p has two dramatic effects on m⁷G methyltransferase activity. First, Trm82p has an important role in regulating the level of Trm8p in the cell, since *trm82-Δ* strains have little, if any, endogenous Trm8p (<6% of wild-type amounts, Fig. 4). Second, Trm82p has an important additional role in promoting m⁷G methyltransferase activity, since Trm8p produced in the absence of Trm82p, either in *E. coli* (Alexan-

FIGURE 6. Trm8p cross-links to pre-tRNA^{Phe} in the presence and absence of Trm82p. Preparations of yeast Trm82p, Trm8p, Trm8p/Trm82p, or similarly purified control protein 3C protease were subjected to UV cross-linking with pre-tRNA^{Phe} as described in Materials and Methods, followed by RNase treatment and resolution by 10% SDS-PAGE. (A) Analysis of proteins after SDS-PAGE and Coomassie staining. (B) Analysis of radioactivity incorporated in gel shown in A.

drov et al. 2002) or in yeast (Fig. 5), has <1% of normal activity.

The exact mechanism by which Trm82p exerts these effects is unclear. Trm82p might affect Trm8p levels by affecting the translation of Trm8p, or by controlling aspects of its targeting for degradation, and Trm82p might affect activity in any number of ways. Trm82p might have a chaperone-like function that protects Trm8p from degradation and stabilizes Trm8p in an active conformation. This would explain both the lack of Trm8p in a *trm82-Δ* strain and the lack of activity of Trm8p purified from a *trm82-Δ* strain, and would be consistent with our inability to reconstitute activity by mixing appropriate extracts or purified components (Alexandrov et al. 2002). Future experiments will undoubtedly cast more light on the precise roles of Trm82p, and may reveal the regulatory basis for the existence of the two-subunit m⁷G methyltransferase in eukaryotes, as opposed to the single-subunit protein in bacteria.

Based on the evidence presented here, Trm8p/Trm82p activity has at least one important physiological role that imparts a selective advantage to cells with m⁷G in their RNA, and which may explain the high degree of evolutionary conservation of both the proteins and the modification. The exact nature of the process impaired by the lack of m⁷G modification remains to be determined.

MATERIALS AND METHODS

Yeast strains

Homozygous diploid *trm8-Δ/trm8-Δ* and *trm82-Δ/trm82-Δ* deletion strains and the corresponding wild-type diploid parent BY4743 were previously described (Alexandrov et al. 2002). Homozygous diploid *trm8-Δ/trm8-Δ trm82-Δ/trm82-Δ* double deletion strain AA0249 (BY4741/BY4742, *trm8-Δ0*, *trm82-Δ::kanMX*) was constructed by PCR-based disruption of *TRM8* using *URA3* from YEplac182 (Gietz and Sugino 1988) in haploid strains BY4741 (*MATa*, *his3-Δ1*, *leu2-Δ0*, *ura3-Δ0*, *met15-Δ0*) and BY4742 (*MATα*, *his3-Δ1*, *leu2-Δ0*, *ura3-Δ0*, *lys2-Δ0*), followed by selection on 5-fluoroorotic acid media to allow excision of *URA3*, as previously described (Xing et al. 2002), disruption of *TRM82* with the appropriate *kanMX* cassette, mating, and diploid selection on synthetic media lacking methionine and lysine. Primers were used as follows: PCR of *URA3* with flanking sequences, 1st round, Trm8_D1F (5'-ACTAGAACAATTACACATATACGCTTTTCAATTCAATTCATC) and Trm8_D1R (5'-CGTTGGTAATCTTGTAAGACCCGATGATAAGCTGTCAAAC), 2nd round, Trm8_D2F (5'-GTTTATTGTGAAGCATAGATGTATAACTAGACAATTTACCATATACGC) and Trm8_D2R (5'-TTACAAATATGGCTGGCGTTGGTAATCTTGTAAGACCCG), 3rd round Trm8_D3F (5'-CCATAGGATAAAATTTCAAGCGTTTATTGTTAAGCATAGATGTATAACTAGAAC) and Trm8_D2R; PCR of *kanMX* with flanking sequences: 165D_F (5'-TTGCGAGAACATAAGACGACG) and 165D_R (5'-GCTTTAGAATTGGGCCTCAG) using genomic DNA from strain 33523 (BY4743, *trm82-Δ::kanMX*), obtained from Research Genetics, as a template.

Yeast strain EJG758 (Martzen et al. 1999) was used for Western

and Northern blot experiments. PCR-based deletion of *trm8* and *trm82* with *kanMX* in this genetic background was done using primer pairs: 201D_F3 (5'-GACTCTCCCCACAAAGCCGG) and 201D_R3 (5'-CCACGTCGTAACATATGGTGATATTGC), and 165D_F and 165D_R (see above), using genomic DNA from strains 33899 (BY4743, *trm8-Δ::kanMX*) and 33523 (BY4743, *trm82-Δ::kanMX*), respectively, as templates. Chromosomal tagging of *TRM8* at its C terminus was achieved in two rounds of PCR using TAP-cassette pAVA0258 as a template and primers TAP_3 (5'-GAGGGCGGTGTCGTGTACAC) and TAP_32 (5'-CTAGTTATACATCTATGTACGACTCACTATAGGG), TAP_3 and TAP_33 (5'-TGTATATGTGGTAAATTGTTCTAGTTATACATCTATGTAC). TAP-cassette pAVA0258, contained in succession *TRM8*, His₆, HA epitope, the rhinovirus 3C protease site, ZZ domain derived from protein A of *Staphylococcus aureus*, and *URA3* gene derived from *Kluyveromyces lactis*. It was constructed by PCR-amplification of the *TRM8*-His₆-HA-3C^{protease site}-ZZ region (which will be described in detail elsewhere) with primers TAP_30 [5'-CTTATTGCCATGGGAGGGCGGTGTCGTGTACAC-3'] and TAP_31 [5'-ATAGTAAGTCAGCTCACTGATGATTCCGGTCTACTTTCCGG-3'] using P_{GAL10}-*TRM8*-His₆-HA-3C^{protease site}-ZZ plasmid (Alexandrov et al. 2002) as a template, followed by digestion of the product with NcoI and PstI and ligation into the vector pBS1539 (Puig et al. 2001). Results of all genetic manipulations were confirmed by PCR.

Plasmids for protein expression in yeast

Open reading frames of *TRM8* and *TRM82* containing 600–650 base pairs of their upstream regions were amplified from yeast genomic DNA using corresponding primer pairs (201_own_F: 5'-TTCACATGCATGCCAGCACAAAGACAAGCTGATGGTG-3', 201_own_R: 5'-GACTAATTCGAGCTCGTTACAATATGGCTGGCGTTGG-3', 165_own_F: 5'-TTCACATGCATGCCCCGCCAAACC AAGCATGTGC-3', 165_own_R: 5'-GACTAATTCGAGCTCCTGCTATTCAATTCGCCGCCCTTC-3'), digested with SphI and SacI and ligated into the *URA3 CEN* vector yCPlac33 and *LEU2 CEN* vector yCPlac111 (Gietz and Sugino 1988) to express these ORFs under their own promoters using a single-copy plasmid.

Expression of proteins under control of P_{CUP1} promoter was achieved in a P_{CUP1}-GST-3C^{protease site}-LIC vector pAVA0262, which was constructed from the plasmid pYEX 4T-1 (Martzen et al. 1999) by replacement of the thrombin site/MCS region (EcoRI, PstI) with 3C^{protease site}/LIC-cloning region: 5'-GAATTCCTGGAAGTTCTGTTCCAGGGTCTGTTTCGCGAATATTCTAGCTTTGTTTAAACAGCACGAACAAGTTCTGCAG-3'. Strains expressing proteins under P_{CUP1} control were grown for extract preparation or protein purification (Figs. 3, 5) as previously described (Martzen et al. 1999). Basal level of expression from P_{CUP1} plasmids was used for complementation experiments (Fig. 2), since overexpression of some protein variants resulted in inhibition of growth. *TRM8* and *TRM82* were amplified from yeast genomic DNA; YggH was amplified from *E. coli* genomic DNA, and *T. maritima* TM0925 was amplified from American Type Culture Collection clone 633437 using appropriate primer pairs:

(201_F_SG: 5'-GGGTCCTGGTTCGATGAAAGCCAAGCCACTAAGCC;
201_R_SG: 5'-CTTGTTTCGTGCTGTTTATTACAATATGGCTGCGTGGTAATC;

165_F_SG: 5'-GGGTCCTGGTTCGATGAGCGTCATTCATC
CTTTGCAG;
165_R_SG: 5'-CTTGTTCTGCTGCTTTACGCCGCTTCAGC
TAGAAACAGAG;
YGGH_F_LIC: 5'-GGGTCCTGGTTCGATGAAAAACGACGTCA
TCTCACCG;
YGGH_R_LIC: 5'-CTTGTTCTGCTGCTTTATTATTTACCCCTC
TCGAACATTAAGTCC;
TM_F_SG: 5'-GGGTCCTGGTTCGATGGTTGTAACAGAATAC
GAACTG; and
TM_R_SG: 5'-CTTGTTCTGCTGCTTTATTAAGGAGAGCCCT
GAGCGGTAACTC).

ORFs were cloned into NruI and PmeI-digested and T4 DNA polymerase-treated (with 1 mM dATP) pAVA0262 using a standard LIC-cloning procedure (Aslanidis and de Jong 1990). Primer pairs used for mutagenesis:

Trm8_G/A_mut_F (5'-GCTGATATTGGCTGTGCATTCGGTG
GGTTGATGATAGATTTATC) and Trm8_G/A_mut_R (5'-
CCCACCGAATGCACAGCCAATATCAGCAATCGTCACCTTC);
Trm8_mut_F (5'-GCTGATATTGCCTGTGCATTCGGTGGGTT
GATGATAGATTTATC) and Trm8_mut_R (5'-CCCACCGA
ATGCACAGGCAATATCAGCAATCGTCACCTTC);
YggH_G/A_mut_F (5'-GCTTGAGATTGGTTTTGCCATGGGG
GCGTCGCTGGTG) and YggH_G/A_mut_R (5'-CGCCCC
CATGGCAAACCAATCTCAAGCGTCACCGGCG);
YggH_mut_F (5'-GCTTGAGATTGCTTTTGCCATGGGGGCGT
CGCTGGTG) and YggH_mut_R (5'-CGCCCCATGGCAA
AAGCAATCTCAAGCGTCACCGGCG);
TM_G/A_mut_F (5'-GGTTGAGATTGGTTTTGCAAACGGGGA
ATTTCTGGCAGAACTTGC) and TM_G/A_mut_R (5'-CCC
GTTTGCAAACCAATCTCAACCACTATCTTTGCCTTTCTG);
and
TM_mut_F (5'-GTGGTTGAGATTGCTTTTGCAAACGGGGAA
TTTCTGGCAGAACTTGC) and TM_mut_R (5'-CCCGTTTG
CAAAGCAATCTCAACCACTATCTTTGCCTTTCTG).

All the resulting clones were sequenced.

Assay for m⁷G methyltransferase activity

[α -³²P]GTP-labeled *S. cerevisiae* pre-tRNA^{Phe} was incubated with protein in the presence of 1 mM S-adenosylmethionine, followed by P1 nuclease treatment of tRNA and thin layer chromatography of modified nucleotides, as described (Alexandrov et al. 2002).

Preparation of low-molecular-weight RNA

RNA was prepared by hot phenol extraction of harvested cells, as described (Rubin 1975).

Purification of individual tRNA^{Phe} from RNA and HPLC analysis of modified nucleosides

Purification of tRNA was carried out as described (Alexandrov et al. 2002; Xing et al. 2004) using 5'-biotinylated DNA oligomer 5Bio-F1 (5'/Biotin/GTGGATCGAACACAGGACCT).

Western blot analysis

Fifteen OD₆₀₀ units of yeast cells were lysed by vigorous shaking with glass beads in the presence of 100 μ L of 2 $\times$ SDS PAGE

loading buffer (100 mM Tris pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), incubated for 5 min in a boiling water bath, centrifuged for 10 sec at 16,000g, and resolved by 4%–15% SDS-PAGE. Western blot was performed using nitrocellulose membrane with peroxidase antiperoxidase as described (Puig et al. 2001).

Northern blot analysis

Northern blot analysis was performed using TRM8- and ACT1-specific DNA oligomers TRM8_N1R (5'-CATCCCTAAGATAA GATCTTCAGGGAAGGCTGG) and ACT13 (5'-TTAGAAA CACTTGTTGGTGAACGATAG) with Bright Star-Plus membrane (Ambion) according to the manufacturer's protocol.

Cross-linking

Thirty thousand cpm of [α -³²P]UTP-labeled intron-containing pre-tRNA^{Phe} (Reyes and Abelson 1987) was incubated for 20 min on ice with metal affinity-purified fractions of His₆-MBP-Trm8, His₆-MBP-Trm82, His₆-MBP-Trm8/His₆-MBP-Trm82 (Alexandrov et al. 2004), or control protein His₆-MBP-3Cpro, and exposed to 254 nm UV light for another 20 min at 26°C. The reactions were treated with 0.5 μ g of DNase-free RNase from bovine pancreas (Roche), and the products were separated by 5%–15% SDS-PAGE. Radioactivity incorporation was visualized using a phosphorimager (Molecular Dynamics).

ACKNOWLEDGMENTS

We thank L. Kotelawala for valuable early contributions, and N. Shull, F. Xing, J. Jackman, and Y. Kon for valuable help and advice. This research was supported by NIH grant GM52347 to E.M.P.

Received January 5, 2005; accepted February 8, 2005.

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ANDREI ALEXANDROV, ELIZABETH J. GRAYHACK and ERIC M. PHIZICKY

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