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Two proteins that form a complex are required for 7-methylguanosine modification of yeast tRNA

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ABSTRACT

7-methylguanosine (m^7G) modification of tRNA occurs widely in eukaryotes and bacteria, is nearly always found at position 46, and is one of the few modifications that confers a positive charge to the base. Screening of a *Saccharomyces cerevisiae* genomic library of purified GST-ORF fusion proteins reveals two previously uncharacterized proteins that copurify with m^7G methyltransferase activity on pre-$tRNA^{Phe}$. ORF YDL201w encodes Trm8, a protein that is highly conserved in prokaryotes and eukaryotes and that contains an S-adenosylmethionine binding domain. ORF YDR165w encodes Trm82, a less highly conserved protein containing putative WD40 repeats, which are often implicated in macromolecular interactions. Neither protein has significant sequence similarity to yeast Abd1, which catalyzes m^7G modification of the 5′ cap of mRNA, other than the methyltransferase motif shared by Trm8 and Abd1. Several lines of evidence indicate that both Trm8 and Trm82 proteins are required for tRNA m^7G-methyltransferase activity: Extracts derived from strains lacking either gene have undetectable m^7G methyltransferase activity, RNA from strains lacking either gene have much reduced m^7G, and coexpression of both proteins is required to overproduce activity. Aniline cleavage mapping shows that Trm8/Trm82 proteins modify pre-$tRNA^{Phe}$ at G46, the site that is modified in vivo. Trm8 and Trm82 proteins form a complex, as affinity purification of Trm8 protein causes copurification of Trm82 protein in approximate equimolar yield. This functional two-protein family appears to be retained in eukaryotes, as expression of both corresponding human proteins, METTL1 and WDR4, is required for m^7G-methyltransferase activity.

Keywords: 7-methylguanosine; S-adenosylmethionine; *S. cerevisiae*; Trm8; Trm82; tRNA methyltransferases; tRNA processing

INTRODUCTION

Virtually every tRNA in every organism carries multiple modifications of its nucleotides (Nishimura, 1979b), and the average yeast tRNA contains about 11 modifications. More than 80 different modifications have been documented so far (Limbach et al., 1994; Bjork, 1995), of which 22 have been found in yeast (Hopper & Martin, 1992). The most widely distributed and prevalent tRNA modification is methylation, which occurs in yeast tRNAs on the 2′-OH of specific nucleotide residues, and at multiple base positions, including the 1 position of adenine, the 5 position of uracil, the 3 and 5 positions of cytosine, and the 1, 2, and 7 positions of guanine (Nishimura, 1979b; Sprinzl et al., 1998).

7-methylguanosine (m^7G) modification of tRNA is of particular interest for several reasons. First, like a number of other modifications, m^7G-modified tRNA is widely found in prokaryotes and eukaryotes (Sprinzl et al., 1998), as well as in some archaea (Edmonds et al., 1991), underscoring its likely importance. In yeast, m^7G has been reported in at least 11 tRNA species, including $tRNA^{Cys}$, $tRNA^{Trp}$, $tRNA^{Pro}$, $tRNA^{Met}$, $tRNA^{Met\text{-}i}$, and two each of $tRNA^{Val}$, $tRNA^{Phe}$, and $tRNA^{Lys}$ (Sprinzl et al., 1998). Second, in almost every tRNA in which m^7G is found, it occurs at position 46 in the extra loop (Fig. 1B), a site that is known to form tertiary interactions with the bases C-13 and G-22 in the crystal structure of yeast $tRNA^{Phe}$ (Kim et al., 1974; Robertus et al., 1974). Two rare exceptions include the finding of m^7G at position 36 in some chloroplast species of $tRNA^{Leu}$ (Sprinzl et al., 1998) and at position 34 in $tRNA^{Ser}_{GCU}$ of starfish mito-

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chondria (Matsuyama et al., 1998). Third, m^7G, like m^1A (Agris et al., 1986), is one of the few tRNA modifications that produces a positively charged base under physiological conditions (see Fig. 1A). Fourth, the same m^7G modification occurs in every eukaryote as part of the mRNA cap; in yeast, this reaction is catalyzed by the essential Abd1 protein (Mao et al., 1995).

To begin to understand the role of m^7G modification of tRNA, we sought to identify the corresponding structural gene from yeast. Previously, a gene encoding a ribosomal RNA m^7G methyltransferase associated with aminoglycoside resistance was cloned from *Streptomyces tenebrarius* (Beauclerk & Cundliffe, 1987; Holmes & Cundliffe, 1991), but this gene bears little resemblance to any yeast gene. Purification of tRNA m^7G methyltransferase activity implicated a 100-kDa fraction and a 300-kDa fraction from *Escherichia coli* (Aschhoff et al., 1976) and a 25-kDa polypeptide from *Salmonella typhimurium* (Colonna et al., 1983), but the responsible gene(s) were not identified. In addition, an *E. coli* mutant strain was previously isolated that is partially defective in tRNA m^7G methyltransferase activity, and the mutation was partially mapped (Marinus et al., 1975), but the gene was not subsequently identified. We show here that two yeast gene products, open reading frames (ORFs) YDL201w and YDR165w (called Trm8 and Trm82) copurify with $tRNA^{Phe}$ m^7G methyltransferase activity, using a previously described biochemical genomics approach (Martzen et al., 1999). Several lines of evidence indicate that these two proteins act together in a complex for efficient m^7G modification of yeast tRNA in vitro and in vivo.

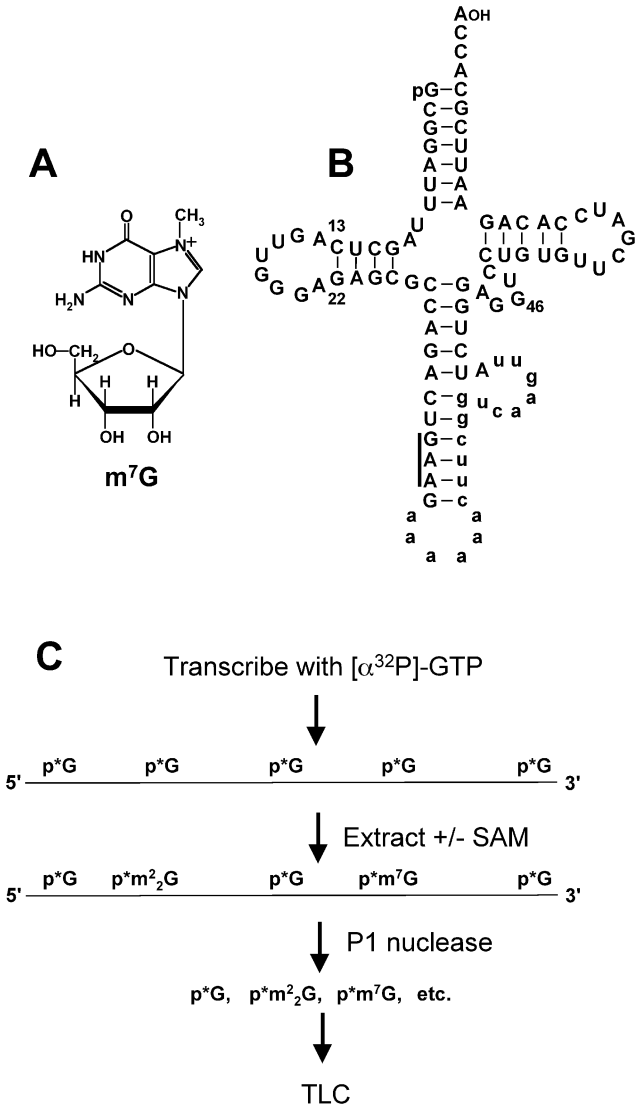

FIGURE 1. A: Structure of m7-Guanosine. **B:** Secondary structure of yeast $tRNA^{Phe}$. m^7G modification occurs at nt 46 in the extra loop. **C:** Schematic of assay for m^7G methylation of pre-$tRNA^{Phe}$.

RESULTS

Detection of tRNA m^7G methyltransferase activity in yeast extract

We employed an assay for the detection of m^7G modification of tRNA, using S-adenosylmethionine (SAM) as a methyl group donor and [α-^{32}P]GTP-labeled *Saccharomyces cerevisiae* intron-containing pre-$tRNA^{Phe}$ as acceptor (Fig. 1). S-adenosylmethionine is used as the methyl donor by most known methyltransferases (Schluckebier et al., 1995), and pre-$tRNA^{Phe}$ is known to be a substrate for m^7G formation in vivo and in vitro (Knapp et al., 1978; Jiang et al., 1997). After incubation of RNA with crude extract and methyl donor, and subsequent P1 nuclease treatment, pG and modified pG derivatives are readily separated by two-dimensional thin layer chromatography (TLC). As shown in Figure 2, several modified guanine nucleotides are formed in the presence of crude extract (compare Fig. 2A, panels a and c). We assigned the second fastest migrating spot in the first dimension to m^7G. This assignment was based on three criteria: comparison with the published two-dimensional map shown in Figure 2B (Nishimura, 1979a); the observed stimulation of formation of this material by exogenous SAM (Fig. 2A, panels c and d); and the observation that $m^2{}_2G$ formed by purified Trm1 protein (Ellis et al., 1986) migrates slightly faster in the first dimension than does the spot corresponding to m^7G (Fig. 2A, panel e). The other guanine nucleotide derivatives, which migrate slower than pm^7G in the first dimension, correspond to pGp, pm^1G, and pm^2G, as indicated in the reference map (Fig. 2B), and possibly pGm (Jiang et al., 1997). We will provide further evidence below to support our assignment of m^7G. As shown in Figure 2C, the separation of m^7G from other modified guanine nucleotides was easily observed if only the first dimension of the two-dimensional TLC was used.

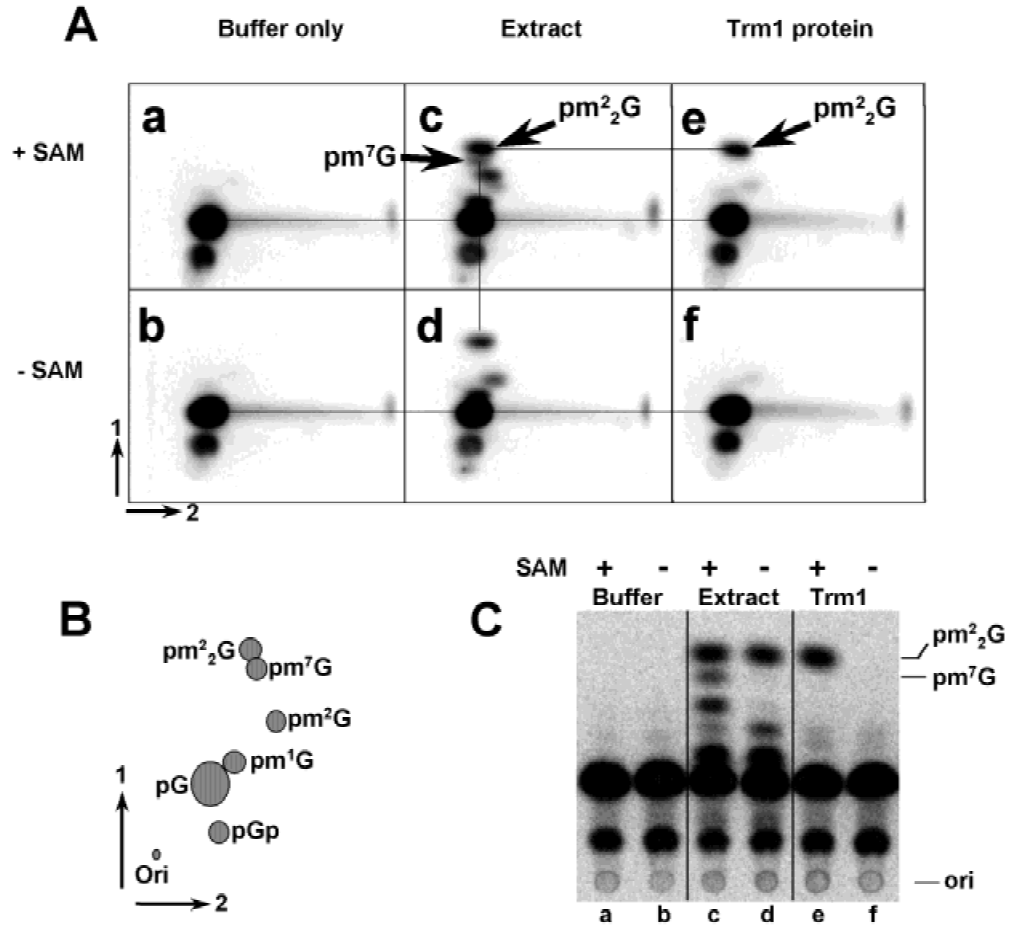

FIGURE 2. A: Detection of m^{7}G methyltransferase activity in yeast extracts. [α-^{32}P]-GTP labeled pre-tRNA^{Phe} was incubated with buffer (a, b), 1 μL of 25 mg/mL extract (c, d) or 1 μL of 0.5 mg/mL Trm1 protein (e, f) in the presence of 1 μL SAM (a, c, e) or buffer (b, d, f) for 6 h at 30 °C. Then samples were treated with P1 nuclease, and modified nucleotides were separated by two-dimensional TLC as described in Materials and Methods. **B:** Schematic of expected position of G-modified nucleotides. The two-dimensional map is adapted from Nishimura (1979a). **C:** One-dimensional chromatography separation of modified guanine nucleotides. Methyltransferase reactions described in **A**, separated in the first dimension of the TLC system described above.

m^{7}G methyltransferase activity is associated with two yeast ORFs

To identify the yeast gene(s) associated with tRNA m^{7}G methyltransferase activity, we screened a genomic set of purified yeast GST-ORF fusion proteins (Martzen et al., 1999). These proteins are derived from a collection of 6,144 yeast strains, each expressing a unique GST-ORF, which are purified in pools after growth of 64 mixtures of 96 strains each. As shown in Figure 3, m^{7}G methyltransferase activity with pre-tRNA^{Phe} was detected in both pools 47 and 51. Deconvolution of these

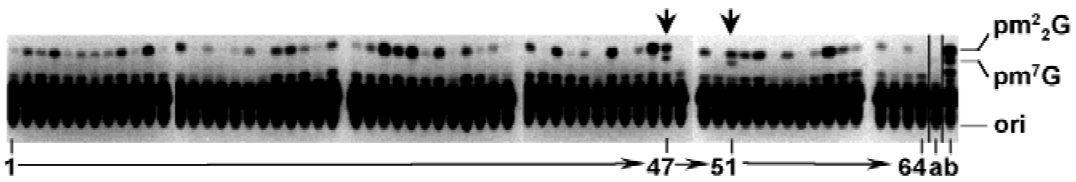

FIGURE 3. Assay of genomic collection of GST-ORF pools for m^{7}G methyltransferase activity implicates pools 47 and 51. Four microliters of each GST-ORF pool, as indicated, were assayed for m^{7}G methyltransferase activity with [α-^{32}P]-GTP labeled pre-tRNA^{Phe} and SAM for 8 h at 30 °C, and then modified nucleotides generated in each pool were separated, as described in Materials and Methods. Lane a: buffer control; lane b: yeast extract. Arrows at pools 47 and 51 indicate positive fractions.

pools, by growth of subpools of the strains and analysis of the corresponding GST-ORF preparations (Martzen et al., 1999), implicated strains MRM 4465 (expressing ORF YDL201w) and MRM 4822 (expressing ORF YDR165w) that were associated with the activity (data not shown). In each case, the corresponding plasmid DNA was transformed into *E. coli* and sequenced to confirm its identity, and then transformed back into yeast and retested to confirm that the observed m^7G-methyltransferase activity was due to the implicated GST-ORFs. We assigned the name *TRM8* to ORF YDL201w, and *TRM82* to YDR165w. We show below that Trm8 and Trm82 are both required for m^7G methyltransferase activity.

Database analysis of Trm8 and Trm82 proteins

Both Trm8 and Trm82 are widely conserved proteins that have not previously been ascribed a function, as determined by a search of the Saccharomyces Genome Database (Cherry et al., 2002), SwissProt (Gasteiger et al., 2001), TrEMBL (Bairoch & Apweiler, 2000), and CDD (Marchler-Bauer et al., 2002). A BLAST search (Altschul et al., 1997) reveals that Trm8 is a likely methyltransferase with putative orthologs in a number of eukaryotes (Fig. 4A) including *Caenorhabditis elegans* ($2e^{-82}$), *Neurospora crassa* ($2e^{-82}$), human ($5e^{-79}$), mouse ($9e^{-79}$), and *Drosophila melanogaster* (2^{e-77}). Furthermore, the protein families database of alignments (Pfam) identified the region from amino acids 77 to 280 as belonging to a highly conserved methyltransferase domain (Methyltransf_4, e^{-54}) that is widely found in prokaryotes such as mycoplasma, cyanobacteria, and *E. coli*, as well as in eukaryotes. This domain contains the SAM-binding motif GXGXG. Trm82 is also conserved in different organisms. As revealed by BLAST analysis, putative orthologs are found in *Schizosaccharomyces pombe* ($7e^{-23}$), mouse ($4e^{-18}$), human ($1e^{-15}$), *D. melanogaster* ($8e^{-14}$), *C. elegans* ($7e^{-10}$), and *Arabidopsis thaliana* ($3e^{-9}$), but not apparently in prokaryotes. There is no obvious indication of the presence of the SAM-binding motif in this group of proteins. Rather, Trm82 contains four or five putative WD40 repeats (Fig. 4B), as predicted by the Protein Sequence Analysis program (Stultz et al., 1997), which are often implicated in macromolecular interaction and regulatory functions (Neer et al., 1994). Neither Trm8 nor Trm82 is significantly related to KgmB from *S. tenebrarius*, a rRNA m^7G methyltransferase associated with aminoglycoside resistance (Beauclerk & Cundliffe, 1987; Holmes & Cundliffe, 1991) or to Abd1, which catalyzes m^7G formation in mRNA cap structures (Mao et al., 1995), other than the methyltransferase domain shared by Trm8, Abd1, and numerous other methyltransferases (Niewmierzycka & Clarke, 1999).

Diploid strains carrying homozygous deletions of either *TRM8* or *TRM82* are viable (Winzeler et al., 1999), and closer examination of growth reveals no measurable growth rate differences, compared to wild-type control strains, in rich media containing glucose at 30 °C.

Strains lacking either Trm8 or Trm82 yield extracts with no detectable tRNA m^7G methyltransferase activity, and have severely reduced m^7G-modified tRNA in vivo

The observation that two GST-ORFs each copurify with tRNA m^7G methyltransferase activity suggests one of two different possibilities: Either each GST-ORF protein separately catalyzes the observed activity, or the two ORFs are part of the same complex of copurifying proteins that catalyze a single activity. To distinguish between these possibilities, we analyzed m^7G-methyltransferase activity in extracts from *trm8*-Δ/*trm8*-Δ, *trm82*-Δ/*trm82*-Δ, and wild-type control strains. If each ORF was associated with a different activity, then lack of one ORF would partially reduce activity. However, if each ORF was part of a complex required for activity, then elimination of either ORF would eliminate the activity. As shown in Figure 5, extracts from either *trm8*-Δ/*trm8*-Δ or *trm82*-Δ/*trm82*-Δ strains have less than 1/1,000th of the m^7G methyltransferase activity observed in control extracts, but nearly the same levels of $m^2{}_2G$ methyltransferase and m^1G methyltransferase activities. This result demonstrates that both proteins are necessary for a single m^7G methyltransferase activity in vitro.

Consistent with our results in vitro, strains lacking Trm8 or Trm82 each have very low levels of m^7G in their tRNA. To analyze the m^7G content of tRNA, we prepared low molecular weight RNA, treated the RNA with P1 nuclease, and then subjected the resulting nucleotides to DEAE-Sepharose chromatography to separate pm^7G (and pC) from the bulk of the pG. Then nucleotides were converted to nucleosides, which were separated on a reverse phase C18 column (Gehrke & Kuo, 1989). As shown in Figure 6, the level of m^7G was much reduced in the low molecular weight RNA from *trm8*-Δ/*trm8*-Δ and *trm82*-Δ/*trm82*-Δ strains, relative to that in the control strain, whereas the rest of the profiles are very similar. Quantitation shows that m^7G was reduced 6–12-fold in RNA from strains lacking Trm8 or Trm82 proteins, whereas the area of the control cytidine peak varied by less than 3% in the different samples. Moreover, we doubt that the material from *trm8*-Δ/*trm8*-Δ and *trm82*-Δ/*trm82*-Δ strains that migrates near m^7G is really that nucleoside, as its peak is offset from m^7G found in wild-type strains (compare Fig. 6A with B and C). The amount of this material was too low to determine a spectrum. Thus, we conclude that both proteins are necessary for the vast majority of m^7G modification of tRNA in vivo.

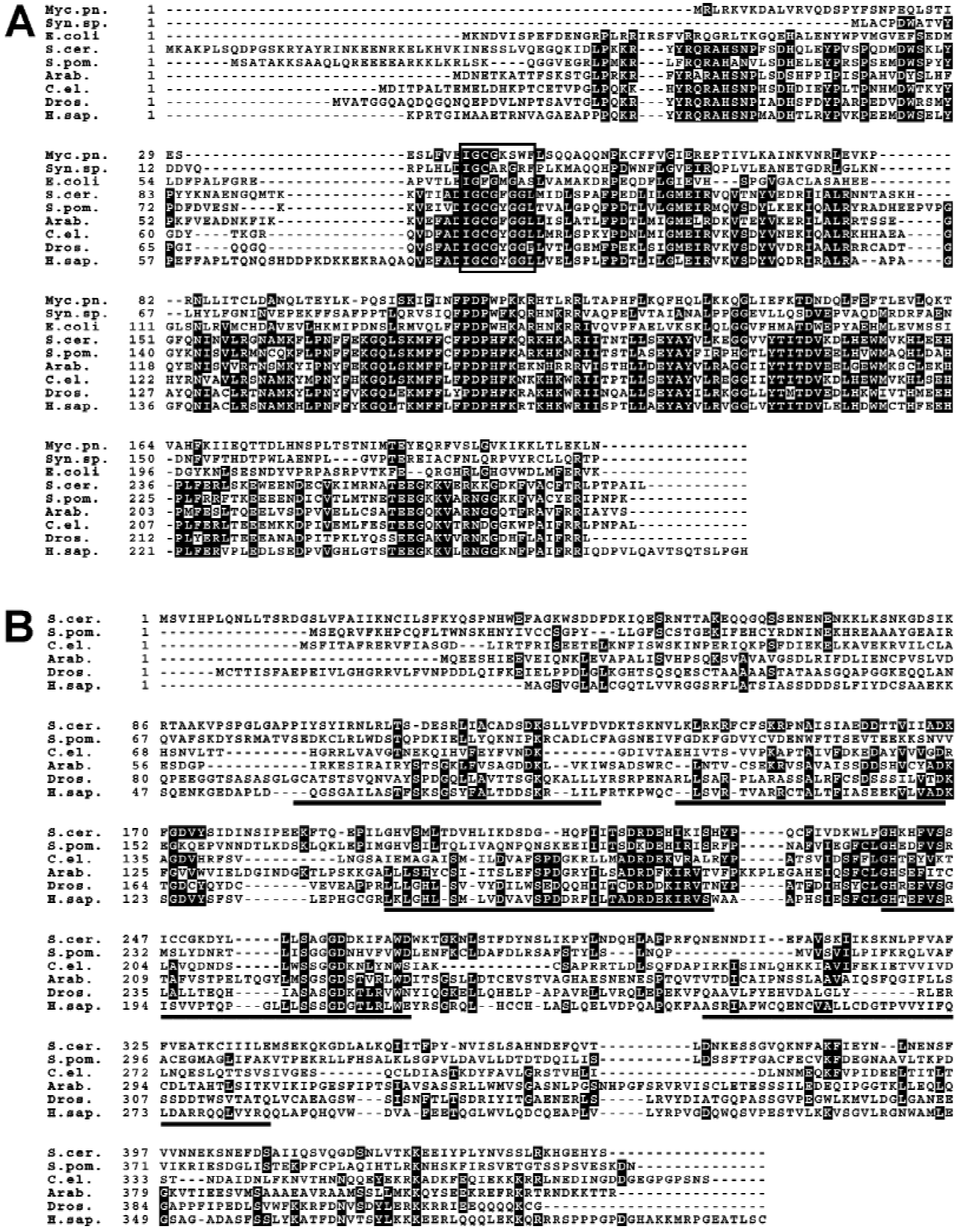

FIGURE 4. A: Amino acid sequence alignment of *S. cerevisiae* protein Trm8 with putative proteins from *Mycoplasma pneumoniae*, *Synechocystis sp.*, *E. coli*, *S. pombe*, *C. elegans*, *A. thaliana*, *D. melanogaster*, and *Homo sapiens*. Boxed: predicted SAM-binding domain. **B:** Amino acid sequence alignment of *S. cerevisiae* protein Trm82 with putative proteins from *S. pombe*, *C. elegans*, *A. thaliana*, *D. melanogaster*, and *H. sapiens*. Underlined: WD40 repeats predicted for Trm82 protein by Protein Sequence Analysis (PSA) program.

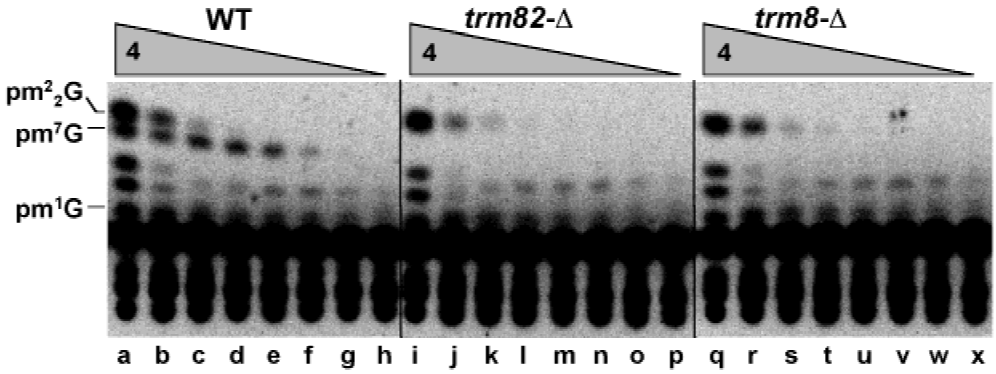

FIGURE 5. m^{7}G-methyltransferase activity is reduced in extracts from *trm8*-Δ/*trm8*-Δ and *trm82*-Δ/*trm82*-Δ strains. Extracts from homozygous diploid strains lacking *TRM8* or *TRM82* (relevant genotype: *trm8*-Δ/*trm8*-Δ or *trm82*-Δ/*trm82*-Δ) or from wild-type control strains, were serially diluted fourfold beginning with 6 μg of protein, and assayed for m^{7}G methyltransferase activity as described in Materials and Methods.

Production of both Trm8 and Trm82 proteins is sufficient for m^{7}G methyltransferase activity

To test whether Trm8 and Trm82 proteins are sufficient for m^{7}G-methylation of tRNA, we expressed both proteins in *E. coli* as His6-fusion proteins under control of the P_{tac} promoter. We find that expression of both proteins in *E. coli* yields extracts with 100-fold more activity than that in extracts from cells overexpressing either His6-fusion protein alone (data not shown). Similarly, copurified His6-Trm8 and His6-Trm82 proteins from cells simultaneously overexpressing both proteins results in preparations with 1,000-fold more activity than that obtained from mock purification or from purification of His6-Trm82 protein alone, and 250-fold more activity than that from purification of His6-Trm8 protein (Fig. 7). We conclude that expression of both Trm8 and Trm82 is sufficient for m^{7}G methyltransferase activity, although the 0.4% basal activity produced by Trm8 protein alone suggests that the presence of Trm82 is not an absolute requirement for activity in vitro.

The methyltransferase activity of purified Trm8/Trm82 proteins is the same as that identified as m^{7}G by modification of pre-tRNA^{Phe} in crude extracts, as determined by two-dimensional TLC (Fig. 7C).

Purified Trm8/Trm82 proteins modify G46 of pre-tRNA^{Phe} to m^{7}G

Because m^{7}G modification of tRNA from different organisms is almost invariably found in the extra loop at G46 (Grosjean et al., 1995), it seemed highly likely that Trm8/Trm82 proteins were acting at the same site on pre-tRNA^{Phe}. To map the site of m^{7}G modification, and to confirm the identity of the modification, we exploited the known sensitivity of m^{7}G to borohydride reduction followed by aniline cleavage of the RNA (Wintermeyer & Zachau, 1975). After incubation of 3′-end-labeled pre-tRNA^{Phe} with Trm8/Trm82 proteins, aniline cleavage yielded a single band (Fig. 8). Comparison of the size of the cleaved product with a guanine cleavage ladder generated from pre-tRNA^{Phe} (Peattie, 1979) demonstrates that cleavage occurs at G46, the same site that is modified to m^{7}G in vivo. Since m^{7}G is the only common modified guanine nucleotide that is sensitive to aniline, this experiment also provides further indication that the modification formed by Trm8/Trm82 is m^{7}G.

Trm8 and Trm82 proteins form a complex

Because both Trm8 and Trm82 proteins are required for a single m^{7}G methyltransferase activity (see Figs. 5 and 6), it seems likely that these two proteins form a complex. Indeed, the identification of a single m^{7}G methyltransferase activity associated with the two different GST-ORFs (Fig. 3), and the increased activity observed when both proteins are coexpressed and copurified (Fig. 7), argues that such a complex exists. To determine explicitly if there is a complex between Trm8 and Trm82 proteins, we constructed a strain expressing both affinity-tagged Trm8 protein and myc-tagged Trm82 protein, and examined affinity-purified Trm8 protein for copurifying myc-Trm82 (see Materials and Methods). As shown in Figure 9A, lane a, the resulting purified Trm8 protein was devoid of visible contaminants, except for one polypeptide. This other polypeptide was present in near stoichiometric amounts, had the same apparent molecular weight as myc-Trm82, and was reactive with anti-[c-myc] antibodies (Fig. 9B, lane a). Parallel purification of a control protein, Tpt1, from a strain coexpressing myc-Trm82, failed to cause copurification of any detectable amount of myc-Trm82 (Fig. 9A, B, lane b). Thus, we conclude that there is a complex between Trm8 and Trm82 proteins that is responsible for copurification of the two proteins.

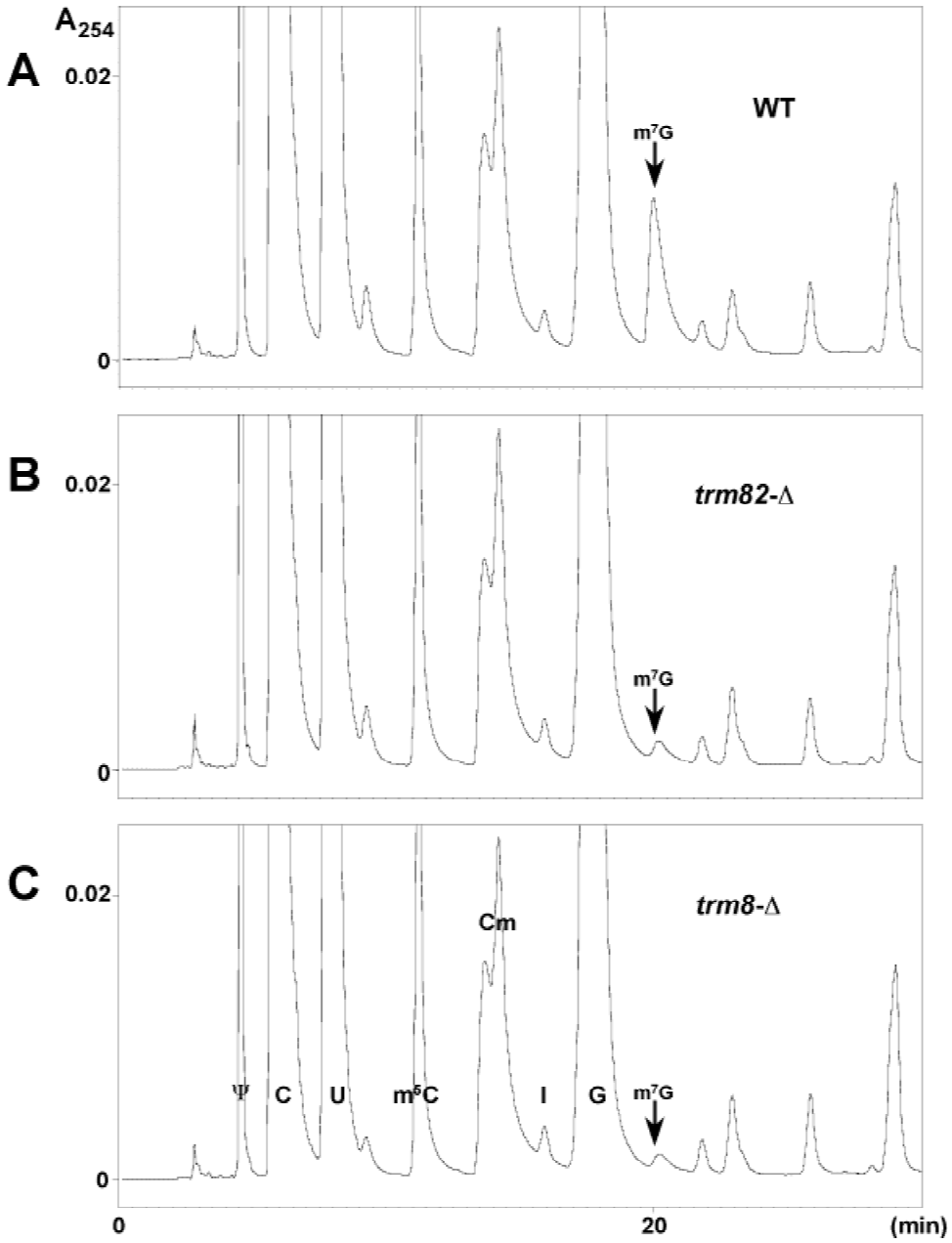

FIGURE 6. m^{7}G content of tRNA from *trm8-Δ/trm8-Δ* or *trm82-Δ/trm82-Δ* strains is reduced. Low molecular weight RNA was purified from wild type (**A**), *trm82-Δ/trm82-Δ* (**B**), and *trm8-Δ/trm8-Δ* (**C**) strains, nucleotides were prepared, and the subfraction of modified nucleosides containing m^{7}G and cytidine was prepared and then separated on HPLC as described in Materials and Methods. Arrow in **B** and **C** indicates elution position of m^{7}G in **A**.

The function of Trm8/Trm82 proteins in tRNA modification is conserved in higher eukaryotes

We tested if the Trm8/Trm82 proteins are conserved in functional form by expressing the corresponding putative human orthologs (METTL1 and WDR4) in yeast, and assaying extracts for activity. In a functional complementation test, we found that expression of each human protein alone failed to restore m^{7}G methyltransferase activity to yeast extracts derived from strains missing the corresponding *TRM8* or *TRM82* yeast gene (Fig. 10A, B). However, coexpression of both METTL1 and WDR4 proteins restored the ability of yeast *trm8-Δ* extracts to m^{7}G modify yeast tRNA^{Phe} (Fig. 10C). This result supports the orthologous nature of the two human proteins with their counterpart yeast proteins, and suggests that the Trm8/Trm82 protein family occurs widely in eukaryotes to form m^{7}G in tRNA. We note that extracts expressing both human proteins METTL1 and WDR4 have about 30-fold higher activity when assayed at 37 °C than at 30 °C (data not shown),

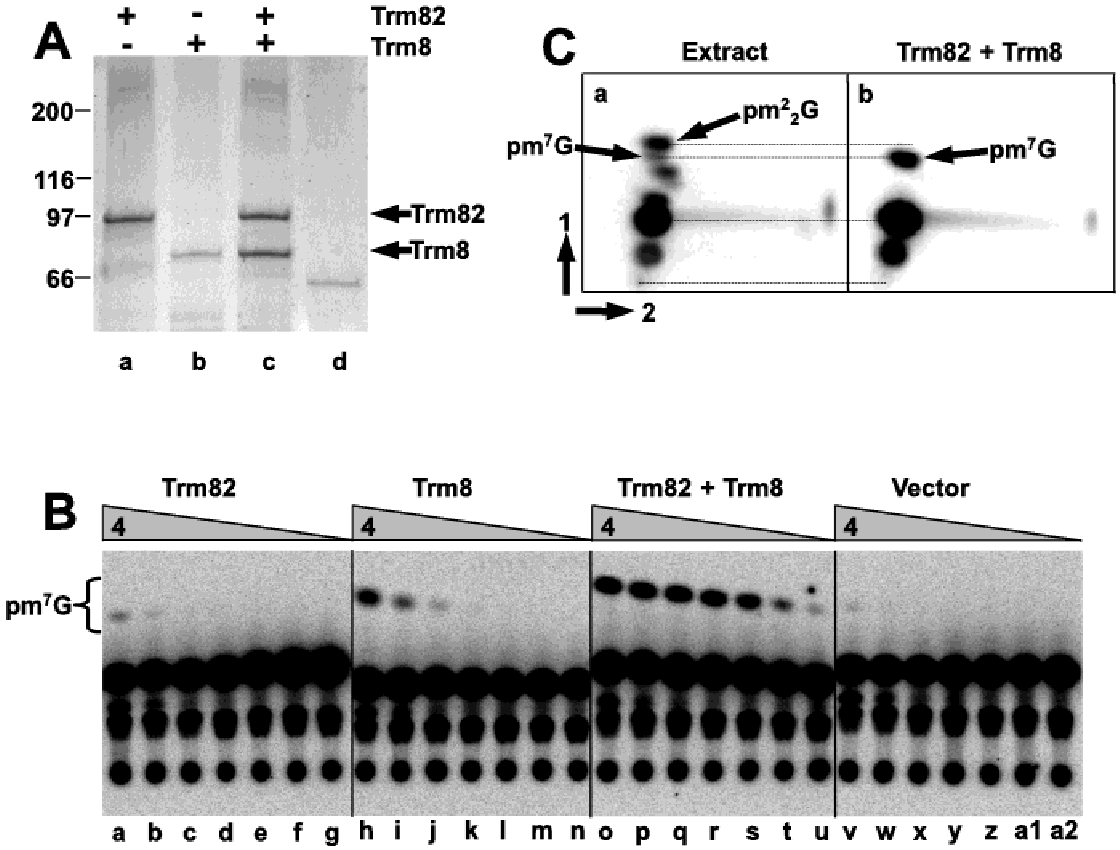

FIGURE 7. A: SDS-PAGE analysis of Trm8 and Trm82 proteins purified from *E. coli*. Yeast Trm8 and/or Trm82 proteins, as well as His6-MBP-3C protease, were purified from *E. coli* strains expressing His6-MBP-ORF fusion proteins under P_{tac} control, as described in Materials and Methods, analyzed on a 4–15% SDS polyacrylamide gel, and stained with silver. Lane a: His-MBP-Trm82 protein; lane b: His-MBP-Trm8 protein; lane c: coexpressed and copurified His-MBP-Trm82 and His-MBP-Trm8 proteins; lane d: His6-MBP-3C protease. **B:** m^7G methyltransferase activity of Trm82 and Trm8 proteins purified from *E. coli* is stimulated when both proteins are copurified. Yeast Trm8 and/or Trm82 proteins, purified from *E. coli*, as well as His6-MBP-3C protease, were assayed for m^7G methyltransferase activity by serial fourfold titrations of purified samples, as described in Materials and Methods. Lanes a–g: titration of Trm82 protein, beginning with 0.1 μg protein; lanes h–n: titration of Trm8 protein, beginning with 0.1 μg protein; lanes o–u: titration of coexpressed and copurified Trm82 and Trm8 proteins, beginning with 0.1 μg total protein; lanes v–a2: titration of control protein, His-MBP-HRV 3C protease, beginning with 6.0 μg protein. **C:** Analysis of modification product produced by purified Trm8/Trm 82 protein. Yeast extract and purified Trm8 and Trm82 proteins were assayed for m^7G methyltransferase activity, and reaction products were resolved by two-dimensional TLC as described in Figure 2A.

whereas control yeast extracts have almost no m^7G methyltransferase activity when assayed at 37 °C (data not shown).

DISCUSSION

We have identified two yeast ORFs, YDR165w and YDL201w (encoding Trm82 and Trm8, respectively), associated with tRNA m^7G-methyltransferase activity, using pre-$tRNA^{Phe}$ as a substrate and S-adenosylmethionine as donor. The product formed by this reaction is m^7G-modified pre-tRNA, as shown by the migration of pm^7G at the expected position in two-dimensional TLC, the characteristic aniline-promoted cleavage of m^7G-modified RNA, and mapping of the modification to the known in vivo site, position 46.

We have provided two lines of evidence that both Trm8 and Trm82 proteins are necessary for m^7G modification of tRNA: First, extracts from strains deleted for either gene have less than 0.1% of the normal amount of m^7G methyltransferase activity with pre-$tRNA^{Phe}$ as substrate. Second, strains lacking either gene each have less than 16% of the normal amount of m^7G in their low molecular weight RNA, and the residual material present from *trm8*-Δ/*trm8*-Δ and *trm82*-Δ/*trm82*-Δ strains may not be m^7G, because of its slightly different mobility. If present, small amounts of m^7G found in RNA from *trm8*-Δ/*trm8*-Δ and *trm82*-Δ/*trm82*-Δ strains could be from capped mRNA, 18S rRNA, or tRNA. Residual m^7G from capped mRNA is not likely because the cap is not sensitive to P1 nuclease (Winicov & Perry, 1976), there are not that many moles of mRNA present in the cell com-

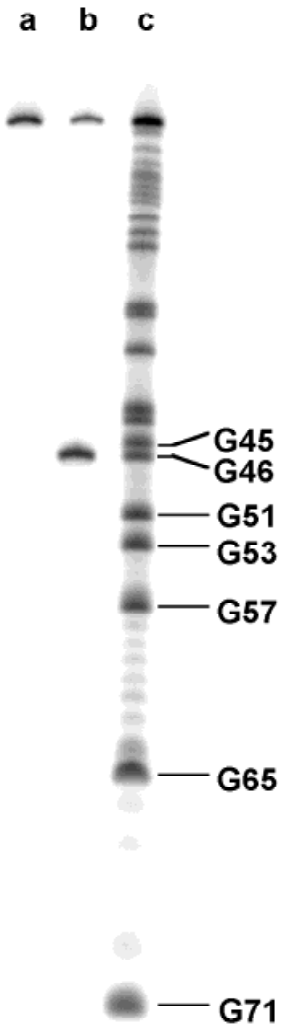

FIGURE 8. Trm8/Trm82 protein modifies the correct guanine residue to m^{7}G in tRNA^{Phe}. 3′-end-labeled with [5′-^{32}P]pCp pre-tRNA^{Phe} was treated with SAM and either Trm8/Trm82 protein or buffer for 8 h at 30 °C, and then analyzed for the site of modification by reduction with borohydride, aniline cleavage, and resolution on a 15% polyacrylamide sequencing gel containing 4 M urea. Lane a: buffer-treated pre-tRNA^{Phe}; lane b: pre-tRNA^{Phe} treated with Trm8/Trm82; lane c: G-ladder, obtained from end-labeled pre-tRNA^{Phe} by dimethylsulfate treatment followed by aniline cleavage.

pared to tRNA (Waldron & Lacroute, 1975; Ares et al., 1999), and because our preparations are enriched for low molecular weight RNA. However, it is formally possible that residual m^{7}G in RNA from *trm8*-Δ/*trm8*-Δ and *trm82*-Δ/*trm82*-Δ strains could arise from one or more of the 10 other tRNA species known to have m^{7}G in their tRNA (Sprinzl et al., 1998), or from rRNA (Zueva et al., 1985b), as we have not explicitly assessed their m^{7}G content. Nonetheless, it seems more likely to us that the m^{7}G modification at position 46 in different tRNAs is mediated by one Trm8/Trm82 protein complex, much as i^{6}A (Dihanich et al., 1987), m$^2{}_2$G (Ellis et al., 1986), and m^{5}C (Motorin & Grosjean, 1999) modifications are each formed in different tRNAs by a single protein.

We have provided three lines of evidence that Trm8 and Trm82 proteins are present in a complex. First, a complex is strongly implied by the observed association of a single methyltransferase activity with two GST-ORF fusion proteins. Second, substantial m^{7}G methyltransferase activity is obtained only when both Trm8 and Trm82 are simultaneously expressed and copurified. Interestingly, we could not reconstitute tRNA m^{7}G methyltransferase activity by mixing of extracts from *trm8*-Δ/*trm8*-Δ and *trm82*-Δ/*trm82*-Δ strains, by mixing extracts from strains overproducing the corresponding proteins, or by mixing the individual purified proteins. This result suggests either that the functional complex between Trm8 and Trm82 proteins is only formed during translation, or that one of the two proteins is inherently unstable in the absence of its interacting partner. Third, myc-Trm82 copurifies in equimolar amounts with Trm8, demonstrating directly the existence of a complex under these conditions.

We note that Trm8/Trm82 proteins purified from *E. coli* and from yeast have nearly identical specific activities under our assay conditions. This implies that there is unlikely to be another yeast subunit that is necessary for activity, unless it is fortuitously present in *E. coli*.

There are interesting similarities between m^{7}G modification and m^{1}A modification of tRNA. First, both modifications introduce a positive charge on the tRNA, and are the only known yeast tRNA modifications that have this property (Agris et al., 1986). Second, m^{1}A modification also requires two proteins in a complex, Gcd10 and Gcd14 (Anderson et al., 1998). The only other yeast tRNA modification enzyme known to be comprised of two polypeptides is Tad2/Tad3, which deaminates A to I at position 34 (Gerber & Keller, 1999). For m^{1}A modification of tRNA, RNA binding depends on Gcd10p, and methyltransferase activity is catalyzed by Gcd14p subunit (Anderson et al., 1998), whereas for inosine modification by Tad2/Tad3, both proteins are necessary for crosslinking to substrate tRNA (Gerber & Keller, 1999). In the case of m^{7}G methyltransferase, the roles of the two proteins are currently being investigated. Based on the presence of a putative SAM-binding domain, it seems likely that Trm8 is the catalytically active subunit, but the role of Trm82 and the source of tRNA binding is not yet known (L. Kotelawala, A.V. Alexandrov, and E.M. Phizicky, data not shown).

Our results suggest that the functional Trm8/Trm82 protein family, and not just the individual proteins, is conserved in higher eukaryotes, as expression of both human homologs is required for activity in the corresponding yeast extracts. However, this conserved two-protein family for m^{7}G modification of tRNA must be different in bacteria, because Trm82 has no obvious bacterial orthologs. It is possible that a different protein substitutes for Trm82 in prokaryotes. Alternatively, orthologs of Trm8 may have evolved in prokaryotes that can effect m^{7}G modification alone. We note that

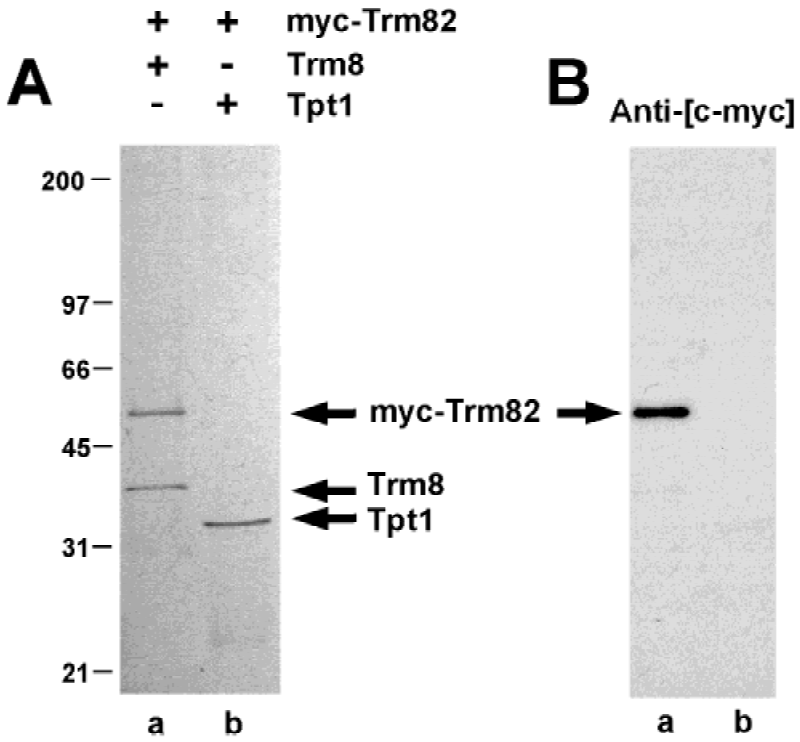

FIGURE 9. Trm82 protein copurifies with affinity-purified Trm8 protein. Extract was prepared from strains expressing myc-Trm82 protein and either Trm8 or Tpt1 protein as the corresponding ORF-HA-3C site-protein A fusion protein, and fusion proteins were purified by IGG-Sepharose chromatography, followed by treatment with 3C protease, as described in Materials and Methods, and preparations were analyzed on 4–15% SDS-PAGE stained with silver (**A**) or anti-[c-myc] immunostaining (**B**).

Trm8 protein has a small residual amount of m^7G methyltransferase activity under conditions we have used. Indeed, a 25-kDa protein from *S. typhimurium* was previously purified to near homogeneity and implicated in tRNA m^7G methyltransferase activity (Colonna et al., 1983).

Although the widespread occurrence of m^7G modified tRNA in eukaryotes and prokaryotes and some archaea argues for its evolutionary importance, its apparent absence in some organisms also argues that it is not crucial. Because there is no obvious growth phenotype of *trm8*-Δ/*trm8*-Δ and *trm82*-Δ/*trm82*-Δ strains in rich media, the exact role of this modification remains to be elucidated.

MATERIALS AND METHODS

Preparation of substrates

[α-^{32}P]GTP-labeled *S. cerevisiae* pre-tRNA^{Phe} (1,500 Ci/mmol of pre-tRNA^{Phe}) was prepared by T7 polymerase transcription of a plasmid-borne copy of the end-matured intron-containing pre-tRNA^{Phe} gene (Reyes & Abelson, 1987). To prepare 3′ end-labeled pre-tRNA, RNA transcribed in vitro was gel purified and then labeled using [^{32}P]pCp and T4 RNA ligase as described (Butler et al., 1997), followed by gel purification to separate the labeled transcript from the common "n + 1" contaminant that arises during transcription (Moran et al., 1996).

Assay for m^7G methyltransferase activity

Reaction mixtures of 20 μL contained 85 mM Tris, pH 8.0, 1.4 mM DTT, 0.07 mM EDTA, 1 mM spermidine, 30,000 cpm [α-^{32}P]GTP-labeled intron-containing pre-tRNA^{Phe}, and, where indicated, 1 mM SAM, 1 μL protein, or appropriate buffer, and were incubated 4 to 8 h at 30 °C for yeast proteins or at 37 °C for human proteins. Then tRNA was extracted with phenol/chloroform, ethanol precipitated, resuspended in 5 μL buffer containing 30 mM sodium acetate, pH 5.2, and 200 μM $ZnCl_2$, and treated with 1 μg P1 nuclease (Calbiochem) for 1 h at 37 °C. The digestion products were resolved on cellulose TLC plates (Merck) separated as described (Nishimura, 1979a), using isobutyric acid:0.5 M ammonium hydroxide, 5:3 (v/v) as the first-dimension solvent, and isopropanol:concentrated HCl:water, 70:15:15 (v/v/v) as the second-dimension solvent. Radioactive species were visualized and quantitated using a phosphorimager (Molecular Dynamics). One unit of activity is defined as the amount of protein required to convert half of the substrate tRNA to m^7G-modified product.

Yeast strains, plasmids, and growth

Homozygous diploid deletion strains and the corresponding wild-type parents were made as described (Winzeler et al., 1999) and obtained from Research Genetics: BY4743 (*MATa*/*MATα*, *his3*-Δ*1*/*his3*-Δ*1*, *leu2*-Δ*0*/*leu2*-Δ*0*, *ura3*-Δ*0*/*ura3*-Δ*0*, *met15*-Δ*0*/+ *lys2*-Δ*0*/+); strain 33523 (BY4743, *YDR165w*-Δ*::kanmx*); strain 33899 (*BY4743*, *YDL201w*-Δ*::kanmx*). Strain YM317 (*MATx*, *leu2*, *trp*, *ura3*, *prb1-1122*, *pep4-1*, Δ*his3::pGAL10::GAL4*) was obtained from H. Madhani and

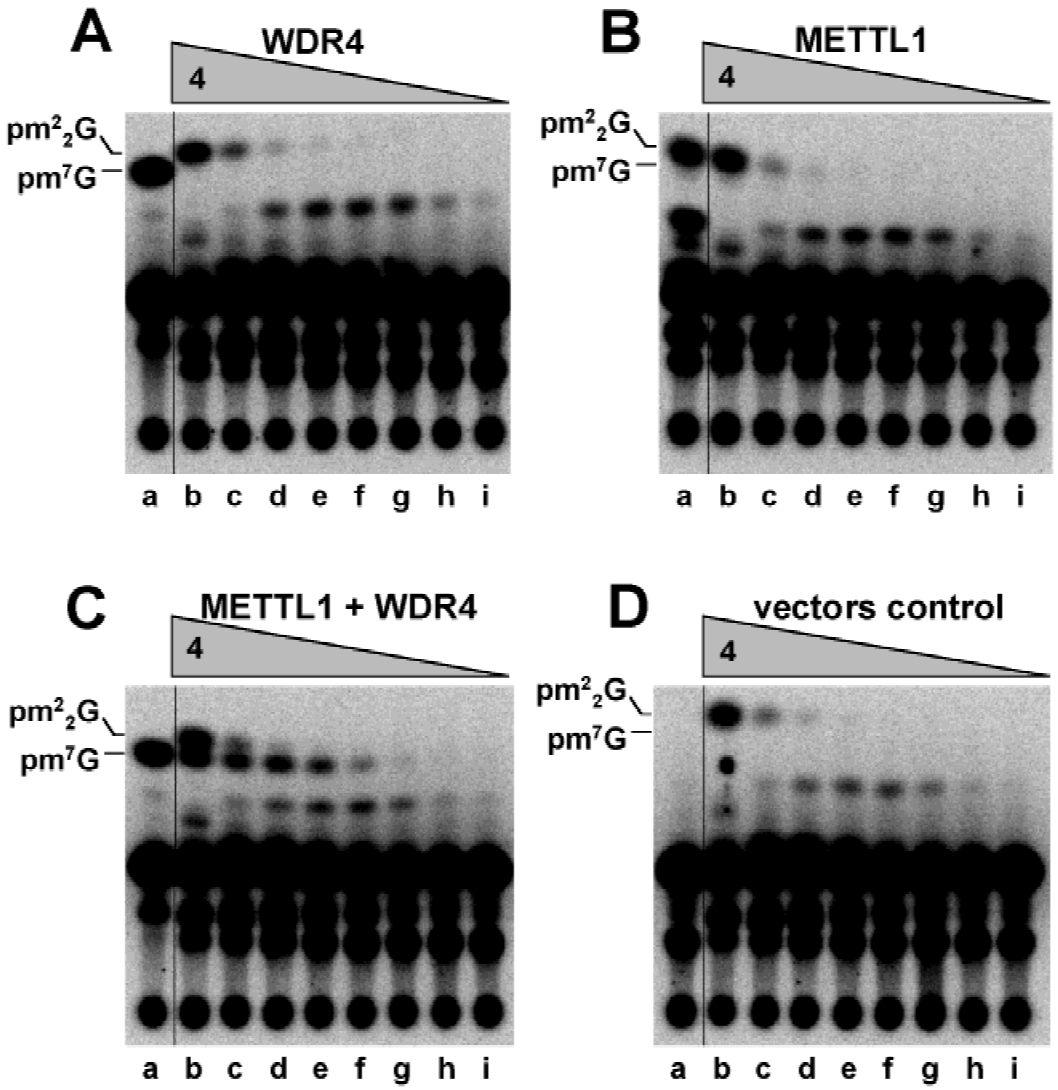

FIGURE 10. Identification of likely human orthologs responsible for m^{7}G modification of tRNA. Yeast extracts prepared from a *trm82*-Δ/*trm82*-Δ (**A**) or a *trm8*-Δ/*trm8*-Δ yeast strain (**B**, **C**, **D**), expressing the putative human ortholog of yeast Trm8 (METTL1) and/or the putative human ortholog of yeast Trm82 (WDR4), were assayed for m^{7}G methyltransferase activity at 37 °C, as described in Materials and Methods, and indicated in the figure, by serial fourfold titration of protein. **A:** Strain expressing WDR4 protein. Lane a: Pre-tRNA^{Phe} incubated with Trm8/Trm82 proteins. **B:** Strain expressing METTL1 protein. Lane a: Pre-tRNA^{Phe} incubated with yeast Trm1 (m$^2_2$G tRNA methyltransferase) **C:** Strain expressing METTL1 and WDR4 proteins. Lane a: pre-tRNA^{Phe} incubated with Trm8/Trm82 proteins. **D:** Extract from a *trm8*-Δ/*trm8*-Δ strain. Lane a: no protein control.

used for galactose-induced expression for immunopurification experiments.

ORFs were expressed under P_{GAL} control using the *Bam*HI-*Eco*RI *GAL1-GAL10* promoter region from pBM258 (Johnston & Davis, 1984), which was ligated into the *URA3 CEN* vector yCPlac33 and *LEU2 CEN* vector yCPlac111 (Gietz & Sugino, 1988) to produce vectors pAVA0040 and pAVA0042, respectively. ORFs YDL201w and YDR165w were PCR-amplified from yeast genomic DNA with appropriate primer pairs (201_F2, 5′-TACATGCCATGGGATCCAAGATGAAAGCCAAGCCACTAAGCC-3′; 201_R, 5′-CATCGCGGATCCCTGCAGCATCTATGTTACAATATGGCTGGCGTTGG-3′; 165-F2, 5′-ATCATGCCATGGGATCCACAATGAGCGTCATTCATCCTTTGCAG-3′; and 165_R, 5′-CATCGCGGATCCCTGCAGCGCCGCCTTCAGCTAGAAACAGAG-3′) and ORFs of human proteins METTL1 (Bahr et al., 1999) and WDR4 (Michaud et al., 2000) were PCR-amplified from I.M.A.G.E. Consortium clones 3163932 and 4080041 using appropriate primer pairs (METTL1_Fwd: 5′-ATCATGCCATGGGATCCACAATGGCAGCCGAGACTCGGAAC-3′; METTL1_Rev: 5′-CATCGCGGATCCGTCGACTCAGTGACCAGGCAGGCTGG-3′; WDR4_Fwd: 5′-ATCATGCCATGGGATCCACAATGGCGGGCTCTGTGGGAC-3′; and WDR4_Rev: 5′-CATCGCGGATCCGTCGACTCAGCAACTTAGCGTCGCCTCC-3′). ORFs YDL201w and YDR165w were digested with *Bam*HI and *Pst*I, and ORFs of METTL1 and WDR4 were digested with *Bam*HI and *Sal*I. Digested ORFs were ligated into vectors pAVA0040 and pAVA0042 to place the genes under P_{GAL1} control.

The sequence encoding c-myc epitope (EQKLISE) was added to *Bam*HI-*Eco*RI *GAL1-GAL10* promoter region by

PCR using primer pairs (Myc_R: 5′-AATTGTGGATCCTT CAGAGATGAGTTTCTGCTCCATGGGGTTTTTTCTCCTTG ACGTTAAAGTATAGAGG-3′, and M13_R: 5′-GTAAAACG ACGGCCAGTGAATTC-3′), and plasmid pAVA0040 as a template, followed by restriction digestion with *Bam*HI and *Eco*RI, and ligation into plasmid pAVA0042 that contained YDR165w, to generate a plasmid with myc-YDR165w under P_{GAL1} control.

Trm8 and Tpt1 for immunopurification experiment were expressed under P_{GAL10} control as fusion proteins containing in succession ORF, HA epitope, the rhinovirus 3C protease site, and the ZZ domain derived from protein A of *Staphylococcus aureus*. Construction of this plasmid will be described later.

Yeast strains expressing proteins under P_{GAL} control were grown in synthetic −Leu, or −Ura −Leu media containing 2% raffinose (Sherman, 1991) to 1.2×10^7 cells/mL, followed by addition of galactose (or dextrose) to 2% and growth for two further generations before harvest. Strains expressing proteins under P_{CUP1} control were grown as previously described (Martzen et al., 1999), and strains without plasmids were grown at 30 °C in YPD media to 6×10^7 cells/mL before harvest.

E. coli strains, plasmids, and growth

Proteins were expressed in *E. coli* as N-terminal His6-maltose-binding protein-ORF fusion under control of the P_{tac} promoter, after ligation of *Bam*HI-*Pst*I digested PCR amplification products of YDL201w and YDR165w genes into the multicloning site of the His-MBP-3C vector (Alexandrov et al., 2001), followed by transformation and then sequencing of the ORFs. Coexpression of His-MBP-3C-YDL201w and His-MBP-3C-YDR165w proteins was achieved by construction of a single plasmid expressing both proteins under P_{tac} control; this plasmid was formed by digestion of His-MBP-3C-YDL201w and His-MBP-3C-YDR165w vectors in the unique *Pst*I site and ligation of the two vectors together head to tail. To express proteins, plasmids were transformed into *E. coli* BL21 Codon Plus (DE3) cells (Stratagene) and 400-mL cultures were grown at 37 °C to $A_{600} = 0.6$, induced with IPTG at 0.8 mM for 4 h at 32 °C, harvested, frozen in dry ice, and stored at −70 °C.

Preparation of extracts

Crude yeast extracts were prepared with glass beads in buffer containing 50 mM Tris, pH 7.5, 1 mM EDTA, 5 mM DTT, 10% glycerol, 1 M NaCl, followed by supplementation with protease inhibitors and centrifugation, essentially as described (McCraith & Phizicky, 1990; Martzen et al., 1999). Extracts contained 24–29 mg/mL protein as determined by assay (Bradford, 1976).

Crude *E. coli* extracts were prepared from frozen cells after thawing, resuspension of cells in 25 mL Buffer A (50 mM Tris, pH 7.5, 5 mM β-mercaptoethanol, 10% glycerol), cell lysis by three passages through a French press, and clarification of the lysate by centrifugation and then passage through a 0.2 μm filter.

Purification of fusion proteins

Purification of GST-ORF fusion proteins was previously described (Martzen et al., 1999).

Purification of ORF-3C site-ZZ fusion proteins was achieved by IgG-sepharose chromatography (Amersham Pharmacia Biotech) as described (Nilsson et al., 1985), followed by cleavage with GST-protease 3C fusion protein to release bound Trm8-protein (Walker et al., 1994). Then GST-protease 3C was removed with glutathione sepharose resin, and the preparation was dialyzed in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 50% glycerol, and stored at −20 °C.

His6-fusion proteins were purified from extracts by chromatography on TALON cobalt-affinity resin (Clontech) as previously described (Alexandrov et al., 2001), followed by desalting with a Pharmacia G-25 Sephadex column, supplementation with glycerol to 40%, and storage at −20 °C.

Western blot

Detection of the c-myc epitope (EQKLISE) fusion proteins was performed using mouse monoclonal anti-[c-myc] antibody (clone 9E10) obtained from Roche and the ECL+plus Western Blotting Detection System from Amersham-Pharmacia.

Analysis of modified nucleosides

Low molecular weight RNA was prepared by hot phenol extraction of harvested cells, as previously described (Rubin, 1975). A total of 1.5 mg RNA was digested with 10 μg P1 nuclease overnight at 37 °C in 450 μL buffer containing 50 mM ammonium acetate, pH 5.2, and 200 μM $ZnCl_2$. Reaction mixtures were adjusted to pH 3.5 by addition of acetic acid, and pm^7G was separated from pG by chromatography of the nucleotides through a 5-mL DEAE-Sephadex A-50 column (Pharmacia Fine Chemicals, Inc.) preequilibrated and resolved in buffer containing 50 mM ammonium acetate buffer, pH 3.5. pm^7G (and pC) flow through this column in the first 3 mL, whereas pG is strongly retained on the column. This procedure was prompted by TLC results obtained by Bochner and Ames (1982). Fractions containing pm^7G and pC were collected, concentrated to 160 μL in a speed-vac, adjusted to pH 7.0 by addition of ammonium hydroxide, incubated at 37 °C for 16 h with 1.5 units of calf intestine phosphatase (Roche Diagnostics), and 30 μL of the resulting nucleosides were resolved on a Supercosil LC-18S HPLC column as previously described (Gehrke & Kuo, 1989). The identity of the m^7G peak was confirmed by comparison of the retention time and absorbance spectra with commercially obtained m^7G (Fluka) and previously published values (Dawson et al., 1989). The areas of the eluted cytidine peaks from different strains varied by no more than 3%.

Aniline cleavage

pCp-labeled pre-$tRNA^{Phe}$ was analyzed for the site of m^7G modification by borohydride reduction (Wintermeyer & Zachau, 1975) in the presence of uniformly methylated tRNA carrier (Zueva et al., 1985a), followed by aniline cleavage of tRNA chain performed as described (Wintermeyer & Zachau, 1975), and separation of products on a 15% polyacrylamide 4 M urea-containing sequencing gel. The G ladder was obtained

by dimethylsulfate (DMS) treatment of pre-$tRNA^{Phe}$ (Peattie, 1979), followed by the same cleavage procedure.

Software used

Sequences were aligned using Mutalin (Corpet, 1988), and shadowed with Boxshade 3.21. The WD40 motifs were predicted with the program Protein Sequence Analysis (PSA) http://bmerc-www.bu.edu/psa/request.htm (Stultz et al., 1997).

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