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Biochemical and Biophysical Research Communications 334 (2005) 433–440
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A novel methyltransferase required for the formation of the hypermodified nucleoside wybutosine in eucaryotic tRNA ☆

Hamid R. Kalhor, Mahmud Penjwini, Steven Clarke *

Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, CA 90095-1560, USA

Received 14 June 2005
Available online 29 June 2005

Abstract

We demonstrate that the product of the yeast open reading frame *YML005w* is required for wybutosine (yW) formation in the phenylalanine-accepting tRNA of the yeast *Saccharomyces cerevisiae*. tRNA isolated from a deletion mutant of the *YML005w* gene accumulates 4-demethylwyosine (ImG-14), a precursor lacking three of the methyl groups of the yW hypermodified base. Since the amino acid sequence of the *YML005w* gene contains the signature motifs of the seven β-strand methyltransferases, we now designate the gene *TRM12* for tRNA methyltransferase. Using pulse-chase labeling of intact yeast cells with *S*-adenosyl-L-[*methyl*-^{3}H]methionine, we show that the methylesterified form of yW is metabolically stable.

Keywords: tRNA methylation; Wybutosine; Modification of tRNA bases

Posttranscriptional covalent modifications of bases in tRNAs are important elements for their functions, especially in the proper recognition of mRNA codons on the ribosome [1,2]. One of the most highly modified tRNA species recognizes codons for the amino acid phenylalanine [3]. A common site of modification in this and many other tRNA species is the nucleotide adjacent to the 3′ end of the anticodon. In eucaryotic tRNA^{Phe}, the guanosine-37 residue at this position is hypermodified to form the branched tricyclic wybutosine nucleoside, generally designated yW, with the base itself sometimes designated Y or Wye [3–5]. This modification promotes accurate translation by stabilizing the appropriate codon–anticodon interaction [6,7]. In tumor cells, the modification is often incomplete and the resulting hypomodified tRNA may be used preferentially in translation [8]. Increased frameshifting occurring when yW is replaced by hypomodified species such as 1-methylguanosine may lead to the expression of alternative reading frames in tumor cells and in cells expressing retroviruses, suggesting that the full modification may help protect cells from both cancerous growth and viral infection [9]. The yW modification appears to be present only in eucaryotic cells, although the unbranched tricyclic base has been found in archael tRNAs [10].

Although pathways for the total chemical synthesis of wybutosine have been developed [11], the biosynthetic pathway has not been established yet. In the yeast *Saccharomyces cerevisiae*, it is known that the initial step is the *S*-adenosylmethionine (AdoMet)-dependent methylation of the *N*1-atom of the guanosine residue [12]; this reaction is catalyzed by the *TRM5* gene product [5]. [*methyl*-^{13}C]Methionine labeling of yeast cells indicates that the methyl group of AdoMet is also utilized for the *N*3-methyl group and for the two methyl groups in ester linkages [13]. Additionally, the Y-side chain may be derived from AdoMet from its the α-amino butyric acid moiety [13]. Interestingly, the methyl group

☆ *Abbreviations:* AdoMet, *S*-adenosyl-L-methionine; [^{3}H]AdoMet, *S*-adenosyl-[*methyl*-^{3}H]-L-methionine.

* Corresponding author. Fax: +1 310 825 1968.
E-mail address: clarke@mbi.ucla.edu (S. Clarke).

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doi:10.1016/j.bbrc.2005.06.111

attached to the third ring does not appear to come from the methyl group of AdoMet; the origin of this atom and the carbon atom attached to it is still unknown [13].

Our laboratory has been interested in identifying new methyltransferases from the genome of *S. cerevisiae*, particularly in those enzymes that catalyze methyl esterification reactions. These modifications can be potentially metabolically reversed by the action of specific esterases in cells and can provide a means for the modulation of function [14]. We had previously identified the yeast enzyme Trm9 that catalyzes methyl esterification reactions resulting in the formation of 5-methoxycarbonylmethyluridine (mcm^5U) in $tRNA^{Arg3}$ and 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) in $tRNA^{Glu}$ [15]. In that study, we labeled intact yeast cells with [*methyl*-^{3}H]AdoMet and also observed the formation of additional radiolabeled methyl groups as ester linkages in nucleosides that eluted in similar positions to those expected for wybutosine and its derivatives [5,15].

We thus became interested in identifying the genes and enzymes that would participate in the formation of the two methyl esters in wybutosine. Our approach has been to identify mutant strains lacking putative methyltransferase genes that would be unable to form wybutosine methyl ester linkages. Because of the diversity of AdoMet-dependent methyltransferases, it has been challenging to identify methyltransferase genes and at least five structural classes of these enzymes occur [16]. However, it appears that the bulk of the methyltransferases are members of a family with a seven β-strand structure and conserved sequence motifs that make it possible to identify putative methyltransferases from their genomic sequence alone [17,18]. Here, we have examined mutants in the previously identified candidate methyltransferases. We demonstrate that a *YML005w* gene deletion mutant is defective in methylesterification of wybutosine, and suggest that this gene product catalyzes the transfer of one or more methyl groups in wybutosine biosynthesis.

Experimental procedures

Yeast strains. *Saccharomyces* Genome Deletion Project strains were obtained from the Invitrogen (Carlsbad, CA) and included the *S. cerevisiae* parent strain BY4742 (*MATαhisΔ1*, *leu2Δ0*, *lys2Δ0*, and *ura3Δ0*) and the *YML005w* kanamycin-insert deletion strain in the BY4742 background.

In vivo labeling and preparation of cell extracts. *S. cerevisiae* strains were grown at 30 °C to early log phase (OD_{600nm} between 0.6 and 0.8) in 50 ml YPD medium (1% yeast extract (Difco, Detroit, MI), 2% peptone (Difco, Detroit, MI), and 2% of D-glucose. An aliquot of 20 OD_{600nm} cells was collected by centrifugation at 110*g* for 5 min at 4 °C and the cells were washed 3 times with 10 ml of YPD medium. The cell pellet was resuspended in 900 μl of YPD medium and 100 μl of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (80 Ci/mmol, in dilute hydrochloric acid/ethanol 9:1 (pH 2.0–2.5), Amersham Biosciences, Piscataway, NJ) to give a final [^{3}H]AdoMet concentration of 1.25 μM. Cells were incubated in a gyratory shaker at 225 rpm for 30 min at 30 °C and pelleted as above and washed twice with 1 ml water.

tRNA extraction and digestion. Yeast cells were resuspended in 300 μl of 0.9% NaCl per 10 OD of cells and 2 volumes of phenol were then added to the suspension. The mixture was rotated gently at room temperature for 30 min. To the mixture, 0.1 volume of chloroform was then added, and the samples were incubated for additional 15 min at room temperature. The samples were spun down at 10,600*g* for 20 min. The aqueous phase was collected and mixed with 2.5 volumes of ethanol and 0.1 volume of 20% potassium acetate to precipitate RNA. tRNA was purified with the 2 M LiCl extraction method as described previously [15,19]. The tRNA pellet was washed twice with the 80% ethanol and finally dissolved in water or 2.4 M tetraethylammonium chloride and digested as previously described [15,20]. Briefly, 50 μl (100–200 μg RNA by A_{260nm}) of tRNA was heat-denatured at 90 °C for 2 min, and 5 μl of 10 mM zinc sulfate and 10 μl nuclease P1 (200 U/ml; Boehringer–Mannheim, Germany) were added. The mixture was incubated at 37 °C for 16 h. To the sample, 10 μl of 0.5 M Tris buffer, pH 8.0, and 10 μl alkaline phosphatase (100 U/ml; Bacterial type III, Sigma, St. Louis, MO) were added. The mixture was incubated at 37 °C for 2 h.

Purification of $tRNA^{Phe}$. To isolate specific tRNA species, the total tRNA extract described above was hybridized to matrix-bound oligonucleotides. Biotinylated primers were synthesized specific for $tRNA^{Phe}$ containing 30 nucleotides complementary to the 3′ end. The probe (0.5 nmol) was bound to Dynabeads M-280 with streptavidin covalently attached to the surface, according to manufacturer's recommendation (Dynal A.S; Olso, Norway). Briefly, the beads were washed three times in the high salt buffer (5 mM Tris–HCl, 0.5 mM EDTA, and 1 M NaCl). The beads were resuspended in 250 μl of the high salt buffer and 10 μl (1 nmol) of biotinylated synthetic probes and incubated at room temperature for 15 min. Beads were washed three times with the high salt buffer. The hybridization method used here is described elsewhere [15]. Briefly, tRNA (50–200 μg), dissolved in 2.4 M tetraethylammonium chloride, was denatured at 60 °C for 3 min and then mixed with the streptavidin bound-oligonucleotide beads at 15 °C for 30 min. The beads were washed three times with 2.4 M tetraethylammonium chloride, and then bound tRNA was eluted after heating at 60 °C for 3 min in 100 μl of 2.4 M tetraethylammonium chloride. tRNA was concentrated and desalted by centrifugation using Centricon 10 columns (Amicon, MA).

HPLC. In a modification of the method of Pomerantz and McCloskey [21], digested tRNA was injected onto a C18 reverse-phase column (Supelcosil, 5 μm beads, 4.6 mm inside diameter, 250-mm length) equilibrated in solvent A (5 mM ammonium acetate, pH 6.0) for 15 min and eluted at 1 ml/min at 25 °C with increasing solvent B (40% acetonitrile in 60% water) (time, %A, %B: 0, 100, 0; 3, 100, 0; 5.8, 98, 2; 7.2, 97, 3; 10, 95, 5; 25, 75, 25; 30, 50, 50; 34, 25, 75; 37, 25, 75; 43, 0, 100; 48, 0, 100). Nucleosides were monitored using a UV detector (Waters Lambda-Max model 489) set at 254 nm. Fractions were collected at either at 1 or 0.5 min intervals and used directly for radioactive methyl ester determination or dried overnight under vacuum for mass spectrometry.

HPLC fractions were analyzed for [^{3}H]methyl esters by mixing 100–150 μl with NaOH to give a 1 M solution in a final volume of 200 μl to form [^{3}H]methanol. The hydrolysate was spotted on a 1.5 × 8 cm piece of Whatman 3MM paper and suspended in the neck of a 20 ml scintillation vial above 5 ml of Safety Solve scintillation fluid. After 2 h at room temperature, the paper was removed and the vial counted for the [^{3}H]methanol that had transferred to the fluid through the vapor phase.

Mass spectrometry. A Perkin-Elmer Sciex API III triple quadrupole mass spectrometer was used as previously described [22]. HPLC fractions containing nucleosides were analyzed by direct injection (15 μl) in a mixture of water/acetonitrile/formic acid (50/50/0.1; v/v/v). Normal spectra were obtained by scanning from *m/z* 200–600 (0.3 Da step size;

30-ms dwell time; 6.0 s/scan; orifice voltage 60). Ion series were transformed using version 3.3 of MacSpec software. For MS/MS analysis, positive ion spectra of Q1 preselected parent ions were generated by collisionally induced dissociation (10% nitrogen in argon) with a thickness instrument setting (CGT) of 100, R_0–R_2 offset of 20 V) via scanning Q3 from m/z 50–400 (step size 0.3 Da, dwell time, 30 ms, 5.02 s/scan, orifice voltage, 60).

Pulse-chase analysis of [^{3}H]methyl ester turnover in intact cells. Yeast strains were grown to log phase in 50 ml YPD medium at 30 °C. Cells were harvested and washed twice with YPD medium, then resuspended in 4 ml YPD containing 1.4 μM [^{3}H]AdoMet (80 Ci/mmol) and 1 mM L-Met. Cells were incubated at 30 °C or 37 °C for 10 min, pelleted at 14,000g for 20 s, and washed once with YPD. Pelleted cells were resuspended in 3 ml of YPD containing 1 mM non-isotopically labeled AdoMet and 1 mM L-Met, and the cells were placed either at 30 or 37 °C. At time points of 10, 20, 30, and 240 min, 500 μl of cells was collected and tRNA was extracted, digested, and fractionated as described above. The [^{3}H]methyl ester content of the desired fractions was measured as described above.

Results and discussion

Identification of a novel methyltransferase required for wybutosine synthesis in the yeast S. cerevisiae

tRNAs from yeast cells contain at least three modified nucleosides that contain methyl ester linkages, including 5-methoxycarbonylmethyluridine (mcm^{5}U) [23], 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U) [24], and wybutosine (yW) [25]. We have previously identified the Trm9 methyltransferase that catalyzes the methylesterification of both mcm^{5}U and mcm^{5}s^{2}U [15]. We are now interested in identifying one or more methyltransferases responsible for the methylesterification of yW.

To confirm the elution position of the yW nucleoside in our HPLC separation system, the BY4742 parental strain was in vivo radiolabeled with [^{3}H]AdoMet. Total tRNA was extracted from the cells, and using a synthetic biotinylated oligonucleotide complementary to the 3′ end of yeast tRNA^{Phe}, radiolabeled tRNA^{Phe} was purified and treated with P1 nuclease and alkaline phosphatase to generate free nucleosides. HPLC fractionation revealed a single [^{3}H]methyl esterified peak at 40–42 min that corresponded to the expected elution position of yW [5,15]. To directly demonstrate that yW is indeed eluting at this position, yeast commercial tRNA was hydrolyzed to nucleosides, and after nucleobases were separated by HPLC, the eluted fractions were subjected to mass spectrometry analysis [15]. The mass spectrum of the 40 min fraction contained a major ion at a m/z value of 509.3 corresponding to that expected for the M+H$^+$ species of yW (509.4). MS/MS analysis of this ion confirmed the chemical structure, giving fragments corresponding to the base (m/z 377.3) and the base with loss of part of the side chain (m/z 345.3) (Fig. 1).

To identify potential methyltransferases responsible for yW formation, [^{3}H]methyl esters eluting with yW and its derivatives were measured in nucleoside hydrol-

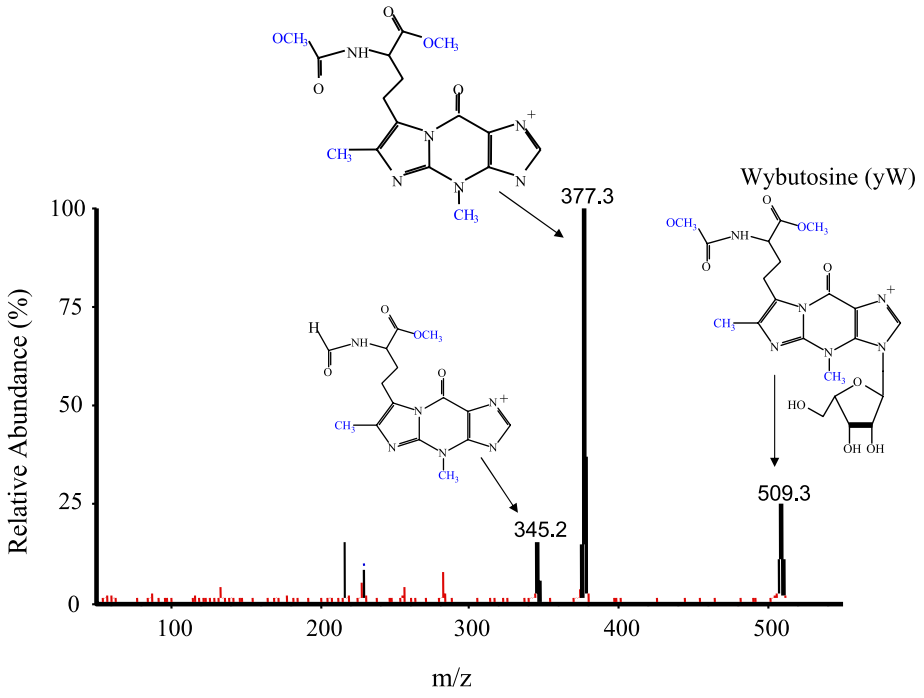

Fig. 1. Mass spectrometry of the modified nucleoside in the [^{3}H]methyl ester-containing nucleoside purified from the digest of [^{3}H]AdoMet labeled-yeast tRNA^{Phe}. The MS/MS spectrum is shown for an m/z 509.3 ion corresponding to wybutosine (yW). Characteristic fragment ions corresponding to the "Y base" (m/z 377.3) and its degradation product (m/z 345.2) were found.

ysates from a number of [^{3}H]AdoMet-labeled mutant strains. A collection of deletion mutants of putative yeast methyltransferases whose sequences contain the seven β-strand methyltransferase motif was obtained from the *Saccharomyces* Genome Deletion Project and other sources [17,18]. In preparations from wild type cells, two peaks of methyl ester radioactivity at 38 min (major) and 40 min (minor) were generally found under our chromatography conditions. The earlier eluting peak is consistent with the partial digestion dinucleotide product yWpA that has been previously observed [5,20] and the latter peak is consistent with the with yW mononucleoside. We found that total nucleoside extracts from a number of strains with candidate open reading frame deletions contained the radioactive peaks of methyl esters at the elution positions of yW/yWpA, including *YDR140w*, *YDR316w*, *YNL022c*, *YNL063w*, *YOL124c*, and *YPL201w* (data not shown). We also found radiolabel as methyl esters here for deletion mutants of the Trm8 methyltransferase for 7-methylguanosine synthesis [26] and the Tgs1 methyltransferase for 2,2,7-trimethylguanosine synthesis [27]. However, in the extract of the deletion mutant of the *YML005w* gene, no methyl ester radioactivity was found at the elution position of either yW or yWpA (Fig. 2).

The hypermodified yW nucleoside has been found at position 37 of tRNA^{Phe} in yeast [3]. Thus, one would expect that in the *YML005w* mutant tRNA^{Phe} would not contain the methylesterified yW. Radiolabeled tRNA^{Phe} from the parent and *YML005w* deletion strain that were grown to log phase was purified, hydrolyzed to nucleosides, and their nucleosides fractionated by HPLC (Fig. 3). As expected, nucleosides from tRNA^{Phe} purified from *YML005w* cells did not contain the radiolabel methyl ester peak corresponding to yW that was found in nucleosides of the parent cells. We conclude that the *YML005* gene encodes a methyltransferase essential for the synthesis of wybutosine, and have designated this gene *TRM12* (tRNA methyltransferase).

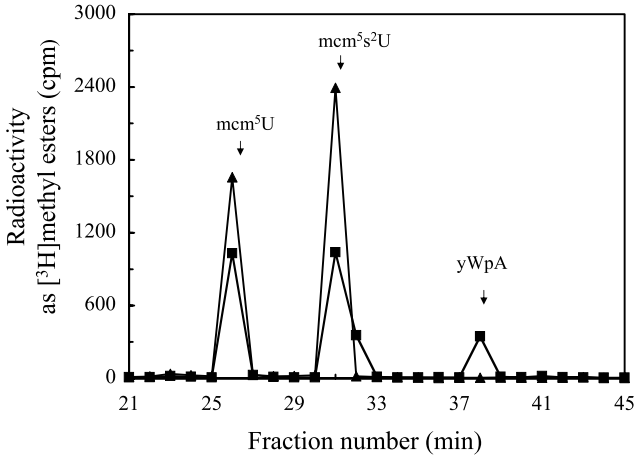

Fig. 2. HPLC analysis of [^{3}H]methyl-esterified nucleosides from the total tRNA pool of yeast cells labelled in vivo with [^{3}H]AdoMet. tRNA was prepared, hydrolyzed to nucleosides, and fractionated by HPLC as described under Experimental procedures. Radioactivity was measured as volatile radioactivity from the methyl esters of 100 μl of each fraction that had been treated with base to form [^{3}H]methanol. (■) parental wild type strain BY4742; (▲) deletion mutant *YML005w*. The elution position of the wybutosine dinucleotide yWpA is indicated, along with those of previously identified methylesterified tRNA nucleosides mcm^{5}U and mcm^{5}s^{2}U [15].

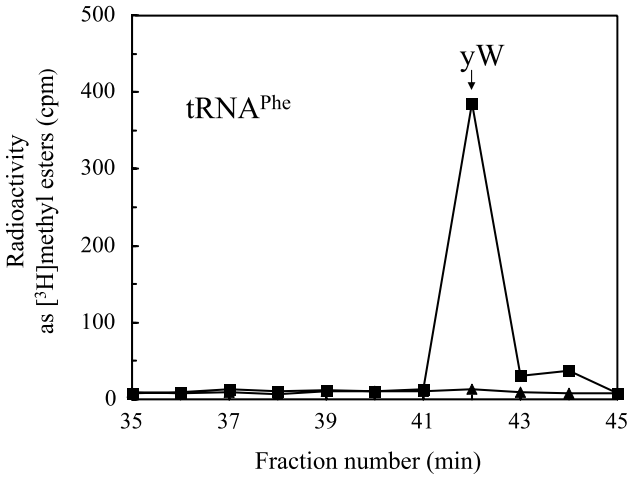

Fig. 3. Purification and characterization of [^{3}H]methylesterified nucleosides from tRNA^{Phe} from in vivo-radiolabeled yeast cells. Total tRNA was isolated as described in the legend of Fig. 2. A biotinylated primer specific for tRNA^{Phe} was used to isolate the tRNA from both wild type and *trm12* mutant, which were then hydrolyzed and fractionated by HPLC as described in Fig. 2. One hundred microliters of each fraction was used to measure the methyl ester radioactivity. (■) parental strain; (▲) deletion mutant *YML005w* (*trm12* mutant). The elution position of the wybutosine (yW) is indicated.

Identification of ImG-14 as an intermediate that accumulates in trm12-deficient yeast and the pathway of wybutosine biosynthesis

To identify the hypomethylated nucleosides that may occur in the *trm12* mutant, unlabeled tRNA was purified from both the *trm12* mutant and its isogenic parental strain, hydrolyzed to nucleosides, and fractionated by HPLC (Fig. 4). We first analyzed the HPLC UV peak at 39.4 min from the wild type fractionation by electrospray mass spectrometry. We found that fractions containing this peak gave the M+H$^+$ 838.5 *m*/*z* ion expected for the yWpA dinucleotide; no comparable ion was present in the corresponding fractions from the *trm12* strain. MS/MS analyses of the 39.4 min peak were consistent with the fragmentation products of yWpA, including yW at 529.1 and ribose-pA at 462.2 (data not shown). The UV profile of nucleosides from the *trm12* cell hydrolysate was very similar to that obtained from the wild type cell hydrolysate with the exception that the 39.4 min peak identified as yWpA

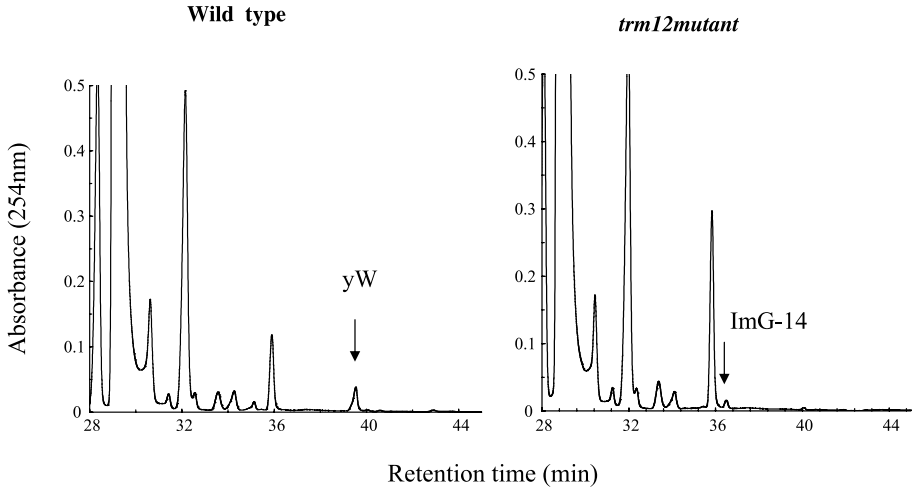

Fig. 4. Large scale HPLC separation of nucleoside-digested tRNA. Total tRNA from wild type and *trm12* yeast cells (500–800 OD units each) was extracted and hydrolyzed to generate nucleosides as described under Experimental procedures. The arrow indicates the position of wybutosine in the left panel and the ImG-14 intermediate in the right panel.

was absent, and that a new peak at 36.4 min was present (Fig. 4). Mass spectrometric analysis of fractions containing the 36.4 min peak from the *trm12* digest and the corresponding fractions of the wild type digest were performed. We detected a 322.0 *m*/*z* ion in the mutant but not in wild type cells, suggesting that we had isolated an intermediate in the biosynthesis of yW that accumulates in the absence of the Trm12 methyltransferase. The 322 *m*/*z* ion is consistent with the structure of the ImG-14 nucleoside. This identification was confirmed by MS/MS analysis of the 322 *m*/*z* ion; the fragments that we obtained exactly matched those observed previously for this nucleoside (Fig. 5; compare with Fig. 3 of reference [10]).

Possible biosynthetic pathways for wybutosine synthesis from guanosine are shown in Fig. 6. The first step in yeast is the formation of the *N*-1 methylated guanosine product [5,12]. The accumulation of ImG-14 shown in this work now suggests that the second step(s) is likely to be the formation of the third ring. Three additional methylation reactions are required to form yW from ImG-14. At this point it is not possible to delineate which one(s) of these steps are catalyzed by the Trm12 methyltransferase. It is formally possible that Trm12

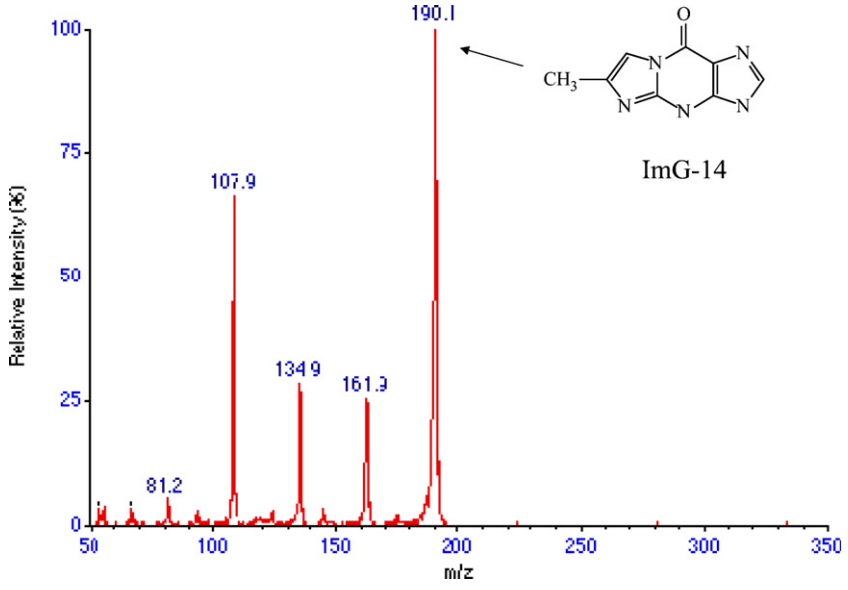

Fig. 5. Mass spectral identification of 4-demethylwyosine (ImG-14) as the intermediate accumulating in the *trm12* deletion mutant. The material in the 37 min peak of UV absorbance seen only in the HPLC analysis of the *trm12* deletion mutant was analyzed by mass spectrometry. MS/MS analysis demonstrated the *m*/*z* 190.1 ion of the base of ImG-14, as well as characteristic fragmentation ions of *m*/*z* 107.9, 134.9, and 161.9 [10].

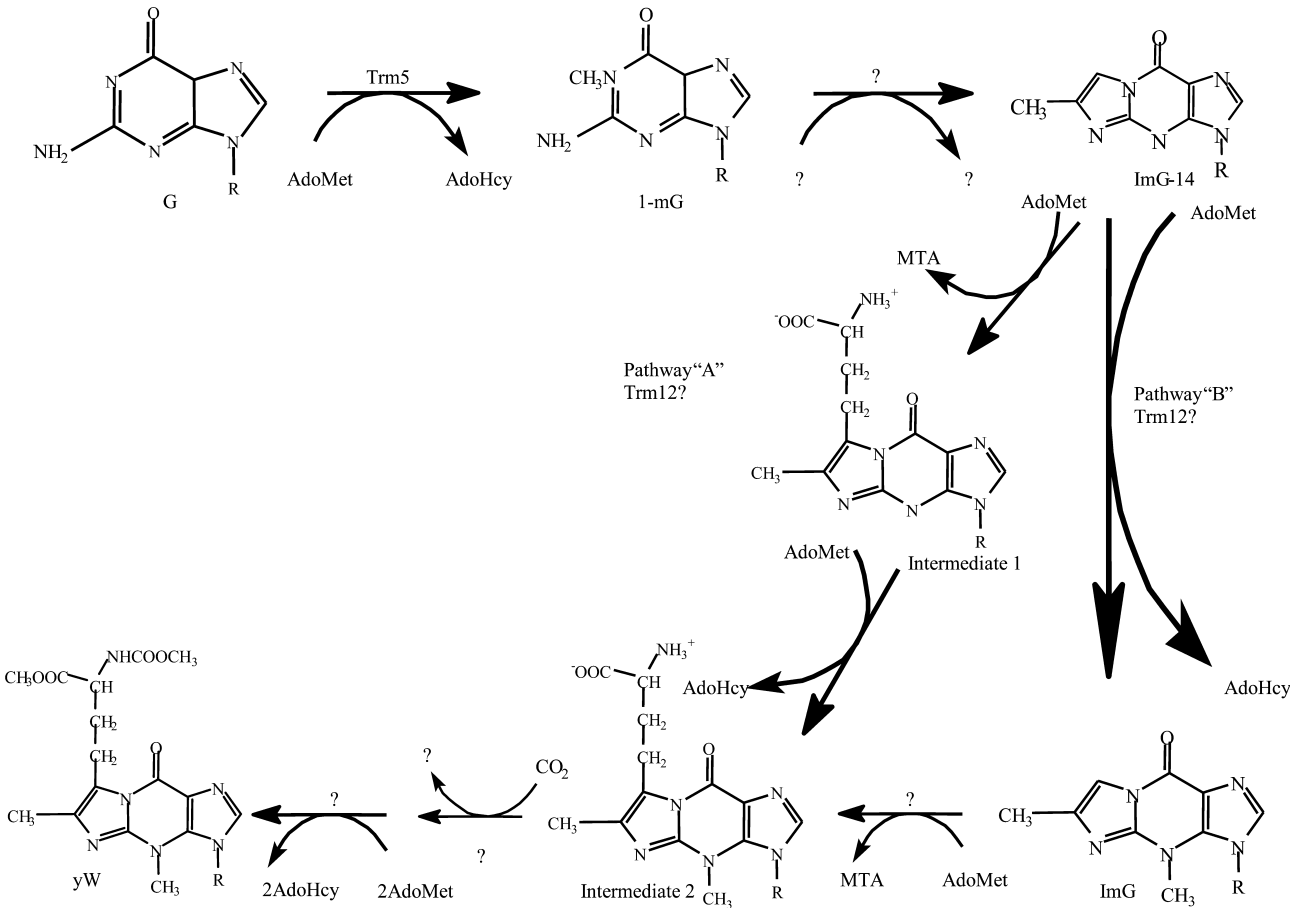

Fig. 6. Possible pathways for wybutosine biosynthesis. In yeast, the initial step is the formation of 1-methylguanosine by the Trm5 methyltransferase [5,12]. The remainder of the pathway is poorly understood. The accumulation of 4-demethylwyosine (ImG-14) in *trm12* mutants suggests that Trm12 catalyzes one or more downstream steps.

may even catalyze the transfer of the aminobutryl moiety of AdoMet to generate the branch structure. Further work will be needed to identify the specific step or steps that Trm12 catalyzes.

A complex of different gene products may be required for the synthesis of yW from ImG-14 and the loss of the Trm12 methyltransferase may compromise the function of the other enzymes. Such a situation has been described for yeast ubiquinone synthesis where the loss of a downstream enzyme can result in the failure to catalyze upstream reactions [28]. In this scenario, Trm12 may catalyze only one or both of the methylesterification reactions; the failure to generate intermediate 2 is possibly explained by the inability to form an active complex of enzymes that can catalyze the reactions from ImG-14 to intermediate 2. Yeast extracts supplemented with AdoMet can catalyze the in vitro formation of most of the modified bases of $tRNA^{Phe}$ but only 1-methylguanosine is formed at position 37, not yW [29]. It is clear that additional studies will be needed to elucidate the full pathway.

Methyl ester turnover

To test the physiological reversibility of methyl ester modifications of tRNA nucleosides in yeast, wild type cells were pulse-labeled with [^{3}H]AdoMet and chased with non-isotopically labeled AdoMet for up to 240 min. Total tRNA was isolated at each time point, hydrolyzed and the nucleosides then examined by HPLC. We detected little or no turnover of the [^{3}H]methyl esters in the mcm^5U and mcm^2s^5U species that are formed by the Trm9 methyltransferase. In addition, we did not detect any significant turnover of [^{3}H]methyl esters in the peaks corresponding to yW and yWpA (data not shown). These results suggest that either these methyl esters are physiologically stable, or that demethylation reactions may occur in vivo, but only under specific environmental conditions.

Phenotype of the trm12 mutant in yeast

The growth rate of the *trm12* deletion mutant was compared to that of its isogenic parental strain under

various conditions including normal (30 °C) and elevated temperature (37 °C) in YPD, as well as when D-glucose was replaced by D-galactose or the non-fermentable carbon source ethanol. No differences were observed.

Homologs of the Trm12 methyltransferase in other species

A BLAST search of the GenBank non-redundant database with the yeast Trm12 amino acid sequence revealed a number of close homologs in hypothetical open reading frames from other fungal species, as well as in higher eucaryotes, including several plant species, mice, and humans. The alignment of the most similar portion of the sequences in the methyltransferase catalytic domain is shown in Fig. 7. There are a number of identities in the motif I and motif post-I sequences characteristic of seven β-strand methyltransferases. However, no clear motif II sequence is apparent in the alignment, and the motif III sequence fits the consensus poorly [18]. Nevertheless, there are also regions of near identity in regions outside of the signature methyltransferase motifs, suggesting that these species may catalyze the same tRNA modification reaction as yeast Trm12. We find no evidence for Trm12 homologs in bacteria or archaea, which is consistent with the presence of the yW only in eucaryotes [10]. Interestingly, we find no evidence for a homolog of the *TRM12* gene in the genome of the insect *Drosophilia melanogaster* or the nematode

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229 Q E Q L N S S I W C K V I Q N G V T Q I W S P V F T M F S R  S. cerevisiae
200 E Q D F Q K A F W V S C R Q N G I Y Q T W A P L Y T M F S R  S. pombe
793 E I L V G D N G W V N H R E N G I L Y S F D A T K C M F S W  A. thaliana
180 T L L L G D H G W V E H M D N G I R Y K F D V T Q C M F S F  M. muscalus
190 T L L L G D H G W V E H V D N G I R Y K F D V T Q C M F S F  H. sapiens

                                                  ---- Motif I ---
259 G N I K E K K R V L T T F P D I C N N D V V D L Y A G I G Y  S. cerevisiae
230 G N S I E K A R V L N - F S Y I K D E I I A D L Y A G I G Y  S. pombe
823 G N L S E K L R M G N M - - A C E N E V V V D L F A G I G Y  A. thaliana
210 G N I T E K L R V A S L - - S C A G E V L V D L Y A G I G Y  M. muscalus
220 G N I T E K L R V A S L - - S C A G E V L V D L Y A G I G Y  H. sapiens

                                  Motif
                               --Post I-
289 F T F S Y L - T K G A R T L F A F E L N P W S V E G L K R G  S. cerevisiae
259 F T F S Y V - K A G A S T V F C W E I N P W S V E A L R R A  S. pombe
851 F V L P F L V R A K A K L V Y A C E W N P H A I E A L R R N  A. thaliana
238 F T L P F L V H A G A A F V H A C E W N P H A V V A L R N N  M. muscalus
248 F T L P F L V H A G A A F V H A C E W N P H A V V A L R N N  H. sapiens


318 L K A N G F N - - - - K S G N C H V F Q E S N E M C V Q R L  S. cerevisiae
288 A L K N G W S I Y V V R N G E N Y E F K V G T H R I V V F L  S. pombe
881 V E A N S - - - - - - - - - - - - - - - - V S E R C I I L E  A. thaliana
268 L E I N G - - - - - - - - - - - - - - - - V A D R C Q I H F  M. muscalus
278 L E I N G - - - - - - - - - - - - - - - - V A D R C Q I H F  H. sapiens


344 T E F L S Q N P G F - R L R I - - R H I N L G L L P S S K Q  S. cerevisiae
318 E S N V Y A A E R F S K M N I S A R H I N L G M L P S S E K  S. pombe
895 G D - - - - - N R I T A P K G V A D R V N L G L I P S S E G  A. thaliana
282 G D - - - - - N R K L K L S D I A D R V N L G L I P S S K E  M. muscalus
292 G D - - - - - N R K L K L S N I A D R V I L G L I P S S E E  H. sapiens

                        ---Motif III?----
371 G W P L A I K L I Y L Q G A S L E K V T M H I H E N V H I D  S. cerevisiae
348 S W S T A T S I L K R E S H S F - - - - I H V H E N V K D E  S. pombe
920 S W V T A I Q A L R P E G G I - - - - - L H V H G N V K D S  A. thaliana
307 G W P V A C Q V L R K D V G G I - - - - L H I H Q N V E S F  M. muscalus
317 G W P I A C Q V L R Q D A G G I - - - - L H I H Q N V E S F  H. sapiens
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Fig. 7. Manual alignment of the amino acid sequence of the catalytic domain of the *Saccharomyces cerevisiae* Trm12 methyltransferase with portions of hypothetical proteins of *Schizosaccharomyces pombe* (SPAC4G8.06c), *Arabidopsis thaliana* (At4g04670.1), *Mus muscalus* (RIKEN cDNA 4632406NP1), and *Homo sapiens* (FLJ20772). Residue numbers are given on the left. Positions where there are at least three identities in the five species are underlined.

worm *Caenorhabditis elegans*. The *TRM12* methyltransferase sequence may have diverged more rapidly in some evolutionary lines than others; alternatively, not all eucaryotes may synthesize yW [30] or an alternate biosynthetic route may be taken in some organisms [31].

Acknowledgments

This work was supported by NIH Grants GM26020 and AG18000. We are grateful to Dr. Kym Faull of the UCLA Pasarow Mass Spectrometry Laboratory for his continuing expert advice.

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