Yeast Mitochondrial Initiator tRNA Is Methylated at Guanosine 37 by the Trm5-encoded tRNA (Guanine-N1-)-methyltransferase* [5]

Received for publication, June 4, 2007, and in revised form, July 5, 2007. Published, JBC Papers in Press, July 25, 2007, DOI 10.1074/jbc.M704572200

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The TRMS gene encodes a tRNA (guanine-N1-)–methyltransferase (Trm5p) that methylates guanosine at position 37 (m1G37) in cytoplasmic tRNAs in Saccharomyces cerevisiae. Here we show that Trm5p is also responsible for m1G37 methylation of mitochondrial tRNAs. The TRMS open reading frame encodes 499 amino acids containing four potential initiator codons within the first 48 codons. Full-length Trm5p, purified as a fusion protein with maltose-binding protein, exhibited robust methyltransferase activity with tRNA isolated from a Δtrm5 mutant strain, as well as with a synthetic mitochondrial initiator tRNA (tRNAMet1–33). Primer extension demonstrated that the site of methylation was guanosine 37 in both mitochondrial tRNA Met1–33 and tRNAPhe. High pressure liquid chromatography analysis showed the methylated product to be m1G. Subcellular fractionation and immunoblotting of a strain expressing a green fluorescent protein-tagged version of the TRMS gene revealed that the enzyme was localized to both cytoplasm and mitochondrion. The slightly larger mitochondrial form was protected from protease digestion, indicating a matrix localization. Analysis of N-terminal truncation mutants revealed that a Trm5p active in the cytoplasm could be obtained with a construct lacking amino acids 1–33 (Δ1–33), whereas production of a Trm5p active in the mitochondria required these first 33 amino acids. Yeast expressing the Δ1–33 construct exhibited a significantly lower rate of oxygen consumption, indicating that efficiency or accuracy of mitochondrial protein synthesis is decreased in cells lacking m1G37 methylation of mitochondrial tRNAs. These data suggest that this tRNA modification plays an important role in reading frame maintenance in mitochondrial protein synthesis.

One of the most ancient tRNA modifications, present in all organisms as well as mitochondria and chloroplasts, is methylation of the N1 atom of guanosine at position 37 (m1G37)3 (1). The m1G37 modification is catalyzed by a tRNA (guanine-N1-)–methyltransferase (EC 2.1.1.31) encoded by trmD in bacteria or TRMS in archaea and eukaryotes (1–3). Remarkably, the bacterial trmD gene is not homologous to TRMS; thus, the m1G37-modifying enzyme evolved twice. Trm5p has been shown to be responsible for m1G37 methylation of at least eight cytoplasmic tRNAs in Saccharomyces cerevisiae (1, 4). trm5 mutants that lack this modification exhibit a severe growth defect (1), consistent with the important role of m1G37 methylation in reading frame maintenance (5).

S. cerevisiae also has at least eight mitochondrially encoded tRNAs that carry the m1G37 modification (6), including the initiator tRNA (tRNA Met1–33) (7) and tRNA Phe (8). The enzyme(s) responsible for modifying these mitochondrial tRNAs has not been identified. There is no apparent homolog of bacterial trmD in eukaryotic or mitochondrial genomes, so it has been proposed (1, 9) that Trm5p might also be responsible for methylation of mitochondrial tRNAs. To date, however, only cytoplasmic and nuclear localization of Trm5p has been reported (10). Therefore, it is possible that yeast encode a separate mitochondrial m1G37 methyltransferase enzyme.

To address this question, we have cloned, purified, and characterized the S. cerevisiae TRMS-encoded protein. We show that it possesses tRNA methyltransferase activity on both natural and synthetic mitochondrial tRNA substrates and is specific for methylation of the N1 atom of guanosine at position 37. Furthermore, we show that Trm5p is localized to both the cytoplasm and mitochondria in yeast. Thus, the protein encoded by the TRMS gene in yeast represents yet another example of dual protein localization from a single gene (11, 12).

EXPERIMENTAL PROCEDURES

Chemicals, Reagents, and Strains—Reagent grade chemicals were purchased from Sigma, Fisher, or VWR and used without further purification. Geneticin (G-418 sulfate) was obtained from American Bioanalytical. SUPERase-in, RPA III ribonuclease protection assay kit, and RNA marker templates were from Ambion. The commercially produced enzymes (and their distributors) were ribonuclease P1 (Sigma), phosphodiesterase 1

3 The abbreviations used are: m1G, N1-methylguanosine; AdoMet, S-adenosyl-L-methionine; GFP, green fluorescent protein; HPLC, high pressure liquid chromatography; MBP, maltose-binding protein; MBP-Trm5, MBP-Trm5 fusion protein; ORF, open reading frame; UTR, untranslated region; RACE, rapid amplification of cDNA ends.
(Worthington), bacterial alkaline phosphatase (Fermentas), T7 RNA polymerase Plus (Ambion), Moloney murine leukemia virus reverse transcriptase (Ambion), T4 DNA ligase (Epicentre), T4 polynucleotide kinase (New England Biolabs), KOD RNA polymerase Plus (Ambion), Moloney murine leukemia virus reverse transcriptase (Ambion), T4 DNA ligase (Epicentre), T7 bacterial alkaline phosphatase (Fermentas), T7 polymerase (Ambion), and pRK793 (14) were generous gifts from Dr. John Tesmer and pRK793 (14) were generous gifts from Dr. John Tesmer.

**TABLE 1**

Yeast strains and plasmid constructs

| Yeast strains | Relevant genotype | Source |
|---------------|-------------------|--------|
| Y21898a       | a/α his3/his3 leu2/leu2 lys2/LYS2 MET15/met15 ura3/ura3 trm5::KanMX4/TRM5 | EUROSCARF |
| DLY1*         | a/α his3 leu2 lys2 ura3 trm5::KanMX4 | This work |
| EY0986/TRM5-GFP | a/α his3 leu2 met15 ura3 TRM5-GFP | Invitrogen and Ref. 10 |
| DAY4          | α ser1 leu2 trp1 ura3-52 his4 | Ref. 44 |

* Maintained with plasmid pRS416-TRM5 complementing the trm5 deletion.

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ribosomal RNA. Isopropanol (0.5 ml) was added to the aqueous phase followed by 0.5 ml of 0.8 M sodium citrate, 1.2 M NaCl. After 10 min at room temperature, the sample was centrifuged for 10 min at 12,000 × g at 4 °C. Isopropanol (0.7 volumes) was added to the supernatant to precipitate the tRNA. After 10 min at room temperature, tRNA was collected by centrifugation (15 min at 12,000 × g at 4 °C). The tRNA pellet was washed with 70% cold ethanol and then resuspended in 100 μl of 10 mM ammonium acetate (pH 5). About 50 μg of yeast mitochondrial tRNA was obtained.

Low molecular weight RNA containing both cytoplasmic and mitochondrial tRNA was isolated from haploid Δtrm5 cells (strain DLY1). Haploid spores were first grown on a YPD agar plate for 9 days at 30 °C; then the cells were grown in YPD broth in the presence of 300 μg/ml geneticin for at least 48 h followed by growth in YPD without geneticin for 48 h. Cells from a 200-ml culture were harvested and extracted with TRI reagent (8 ml of TRI reagent was used for 4–5 ml of packed cell pellet). The mixture was kept at 37 °C for 5 min before chloroform was added. Then the manufacturer’s procedure was followed with no high salt precipitation step. This procedure gave mainly tRNA (analyzed by polyacrylamide-urea gel electrophoresis) with a yield of 2.3 mg. For further purification, a second ethanol precipitation was carried out. The tRNA was resuspended in sterile 10 mM ammonium acetate and stored frozen in aliquots. The same method was used to isolate total tRNA from DLY1 harboring various TRM5 plasmids.

**Methyltransferase Assay**—Methyl group incorporation from [3H]methyl-S-adenosyl methionine ([3H]AdoMet) into tRNA was measured by determining radioactivity in acid-precipitable product (20). The assay was carried out in a total volume of 30 μl containing 100 mM Tris-HCl (pH 8), 5 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 600–700 pmol of Δtrm5 tRNA, or 20–30 pmol synthetic tRNA<sup>Met</sup>₅₄ unless otherwise stated, various amounts of enzyme, and 52 μM [3H]AdoMet (300–500 Ci mol⁻¹). The synthetic tRNA<sup>Met</sup>₅₄ was heated for 5 min at 70 °C and cooled at room temperature before being added to the reaction mixture. Incubation was maintained at 37 °C for the indicated time, usually 30 min. Then 25 μl of the reaction mixture were added to 40 μg of bovine serum albumin in a glass tube. Protein was precipitated with 2 ml of cold 10% trichloroacetic acid. The tube was kept in ice for 10 min before the precipitate was collected on a 34 glass fiber filter (Schleicher & Schuell) using a Millipore manifold. The filters were washed three times with 3 ml of 10% trichloroacetic acid and dried for 15 min at 150 °C, and the bound radioactivity was determined by liquid scintillation counting. A reaction mixture without tRNA was included as negative control.

**Synthesis of 1-Methylguanosine**—Methyl iodide was used to methylate guanosine, producing m<sup>G</sup> as described by Broom et al. (21). The structure of the synthetic product was confirmed using liquid chromatography-electrospray ionization-tandem mass spectrometry at the Mass Spectrometry Facility of the Department of Chemistry and Biochemistry, University of Texas at Austin. Negative ionization mode identified the molecular ion [M – H]⁻ at m/z 296 and the chloride adduct [M + Cl]⁻ at m/z 332. Tandem mass spectrometry product ion analysis of the peak at m/z 296 produced the 1-methylguanine base fragment ion [B – H]⁻ at m/z 164 and the ribosyl fragment ion [Ribose – H]⁻ at m/z 133. Subsequent fragmentation of the ion at m/z 164 produced characteristic fragment ions [B – NH₄]⁻ at m/z 149 and [B – NH₄ – CO]⁻ at m/z 121. The UV absorbance spectrum of the purified m<sup>G</sup> dissolved in 50 mM sodium phosphate (pH 7.2) showed absorbance maxima at 256 and 270 nm, consistent with the previously reported spectrum (22).

**HPLC Analysis of Nucleosides**—The reverse phase HPLC protocol used to separate nucleosides was modified from one described by Pomerantz and McCloskey (23). Buffer A contained 250 mM ammonium acetate (pH 6.0), and buffer B contained 60% (v/v) acetonitrile in water. Samples (20 μl) were applied to a reversed phase C<sub>18</sub> column (4.6 × 250 mm, 5 μm; Axxium) with a Security Guard ODS cartridge (4 × 3 mm; Phenomenex) that was equilibrated in mobile phase containing 8% buffer B at a flow rate of 1 ml min⁻¹ at 35 °C. After 10 min, a gradient from 8 to 15% buffer B was applied. UV absorbance was monitored using a System Gold 168 photodiode array detector (Beckman). The data were collected, and chromatograms were integrated using the 32 Karat software (Beckman). For the analysis of radioisotopes, fractions (0.5 ml) were collected and diluted with 3 ml of ScintiVerse scintillation fluid (Fisher). Radioactivity measurements were made using a LS 6000SC liquid scintillation counter (Beckman). Using this HPLC method, nucleosides eluted with the following retention times: cytidine (3.4 min), uridine (3.5 min), 5-methylcytidine (4.7 min), 7-methylguanosine (4.7 min), guanosine (4.7 min), 1-methylguanosine (6.7 min), 2-methylguanosine (7.4 min), and adenosine (8.9 min). Incompletely hydrolyzed nucleotides and oligonucleotides eluted before 3 min.

**Subcellular Fractionation and Immunoblotting**—Yeast mitochondria were isolated from strain EY0986/TRM5-GFP. The post-mitochondrial supernatant was used as the cytoplasmic fraction. The samples were analyzed by SDS-PAGE on 10% gels, and immunoblotting was performed as described (24) using anti-GFP primary antibodies (1:500 dilution) or anti-Hsp60 antibodies (1:50,000 dilution). For proteinase K treatment, 50-μg aliquots of mitochondria were resuspended in 100 μl of isotonic buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4). Five μl of proteinase K (1 mg ml⁻¹) was added to samples and incubated for 15 min on ice. In some digestions, the membranes were solubilized by addition of Triton X-100 (final concentration, 0.5%) during the 10-min proteinase K treatment. Digestion was stopped by addition of 1 μl of 100 mM phenylmethylsulfonyl fluoride in ethanol followed by a further 10-min incubation on ice. Mitochondria were collected by centrifugation at 12,000 × g for 15 min, the pellets were resuspended in SDS sample buffer, and protein was analyzed by immunoblotting.

**TRM5 Transcript Mapping**—The 5’ end of the TRM5 transcript was mapped by a primer extension-based method (RNA ligase-mediated rapid amplification of cDNA ends (RACE)) using the FirstChoice RNA ligase-mediated RACE kit from Ambion. Total RNA was isolated from yeast strain DAY4 using the RNAeasy Mini Kit (Qiagen). DNA contamination was eliminated using a Turbo DNA-free kit (Ambion). Nested antisense
primers specific to the TRM5 transcript were designed for use with the two nested 5'-RACE primers provided in the kit. The TRM5-specific inner primer (5R_AGA_IP; supplemental Table S1) was complementary to nucleotides +183 to +161. The TRM5-specific outer primer (5R_AGA_OP; supplemental Table S1) was complementary to nucleotides +283 to +264. The 5'-RACE inner primer and the TRM5-specific inner primer had BamHI and HindIII sites, respectively, at their 5’ ends to facilitate cloning. PCR fragments generated in the “inner” PCRs were cloned and sequenced as described previously (24).

Construction and Expression of TRM5 Truncation Mutants in Yeast—The complete sequence of the yeast TRM5 gene including 300 base pairs 5’ of the first AUG codon was amplified from yeast genomic DNA (strain DAY4) by PCR using KOD Hot Start DNA polymerase and primers TRM5_FOR and TRM5_REV containing HindIII and BamHI restriction sites, respectively (supplemental Table S1). The resulting PCR product was gel-purified, digested, and ligated into the yeast low copy vectors, URA3-containing pRS416 and LEU2-containing pRS415 (25), to produce the pRS416-TRM5 and pRS415-TRM5 wild-type constructs (Table 1). SLIC (sequence and ligation-independent cloning) (26) was used to generate the construct with codons 1–19 deleted, using T7 forward primer versus T5_1/20_2 and T5_1/20_3 versus T3 reverse primer (supplemental Table S1). The two PCR products and the linearized pRS415 vector (digested with HindIII and BamHI restriction enzymes) were gel-purified and treated with 0.5 unit of T4 DNA polymerase. The two PCR products and the vector were annealed together at a 2:2:1 molar ratio with 20 ng of RecA protein at room temperature for 30 min, and the recombined plasmid (pRS415-Δ1–19TRM5; Table 1) was recovered from transformed E. coli cells. Splice overlap extension PCR (PCR) was used to generate the other truncations. For the construct with codons 1–33 deleted (pRS415-Δ1–33TRM5; Table 1), the TRM5 gene containing 300 bp of 5’-UTR in pRS416-TRM5 was amplified with primers T7 forward primer (upstream vector primer) and T5_1/34_2 (supplemental Table S1). The DNA including the region between codon 34 and the stop codon of TRM5 was PCR-amplified using primers T5_1/34_3 and T3 reverse (downstream vector primer). Primers T5_1/34_3 and T5_1/34_2 contain 18 bp of complementarity at their 5’ ends for the second splice overlap extension PCR. The resulting PCR products from two separate reactions were purified by a PCR purification kit (Qiagen) and combined together for second PCR with T7 forward primer and T3 reverse primer. This product was gel-purified, digested with HindIII and BamHI, and cloned into pRS415 (25). The construct with codons 1–47 deleted was constructed similarly using primers T7 forward versus T5_1/48_2 and T5_1/48_3 versus T3 reverse for the first PCR. Primers T5_1/48_3 and T5_1/48_2 contain 18 bp of complementarity at their 5’ ends for the second splice overlap extension PCR. The procedure described above was used for the second PCR, followed by cloning into pRS415. These truncation constructs were transformed into DLY1 harboring pRS416-TRM5 for subsequent analysis.

RESULTS

Expression of Trm5p as a Fusion Protein—Attempts to express S. cerevisiae Trm5p in E. coli fused to N- or C-terminal polyhistidine tags produced mostly insoluble protein (data not shown). Therefore the full-length TRM5 ORF (499 amino acids) was cloned into the vector pMALc2H10T to express N-terminal maltose-binding protein, decahistidine tag, and tobacco etch virus protease cleavage site fused to Trm5p (MBP-TRm5). E. coli cells expressed this fusion protein at high levels in soluble form. MBP-Trm5 was purified by nickel affinity chromatography, resulting in nearly homogeneous preparation of fusion protein that had an apparent mass of 97 kDa, similar to its calculated mass of 101.4 kDa (data not shown).

Methyltransferase Activity of the MBP-TRm5 Fusion Protein and Its Cleavage Product—The fusion protein could be partially cleaved with tobacco etch virus protease to generate Trm5p and MBP polypeptides. The Trm5p fragment migrated on an SDS-PAGE gel with an apparent mass of 61 kDa, close to its predicted mass of 57.2 kDa, whereas the MBP fragment had an apparent mass of 42 kDa, similar to its predicted mass of 44.2 kDa. The MBP-Trm5 protein had substantial methyltransferase activity in assays using trm5 tRNA as substrate (supplemental Table S2). However, preincubation with buffer alone or tobacco etch virus protease reduced the activity considerably; this appears to be due to the inherent instability of the enzyme. Thus, all subsequent experiments described here were carried out with the uncleaved fusion protein (MBP-Trm5). As a control experiment, maltose-binding protein alone was purified from empty pMALc2H10T vector. The MBP protein had no methyltransferase activity when incubated with Δtrm5 tRNA and AdoMet (data not shown).

A linear relationship was observed between the amount of substrate (Δtrm5 tRNA) and the incorporation of methyl groups when a saturating amount of enzyme and 50 μM [3H]AdoMet were used in the assay (Fig. 1A). From initial rate conditions with a small amount of enzyme and saturating concentrations of the substrates, a specific enzymatic activity of 30 ± 1.3 nmol of methyl incorporated per min per mg fusion protein was calculated. MBP-Trm5 did not catalyze the methylation of wild-type S. cerevisiae total tRNA (data not shown), presumably because it was already fully methylated.

In Vitro Synthesis of Yeast Mitochondrial Initiator tRNA and Methylation by MBP-TRm5—To produce a synthetic transcript of yeast mitochondrial trNA<sup>Met</sup><sub>f</sub> with its native 5’ uridylic nucleotide, a self-cleaving hammerhead ribozyme construct was produced using eight overlapping oligonucleotides (28, 29) (supplemental materials). T7 RNA polymerase efficiently transcribed the template, and the ribozyme self-cleaved to produce 25 μg of tRNA from 200–300 ng of template. After denaturation at 70 °C for 5 min, followed by slow cooling to room temperature, the synthetic yeast mitochondrial tRNA<sup>Met</sup><sub>f</sub> was a good substrate for methylation by MBP-TRm5. In reactions containing high levels of AdoMet and enzyme, up to 50% of the tRNA was methylated, as shown in Fig. 1B. The extent of this reaction is comparable with that reported for a homologous archaeal enzyme methylating a synthetic transcript (3). With-
out the tRNA refolding procedure, methyl group incorporation was very poor into the synthetic yeast mitochondrial tRNA<sup>Met</sup>.

Identification of the Trm5 tRNA Methylation Site and Product—Two complementary approaches were taken to demonstrate that the MBP-Trm5 protein catalyzed the methylation of guanosine at tRNA position 37, on N1 of the guanine base: primer extension and chromatographic analysis of yeast mitochondrial tRNA<sup>Met</sup>. Because the m<sup>1</sup>G modification interferes with Watson-Crick base pairing, reverse transcriptase is unable to incorporate a complementary cytidylate, blocking further primer extension (30). Primer extension experiments were carried out as described under “Experimental Procedures.” Two different primers specific for yeast mitochondrial RNAs were used: one for the initiator tRNA<sup>Met</sup> (supplemental Fig. S1) and another for tRNA<sup>Phe</sup>, which also in its native form has a m<sup>1</sup>G<sup>37</sup> following A<sup>36</sup> (8).

Total tRNA isolated from wild-type or Δtrm5 cells was annealed to 32P-labeled primer, then the reverse transcriptase reaction was carried out, and the samples were analyzed by denaturing gel electrophoresis (Fig. 2, lanes 1–3 (tRNA<sup>Met</sup> primer) and lanes 4–6 (tRNA<sup>Phe</sup> primer)). With the tRNA<sup>Met</sup> primer, the bands are weaker than with the tRNA<sup>Phe</sup> primer; this may reflect lower abundance of initiator tRNA<sup>Met</sup> in the Δtrm5 tRNA sample. Reverse transcriptase extends the annealed primers up to m<sup>1</sup>G<sup>37</sup> in the wild-type tRNAs, terminating opposite the A at position 38 (Fig. 2, lanes 1 and 4). In contrast, full-length transcripts are present in reactions containing undermodified Δtrm5 tRNA (Fig. 2, lanes 3 and 6). When Δtrm5 tRNA was first methylated in vitro by MBP-Trm5, a significant portion of the transcripts terminated at the same position observed in reactions with wild-type tRNA (Fig. 2, lanes 2 and 5). A similar result was observed comparing modified and unmodified synthetic tRNA<sup>Met</sup> transcripts (supplemental Fig. S2). The samples methylated by MBP-Trm5 show a mixture of full-length and m<sup>1</sup>G<sup>37</sup>-terminated transcripts, consistent with the proportion of modified tRNAs shown in Fig. 1B. This has been observed with other synthetic tRNA<sub>s</sub> (31).

To map the termination site of reverse transcription after methylation with MBP-Trm5, the primer extension reaction was modified to include dideoxynucleoside triphosphate terminators (supplemental materials). The tRNA<sup>Met</sup> primer was annealed to Δtrm5 tRNA, and then the reverse transcriptase reaction was carried out in the absence or presence of the four different ddNTPs. The termination product observed with wild-type yeast tRNA corresponds to dideoxy T incorporation complementary to A<sup>38</sup> (supplemental Fig. S3). As expected, the

FIGURE 1. MBP-Trm5 methyltransferase activity. A, Δtrm5 tRNA as substrate. The methyltransferase assay was carried out as described under “Experimental Procedures” with 0.8 μg of MBP-Trm5 enzyme, 50 μM [methyl-<sup>3</sup>H]AdoMet, and Δtrm5 tRNA. B, synthetic tRNA<sup>Met</sup> as substrate. Synthetic tRNA<sup>Met</sup> was heated at 70 °C for 5 min, and then cooled to room temperature immediately before the reaction mixtures were assembled. The reactions contained 0.8 μg of MBP-Trm5 enzyme and the indicated amount of the tRNA<sup>Met</sup> in the standard assay. The results from duplicate reactions are shown fit to a linear model (R<sup>2</sup> = 0.98).

FIGURE 2. Primer extension demonstrates methylation of yeast mitochondrial tRNA. Total tRNA (cytoplasmic + mitochondrial) from wild-type (wt) or Δtrm5 cells was incubated in the methyltransferase assay with or without MBP-Trm5 as indicated. <sup>3</sup>P-Labeled primers specific for yeast mitochondrial initiator tRNA<sup>Met</sup> (Met, lanes 1–3) or mitochondrial tRNA<sup>Phe</sup> (Phe, lanes 4–6) were then annealed and primer extension reactions performed. The arrows indicate the primer extension termination products caused by the presence of m<sup>1</sup>G for tRNA<sup>Met</sup> (left) or tRNA<sup>Phe</sup> (right).
Mitochondrial Initiator tRNA Methylation

Hydrolysates of total tRNA isolated from the haploid Δtrm5 yeast contained significant levels of m1G (data not shown). This m1G is likely due to the activity of the m1G (9) tRNA methyltransferase encoded by the TRM10 gene (30).

Mitochondrial Localization of Trm5p—These results confirm that the Trm5p methyltransferase is capable of methylating the N1 of guanosine at position 37 in mitochondrial tRNAs (tRNA<sup>Met</sup><sub>f</sub> and tRNA<sup>Met</sup><sub>Ch</sub>), at least in vitro. These mitochondrial tRNAs, encoded and synthesized in the mitochondrion, are known to be modified in vivo. If Trm5p is responsible for this modification in vivo, the enzyme must also exist in mitochondria. To address this possibility, S. cerevisiae strain EY0986/TRM5-GFP was used (Table 1). This strain carries a GFP-tagged TRM5 gene integrated at its normal chromosomal locus, under control of the endogenous TRM5 promoter. EY0986/ TRM5-GFP expresses Trm5p with GFP fused to its C terminus. The cells were grown in a semisynthetic galactose medium, fractionated by differential centrifugation into cytoplasmic and mitochondrial fractions, and analyzed by immunoblotting with antibodies against GFP. Fig. 4A shows that Trm5-GFP is detected in both the cytoplasmic and mitochondrial fractions, with the cytoplasmic protein migrating slightly faster than the mitochondrial protein. Antibodies against the mitochondrial matrix marker Hsp60 confirm the lack of contamination between the two fractions (Fig. 4A). Mitochondrial Trm5-GFP was protected from digestion by proteinase K, unless the membranes were solubilized by Triton X-100, similar to the matrix marker Hsp60 (Fig. 4B). Taken together, these data indicate that the single TRM5 gene encodes a protein that localizes both to the cytoplasm and mitochondrial compartments, with the mitochondrial form residing in the matrix.

Analysis of TRM5 Truncation Mutants—There are several mechanisms known that can generate dual localization of a protein encoded by a single gene (11, 12). In most cases, alternative translational start site use results in alternative N-terminal sequences of the proteins, which in turn targets the proteins to different compartment (e.g. cytoplasm versus mitochondria). Inspection of the 5′ end of the proposed TRM5 ORF reveals four in-frame AUG codons (positions 1, 20, 34, and 48) within the first 48 codons (Fig. 5). To try to identify the translational start site(s) used in vivo, we constructed three N-terminal truncation mutants of Trm5p, in which amino acids 1–19, 1–33, or 1–47 were deleted. The 5′-UTR up to position 1 adjacent to the first AUG codon remained intact in each construct. Each construct was expressed from the single-copy vector pRS415 (LEU2 vector), driven by the TRM5 promoter. These pRS415 constructs were transformed into the Δtrm5 haploid mutant strain (DLY1) harboring a wild-type copy of TRM5 integrated at its normal chromosomal locus, under control of the endogenous TRM5 promoter (30). Inspection of the 5′ end of the proposed TRM5 ORF reveals four in-frame AUG codons (positions 1, 20, 34, and 48) within the first 48 codons (Fig. 5). To try to identify the translational start site(s) used in vivo, we constructed three N-terminal truncation mutants of Trm5p, in which amino acids 1–19, 1–33, or 1–47 were deleted. The 5′-UTR up to position 1 adjacent to the first AUG codon remained intact in each construct. Each construct was expressed from the single-copy vector pRS415 (LEU2 vector), driven by the TRM5 promoter. These pRS415 constructs were transformed into the Δtrm5 haploid mutant strain (DLY1) harboring a wild-type copy of TRM5 integrated at its normal chromosomal locus, under control of the endogenous TRM5 promoter (30).
for the expression of functional Trm5p, because trm5 null mutants exhibit a severe growth defect on glucose (1). The simplest interpretation of these data is that translation initiates at the third AUG codon (codon 34) to produce a Trm5p that functions in the cytoplasm to methylate cytoplasmic tRNAs.

To try to determine the translation start site of the mitochondrial form of Trm5p, we examined the methylation status of mitochondrial tRNAs in DLY1 cells expressing the \( \text{trm5} \) null mutants. The termination product indicative of methylation at position 37 was undetectable in mitochondrial tRNAMet and tRNAPhe isolated from the two truncation mutants (Fig. 7). Overexposure of the gel revealed a faint signal in the tRNAMet isolated from the \( \text{trm5} \) null 1–19 mutant (lane 3), but this could not be replicated in subsequent experiments. These data suggest that initiation at either the first or second AUG codon can produce a functional mitochondrial form of Trm5p, although use of the second AUG (codon 20) is much less efficient.

**FIGURE 4.** Trm5p shows dual localization to cytoplasm and mitochondria. A, cytoplasmic (C) and mitochondrial (M) fractions prepared from cells expressing Trm5-GFP were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted with antibodies against GFP. Each lane contains 75 μg of protein. Locations and size (kDa) of molecular mass markers are shown on the right. Full-length Trm5p (499 amino acids) fused to GFP (238 amino acids) would have a molecular mass of ∼83.3 kDa. A duplicate gel was immunoblotted with antibodies against mitochondrial marker Hsp60 as a control.

B, mitochondria (50 μg protein) were incubated with or without proteinase K (PK, 1 mg ml\(^{-1}\)) in the presence or absence of 0.5% Triton X-100 and immunoblotted with antibodies against GFP. A duplicate gel was immunoblotted with antibodies against mitochondrial matrix marker Hsp60 as a control.

**FIGURE 5.** 5′-UTR and potential transcription and translation start sites of yeast TRM5 gene. Nucleotides are numbered with the A of the first ATG codon as 1. Codons are numbered with the first potential ATG start codon as 1. In-frame ATG codons (codons 1, 20, 34, and 48) are indicated in bold type. The arrows beginning at nucleotides −24, −18, +12, and +21 indicate transcriptional start sites based on 5′-RACE (this work). The diamond at −94 indicates the 5′ end of the TRM5 transcript mapped using a high density oligonucleotide array (36).

**FIGURE 6.** Growth phenotypes and oxygen consumption of Trm5p truncation mutants. A, a Δtrm5 haploid strain (DLY1) harboring full-length TRM5 on a URA3 plasmid (pRS416-TRM5) was transformed with various TRM5 constructs in LEU2 plasmids (pRS415). Levi \(^{+}\) transformants were then streaked onto 5-fluoroorotic acid (5-FOA)/YMD or 5-fluoroorotic acid/YPEG plates and incubated at 30 °C for 4 or 5 days, respectively. Sector 1, full-length TRM5 (pRS415-TRM5); sector 2, pRS415-Δ1–33TRM5; sector 3, pRS415-Δ1–19TRM5; sector 4, pRS415-Δ1–47TRM5; sector 5, pRS415 empty vector (negative control). B, oxygen consumption of strain DLY1 harboring various TRM5 constructs grown in minimal medium with glucose as carbon source (YMD). All of the rates (nmol oxygen min\(^{-1}\)) were normalized to the A\(_{600}\) of the culture, and the mean oxygen consumption rate (nmol min\(^{-1}\) A\(_{600}\)) of the pRS416-TRM5 (wild-type) culture was assigned a value of 1. Each column represents the mean ± the S.E. from two independent experiments (\( n = 2 \)). *, \( p < 0.02 \) versus pRS416-TRM5, unpaired t test.
Although neither the Δ1–19 or Δ1–33 truncation mutants showed a growth defect on YPEG plates, the lack of detectable methylation prompted us to take a closer look at mitochondrial function in these two mutants. Oxygen consumption rates were measured on DLY1 cells expressing either the full-length or the Δ1–19 and Δ1–33 truncation constructs. As shown in Fig. 6B, cells expressing the Δ1–33 construct exhibited a significantly lower rate of oxygen consumption compared with cells expressing full-length TRMS. Cells expressing the Δ1–19 construct exhibited a smaller, nonsignificant decrease in oxygen consumption rate.

**TRMS Transcript II**—To determine whether the TRMS gene produces multiple transcripts, we used RNA ligase-mediated RACE to attempt to map the 5′ end of the TRMS transcript. The final PCR products were cloned into pBluescript II. Sequencing of 10 clones revealed four start sites: −24, −18, +12, and +21 (Fig. 5).

In primer extension-based methods, reverse transcriptase can pause or prematurely terminate in regions of high GC content or extensive secondary structure in the target mRNA. Therefore, we also tried a complementary transcript mapping method, ribonuclease protection analysis. In this method, a single-stranded RNA probe, covering nucleotides −300 to +180 of the TRMS gene, is hybridized to the 5′ end of the mRNA, forming a duplex RNA that is resistant to digestion by single strand-specific ribonucleases. The length of the protected fragment is then determined by gel electrophoresis. However, we were unable to reproducibly detect protected fragments, apparently because of the presence of stable secondary structure(s) in the probe RNA.

**DISCUSSION**

At least eight mitochondrially encoded tRNAs are known to be methylated at G37 in vivo (6), yet the enzyme(s) responsible for modification of these mitochondrial tRNAs has not been previously identified. We have shown here that the tRNA(guanine-N1-)–methyltransferase encoded by the yeast TRM5 gene is responsible for methylation of G37 in mitochondrial initiator tRNA (tRNA^Met_p). Similar to the human Trm5p (9), the recombinant yeast enzyme uses S-adenosyl-l-methionine as a methyl donor and is specific for G residues at position 37 in natural tRNAs. In particular, yeast Trm5p efficiently methylates G37 in mitochondrial tRNA^Met, which contains U at position 36. Thus, like the human Trm5p and in contrast to prokaryotic TrmD (9, 32), the yeast enzyme appears to tolerate any nucleotide at position 36. The product of the reaction catalyzed by yeast Trm5p was shown to be m1G by HPLC.

Because Trm5p is also responsible for m1G37 modification of cytoplasmic tRNAs (1), the enzyme must have a dual localization to both cytoplasm and mitochondria. This was confirmed in yeast using a GFP-tagged version of Trm5p and antibodies against GFP. Proteinase K digestion showed that the mitochondrial form resides in the mitochondrial matrix, where it can access the mitochondrially encoded tRNAs. Early work in yeast indicated the existence of two tRNA(guanine-N1-)–methyltransferases in that organism, specific for tRNA sites 9 and 37 (2, 33). These two distinct enzymes are now known to be encoded by TRM10 (30) and TRMS (1), respectively. Sindhupak et al. (2) even detected two peaks of m1G37 methyltransferase activity on a tRNA affinity column, foreshadowing the two forms of Trm5p (cytoplasmic and mitochondrial) reported here.

The predicted amino acid sequence of Trm5p reveals four potential AUG start codons in the N terminus (codons 1, 20, 34, 48) (Fig. 5). Growth analysis (Fig. 6A) revealed that a Trm5p active in the cytoplasm (as determined by normal growth on glucose) could be obtained with a construct lacking amino acids 1–33, whereas production of a Trm5p active in the mitochondria (as determined by the methylation status of mitochondrial tRNAs) required these first 33 amino acids. Oxygen consumption assays (Fig. 6B) revealed decreased respiration efficiency for both the Δ1–19 and Δ1–33 constructs, although only the Δ1–33 construct was significantly reduced. These data suggest a model in which the mitochondrial form initiates at the first AUG codon, producing a protein with an N-terminal mitochondrial targeting sequence. Helical wheel analysis indicates that residues 1–19 could fold into an amphipathic α-helix, with a hydrophobic face and a basic face (cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html). Our data cannot deduce whether or where the presequence is cleaved upon translocation into the mitochondrion. PSORT II analysis (psort.hgc.jp/form2.html) predicts mitochondrial localization only for the full-length ORF; initiation at any of the downstream AUG codons predicts a cytoplasmic localization. In our model, the shorter cytoplasmic form initiates at the third AUG codon, producing a protein lacking a mitochondrial targeting sequence. This model is consistent with the slightly larger size of the mitochondrial form compared with the cytoplasmic form on SDS
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gels (Fig. 4). However, we cannot rule out the possibility that a non-AUG codon is used to initiate translation, as occurs in the dual localization of the GRS1 and ALA1 gene products (34, 35). Attempts to address this question with N-terminal amino acid sequencing of Trm5p isolated from yeast are underway.

Mapping of the 5′ ends of the TRM5 transcript suggests how this differential translation initiation might occur. We observed two sets of transcriptional start sites: nucleotides −24 and −18 (upstream of the first AUG codon) and nucleotides +12 and +21 (between the first and second AUG codons). Transcriptional initiation at the upstream sites would allow translation to initiate from the first AUG, producing a protein with a mitochondrial presequence to direct translocation into the mitochondrion. Transcriptional initiation at the downstream sites would force translation initiation to the second or third AUG codon, bypassing the mitochondrial presequence, to yield a cytoplasmic protein.

A transcription map of the yeast genome obtained using a high density oligonucleotide array (36) indicates the 5′ end of the TRM5 transcript at nucleotide −94, although this mapping method would not detect transcripts from the same gene with shorter 5′ ends, such as those we observed with 5′-RACE mapping. Because we were unable to obtain reproducible results with ribonuclease protection mapping, the transcriptional start site(s) of the TRM5 gene remain equivocal.

It is also possible that the two Trm5p isoforms are produced from the same transcript, as a result of “leaky” ribosome scanning. Leaky scanning can occur when the first AUG resides in a suboptimal sequence context, leading to its inefficient utilization as initiator and shifting initiation to an AUG at a downstream site, such as those we observed with 5′-RACE mapping. Because we were unable to obtain reproducible results with ribonuclease protection mapping, the transcriptional start site(s) of the TRM5 gene remain equivocal.

Finally, it is possible that mRNA localization plays a role as well. Total RNA isolated from a crude mitochondrial fraction was enriched for TRM5 mRNA (data not shown). A number of nuclear-encoded mitochondrial proteins are known to be translated from mitochondria-associated mRNAs (40), and the TRM5 transcript has a fairly high mitochondrial localization of mRNA index level of 63 (41). Thus, it may be that the mitochondrial form of Trm5p is translated on cytoplasmic ribosomes elaborately to the surface of mitochondria.

It is likely that the m1G37 tRNA modification plays the same role in mitochondria as it does in cytoplasmic and bacterial translation systems, i.e. reading frame maintenance (5). The results from the truncation mutants showed that m1G37 methylation of mitochondrial tRNAs is not essential for mitochondrial protein synthesis, because the Δ1–33 mutant, which lacks this modification, can still respire (as evidenced by growth on ethanol + glycerol; Fig. 6A). However, loss of mitochondrial m1G37 tRNA methylation did correlate with decreased oxygen consumption (Fig. 6B), indicating a defect in oxidative phosphorylation. This suggests that the efficiency or accuracy of mitochondrial protein synthesis is decreased in cells lacking m1G37 methylation of mitochondrial tRNAs, probably because of accumulation of translational frameshift mutations.

This same phenomenon is also seen in mutants of the MSSI and MTO1 genes involved in 2-thiolation of the wobble position in yeast mitochondrial tRNAs (42). Thus, whereas lack of these tRNA modifications may not cause observable growth defects in a laboratory setting, they can cause decreased mitochondrial translation efficiency. If these tRNA modifications provide even the slightest growth advantage to the organism, they would be retained, reflecting what has been called “the ruthless delicacy of selection” (43).

The bacterial ancestor of mitochondria probably had a full complement of tRNA-modifying enzymes. During their organellar evolution, mitochondria have maintained distinct transcription, translation, and tRNA genes, even as their aminoacyl-tRNA synthetases and RNA-modifying enzymes were displaced by nuclear-encoded eukaryotic forms. Similar to the TRM1 gene, whose product introduces the N2,N2-dimethylguanosine modification in cytoplasmic and mitochondrial tRNAs (11), TRM5 encodes multiple targeting sequences. This organization simplifies and consolidates the RNA-modifying systems but constrains the evolution of RNA processing and translation in both the nucleus and the mitochondria.

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