Trmt61B is a methyltransferase responsible for 1-methyladenosine at position 58 of human mitochondrial tRNAs

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ABSTRACT

In human mitochondria, 1-methyladenosine (m¹A) occurs at position 58 of tRNA^{Leu(UUR)}. In addition, partial m¹A58 modifications have been found in human mitochondrial tRNA^{Lys} and tRNA^{Ser(UCN)}. We identified human Trmt61B, which encodes a mitochondria-specific tRNA methyltransferase responsible for m¹A58 in these three tRNAs. Trmt61B is dominantly localized to the mitochondria. m¹A58 formation in human mitochondrial tRNA^{Leu(UUR)} could be reconstituted in vitro using recombinant Trmt61B in the presence of Ado-Met as a methyl donor. Unlike the cytoplasmic tRNA m¹A58 methyltransferase that consists of an $\alpha 2\beta 2$ heterotetramer formed by Trmt61A and Trmt6, Trmt61B formed a homo-oligomer (presumably a homotetramer) that resembled the bacterial homotetrameric m¹A58 methyltransferase. The bacterial origin of Trmt61B is supported by the results of the phylogenetic analysis.

Keywords: Trmt61B; 1-methyladenosine; tRNA; RNA modification; mitochondria

INTRODUCTION

RNA modifications play pivotal roles in the biogenesis, structural integrity, function, and metabolism of RNA molecules. To date, more than 100 site-specific RNA modifications have been identified (Czerwonec et al. 2009), the majority of which occur in tRNAs (Juhling et al. 2009).

1-Methyladenosine (m¹A) (Fig. 1A) has been observed at positions 9, 14, 22, and 58 of tRNAs and is thought to be a primordial RNA modification based on its widespread occurrence in tRNAs in each of the three major phylogenetic domains, as well as in organellar tRNAs (Juhling et al. 2009; Suzuki et al. 2011). m¹A at position 58 forms a reverse-Hoogsteen base-pairing with 5-methyluridine (m⁵U) at position 54 in the T-loop of tRNAs (Robertus et al. 1974). The methylation at the N1 position of A58 confers a positive charge to the elbow region of the tRNA (Agris 1996). This positive charge is believed to stabilize the tertiary structure of tRNAs, especially that of eukaryotic initiator tRNA^{Met} (Basavappa and Sigler 1991). m¹A at position 9 in

mammalian mitochondrial tRNAs is known to stabilize the canonical cloverleaf structure; when A9 is not methylated, the tRNA folds into a nonfunctional structure (Helm et al. 1998, 1999). In nematode mitochondrial tRNAs lacking the T-arm, m¹A9 is an indispensable modification for their aminoacylation and EF-Tu binding (Sakurai et al. 2005). In addition to the role of m¹A in tRNA structure and function, m¹A is critical for retrovirus replication. All retroviruses use m¹A58-containing tRNAs as primers to initiate reverse transcription of the minus strand. During replication of the human immunodeficiency virus-1 (HIV-1), the presence of m¹A58 in human tRNA^{Lys} is required for the accurate termination of plus-strand strong-stop DNA synthesis (Gilboa et al. 1979; Burnett and McHenry 1997).

In *Saccharomyces cerevisiae*, the tRNA methyltransferase responsible for m¹A58 formation was identified as a two-subunit enzyme encoded by *TRM61(GCD14)* and *TRM6(GCD10)* (Anderson et al. 1998, 2000; Ozanick et al. 2007), whose gene products form an $\alpha 2\beta 2$ heterotetramer complex. Trm61p functions as the catalytic subunit for m¹A58 formation and is a member of the Rossmann-fold-type tRNA methyltransferase family, which uses S-adenosylmethionine (Ado-Met) as a methyl donor (Anderson et al. 2000; Kozbial and Mushegian 2005). Both Trm61p and Trm6p are required

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FIGURE 1. The chemical structure of 1-methyladenosine and human mitochondrial tRNA^{Leu(UUR)}. (A) Chemical structure of 1-methyladenosine (m¹A). (B) Secondary structure of human mitochondrial tRNA^{Leu(UUR)} with modified nucleosides: 1-methylguanosine (m¹G), 2-methylguanosine (m²G), dihydrouridine (D), pseudouridine (Ψ), 5-taurinomethyluridine (τm⁵U), 5-methylcytidine (m⁵C), and 5-methyluridine (m⁵U). Nucleoside positions are numbered following conventional guidelines (Steinberg et al. 1993).

for tRNA binding (Ozanick et al. 2007). Sequence analysis suggests that *TRM61* and *TRM6* share a common ancestor and arose through gene duplication and divergent evolution (Bujnicki 2001). Both *TRM61* and *TRM6* have been shown to be essential in yeast. When either gene is inactivated, the initiator tRNA^{Met} is 3'-polyadenylated by the Trf4p/Mtr4p complex and subjected to exosome-mediated degradation (Anderson et al. 1998; Kadaba et al. 2004). Thus, m¹A58 is required for the cellular stabilization of initiator tRNA^{Met} in yeast.

The bacterial and archaeal tRNA m¹A58 methyltransferase, TrmI, was identified as a homolog of yeast Trm61p (Droogmans et al. 2003; Roovers et al. 2004). Whereas the eukaryotic enzyme forms a heterotetramer, TrmI is a homotetrameric enzyme (Gupta et al. 2001; Roovers et al. 2004; Varshney et al. 2004). In *Thermus thermophilus*, inactivation of *TrmI* induces a thermosensitive phenotype (Droogmans et al. 2003). Since *TrmI* disruption strongly reduced 2-thiolation of 5-methyl-2-thiouridine (s²T) at position 54 (Shigi et al. 2006b), the decreased thermo-tolerance observed in the Δ*TrmI* strain can be partly explained by the lack of 2-thiolation of s²T54, a key modification required for tRNA thermostability (Yokoyama et al. 1987; Shigi et al. 2006a).

In humans, the majority of cytoplasmic tRNAs bear the m¹A58 modification (Juhling et al. 2009). Trmt61A (hTrm61p) and Trmt6 (hTrm6p), the human homologs of yeast Trm61p and Trm6p, respectively, are responsible for m¹A58 modification of cytoplasmic tRNAs (Ozanick et al. 2005). In human cells, siRNA-mediated knockdown of either subunit results in a slow-growth phenotype, suggesting that Trmt61A and Trmt6 play a role in cell proliferation (Saikia et al. 2010).

In mammalian mitochondria, all 22 tRNA species are encoded in mitochondrial DNA (Anderson et al. 1981). Among them, three mitochondrial tRNAs have been reported to bear m¹A58: human tRNA^{Leu(UUR)} (Fig. 1B),

bovine tRNA^{Ser(UCN)}, and bovine tRNA^{Glu} (Yokogawa et al. 1991; Yasukawa et al. 2000; Nagao et al. 2009). To date, the functional role and biogenesis of m¹A58 in mammalian mitochondrial tRNAs remain to be elucidated (Suzuki et al. 2011). Here, we report on Trmt61B as a mitochondria-specific methyltransferase responsible for m¹A58 formation in human mitochondrial tRNAs.

RESULTS AND DISCUSSION

Subcellular localization of Trmt61B in human mitochondria

Human Trmt61B is a paralog of Trmt61A with unknown function. To determine whether Trmt61B acts as a methyltransferase for m¹A58 in mitochondrial tRNAs, its subcellular localization was examined. Based on an analysis using the WoLF PSORT and Mitoprot programs (Claros and Vincens 1996; Horton et al. 2007), vertebrate Trmt61B proteins were predicted to localize to the mitochondria. Human Trmt61B with a C-terminal Flag tag (Trmt61B-Flag) was transiently expressed in HeLa cells. Immunofluorescence microscopy revealed that Trmt61B-Flag colocalized with GFP-fused MRPL44, a mitochondrial ribosomal protein (Fig. 2; Koc et al. 2001). In addition, we confirmed the mitochondrial localization of Trmt61B, which is fused with C-terminal GFP (Supplemental Fig. 1). For reference, the subcellular localization of transiently expressed Trmt61A-Flag and Trmt6-Flag was also observed in HeLa cells. Both Trmt61A-Flag and Trmt6-Flag localized to the nuclei and not to the mitochondria, in accordance with the nuclear localization of yeast Trm61p and Trm6p (Anderson et al. 1998). These results suggested that Trmt61B might function as a mitochondrial tRNA m¹A58 methyltransferase.

Trmt61B is required for m¹A58 formation in human mitochondrial tRNAs

Next, the capacity of Trmt61B to catalyze m¹A58 formation in human mitochondrial tRNAs was assessed. Since N1-methylation of m¹A inhibits Watson-Crick base-pairing

FIGURE 2. Subcellular localization of human Trmt61B and its paralogs. HeLa cells were cotransfected with plasmids expressing MRPL44-GFP (green) and C-terminal Flag-tagged Trmt61B, Trmt61A, or Trmt6. Microscopic images were obtained after immunostaining of Flag-tagged proteins (red) and DAPI staining (blue). (Yellow–orange) An overlap of Flag-tagged proteins and MRPL44-GFP. (Pink) An overlap of Flag-tagged proteins and DAPI-stained nucleus.

with T, m¹A can be detected by primer extension (Burnett and McHenry 1997). To detect m¹A58 of human mitochondrial tRNA^{Leu(UUR)}, a primer complementary to the 3'-terminal region was designed (positions 61–76). Following siRNA-mediated knockdown of *Trmt61B*, *Trmt6*, or *luciferase* mRNA in HeLa cells, total RNA was extracted and used as a template for primer extension. In the *luciferase* knockdown cells, used as a control, cDNA extension was strongly arrested just before m¹A58 of tRNA^{Leu(UUR)} and produced a clear band corresponding to position 59 (Fig. 3A). When *Trmt61B* was knocked down, the cDNA partially extended past position 58 and was stopped at position 56 by inserting dideoxy guanosine (Fig. 3A). Repetitive knockdown of *Trmt61B* resulted in reduction of the cDNA arrest at position 59. Thus, unmodified adenosine at position 58 becomes evident upon knockdown of *Trmt61B*. Two different siRNAs (si1 and si2) were used to target *Trmt61B* mRNA to confirm that the changes in m¹A58 level were not due to off-target effects. In contrast, knockdown of *Trmt6* did not affect cDNA arrest just before m¹A58 of tRNA^{Leu(UUR)} and produced no long cDNA. Instead, when cytoplasmic tRNA^{His} was analyzed to confirm the results of RNAi knockdown, the long cDNA that passed over A58 became evident upon

Trmt6 knockdown (Fig. 3D). No involvement of *Trmt6* in mitochondrial m¹A58 formation was apparent. These results demonstrate that *Trmt61B* is responsible for m¹A58 formation in tRNA^{Leu(UUR)}.

In human mitochondria, tRNA^{Leu(UUR)} is the only tRNA bearing m¹A58 reported so far (Suzuki et al. 2011). Because bovine mitochondrial tRNA^{Ser(UCN)} also contains m¹A58 (Yokogawa et al. 1991), human mitochondrial tRNA^{Ser(UCN)} was examined by primer extension. As expected, an arrest band corresponding to position 59 was observed, as well as a long cDNA band at position 56 of tRNA^{Ser(UCN)}. When *Trmt61B* was knocked down, the arrest band disappeared, suggesting that tRNA^{Ser(UCN)} contains partially modified m¹A58 (Fig. 3B). Using the same approach, a partial m¹A58 modification was also detected in human mitochondrial tRNA^{Lys} (Fig. 3C). By quantifying the arrest band and long cDNA bands obtained from primer extension (Fig. 3A–C), the frequencies of m¹A58 in mitochondrial tRNA^{Leu(UUR)}, tRNA^{Ser(UCN)}, and tRNA^{Lys} were estimated to be 97%, 48%, and 27%, respectively. *Trmt61B* is responsible for m¹A58 formation in these three tRNAs.

Because mitochondrial tRNA^{Leu(UUR)} contains fully modified m¹A58, we speculated that *Trmt61B* might enhance the processing, stability, or function of tRNA^{Leu(UUR)}, which is required for translation of the mitochondrial respiratory machinery. To investigate the physiological role of *Trmt61B*, *Trmt61B* was knocked down in HeLa cells, and the steady-state levels of mitochondrial tRNA^{Leu(UUR)}, its processing intermediate (RNA19), and the cellular ATP level were quantified (King et al. 1992). Although a slight increase in RNA19 (~1.5-fold) was observed, neither a change in the steady-state level of tRNA^{Leu(UUR)} nor any measurable loss of ATP level was measured (data not shown). Further studies (e.g., using knockout mice, etc.) will be necessary to identify any physiological roles of m¹A58 and *Trmt61B*.

FIGURE 3. Detection of hypomodified m¹A58 in mitochondrial tRNAs from siRNA-treated cells. After knockdown of *luciferase* (control), *Trmt61B*, or *Trmt6* mRNAs, primer extension was used to detect methylated or nonmethylated A58 in mitochondrial tRNA^{Leu(UUR)} (A), mitochondrial tRNA^{Ser(UCN)} (B), mitochondrial tRNA^{Lys} (C), and cytoplasmic tRNA^{His} (D). Two different siRNAs (si1 and si2) targeting *Trmt61B* mRNA were used. In A, “+” indicates that the cells were transfected once with siRNA and harvested 4 d after transfection. “++” indicates that the cells were transfected with siRNA a total of three times (every 2 d) and harvested 6 d after the first transfection. In B–D, cells were transfected once with siRNA and harvested 4 d after transfection. The knockdown efficiencies of *Trmt61B* and *Trmt6* mRNAs were quantified by qRT-PCR and normalized to *GAPDH* mRNA. The steady-state level of *Trmt61B* mRNA was decreased to 8.0% (si1, +), 8.3% (si1, ++), 18% (si2, +), and 12% (si2, ++), and *Trmt6* mRNA was decreased to 17% (si-Trm6, +) and 17% (si-Trm6, ++), compared with si-Luc cells. The primers are shown as solid lines next to the tRNAs, and nascent cDNAs synthesized from the primers are depicted as gray lines.

Trmt61B is a homo-oligomeric tRNA m¹A58 methyltransferase

To confirm that *Trmt61B* is a methyltransferase for m¹A58 of mitochondrial tRNAs, human *Trmt61B* lacking the predicted mitochondrial targeting sequence at the N terminus (amino acid residues 1–34) was recombinantly expressed in *Escherichia coli* (His-*Trmt61B*). Total RNAs were extracted, and the total tRNA nucleosides were analyzed by liquid chromatography/mass spec-

trometry (LC/MS). No m¹A was detected in total tRNA from wild-type *E. coli* cells (Fig. 4A) because *E. coli* tRNAs do not contain m¹A. In contrast, in *E. coli* overproducing His-Trmt61B, m¹A was apparent (Fig. 4A), suggesting that Trmt61B is able to synthesize m¹A in *E. coli* (Fig. 4A).

His-Trmt61B (52 kDa) was then purified using a Ni²⁺-chelating column (Fig. 4B) and analyzed by gel filtration chromatography (Fig. 4C). Based on the analysis of peak elution volumes, His-Trmt61B was observed to be ~200–300 kDa in size, indicating that Trmt61B forms a homooligomeric complex. The crystal structure of Trmt61B (C-terminal 334 amino acids) was solved as a homotetramer (deposited as unknown protein PDB ID: 2B25), similar to that of bacterial TrmI (Gupta et al. 2001; Varshney et al. 2004). Taken together, these data indicate that the His-Trmt61B construct forms a TrmI-like homotetrameric structure.

m¹A58 formation was then reconstituted in vitro using the recombinant protein. Human mitochondrial tRNA^{Leu(UUR)} transcribed in vitro was used as a substrate for the reconstitution. The arrest band at position 59 generated by primer extension was detected only in the presence of both recombinant His-Trmt61B and Ado-Met (Fig. 5A). Next, the product tRNA was digested by RNase T₁, and the digest was analyzed by LC/MS. In the absence of Ado-Met, the unmodified 23-mer fragment (positions 54–76) was detected as multiply charged negative ions (*m/z* 709.7) (Fig. 5B). In addition to the unmodified fragment, the 23-mer fragment containing a single methyl group (*m/z* 711.0) appeared only in the presence of Ado-Met (Fig. 5B). These results demon-

strate that recombinant Trmt61B catalyzed the formation of m¹A at position 58 of mitochondrial tRNA^{Leu(UUR)} in an Ado-Met-dependent manner. In addition, no additional partner proteins were necessary for Trmt61B to reconstitute m¹A58 in vitro.

Trmt61B originates from bacterial TrmI

Sequence alignment of tRNA m¹A58 methyltransferases from eukaryotes, bacteria, and archaea shows that they possess several conserved motifs (I–VI) specific to Rossmann-fold Ado-Met-dependent methyltransferases (Fig. 6A), including the GxGxG sequence (motif I) for Ado-Met binding, and the conserved carboxylate motif (motif II) for recognition of ribose hydroxyls. Trmt61B homologs were also predicted to contain N-terminal mitochondria-targeting sequences (Fig. 6A). According to the neighbor-joining phylogenetic tree, eukaryotic Trmt61B is closer to bacterial TrmI than to eukaryotic Trmt61A, whereas Trmt61A showed more similarity to the archaeal TrmI (Fig. 6B). The homotetrameric character of functional Trmt61B without additional partner proteins is consistent with the evolutionary assessment of its bacterial origin.

MATERIALS AND METHODS

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, at 37°C under a humidified atmosphere with 5% CO₂.

Fluorescence microscopy

The cDNAs for *Trmt61B*, *Trmt61A*, and *Trmt6* were amplified by reverse-transcription PCR of total RNA from HeLa cells using the primers listed in Supplemental Table S1 and cloned using the Directional TOPO Cloning Kit (Invitrogen). C-terminal Flag-tagged Trmt61B, Trmt61A, and Trmt6 expression vectors were generated by LR reaction of pDEST 12.2 Flag and directional TOPO clones using LR Clonase (Invitrogen). The C-terminal GFP-fused Trmt61B expression vector was generated by LR reaction of Trmt61B directional TOPO clone and pcDNA-Dest47 Gateway Vector (Invitrogen). The C-terminal GFP-fused MRPL44 expression vector was generated by LR reaction of Human Gateway Entry Clone FLJ12701AAAF (Goshima et al. 2008) and pcDNA-Dest47 Gateway Vector. HeLa cells were grown on uncoated, glass-bottom dishes (Matsunami). Cells were cotransfected with 0.5 µg of Trmt61B-Flag, Trmt61A-Flag, or

FIGURE 4. Trmt61B is responsible for m¹A formation in *E. coli* cells. (A) LC/MS analysis of total tRNA nucleosides from *E. coli* rosetta cells (top panel) and His-Trmt61B-expressing *E. coli* rosetta strain (middle panel). Mass chromatograms detecting MH⁺ (*m/z* 282) of m¹A, Am, m²A, and m⁶A are shown. Synthetic nucleosides (m¹A, Am, and m⁶A) were also analyzed as a control (bottom panel). (B) Purified His-Trmt61B was resolved by SDS-PAGE and stained with Coomassie brilliant blue. The protein size marker is indicated. (C) Elution profile (UV trace at 280 nm) of His-Trmt61B (bottom) with protein size marker (top) in gel filtration chromatography.

FIGURE 5. In vitro reconstitution of *m*¹A58 with recombinant *Trmt61B*. (A) *m*¹A58 in the tRNA^{Leu(UUR)} transcript was detected by primer extension. (B) LC/MS analysis of RNase T1-digested fragments of the tRNA^{Leu(UUR)} transcript. Mass chromatograms detecting [M-10H]¹⁰⁻ ions for the unmodified 23-mer fragment (positions 54–76, *m/z* 709.7, and MW 7106.9) and the 23-mer fragment containing a single methyl group (*m/z* 711.0 and MW 7120.9).

Trmt6-Flag expression vector and 0.5 μ g of MRPL44-GFP expression vector using FuGENE HD (Roche). Cells were cultivated for 48 h, washed with phosphate-buffered saline (PBS), and fixed with 3.7% formaldehyde in PBS for 30 min at room temperature. Cells were then washed three times with PBS, permeabilized with 0.5% Triton in PBS for 10 min at room temperature, and blocked with 2% fetal bovine serum in PBS for 30 min at room temperature. Cells were incubated for 30 min at room temperature with anti-DYKDDDDK antibody (1:500; Wako) diluted in Can Get Signal solution A (Toyobo), followed by 1 h of incubation at room temperature with Cy3-conjugated anti-mouse secondary antibody (1:1000; Millipore) in Can Get Signal solution A. Nuclei were stained with DAPI. For *Trmt61B*-GFP, 1 μ g of expression vector was transfected into HeLa cells grown on an uncoated, glass-bottom dish (Matsunami). Cells were cultivated for 24 h, and a final concentration of 100 nM Mitotracker Red (Molecular Probes) was added to media for 10 min. Fluorescent images were acquired with a Leica DMI6000B microscope equipped with a DFC360FX cooled CCD camera. Images were processed using AF6500 software (Leica).

Quantitative reverse-transcription real-time PCR (qRT-PCR)

qRT-PCR was conducted as was previously described (Chujo et al. 2012). The sequences of the qPCR primers are listed in Supplemental Table S1.

RNAi

siRNAs targeted to *Trmt61B*, *Trmt6*, and *luciferase* mRNAs were designed using the siRNA design algorithm “siExplorer” (<http://rna.chem.t.u-tokyo.ac.jp/cgi/siexplorer.htm>) (Kato and Suzuki 2007). The siRNAs used here are listed in Supplemental Table S1. RNAi experiments were conducted with 48 pmol of siRNA (4 nM final concentration), as previously described (Chujo et al. 2012).

Primer extension method to detect *m*¹A58 modification

Primer extension was conducted essentially as described previously (Kirino et al. 2005). The 5' ³²P-labeled primer (0.1 pmol) was incubated with 1 μ g of total RNA in a 10- μ L solution containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA for 2 min at 80°C, and then allowed to cool to 42°C. Alternatively, after in vitro reconstitution (Fig. 5), ~0.05 pmol of tRNA transcript was mixed with 1 μ g of total RNA from *E. coli* and was treated in the same manner as described above. Subsequently, 1.5 μ L of ddH₂O, 4 μ L of 5 $\times$ reverse transcription reaction buffer (Invitrogen), 0.5 μ L of 1.5 mM d/ddNTP mix, 3 μ L of 25 mM MgCl₂, and 1 μ L of Moloney murine leukemia virus reverse transcriptase (Invitrogen) were added, and the mixture was incubated for 1 h at 42°C. The d/ddNTP mix consisted of dATP, dTTP, and ddGTP for mitochondrial tRNA^{Leu(UUR)}; dATP, dTTP, dCTP, and ddGTP for mitochondrial tRNA^{Ser(UCN)}; dTTP, dGTP, and ddCTP for mitochondrial tRNA^{Lys}; and dATP, dTTP, dCTP, and ddGTP for cytoplasmic tRNA^{His}. Following incubation, 0.1 μ g of RNase A (Ambion) was added, and the mixture was incubated again for 10 min at 42°C. The reaction mixture was then subjected to 20% polyacrylamide gel electrophoresis (PAGE) containing 7 M urea. Radiolabeled bands were visualized using a FLA-7000 imager (Fujifilm). The DNA primers are listed in Supplemental Table S1.

Expression and purification of the recombinant *Trmt61B* protein

The cDNA of *Trmt61B* lacking the predicted N-terminal mitochondria-targeting signal (amino acid residues 35–477) was amplified by RT-PCR of total RNA from HeLa cells using the primers listed in Supplemental Table S1, which contained HindIII and NheI sites. The PCR product was cloned into the corresponding sites of the pET-28a(+) vector (Novagen) to obtain the expression vector pET-*Trmt61B*, which produced N-terminal hexahistidine tag-fused *Trmt61B* (His-*Trmt61B*). The *E. coli* rosetta (DE3) strain was transformed with pET-*Trmt61B* and cultured in LB media containing 50 μ g/mL kanamycin and 30 μ g/mL chloramphenicol. When the bacteria reached an OD = 0.5, protein expression was induced by the addition of 100 μ M IPTG, and the cells were grown for 3 h at 37°C. Cells were harvested and suspended in buffer A (50 mM HEPES-KOH at pH 7.6, 100 mM KCl, 10 mM MgCl₂, and 7 mM β -mercaptoethanol), followed by sonication on ice. Cell lysates were cleared by ultracentrifugation at 100,000g for 60 min. The supernatant was loaded onto a nickel-charged HiTrap chelating column (GE Healthcare). After washing off unbound proteins with buffer A, recombinant proteins were eluted with a 50-mL linear gradient

FIGURE 6. Sequence alignment and phylogenetic analysis of Trtm61B and TrmI family proteins. (A) Sequence alignment of Trtm61B and its homologs from Eukarya, Bacteria, and Archaea. RefSeq or UniProt IDs of these proteins are listed in Supplemental Table S2. The mitochondria-targeting signal (MTS) was predicted using Mitoprot software (Claros and Vincens 1996) and is shown by green underlines. Human MTS is depicted as a dashed line, because the exact end of the MTS could not be predicted. Motifs I–VI of the Rossmann-fold-type methyltransferase in *S. cerevisiae* Trm61 (Kozbial and Mushegian 2005) are indicated. (B) A nonrooted neighbor-joining tree for Trtm61B and TrmI family proteins was constructed by Clustal_X software (Thompson et al. 1997). The bootstrap value was obtained by 10,000 trials and is indicated at each node. RefSeq or UniProt IDs are listed in Supplemental Table S2.

from zero to 500 mM imidazole in buffer A. Fractions containing the recombinant proteins were pooled and dialyzed overnight against buffer B (50 mM Tris-HCl at pH 8.0, 100 mM KCl, and 1 mM dithiothreitol). The concentration of purified His-Trmt61B was determined by the BCA protein assay (Thermo Scientific) using bovine serum albumin as a standard.

Preparation of the tRNA fraction from Trmt61B-expressing *E. coli* cells

The *E. coli* rosetta (DE3) strain was transformed with pET-Trmt61B and cultured in LB media containing 50 µg/mL kanamycin and 30 µg/mL chloramphenicol. At an OD = 0.5, protein expression was induced by the addition of 100 µM IPTG, and the cells were grown for 3 h at 37°C. Total RNA was extracted from the cells, and the tRNA fraction was purified by denaturing PAGE.

Gel filtration chromatography

Superdex200 (GE Healthcare) was equilibrated with buffer C (50 mM Tris HCl at pH 8.0, 200 mM KCl, and 7 mM 2-mercaptoethanol). Purified His-Trmt61B (25 µg) was injected into the column and eluted with buffer C.

In vitro reconstitution of m¹A58 using recombinant Trmt61B

For in vitro reconstitution of m¹A58, tRNA was prepared by in vitro transcription with T7 RNA polymerase. Template DNA for mitochondrial tRNA^{Leu(UUR)} was generated using the oligo DNAs listed in Supplemental Table S1. tRNA was transcribed by T7 RNA polymerase in vitro (Milligan et al. 1987) and purified by denaturing PAGE. Isolated tRNA was quantified by measuring the optical density at 260 nm. The in vitro reconstitution reaction mixture (50 µL) contained 50 mM NH₄OAc, 3 mM MgCl₂, 50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM Ado-Met, 1.6 µM tRNA^{Leu(UUR)} transcript, and 1 µM His-Trmt61B. The reaction mixture was incubated for 2 h at 37°C. The reaction was terminated by adding phenol:chloroform isoamylalcohol (Nacalai), and the tRNA was recovered by ethanol precipitation.

RNA mass spectrometry

Nucleoside analysis was performed as previously described (Suzuki et al. 2007). In brief, 10 µg of total tRNA was digested to nucleosides by nuclease P1 (Yamasa) and bacterial alkaline phosphatase A19 (Takara), and then analyzed using a LCQ DUO ion-trap (IT) mass spectrometer (ThermoFinnigan) equipped with an electrospray ionization (ESI) source and HP1100 liquid chromatography system (Agilent Technologies). Specific modified nucleosides were monitored on the mass chromatogram.

After in vitro reconstitution, ~1 pmol of mitochondrial tRNA^{Leu(UUR)} transcript was digested by RNase T₁ (Epicentre), and analyzed by capillary liquid chromatography nano-ESI/mass spectrometer (Suzuki et al. 2007; Katoh et al. 2009).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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REFERENCES

- Agris PF. 1996. The importance of being modified: Roles of modified nucleosides and Mg²⁺ in RNA structure and function. *Prog Nucleic Acid Res Mol Biol* **53**: 79–129.
- Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, et al. 1981. Sequence and organization of the human mitochondrial genome. *Nature* **290**: 457–465.
- Anderson J, Phan L, Cuesta R, Carlson BA, Pak M, Asano K, Bjork GR, Tamame M, Hinnebusch AG. 1998. The essential Gcd10p–Gcd14p nuclear complex is required for 1-methyladenosine modification and maturation of initiator methionyl-tRNA. *Genes Dev* **12**: 3650–3662.
- Anderson J, Phan L, Hinnebusch AG. 2000. The Gcd10p/Gcd14p complex is the essential two-subunit tRNA(1-methyladenosine) methyltransferase of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci* **97**: 5173–5178.
- Basavappa R, Sigler PB. 1991. The 3 Å crystal structure of yeast initiator tRNA: Functional implications in initiator/elongator discrimination. *EMBO J* **10**: 3105–3111.
- Bujnicki JM. 2001. In silico analysis of the tRNA:m¹A58 methyltransferase family: Homology-based fold prediction and identification of new members from Eubacteria and Archaea. *FEBS Lett* **507**: 123–127.
- Burnett BP, McHenry CS. 1997. Posttranscriptional modification of retroviral primers is required for late stages of DNA replication. *Proc Natl Acad Sci* **94**: 7210–7215.
- Chujo T, Ohira T, Sakaguchi Y, Goshima N, Nomura N, Nagao A, Suzuki T. 2012. LRPPRC/SLRP suppresses PNPase-mediated mRNA decay and promotes polyadenylation in human mitochondria. *Nucleic Acids Res* **40**: 8033–8047.
- Claros MG, Vincens P. 1996. Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur J Biochem* **241**: 779–786.
- Czerwonec A, Dunin-Horkawicz S, Purta E, Kaminska KH, Kasprzak JM, Bujnicki JM, Grosjean H, Rother K. 2009. MODOMICS: A database of RNA modification pathways. 2008 update. *Nucleic Acids Res* **37**: D118–D121.
- Droogmans L, Roovers M, Bujnicki JM, Tricot C, Hartsch T, Stalon V, Grosjean H. 2003. Cloning and characterization of tRNA (m¹A58) methyltransferase (TrmI) from *Thermus thermophilus* HB27, a protein required for cell growth at extreme temperatures. *Nucleic Acids Res* **31**: 2148–2156.
- Gilboa E, Mitra SW, Goff S, Baltimore D. 1979. A detailed model of reverse transcription and tests of crucial aspects. *Cell* **18**: 93–100.
- Goshima N, Kawamura Y, Fukumoto A, Miura A, Honma R, Satoh R, Wakamatsu A, Yamamoto J, Kimura K, Nishikawa T, et al. 2008. Human protein factory for converting the transcriptome into an in vitro-expressed proteome. *Nat Methods* **5**: 1011–1017.
- Gupta A, Kumar PH, Dineshkumar TK, Varshney U, Subramanya HS. 2001. Crystal structure of Rv2118c: An AdoMet-dependent methyltransferase from *Mycobacterium tuberculosis* H37Rv. *J Mol Biol* **312**: 381–391.

- Helm M, Brule H, Degoul F, Cepanec C, Leroux JP, Giege R, Florentz C. 1998. The presence of modified nucleotides is required for cloverleaf folding of a human mitochondrial tRNA. *Nucleic Acids Res* **26**: 1636–1643.
- Helm M, Giege R, Florentz C. 1999. A Watson-Crick base-pair-disrupting methyl group (m^1A9) is sufficient for cloverleaf folding of human mitochondrial tRNA^{Lys}. *Biochemistry* **38**: 13338–13346.
- Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, Nakai K. 2007. WoLF PSORT: Protein localization predictor. *Nucleic Acids Res* **35**: W585–W587.
- Juhling F, Morl M, Hartmann RK, Sprinzl M, Stadler PF, Putz J. 2009. tRNAdb 2009: Compilation of tRNA sequences and tRNA genes. *Nucleic Acids Res* **37**: D159–D162.
- Kadaba S, Krueger A, Trice T, Krecic AM, Hinnebusch AG, Anderson J. 2004. Nuclear surveillance and degradation of hypomodified initiator tRNA^{Met} in *S. cerevisiae*. *Genes Dev* **18**: 1227–1240.
- Katoh T, Suzuki T. 2007. Specific residues at every third position of siRNA shape its efficient RNAi activity. *Nucleic Acids Res* **35**: e27. doi: 10.1093/nar/gkl1120.
- Katoh T, Sakaguchi Y, Miyauchi K, Suzuki T, Kashiwabara S, Baba T, Suzuki T. 2009. Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev* **23**: 433–438.
- King MP, Koga Y, Davidson M, Schon EA. 1992. Defects in mitochondrial protein synthesis and respiratory chain activity segregate with the tRNA^{Leu(UUR)} mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. *Mol Cell Biol* **12**: 480–490.
- Kirino Y, Goto Y, Campos Y, Arenas J, Suzuki T. 2005. Specific correlation between the wobble modification deficiency in mutant tRNAs and the clinical features of a human mitochondrial disease. *Proc Natl Acad Sci* **102**: 7127–7132.
- Koc EC, Burkhart W, Blackburn K, Moyer MB, Schlatzer DM, Moseley A, Spremulli LL. 2001. The large subunit of the mammalian mitochondrial ribosome. Analysis of the complement of ribosomal proteins present. *J Biol Chem* **276**: 43958–43969.
- Kozbial PZ, Mushegian AR. 2005. Natural history of S-adenosylmethionine-binding proteins. *BMC Struct Biol* **5**: 19. doi: 10.1186/1472-6807-5-19.
- Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC. 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res* **15**: 8783–8798.
- Nagao A, Suzuki T, Katoh T, Sakaguchi Y, Suzuki T. 2009. Biogenesis of glutamyl-mt tRNA^{Gln} in human mitochondria. *Proc Natl Acad Sci* **106**: 16209–16214.
- Ozanick S, Krecic A, Andersland J, Anderson JT. 2005. The bipartite structure of the tRNA m^1A58 methyltransferase from *S. cerevisiae* is conserved in humans. *RNA* **11**: 1281–1290.
- Ozanick SG, Bujnicki JM, Sem DS, Anderson JT. 2007. Conserved amino acids in each subunit of the heterooligomeric tRNA m^1A58 Mtase from *Saccharomyces cerevisiae* contribute to tRNA binding. *Nucleic Acids Res* **35**: 6808–6819.
- Robertus JD, Ladner JE, Finch JT, Rhodes D, Brown RS, Clark BF, Klug A. 1974. Structure of yeast phenylalanine tRNA at 3 Å resolution. *Nature* **250**: 546–551.
- Roovers M, Wouters J, Bujnicki JM, Tricot C, Stalon V, Grosjean H, Droogmans L. 2004. A primordial RNA modification enzyme: The case of tRNA (m^1A) methyltransferase. *Nucleic Acids Res* **32**: 465–476.
- Saikia M, Fu Y, Pavon-Eternod M, He C, Pan T. 2010. Genome-wide analysis of N1-methyl-adenosine modification in human tRNAs. *RNA* **16**: 1317–1327.
- Sakurai M, Ohtsuki T, Watanabe K. 2005. Modification at position 9 with 1-methyladenosine is crucial for structure and function of nematode mitochondrial tRNAs lacking the entire T-arm. *Nucleic Acids Res* **33**: 1653–1661.
- Shigi N, Sakaguchi Y, Suzuki T, Watanabe K. 2006a. Identification of two tRNA thiolation genes required for cell growth at extremely high temperatures. *J Biol Chem* **281**: 14296–14306.
- Shigi N, Suzuki T, Terada T, Shirouzu M, Yokoyama S, Watanabe K. 2006b. Temperature-dependent biosynthesis of 2-thioribothymidine of *Thermus thermophilus* tRNA. *J Biol Chem* **281**: 2104–2113.
- Steinberg S, Misch A, Sprinzl M. 1993. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res* **21**: 3011–3015.
- Suzuki T, Ikeuchi Y, Noma A, Suzuki T, Sakaguchi Y. 2007. Mass spectrometric identification and characterization of RNA-modifying enzymes. *Methods Enzymol* **425**: 211–229.
- Suzuki T, Nagao A, Suzuki T. 2011. Human mitochondrial tRNAs: Biogenesis, function, structural aspects, and diseases. *Annu Rev Genet* **45**: 299–329.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876–4882.
- Varshney U, Ramesh V, Madabushi A, Gaur R, Subramanya HS, RajBhandary UL. 2004. *Mycobacterium tuberculosis* Rv2118c codes for a single-component homotetrameric m^1A58 tRNA methyltransferase. *Nucleic Acids Res* **32**: 1018–1027.
- Yasukawa T, Suzuki T, Suzuki T, Ueda T, Ohta S, Watanabe K. 2000. Modification defect at anticodon wobble nucleotide of mitochondrial tRNAs^{Leu(UUR)} with pathogenic mutations of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. *J Biol Chem* **275**: 4251–4257.
- Yokogawa T, Watanabe Y, Kumazawa Y, Ueda T, Hirao I, Miura K, Watanabe K. 1991. A novel cloverleaf structure found in mammalian mitochondrial tRNA^{Ser(UCN)}. *Nucleic Acids Res* **19**: 6101–6105.
- Yokoyama S, Watanabe K, Miyazawa T. 1987. Dynamic structures and functions of transfer ribonucleic acids from extreme thermophiles. *Adv Biophys* **23**: 115–147.

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