Isolation and Characterization of the Human tRNA-(N^{1}G37) Methyltransferase (TRM5) and Comparison to the *Escherichia coli* TrmD Protein†

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Received February 12, 2004; Revised Manuscript Received May 4, 2004

ABSTRACT: A human TRM5 cDNA has been cloned and recombinant tRNA-N^{1}G37 methyltransferase was produced. The recombinant enzyme methylates the N1 position of guanosine 37 (G37) in selected tRNA transcripts utilizing *S*-adenosyl methionine. The effects of RNA sequence and structure on the methylation reaction in comparison between the *Escherichia coli* TrmD and human TRM5 recombinant enzymes are presented. G37-methylation by TRM5 occurs regardless of the nature of the nucleotide at position 36. TRM5 also methylates inosine at position 37 unlike TrmD, which recognizes the G36pG37 motif preferentially and does not methylate inosine. New evidence is presented concerning TrmD showing that with some tRNA species, A at position 36 is also recognized. The TRM5 enzyme is sensitive to subtle changes in the tRNA−protein tertiary interaction leading to loss of activity. The TrmD enzyme is more tolerant of alterations in tRNA−protein tertiary interactions as long as the core tRNA structure and the G36pG37 are present. The TRM5 enzyme does not have an absolute requirement for magnesium ions, whereas TrmD requires magnesium to express activity. TRM5 demonstrates much higher affinity for substrates with K_m values for tRNA that are nanomolar. TrmD has K_m values for tRNA in the micromolar range. Recombinant TRM5 appears to function as a 60 772 Da monomer, while recombinant TrmD functions as a homodimer of 30 586 Da subunits. Bioinformatic analysis of the human *TRM5* genomic locus (KIAA1393) have identified *TRM5* homologues in eukaryotes and archaea; however, no significantly homologous regions were identified in any prokaryotes including the *TrmD* gene.

Cellular RNAs contain a plethora of modified nucleotides with 96 different types having been characterized. Most of these modifications are found in transfer RNAs, where 81 different modified nucleotides have been identified (*1*). Indeed, an average *Escherichia coli* tRNA containing 77 nucleotides will have approximately 11% (8 to 9) of its residues modified. In mammalian cytosolic tRNAs, these numbers are even higher at 17% (13 to 14) residues per tRNA (*3*). RNA nucleotides are modified posttranscriptionally by enzymes that are highly specific for a given modified nucleotide and its position in the tRNA structure. These enzymes frequently use cofactors such as *S*-adenosylmethionine (AdoMet).[1] In the eubacterium *E. coli*, it is estimated that 45 genes are required to carry out these modifications, or approximately 1% of the bacterial genome is devoted to tRNA modification (*4*).

One of the most thoroughly investigated modifications of tRNA is 1-methylguanosine (m^{1}G) found at position 37 immediately adjacent to the anticodon. The presence of this modification in tRNA^{Pro} has been shown to prevent frameshifting in bacteria by preventing a four-nucleotide base-pairing (extended codon) with mRNA (*5*). In eukaryotes, m^{1}G37 was shown to be necessary and sufficient to prevent incorrect aminoacylation of yeast tRNA^{Asp} by yeast arginyl-tRNA synthetase (*6*). Both mechanisms are important for maintaining accuracy and fidelity of translation; therefore, the absence of m^{1}G37 may lead to significant disturbances in protein synthesis.

The m^{1}G37 modification is performed in bacteria by the product of the *TrmD* gene, a tRNA-(N^{1}G37) methyltransferase (TrmD) (*7*). Although many bacterial orthologs of *TrmD* can be identified in various databases, no eukaryotic or archaeal equivalent can be identified by sequence comparisons (BLAST searches). Recently, Björk et al. isolated an archeal gene from *Methanococcus vannielii* that was able to complement the growth defect of a bacterial strain deficient in TrmD and consequently also in m^{1}G37. Subsequently, these researchers identified both the *Methano-*

† This work was supported by grants from GlaxoSmithKline (Collegeville, PA) and the National Science Foundation (MCB-9816570) to W.M.H., and NHLBI, National Institutes of Health (NIH) Grant HL-45422 to Z.E.Z.

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; m^{1}I: methyl-1 inosine; m^{1}G: methyl-1 guanosine; 1MGT, 1-methyl-G-transferase; ORF, open reading frame; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis: OG, *n*-octyl-*β*-glucopyranoside; RAIV, relative apparent initial velocity.

10.1021/bi049671q CCC: $27.50
Published on Web 06/18/2004

caldococcus jannaschii and yeast orthologs by sequence homology, and designated the gene *TRM5*. The yeast gene was shown to be responsible for m^1G37 formation in vivo (*8*). The amino acid sequence of TrmD and TRM5 are very dissimilar, with only a 7% amino acid homology. However, both genes were shown to be absolutely required for cellular vitality. Disruption of the *TrmD* gene in *Salmonella typhimurium* (*8*) and *Streptococcus pneumoniae* (*9*), and disruption of the *TRM5* gene in *Saccharomyces cerevisiae* (*8*) severely impaired or eliminated growth in all these microorganisms. The apparent vital role of this single modification in both Eubacteria and Eukarya, its role in translational accuracy, and its limited degree of sequence conservation prompted Björk et al. to propose that the two enzymes share a distant common ancestor (*8*).

Early observations suggested that the prokaryotic and eukaryotic enzymes were quite different. Initial studies in bacteria (TrmD) showed that m^1G37 existed only in a subset of tRNAs having the common property of decoding codons starting with C. Therefore, these tRNAs must have a G at position 36 of the anticodon. It was later shown that the presence of a guanosine at position 36 was required for efficient methylation of G37 (*10*), and the simple insertion of G36pG37 into any type of tRNA transcript was sufficient to make it a good substrate as long as the general tRNA tertiary structure was maintained (*11*). Chemical and enzymatic protection studies showed that the *E. coli* TrmD enzyme did in fact bind the anticodon loop, but G36 and G37 were protected only in the presence of the AdoMet analogue Sinefungin (*12*). This suggested that G36 and G37 are free to move (rotate or base flip) into the active site while the rest of the tRNA molecule is firmly anchored to the enzyme.

These characteristics are different for eukaryotes. Experiments involving the microinjection of tRNAs into *Xenopus* oocytes indicated that methylation of G37 occurred regardless of the nature of the nucleotide at position 36 (*13*). A search of the tRNA database revealed that eukaryotic tRNAs having m^1G37 could contain any of the four possible nucleotides at position 36 (although U is very rare) (*3*). The recent identification of *TRM5* by Björk et al. confirmed this proposal, in which a yeast strain with the *TRM5* gene deleted resulted in a dramatic drop in m^1G at position 37. Several of these tRNA species had C or A at position 36. Therefore, it can be concluded that the eukaryotic tRNA-(N^1G37) methyltransferase is *insensitive* to the identity of nucleotide 36.

We have cloned the human ortholog of the yeast *TRM5* gene, overproduced it in *E. coli*, and purified the histidine-tagged recombinant TRM5 protein. Here, we present the first biochemical characterization of a human tRNA-(N^1G37) methyltransferase and compare it to the bacterial TrmD enzyme. *TRM5* is only the third human tRNA modification enzyme to be cloned, expressed, and characterized in vitro, the two other being *hTRM1*, which synthesizes N^2-dimethylguanosine at position 26 of tRNA (*14*), and *hADAT1*, which creates inosine 37 by deamination of a primary transcript adenosine in tRNA (*15*).

MATERIALS AND METHODS

Chemicals and Enzymes. Supplies were obtained from the following sources as indicated: *S*-adenosyl-L-[methyl-^{3}H]-methionine was from Perkin-Elmer; TG1 cells are from Amersham; detergent-compatible Lowry and Quantum Prep Plasmid Miniprep kits are from Biorad; a Quick Ligation kit, T4-polynucleotide kinase, calf intestinal phosphatase, and restriction enzymes are from New England Biolabs; BugBuster 10× Protein Extraction reagent, the Rosetta(DE3)-pLacIRARE strain are from Novagen; a QIAquick gel extraction kit, Ni-NTA agarose, and plasmid Maxi kit are from Qiagen; acetylated bovine serum-albumin (acBSA), *n*-octyl β-D-glucopyranoside (OG), *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), DNAse I (type II, from bovine pancreas), and molecular weight markers for gel filtration were from Sigma; molecular weight markers for SDS−PAGE are from BioRad; *Pfu Turbo* DNA polymerase and buffer are from Stratagene; a cocktail of protease inhibitors (EDTA-free) are from Roche. T7-RNA polymerase was prepared from an IPTG-induced, XL-1 blue, *E. coli* strain carrying a plasmid encoding the enzyme fused to a polyhistidine tag, and purification was done using a Ni-NTA agarose resin.

Cloning Human TRM5. The cDNA clone, KIAA 1393 (*16*), containing the human *TRM5* sequence was obtained from Kazusa DNA Research Institute (Kisarazu, Chiba, Japan) and used as a template for PCR. Appropriate primers were chosen to clone the ORF (nt 3 to 1502) into the pET28a+ vector using *Nhe* I (upstream) and *Hind* III/*Xho* I (downstream) restriction sites. Those primers are respectively: 5′-ATGGCTAGCTTTGGATTCTCAGG-3′, 5′-GCAAGCTTTTAAGTGTTTGAAACAATTTGTG-3′ and 5′-GTGCTCGAGTGCTTTAGTGTTTGAAACAATTTGTG-3′. The two downstream primers yielded an enzyme with a carboxyterminal histidine-tag (5′-GTG...) or without a carboxyterminal histidine-tag (5′GCA...) upon cloning into pET28a+. *Pfu Turbo* DNA polymerase was used according to the manufacturer's instructions. The thermocycling scheme for PCR was 1 cycle of 3′ at 95 °C, 15 cycles of [1′ at 95 °C, 1′ at 56 °C, and 1′30″ at 72 °C], 15 cycles of [1′ at 95 °C, 1′ at 58 °C, and 1′30″ at 72 °C], and 1 cycle of 5′ at 72 °C. Digestion of the PCR product and vector by restriction enzymes, recovery from gel, and ligation were done using standard procedures. Recombinant plasmids were transformed into TG1 cells. Plasmid DNA recovered from TG1 cells was subjected to confirmation by DNA sequence analysis.

Expression and Purification of Recombinant TRM5. Plasmids were transformed into a BL21 derivative overexpressing rare tRNAs (Rosetta(DE3)pLacI *RARE*) to accommodate potential codon bias problems with human gene expression in a bacterial system. Cells were incubated in LB medium containing 50 μg/mL kanamycin and 34 μg/mL chloroamphenicol at 37 °C until they reach mid-log phase (A_{595} = 0.7−0.8 units). Cell cultures were chilled to 27 °C, then induced to express TRM5 with 100 μM IPTG at a temperature of 27 °C for 7 h. Cells were harvested by centrifugation and frozen at −80 °C. For the initial Ni-NTA agarose column purification of histidine-tagged TRM5, the following buffer was used: 50 mM Tris/HCl pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol, 0.05% OG, and 5% glycerol. This basic buffer system was modified for various applications as noted below, and all steps were carried out at room temperature except where noted. Frozen cells (25 g) were resuspended in 200 mL of buffer containing 5 mM imidazole, 25 μg/mL

DNase, 200 μg/mL lysozyme, and protease inhibitors without EDTA. Resuspended cells were passed through a Cell disrupter (Avestin) four times at 15 000 psi. The cell extract was centrifuged at 18000*g* for 30 min at 4 °C, and the supernatant was applied to a gravity-flow column containing 5 mL of packed Ni-NTA-agarose resin, preequilibrated with buffer. The resin was washed extensively with buffer containing 10 mM imidazole and then with additional 400 mM NaCl (final concentration = 500 mM) until $A_{280} \leq 0.05$. The Ni-NTA resin was then reequilibrated in 5 volumes of original low salt buffer, and histidine tagged protein was eluted with 250 mM imidazole/HCl (pH 8.0) in standard low salt buffer. Eluted protein fractions were pooled and dialyzed against buffer without salt for several hours at 4 °C and then again overnight against buffer without salt containing 50% glycerol, then stored at −20 °C. Insertion of TRM5 sequences into the pET28 expression vector as outlined above provided a functional translational start site and added histidine-tagged sequences to both the amino- and carboxyl-terminal ends. The XhoI insertion site provides the C-terminal his tag sequence. Preliminary experiments suggested that N-terminal, histidine-tagged enzyme bound Ni-NTA columns poorly as judged by activity assay and SDS−PAGE gels. Following induction and expression of N- and C-terminal histidine tagged TRM5 in the Rosetta strain (DE3 vector), a prominent band of the expected size (60 kDa) was evident in crude extracts analyzed by SDS−PAGE. Approximately 10% of the crude extract's histidine-tagged protein bound to the Ni-NTA column as determined by SDS−PAGE. However, these partially purified Ni-NTA fractions contained sufficient enzyme and were used to carry out most of the kinetic characterizations and comparisons.

Enzyme prepared as outlined above was dialyzed into 10% glycerol buffer could be further purified using MonoS column chromatography (0.5 × 5 cm Pharmacia). Enzyme was eluted with a linear gradient of KCl ranging from 50 to 700 mM (in 50 mM, $NaPO_4$ buffer pH 6.8, 50 mM KCl, 5 mM $MgCl_2$). Enzyme activity coeluted with a single protein peak at approximately 500 mM KCl. SDS gel analysis showed that these preparations were at least 80% pure as judged by SDS−PAGE. These preparations were used for further studies on the subunit content of native enzyme and for more accurate turnover number calculations.

Molecular Weight Determination of Native TRM5 Protein. Four milligrams of TRM5 protein eluted from the Ni-NTA column and further purified by MonoS ion exchange chromatography were subjected to molecular sieve column chromatography using a Superose 12 column (1 × 30 cm, Pharmacia). Protein was eluted from the column at 0.3 mL/min with 50 mM $NaP0_4$ buffer, pH 6.8, 50 mM KCl, and 5 mM beta mercaptoethanol. An elution volume was used to estimate a molecular weight as compared to known molecular weight standards.

Expression and Purification of Bacterial TrmD. Plasmids and bacterial strains expressing *E. coli* TrmD with a polyhistidine-tag have already been described (*11*). TrmD was expressed, purified, and stored as per the TRM5 protocol. The well-characterized TrmD enzyme was used throughout this project as the representative prokaryotic enzyme in comparative analysis with human TRM5.

Protein Analysis and Quantitation. Protein concentration was determined using a BioRad kit with a bovine serum albumin standard. Qualitative analysis of protein on SDS−PAGE was performed according to standard protocols (*17*). Quantitation of protein in gel was peformed according to ref *18*. The DOC/TCA precipitation method (*19*) was used to remove interfering substances prior to protein quantitation or to concentrate protein samples prior to SDS−PAGE. Human recombinant TRM5 is composed of 533 amino acids, comprising the 500 amino acids derived from the KIAA1393 ORF plus extensions of 23 N-terminal and 10 C-terminal amino acids. The amino acid sequence is given in Figure 4, and the plasmid construct will be referred to as *hTRM5*. The calculated molecular mass of recombinant TRM5 is 60 772 g/mol. *E. coli* recombinant TrmD has a calculated molecular mass of 30 586 g/mol. Native molecular weights were estimated to be 58 246 (TRM5) and 28 422 (TrmD) by bioinformatic approaches using ExPASy (Expert Protein Analysis system) from the Swiss Institute of Bioinformatics.

Template Plasmids for T7 in Vitro Transcription of tRNA Genes. Plasmid pUTL4 encoding *E. coli* $tRNA_1^{Leu}$ as well as some derivatives have been described (*10*, *11*). Plasmids for the production of wild-type, human mitochondrial $tRNA^{Pro}$ and mutant G36A have been described (*20*). Plasmids encoding wild-type yeast $tRNA^{Asp}$ (*21*) as well as several mutants at the level of nucleotide 36 and 37 were a gift from Dr. Catherine Florentz. All these pTFMa constructs differ from the true wild-type in that the first base-pair U1-A72 has been replaced by G1-C72 for improving in vitro transcription. Plasmids with names ending in HB correspond to minor variants from the original plasmids generated in the course of these studies. Plasmid for production of yeast $tRNA^{Phe}$ was a gift from Dr. Mark Helm and corresponds to the original plasmid described by Sampson and Uhlenbeck (*22*). All these plasmids were retransformed into TG1 *E. coli* cells. Plasmids were amplified and purified according to standard procedures and verified by DNA sequencing. All tRNAs used in this study are displayed in Figure 2.

In Vitro Transcription and Purification of tRNA Transcripts. In vitro transcription was carried as described elsewhere (*11*) using *Bst*N 1 or BamHI linearized plasmids. Transcripts were size-purified using 10-cm denaturing urea-PAGE, visualized by UV shadowing, and appropriate bands were excised, electroeluted, precipitated in ethanol, dissolved in RNase-free water, and stored at −20 °C. Quantitation was made by UV spectrophotometry where 1 A_{260} unit corresponds to a concentration of 40 μg/mL (1-cm path length). Molecular weights for the different transcripts were calculated from their sequences.

Chemically Synthesized tRNAs. A tRNA containing inosine at position 37 instead of guanosine was chemically synthesized by Qiagen-Xeragon (Germantown, Maryland, USA). This tRNA corresponds to the yeast $tRNA^{Asp}$ sequence with the following differences: the presence of a G36 instead of C36, the first base pair of the acceptor stem is G1−C72, and inosine inserted at position 37. This tRNA was used in assays without further purification.

Salmonella tRNAs Deficient in m^1G37. Salmonella typhimurium strain GT875 was obtained from Dr. Glenn Björk (Umea University, Umea, Sweden). Cells were grown in LB broth at 37 °C and harvested at mid-log phase. RNAs were isolated by acidic phenol extraction and 2-propanol precipitation. RNAs were purified by urea-PAGE electrophoresis. The two bands migrating above the xylene cyanol tracking dye

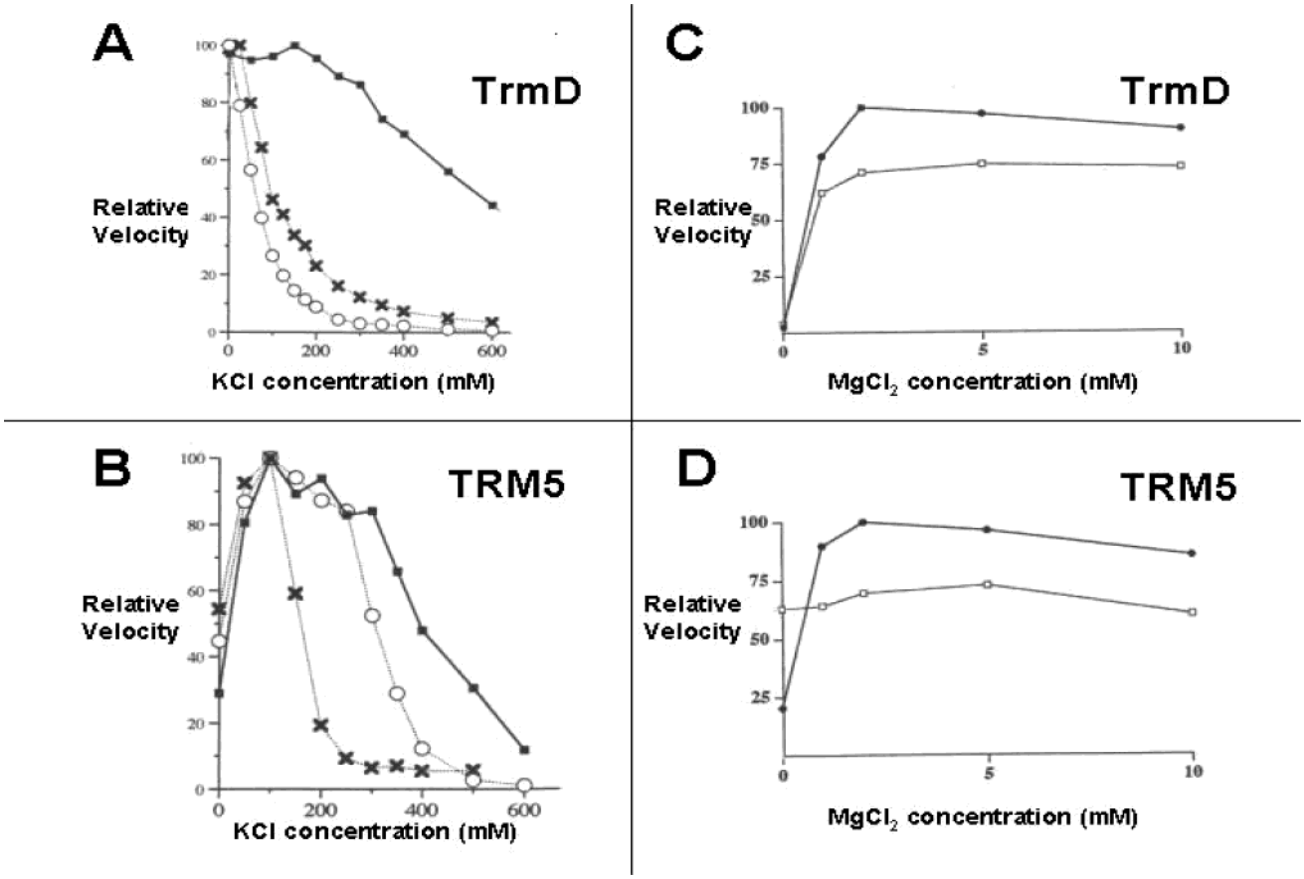

FIGURE 1: Effects of salt and magnesium on TRM5 and TrmD activity. (A, B) Closed squares = $tRNA_1^{Leu}$, X's = mitochondrial $tRNA^{Pro}$, open circles = $tRNA^{Asp}$. (C, D) Closed circle = buffer OO (optimization buffer), open squares = SS buffer (salt plus spermidine).

(class I and II tRNAs) were excised from the gel and purified as described for transcript RNA.

Assay Conditions for In Vitro Methylation of tRNAs. A standard methylation buffer (50 mM Tris-HCl pH 8.0, 24 mM NH_4Cl, 6 mM $MgCl_2$, 1 mM DTE, 0.1 mM Na_3EDTA) was used for initial enzyme activity assays with $tRNA_1^{Leu}$. An optimized methylation buffer was used for kinetic parameter determinations for the two enzymes that differed slightly depending on salt concentration (50 mM BES-NaOH pH 7.34, 5 mM $MgCl_2$, 2 mM DTT, 0.05% OG, 0.1 mM Na_3EDTA, and 100 mM KCl for TRM5, no KCl for TrmD). Besides the buffer, other reaction mixture components were slightly different between TrmD and TRM5. When necessary, the enzyme was diluted in buffer containing 1 mg/mL acetylated BSA to give a final concentration of 0.1 mg/mL acetylated BSA in the reaction. All *S*-adenosylmethionine (AdoMet) dilutions were made and stabilized in HCl pH 2.0 (final concentration 10 mM HCl) and stored at −20 °C. Reagents were combined according to the following protocol: tRNAs were denatured at 63 °C for 3 min in the presence of reaction buffer in two-fifths of the final volume, and then renatured at room temperature for at least 5 min. Enzyme and the remaining buffer were added and the mixture was preincubated at 37 °C for 5 min. Reactions were started by the addition of AdoMet representing one-tenth of the final volume. Reaction volumes were typically 25 to 50 μL. Reactions were incubated at 37 °C, for times ranging between 3 and 50 min. Reaction aliquots were spotted onto 3MM Whatman paper squares (DE81 Whatman circles for $tRNA_1^{Leu}$-minihelix) (*23*), and separation of methylated RNA products from free[^{3}H]-AdoMet was done by 5% TCA precipitation (*6*, *11*, *12*). Quantitation of radioactively labeled tRNA was performed by liquid scintillation counting using an aqueous liquid scintillation cocktail.

Buffer optimization assays for both enzymes were conducted with 100 μM (0.275 Ci/mmol) AdoMet, and 10 μM $tRNA_1^{Leu}$ transcript. KCl testing utilized $tRNA^{Pro}$ and $tRNA^{AspGG}$ at 10 μM final concentration. Glycerol testing was done in standard methylation buffer. Testing optimal pH was done with a reaction mix containing 150 mM KCl, 2 mM DTT, 6 mM $MgCl_2$, 0.1 mM EDTA, and 0.05% OG. Buffering agents tested were (50 mM each) MES/NaOH pHs 5.68, 6.04, 6.39, BES/NaOH pHs 6.71, 7.02, 7.34, Tris/HCL pHs 7.70, 8.02, 8.36, and CHES/NaOH pHs 8.91, 9.25, 9.57. Testing for salt dependency was performed with 50 mM Tris/HCl pH 8.0, 6 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM EDTA, 0.05% OG, plus varying concentrations of KCl. Magnesium and EDTA testing utilized two additional buffer systems, buffer OO: 50 mM BES/NaOH pH 7.34, 150 mM KCl, 2 mM DTT, 0.05% OG, and buffer SS: 50 mM Tris/HCl pH 8.1, 200 mM NaCl, 2 mM DTT, 2 mM spermidine, 0.05% OG. $MgCl_2$ was tested at 1, 2, 5, and 10 mM and Na_3EDTA was tested at 5 mM.

Comparison of RNA transcripts as substrates for TrmD and TRM5 were performed with buffers optimized for each enzyme. Conditions for determination of relative apparent initial velocities (RAIV) are described in the legend to Table 1. Conditions for determining the K_m and V_{max} of TrmD were saturating AdoMet at 100 μM (0.275 Ci/mmol), tRNA range from 0.4 to 60 μM, enzyme from 1 to 20 ng/μL, and

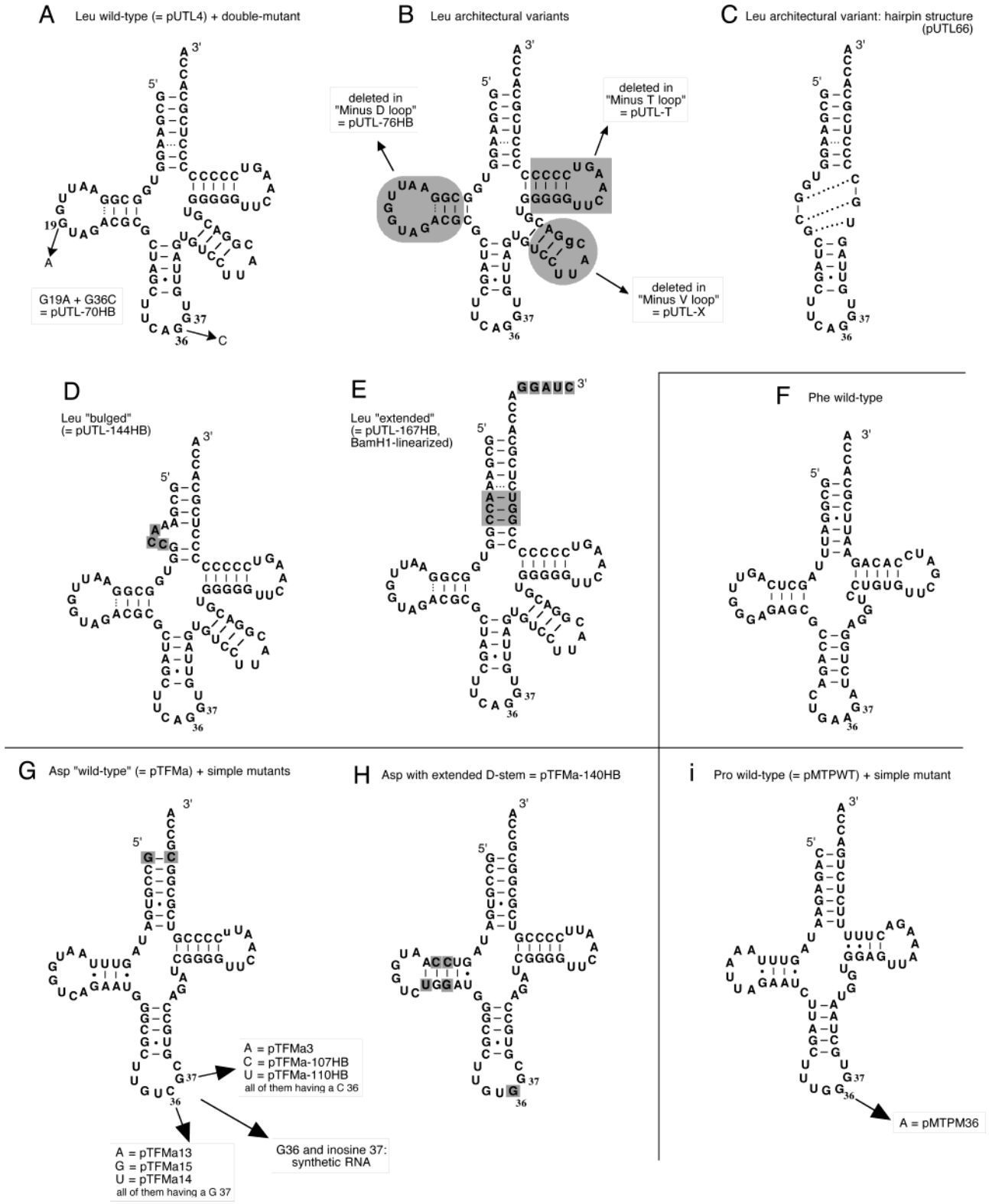

FIGURE 2: Sequence and structures of the RNA substrates used in this study. The target nucleotide 37 and the pivotal nucleotide 36 are numbered. (A−E) *E. coli* $tRNA_1^{Leu}$ and variants. All corresponding plasmid names start with pUTL. In B, three different mutants are depicted on the same figure. Each of them lack one of the highlighted stem−loop but not the two other. Transcript encoded by pUTL-76HB, besides having lost the D-stem−loop, also has a single point mutation in the variable stem, where a G (written in lower case) is mutated into an A. In D and E, differences with A are highlighted on a gray background. (F) Yeast tRNA for phenylalanine. (G, H) Yeast tRNA for aspartic acid and variants. Corresponding plasmid names start with pTFMa. In G, note that the first base-pair which is naturally a U−A has been transformed into a G−C for transcriptional stability reasons. The in "wild-type" are present only to indicate this change. Note that the transcript encoded by pTFMa-107HB, besides having a C37, has also lost U60 in the T-loop (written in lower case). In H, differences with Asp "wild-type" have been highlighted on a gray background. (I) Human mitochondrial tRNA for proline and variant. Both plasmid names start by pMTP.

Table 1: Kinetic Values for tRNA Methylation by Human TRM5 and *E. coli* TrmD

	human TRM5p				*E. coli* TrmDp[f]			
tRNA substrates used[a]	RAIV in %[b]	V_{max}[c]	app K_m (μM)	V_{max}/K_m relative to Leu (%)	RAIV in %[d]	V_{max}[e]	K_m (μM)	V_{max}/K_m relative to Leu (%)
reference tRNAs								
Leu = *E. coli* $tRNA^{Leu}_1$	100/−	0.575	0.0546	100	100/−	(424)	(5.7)	100
Asp = yeast $tRNA^{Asp}$ with G36	14/−	0.475	0.244	18	50/−	286	19	21
Pro = human mitochondrial $tRNA^{Pro}$	0.87/100	0.303	5.46	0.53	63/−	212	8.6	33
Salmonella GT875 bulk tRNAs	6.3/−	0.358	5.27	0.64	7.6/16	NC[g]		0.21
other than G at position 36								
Asp A36	9.9/−	0.487	0.59	7.8	21/−	303	46	8.9
C36 (= Asp wild-type)	11/−	0.355	0.075	45	−/1.7	NM[h]		inactive
U36	10/−	0.513	0.58	8.4	−/1.6	NM		inactive
Pro A36	0.57/58	0.212	18	0.11	1.9/62	NC		0.6
Phe Yeast $tRNA^{Phe}$, has an A36	16/−	0.321	0.0457	67	62/−	(540)	(10)	71
other than G at position 37								
Asp inosine 37	5.0/−	0.245	≤1.23	≥1.9	−/1.7	NM		inactive
A37 (has C36)	−/0.6	NM		inactive	−/0.0	NM		inactive
C37 (has C36)	−/0.5	NM		inactive	−/0.06	NM		inactive
U37 (has C36)	−/0.4	NM		inactive	−/0.25	NM		inactive
architectural variants								
Leu minus D-stem−loop	−/0.8	NM		inactive	3.3/58	ND[i]		[4]
minus T-stem−loop	−/2.7	NM		inactive	8.8/100	ND		[9]
minus V-stem−loop	1.37/176	0.458	6.19	0.70	18/−	ND		[20]
hairpin structure	−/0.0	NM		inactive	2.6/51	ND		[3]
Asp w/extended D-stem	3.4/−	0.355	2.6	1.3	−/6.7	ND		[0.6]
Leu G19A (has C36)	1.9/−	0.383	5.97	0.61	0.9/25	ND		[2]
acceptor stem variants								
Leu w/bulge in acceptor stem	90/−	0.711	0.0499	135	99/−	ND		[100]
Leu + 3 bp in accept stem + 3′ extension	92/−	0.523	0.0567	88	109/−	ND		[100]

[a] All tRNAs have G36 and G37 unless otherwise specified. Reference tRNAs are lead substrates from which other mutants were derived. They correspond to wild-type in the case of $tRNA^{Leu}$ and $tRNA^{Pro}$. The reference $tRNA^{Asp}$ is not the wild-type because it has a C at position 36 and would not be a substrate for bacterial TrmD. *Salmonella* GT875 bulk tRNAs are specifically undermethylated at G37 and comprise a mixture of $tRNA^{LeuCUN}$, $tRNA^{Pro}$, and $tRNA^{ArgCGN}$ isoacceptors which are thought to represent about 10% of total tRNAs. [b] RAIV = relative apparent initial velocity represents initial velocities measured at a single nonsaturating AdoMet concentration (5 μM), a single tRNA, and a single enzyme concentration. For good substrates, tRNA concentration was 500 nM, and results are expressed relative to the best substrate, $tRNA^{Leu}$ (left of column). For bad substrates, tRNA was 20 μM, enzyme concentration was raised, and results are expressed relative to Pro (right of column). [c] Picomol/min/μg. [d] Here AdoMet is saturating at 100 μM final. For good substrates, RNA was 5 μM, enzyme 1 ng/μL (33 nM), and activity is relative to $tRNA^{Leu}$. For bad substrates, RNA was 20 μM, enzyme 16 ng/μL (0.53 μM), and activity is relative to $tRNA^{Leu}$-minus-T-stem−loop. [e] Expressed in picomol/min/μg of enzyme prep. [f] Values in parentheses are imprecise because of substrate inhibition (Leu and Phe). Values in brackets are orders of magnitude simply derived from rounding the RAIV. [g] NC: not calculable (V_{max} and K_m appear infinite (zero order kinetics) or sometimes negative, as a result of a too low tRNA concentration). The slope of the double reciprocal plot however allows one to determine the V_{max}/K_m ratio, which is given relative to $tRNA^{Leu}$. [h] NM: not measurable (counts near or equal to background). [i] ND: not done.

incubations of 12 min at 37 °C. Conditions for determining the K_m and V_{max} of TRM5 were nonsaturating AdoMet at 5 μM (5.5 Ci/mmol), tRNA range from 25 nM to 60 μM, enzyme from 1 to 37 ng/μL, and incubations of 15 min at 37 °C. Studies on the kinetic mechanism for TRM5 were performed with optimized methylation buffer, an enzyme concentration of 17 ng/μL, AdoMet concentrations of 0.5, 1, 2, 5, and 13 μM, $tRNA^{AspGG}$ concentrations of 0.1, 0.2, 0.5, 1, and 2 μM, for 3-min incubations at 37 °C.

In Silico Methods. For identification of the human *TRM5* gene, we utilized the TRM5 peptide sequence of yeast (ORF Yhr070w) and *M. jannaschii* (*8*) to perform BLAST searches against the nucleotide and genome databases at NCBI (www.ncbi.nlm.nih.gov). A BLAST search of the genomic *TRM5* locus against an EST database was done at the Gene2EST server (http://woody.embl-heidelberg.de/) (*24*). Other miscellaneous bioinformatic techniques (peptide alignment with ClustalW 4.0, MW and pI calculation) were performed using MacVector 7.0 software and ExPASy (Expert Protein Analysis System) from the Swiss Institute of Bioinformatics.

RESULTS

Cloning, Expression, and Protein Purification. Using the yeast TRM5 sequence, a BLAST search revealed a highly homologous human sequence encoded by cDNA clone KIAA1393. The first 1505 nucleotides of this cDNA contained an open reading frame (ORF), which could encode a protein 500 amino acids long, but lacking a start methionine. This was supplied by the plasmid pET expression vector system used. Purification of full-length, active protein was achieved with difficulty due to poor yields probably resulting from much of the peptide remaining in inclusion bodies. The addition of a C-terminal histidine tag sequence proved necessary for significant binding to NiNTA columns. Sufficient functional enzyme was isolated using methods outlined here for kinetic analysis and initial enzyme characterization.

Further purification methods using MonoS columns were developed such that sufficient purified enzyme can be isolated to unequivocally determine the subunit content. Using these preparations, a single activity peak with a molecular mass of approximately 60 500 Da was observed

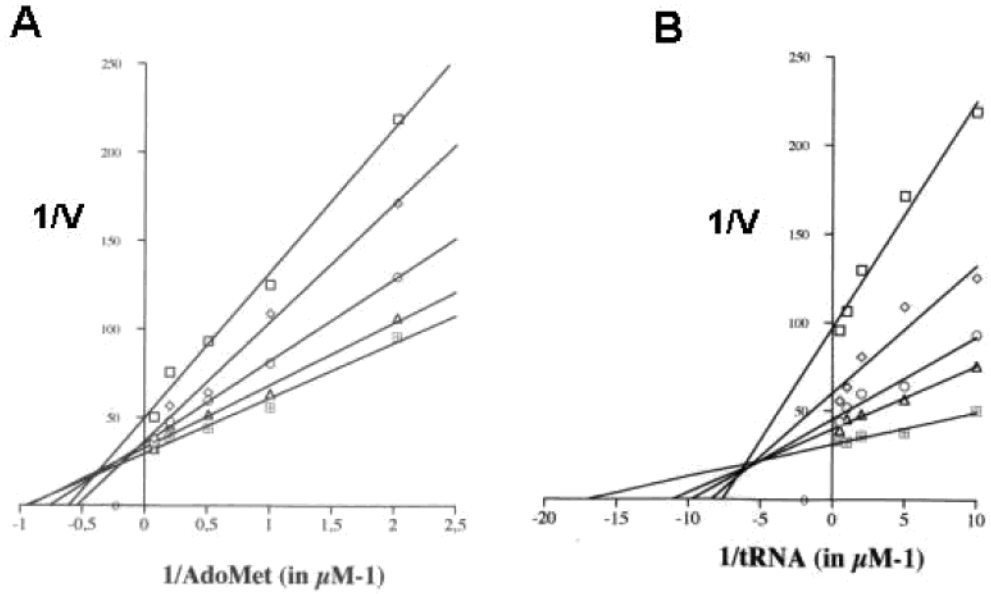

FIGURE 3: (A) From the bottom reciprocal plot up, tRNAAspGG concentrations are 2, 1, 0.5, 0.2 and 0.1 μM, respectively. (B) From the bottom reciprocal plot up, AdoMet concentrations are 13, 5, 2, 1 and 0.5 μM, respectively.

eluting from the Sepharose 12 column. SDS−PAGE also revealed only a single prominent band with this molecular weight. No evidence of higher molecular weight forms was observed in columns or in nondenaturing polyacrylamide gels.

Specific Formation of m^{1}G37 by TRM5. Previous studies with the yeast TRM5 protein indicated methylation of tRNA in vitro using *S*-adenosylmethionine (*13*, *26*). Methylation of tRNA by human TRM5 in vitro also required AdoMet and was proportional to the amount of RNA transcript or enzyme added and linear with time up to 50 min (data not shown). A transcript of tRNA^{Asp} having an A37, C37, or U37 instead of G37 (Figure 2G) could not be methylated (Table 1), indicating that G37 was the target of methylation. Fully modified *E. coli* total tRNAs were not a substrate for TRM5 (data not shown). However, tRNA from a *Salmonella typhimurium TrmD* mutant (GT875) deficient in m^{1}G37 but otherwise fully modified (*5*) was a good substrate for TRM5 enzyme activity (Table 1). These studies confirm that G37 is the target for methylation.

Unlike bacterial tRNAs, which have m^{1}G only at G37, eukaryotic cytosolic tRNAs contain m^{1}G at two positions, G9 and G37 (*25*). In yeast, these two positions are methylated by distinct enzymes (*8*, *26*). Our results with recombinant TRM5 using fully modified *E. coli* tRNAs indicate that the same is true for the human TRM5 protein. TRM5 cannot methylate *E. coli* tRNAs despite the fact that they are predominantly unmodified at G9 (*3*) and are substrates for a G9-specific methylase (*26*). The recent discovery that the *TRM10* gene in yeast codes for a tRNA-(N^{1}G9)-methyltransferase strongly suggests that methylation at G9 is not performed by TRM5 (*28*).

Assay Optimization and Biochemical Characterization of TRM5. We investigated the effect of different assay conditions on the velocity of methylation by human TRM5 and *E. coli* TrmD to compare properties of the two enzymes. The transcript used for most studies was *E. coli* tRNA$_1^{Leu}$ unless otherwise indicated. Additional tRNAs (Figure 2) were used to probe the structural basis of substrate binding.

The effect of glycerol was substantial on both enzymes. TRM5 performs best between 25 and 30% glycerol, and is less active at lower concentrations. The enzyme retains 45% of its activity in the presence of 50% glycerol. The *E. coli* TrmD performs best in the absence of glycerol, its activity decreasing linearly with increasing glycerol concentrations, demonstrating no activity in 50% glycerol (data not shown).

The pH optima for both enzymes are broad and show no remarkable difference. Both enzymes are fairly active between pH 6.4 and 8.4, with an optimum at pH 7.4. On the basis of these studies, BES/NaOH pH 7.35 was chosen for the optimized buffer to run kinetic studies. The reducing agent DTT was investigated at concentrations ranging from 0 to 10 mM. The effect was moderate, but significantly different for both enzymes. TRM5 displays maximal activity above 1 mM DTT and loses 20% of its activity below that value, whereas TrmD has maximal activity below 1 mM DTT and loses 20% of its activity above that value (not shown). The presence of 0.05% OG detergent had no effect on either enzyme (data not shown).

Ionic strength is an important variable for the activity of both bacterial TrmD and human TRM5 proteins. This was especially so when different tRNA substrates were utilized. Both enzymes display similar activity profiles using tRNA$_1^{Leu}$ as the substrate (Figure 1A,B). However, the TRM5 enzyme is stimulated 4-fold by 100 mM KCl, whereas the TrmD protein is most active in the absence of KCl. Overall, it can be seen that the bacterial protein is more salt tolerant when tRNA$_1^{Leu}$ is used, maintaining some activity up to 600 mM KCl. However, TRM5 tends to lose all activity in 600 mM KCl. Interestingly, when substrates other than tRNA$_1^{Leu}$ are used, marked differences in response to salt concentration are seen. In the case of the bacterial enzyme, activity decreases rapidly with increasing salt when either human mitochondrial tRNA^{Pro} or tRNA^{AspG37} are used as substrates (Figure 1A). The loss of TRM5 enzyme activity with these tRNAs is less sensitive to increasing salt than is TrmD, where tRNA^{AspG37} is nearly as good a substrate as tRNA$_1^{Leu}$ (Figure 1B). In view of these results, we elected to use 100 mM

KCl for the TRM5 buffer, and no added KCl for the TrmD buffer in subsequent experiments.

The effect of magnesium is complex and can be modulated by the presence of salt and spermidine. We studied concentrations of $MgCl_2$ ranging from 0 to 10 mM as well as the effect of 5 mM EDTA using buffers with 2 mM spermidine and 200 mM NaCl (SS buffer), or buffers without spermidine and 150 mM KCl (OO buffer) (Figure 1C,D). TRM5 and Trm5 activities in the presence of 5 mM EDTA were similar to those obtained with no added magnesium (not shown).

TRM5 is active in the absence of magnesium with 20 and 56% of maximal activity observed in buffers OO and SS, respectively (Figure 1D). In the presence of magnesium, TRM5 and TrmD activity is quite similar (Figure 1C). TrmD is totally inactive without magnesium. Extended incubation periods of up to 1 h, confirmed that in the absence of magnesium TrmD is totally inactive regardless of the buffer used (data not shown). The fact that magnesium was not absolutely required for the eukaryotic enzyme and that spermidine could increase activity in the absence of magnesium agreed with results reported for the yeast enzyme (*26*). But the spermidine-NaCl containing buffer did not support the maximal activity level seen when using the KCl buffer even in the presence of magnesium (30% reduction, Figure 1C,D). This is either an inhibitory effect exerted by spermidine bound to the tRNAs or by the presence of NaCl instead of KCl.

Methylation of tRNA Variants by TRM5. TRM5 methylation of the four types of tRNA structure used (Figure 2, $tRNA_1^{Leu}$, $tRNA^{Asp}$, $tRNA^{Phe}$, $tRNA^{Pro}$) leads to a great variation in overall efficiency (V_{max}/K_m). As can be seen in Table 1. $tRNA_1^{Leu}$, a class II tRNA, is the best substrate; thus, all V_{max}/K_m ratios were compared to $tRNA_1^{Leu}$ values. The class I tRNA, $tRNA^{AspG36}$, is 5-fold less efficient than $tRNA_1^{Leu}$ and $tRNA^{Asp}$ "wild-type (C36)" is nearly as active as $tRNA_1^{Leu}$ (45%). Note that the yeast $tRNA^{Phe}$ transcript has been grouped in the second row, because it has an A36. It represents a class I tRNA with a 5 nt variable loop, whereas $tRNA^{Asp}$ has a 4 nt variable loop. The catalytic efficiency of $tRNA^{Phe}$ is 67% of that for $tRNA_1^{Leu}$. Hence, it appears that all canonical tRNAs, whatever their class, are good substrates for TRM5. However, mitochondrial $tRNA^{Pro}$ (Figure 2I) is 200-fold less active than $tRNA_1^{Leu}$. This tRNA lacks several conserved residues, notably, G18, G19, and C56, which are involved in conserved tertiary interactions. It also has a shortened T-stem with only 4 bp, which might compromise orthodox D-loop/T-loop interactions (*20*). This suggests that TRM5 activity is susceptible to variations in tRNA core structure.

Because bacterial TrmD prefers a G at position 36 for optimal activity in methylating G37, we investigated the behavior of recombinant human TRM5 regarding nucleotide identity at position 36. All four nucleotides (C, U, G, A) were tested at position 36 in the context of methylation of position G37 in $tRNA^{Asp}$. The wild-type $tRNA^{Asp}$ (C36pG37) was the best substrate in this series (Table 1). Relative to $tRNA^{Asp}$ (C36pG37), $tRNA^{Asp}$ (G36pG37) substrate efficiency was 40%, $tRNA^{Asp}$ (U36pG37) was 19%, and $tRNA^{Asp}$ (A36pG37) was 17%. Results with the $tRNA^{Phe}$ (A36pG37) show substrate efficiency 67% of the best substrate, $tRNA_1^{Leu}$. Using comparatively poor substrates, $tRNA^{Pro}$ (G36pG37) (0.53% of $tRNA_1^{Leu}$) vs $tRNA^{Pro}$ (A36pG37) (0.11% of $tRNA_1^{Leu}$) a 5-fold decrease in TRM5 activity was seen. Therefore, it can be concluded that TRM5 does not require a G36 and can have any nucleotide at position 36 and still methylate G37. Methylation of transcripts is *completely* abolished when G37 is replaced by any of the other three natural nucleotides (A, C, U). This is strong evidence that TRM5 is indeed the eukaryotic tRNA-(N^1G37)-methyltransferase. Surprisingly, replacement of G37 by inosine in $tRNA^{Asp}$ (G36pI37) yields a transcript that is still a reasonably active substrate for TRM5 demonstrating a 10-fold reduction in efficiency from $tRNA^{Asp}$ (G36pG37). This observation together with the fact that TRM5 does not methylate A37 in the same tRNA context supports the previously proposed sequential synthesis of m^1I37 involving deamination of A37, then methylation of inosine by TRM5 (*29*).

Gross structural perturbations of $tRNA_1^{Leu}$ such as removing the D- or the T-stem−loops completely abolish activity of the TRM5 enzyme. Removing the variable loop yields a poor substrate similar to human mitochondrial $tRNA^{Pro}$ in terms of efficiency (Table 1). Another interesting architectural variant is represented by $tRNA^{Asp}$ with a D-stem extended at the expense of the D-loop (Figure 2H). Core tertiary interactions (pair U8−A14, triples 9−23−12 and 13−22−46) are likely impaired with this mutant, but the loop−loop tertiary interactions (G18−U55 and G19−C56) are probably retained. This mutant therefore represents an interesting middle point between canonical and structurally challenged tRNAs. As a result, its catalytic parameters were predictably intermediate (Table 1). These results indicate that a stable native tertiary interaction network is required for tRNA methylation by TRM5.

Finally, a $tRNA_1^{Leu}$ derivative differing from normal $tRNA_1^{Leu}$ by two point mutations (Figure 2A) is a very poor substrate. The change of G36 into a C should not appreciably influence the catalytic efficiency (shown above). If the G36C change is neutral toward TRM5, then most of the loss of activity of transcript pUTL-70HB must be attributed to the single change, G19A. This mutation eliminates the classical tertiary interaction of G19−C56 and weakens the D-loop/T-loop interaction. The effect of this point mutation is quite remarkable, transforming the best substrate of TRM5 into as poor a substrate as human mitochondrial $tRNA^{Pro}$. This mutant adds additional support to the line of evidence derived from the results of $tRNA^{Pro}$, $tRNA_1^{Leu}$-minus-V-loop and $tRNA^{Asp}$-extended D-stem, which suggests an important role for the tertiary interactions (particularly D-loop/T-loop interaction) and resulting structural stability for efficient methylation by TRM5.

The 5′- and 3′-ends of tRNA are probably not in contact with the enzyme, and do not influence the catalytic efficiency, because the "Leu-extended" transcript with a 10-bp acceptor stem instead of 7-bp, and a 9 nt long extended 3′-end instead of 4 nt, is an excellent substrate equivalent to $tRNA_1^{Leu}$ (Figure 2E and Table 1). Furthermore, the introduction of a 3 nt bulge on the 5′-side of the acceptor stem ("Leu-bulged", Figure 2D) does not influence activity supporting the probability that the acceptor-stem is not involved in substrate binding by TRM5.

Methylation of tRNA Variants by TrmD. Three transcripts ($tRNA_1^{Leu}$, $tRNA^{Asp}$, $tRNA^{Pro}$) demonstrate remarkably similar activity with TrmD (Table 1) (*10*, *11*, *20*). The $tRNA^{Asp}$

transcripts with a pyrimidine at position 36 were essentially inactive regarding methylation. Remarkably, $tRNA^{AspA36}$ is an excellent substrate and was only 2-fold less active than $tRNA^{AspG36}$. Likewise, the wild-type yeast $tRNA^{Phe}$ (A36), is an excellent substrate (71% of $tRNA^{Leu}$ activity). Therefore, it is clear that TrmD can accommodate both A and G at position 36 in $tRNA^{Asp}$ and $tRNA^{Phe}$. However, this is not the case with human mitochondrial $tRNA^{Pro}$ where the introduction of A36 leads to a 55-fold loss of activity. Therefore, it appears that TrmD can accept an A36, but only in certain tRNA structural contexts. Guanosine still remains the best overall choice at position 36, and there are no known examples of wild-type transcripts having G36, which are not methylated at G37.

Mutants of $tRNA^{Asp}$ at position 37 including $tRNA^{Asp-inosine}$, are all inactive substrates for TrmD activity. The variants of $tRNA_1^{Leu}$ lacking one stem−loop were all active, but compromised substrates in this study, agreeing with earlier studies (*10*). In general, these tRNAs were much better substrates for TrmD than TRM5. In contrast, lengthening the D-stem to 5 bp at the expense of the D-loop has a very deleterious effect in the $tRNA^{Asp}$ context (Figure 2H) as compared to $tRNA_1^{Leu}$ lacking the entire D-loop. Even removal of all three stem−loops in $tRNA_1^{Leu}$ had a smaller effect. Clearly, TrmD is less stringent with regard to $tRNA_1^{Leu}$ structure than it is to $tRNA^{Asp}$. This is highlighted by the differences in salt sensitivity between these two transcripts (Figure 1A). Finally, $tRNA_1^{Leu}$ "extended" is just as active as $tRNA_1^{Leu}$ wild type (*11*), and the bulge in the acceptor stem does not disturb TrmD. This result is similar to that obtained for TRM5.

Kinetic Analyses of Enzymatic Activity. We investigated the mechanism of the methylation reaction by TRM5 with regard to the binding order of the two substrates. Velocity measurements in the presence of various concentrations of AdoMet (0.5 to 13 μM) and $tRNA^{AspGG}$ transcript (100 nM to 2 μM) were plotted as double reciprocal plots. Graphs showed lines intersecting at the left of the *Y*-axis (Figure 3A,B), in a pattern consistent with a sequential mechanism (random or ordered), and excluding a "ping-pong" mechanism (*30*). A sequential pattern is consistent with a direct S_N2 reaction, a mechanism that has been proposed for most AdoMet-dependent N- and O-methyl-transferases (*31*). In Figure 3A, extrapolation of the results to extreme concentrations of AdoMet (zero or infinite) indicates that for TRM5, $tRNA^{AspGG}$ has an apparent K_m of 163 nM in the absence of AdoMet, and 65 nM in the presence of saturating Adomet. Similarly, from Figure 3B, a replot of the slopes versus 1/tRNA allows calculation of an AdoMet K_m of 2.8 μM (absence of tRNA) and 1.1 μM (saturating tRNA) for TRM5. Therefore, the K_m values of the substrates are co-dependent with an interaction factor equal to 0.4. In the case of TrmD, it appears that substrate binding is independent and random, and sequential kinetics are displayed (*11*).

The TRM5 K_m for AdoMet (1.1−2.8 μM) is consistent with the intracellular concentration of 2−13 μM for AdoMet in eukaryotes (*32*, *33*) and to the K_m of other eukaryotic AdoMet-dependent methylases (*34*). This value is also very close to the K_m values (5 and 15 μM) of AdoMet for TrmD previously reported (*10*, *35*). The turnover number for TRM5 has been calculated using purified enzyme. We find that each molecule of TRM5 can methylate 48 tRNA molecules per minute under the conditions utilized here. This is considerably more active than the TrmD protein which can methylate approximately nine molecules of tRNA per minute per molecule of enzyme.

Bioinformatic Searches. Using the human KIAA1393 translated sequence, protein BLAST searches found numerous *TRM5* orthologs in eukaryotes and archaeae, e.g., *M. jannaschii* (MJ0883) and *S. cerevisiae* (yhr070w), while *Arabidopsis thaliana* and *Pyrococcus abyssi* each have two *TRM5* orthologs. These searches also identified *TRM5*-paralogs in MJ1557 for *M. jannaschii*, Ym1005w for yeast and FLJ20772 for human, all based on conserved domain searches. These sequences possess the so-called "met-10" domain named after a *Neurospora crassa* complementation group (*36*). This domain also was identified in the *TRM5* gene. An AdoMet binding motif can be detected in some *TRM5* orthologs (e.g., *Drosophila* Q9VZU0, *C. elegans* C53A5.2). No portable RNA-binding motif could be detected. Using *trmD* sequences from *E. coli* (CAA25959) and *Thermotoga maritima* (AAD36636), no *trmD* homologous sequences were found in eukaryotes (human or yeast TRM5) or archaea. Predicted physical properties show large differences; bacterial TrmD proteins are small proteins of about 250 amino acids (28 kDa), eukaryotic TRM5 averages 470 amino acids (58 kDa), and archaeal TRM5 is 340 amino acids (38 kDa). TrmD proteins are acidic, with predicted isoelectric points around 5.6, whereas TRM5 are alkaline with pIs between 7.9 and 8.8 for eukaryotes and archaeas, respectively (ExPASy, ProtParam tool).

Genomic Structure of the TRM5 Locus. The KIAA1393 cDNA is 5164 nucleotides long, with the first 1505 nt corresponding to the *TRM5* ORF. A nucleotide BLAST search against the human genome using the *TRM5* ORF shows a match with the long arm of chromosome 14 (14q23.1). The *TRM5* gene is split into several exons (exons 2−5 in Figure 4A). However, the protein made from the KIAA1393 ORF may not be complete for several reasons: First, the sequence is truncated at its 5′-end lacking a start methionine. Second, a methionine residue at position 32 may not represent the translation start site since it is not conserved in the mouse (BAB27710) or *Macaca fascicularis* (BAB46864) orthologs and lacks a Kozak consensus start sequence. Therefore, it is likely that the enzyme may contain amino acids encoded by an additional upstream exon. To address this question, a search for relevant EST sequences was carried out. BG113710 was identified containing an extended version of exon 2 and another upstream exon, which we have chosen to designate exon 1. Together, these sequences encode nine additional amino acids residues (MVLWILWRP) (Figure 4A,B). Thus, it appears possible that some form(s) of the protein might contain the nine residues encoded by the upstream exon 1 and exon 2. The *M. fascicularis* sequence mentioned above harbors exactly the same nine amino acids and supports this idea.

The cDNA clone KIAA1393 (Figure 4) used in this study is unusual because of the very large (3664 nucleotides) 3′ untranslated region following the 1505 nt long ORF. A nucleotide BLAST search against the human genome showed this region contains a potential open reading frame encoding a 73-residue peptide that is homologous to the high mobility group protein HMG17 normally found on chromosome 1 (accession no. X13546).

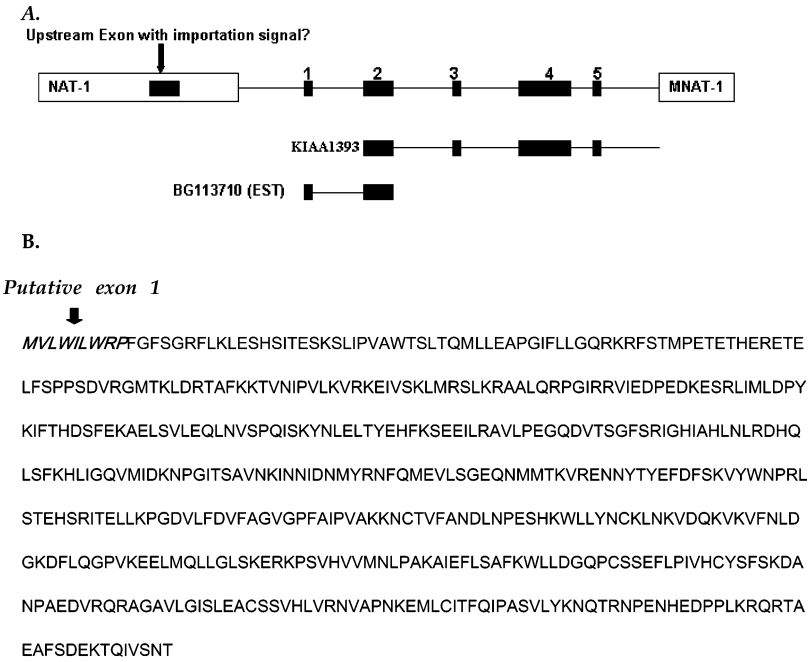

FIGURE 4: Organization of the human *TRM5* gene locus. (A) The *TRM5* locus located on chromosome 14 flanked by the *NAT-1* (N system amino acid transporter) and *MNAT-1* (ménage à trois) loci. KIAA1393 represents the sequences used to produce protein in this study. BG113710 represents an EST which encodes both exon I and 2 of the *TRM5* locus. The region spans nucleotide sequences found on chromosome 14 ranging from nucleotide position 58 775 to 58 764 kb. (B) The amino acid sequence of the TRM5 locus including the putative nine amino terminal residues which might be encoded by exon 1 (bold).

DISCUSSION

Biochemical Comparisons of TRM5 and TrmD. This initial characterization of recombinant human TRM5, a tRNA (N^1G37) methyltransferase, reveals unique features of this new enzyme, confirms some earlier findings regarding 1mG methyltransferases (*13*, *25*, *26*, *29*), and complements in vivo studies performed on the yeast TRM5 (*8*). Comparison of the human TRM5 to the thoroughly characterized bacterial TrmD enzyme suggests the two enzymes are very different and may have evolved independently. The size and subunit composition of the two enzymes are dramatically different, where TrmD appears to function as a heterodimer of 28 422 Da subunits (*37*) (30 586 for recombinant his-tagged constructs used here), and TRM5 is a monomer of 58 246 Da at least in the absence of added ligands (60 772 for recombinant his-tagged constructs used here). Interestingly, the human enzyme has a faster turnover number than the bacterial enzyme.

There are marked differences between TrmD and TRM5 in requirements for KCl concentrations and magnesium ions, where TRM5 prefers KCl and can function without Mg^{2+}, while TrmD works well without KCl but requires Mg^{2+}. TrmD accommodates structural heterogeneity in tRNA substrates, while TRM5 requires strong conservation of the core tRNA structure. TrmD is specific for G36pG37 substrate targets with activity also observed for A36pG37. TRM5 can recognize either G37 or I37 and is not as sensitive to the identity of the nucleotide at position 36. Finally, bioinformatic searches of available databases finds no homology between the bacterial TrmD and human TRM5 gene structure or amino acid sequences. These data strongly suggest that these two enzyme systems are individual, unique, and independently evolved.

Salt concentration, magnesium ions, and spermidine effect enzyme activity differently in TRM5 and TrmD, and raise some interesting mechanistic questions. Several options exist to explain these observations, (i) magnesium is essential for proper tRNA folding and maintaining substrate structure, (ii) magnesium may be directly involved in the catalytic reaction mechanism, (iii) magnesium facilitates substrate tRNA binding to the enzyme. Results show TrmD cannot methylate $tRNA_1^{Leu}$ in the absence of magnesium, whereas TRM5 still maintains 20% of its optimal activity. Spermidine buffer lacking Mg^{2+} improved activity of TRM5 from 20−60% of its optimal activity but TrmD remained inactive in this buffer. Spermidine can substitute for magnesium in assisting tRNA structural stabilization and this stabilization may be what is reflected with the increase in TRM5 activity. However, spermidine did not effect TrmD activity with the same tRNAs; only magnesium induced TrmD activity. Furthermore, spermidine in the presence of magnesium was inhibitory to both TRM5 and TrmD. Both enzymes were inhibited by 30% in the presence of spermidine.

It will be important to determine whether Mg^{2+} plays a catalytic role in the reaction or simply is involved in tRNA substrate conformation for TrmD activity. The mechanism proposed to occur in AdoMet-dependent *O*- and *N*-methyltransferases is a simple S_N2 reaction in which the nucleophilic atom (N1 of the guanosine ring) attacks the carbon of the methyl group attached to the sulfonium center when the three atoms are appropriately aligned (*31*). When the target atom is not nucleophilic, as is the case for the N1 of guanosine at neutral pH, it must be deprotonated first either directly or after tautomerization. This is probably achieved by a nucleophilic amino acid at the active site. Our recent published mutagenesis results suggest strongly that this is the case (*37*). Therefore, in the classical S_N2 reaction, there is no requirement for metal ions. However, in the case of catechol-*O*-methyltransferase (COMT), Mg^{2+} ion was shown to be involved in substrate binding and deprotonation (*34*).

Interestingly, Mg^{2+} is seen bound near the catalytic center of the COMT enzyme, but not that of any TrmD structure so far solved (*37*).

Another explanation might be that the tRNA binding site may require Mg^{2+} and/or increased ionic strength (KCl effects) for proper active site interaction. We have determined the crystal structure of TrmD and found that tRNA must fit into a *negatively* charged, deep cleft to gain a catalytic proximity to the AdoMet buried at the bottom of the cleft (*37*). In this case, a high level of counterion (Mg^{2+} and/or KCl) might be required for proper tRNA interaction within the cleft to position the anticodon loop into the active site with proper orientation. That the same enzyme accommodates different tRNA substrates (different structures and sequences) optimally with different salt concentrations speaks to the existence of optimal ionic interaction between specific substrates and the enzyme.

Synthesis of Methyl Inosine by TRM5. Eukaryotic $tRNA^{Ala}$ contains inosine 37, which is converted to m^1I37. The enzyme ADAT1 forms I37 by deamination of primary transcript adenosine 37 (*15*, *38*). Deletion of the *TRM5* gene abolishes m^1I37 formation in vivo (*8*); therefore, I37 is probably converted into m^1I by TRM5. Although m^1I37 is not found in bacteria, it is formally possible that *E. coli* TrmD might methylate inosine. This possibility was assessed using synthetic yeast $tRNA^{Asp}$ with G36pI37 instead of wild type C36pG37. The results clearly show that TRM5 can methylate inosine in this context and TrmD cannot. The only difference between guanosine and inosine is the lack of the amino group linked to carbon 2 of guanosine. That TrmD is inactive with this substrate suggests either a distinct methylation mechanism from TRM5, or that the N2-amino group of guanosine 37 is required for proper orientation of the base to AdoMet in the active site of TrmD.

Significance and Comparisons of K_m Data. Kinetic analysis indicates that TRM5 and TrmD differ concerning their affinity for tRNA. Substrate $tRNA^{AspGG}$ had apparent K_m's of 244 nM for TRM5 and 19 μM for TrmD. This represents an 80-fold difference in relative affinities. Differences of a similar magnitude between K_m values for TRM5 and TrmD are found for $tRNA_1^{Leu}$ (54 nM and 5.7 μM respectively, 100-fold) and $tRNA^{Phe}$ (45 nM and 10 μM, respectively, 220-fold) (Table 1). No significant difference could be found for the K_m for AdoMet between the two enzymes. It is assumed that the K_m of an enzyme for its substrate reflects the approximate concentration of the substrate in living cells (*30*). This assumption is supported by the finding that concentrations of tRNAs are 290, 36, and 4.2 μM for *E. coli* cytosol, HeLa cell cytoplasm or HeLa mitochondria, respectively (*39*). Interestingly, the ratio of tRNA concentrations between bacteria and mitochondria is 70. This value is compatible with the 80−220-fold difference between K_m's of transcripts for TrmD and TRM5. If TRM5 had to methylate mitochondrial tRNAs in addition to cytosolic tRNAs, it should have evolved to be catalytically competent in the compartment having the lowest concentration of tRNAs (i.e., mitochondria). Therefore, these numbers are consistent with the idea that TRM5 has adapted its RNA binding properties to be efficient in the presence of low levels of tRNAs such as those in the mitochondria. Although these theoretical calculations are appealing, they are challenged by the very poor catalytic efficiency of TRM5 for the only mitochondrial tRNA tested here, $tRNA^{Pro}$. However, in vitro transcripts were used, and it is possible that such tRNA species require additional modifications to function as substrates, or they must be presented to the enzyme in the appropriate context.

Common or Distinct Origins for TrmD and TRM5. Given the ubiquitous occurrence of the 1mG, it is convenient to presuppose that a single primitive gene gave rise to all DNA sequences producing m^1G37 tRNA methyltransferase. Studies presented here indicate otherwise. Several critical distinctions can be made based on physical and enzymatic properties of the two proteins. The molecular mass of *E. coli* TrmD is 28 422 Da (30 586 recombinant form), and it functions as a dimer while the human TRM5 is 58 246 Da (60 772 recombinant form) and functions as a momoner. The predicted isoelectric points are 5.5 for *E. coli* TrmD and 8.78 for human TRM5 (ExPASy). TRM5 will accept any canonical tRNA as substrate whatever the nucleotide at position 36 but cannot modify structurally impaired tRNA molecules. In contrast, TrmD will methylate virtually any tRNA structural analogue as long as it has a G37 and G36, but can also accommodate some tRNA substrates with an A36. The importance of the tertiary interaction network for catalysis by TRM5 (e.g., the effect of the mutation G19A) contrasts with TrmD's relative tolerance for atypical structures such as human mitochondrial $tRNA^{Pro}$. On the other hand, TrmD seems much more discriminating with regard to the anticodon loop sequence. Even though the range of possibilities for substrates was larger than previously thought, it appears that nucleotide 36 still plays a pivotal role in substrate discrimination by TrmD and that more identity elements must be present in the tRNA molecule. The 3′- and 5′-ends of tRNA are not involved in substrate recognition for either enzyme.

Search for Homology between TrmD and Human TRM5. The TrmD protein sequences from *E. coli* (CAA25959) and *Thermotoga maritima* (AAD36636), two widely divergent bacteria, were used for a protein BLAST search against eukaryotic databases. No homologous sequences were discovered in the human or yeast databases. The Björk group reported only a 7% primary sequence homology between *E. coli TrmD* and the *TRM5* for archaea, *M. jannaschii* (*8*). A protein BLAST search using human TRM5, discovered TRM5-like proteins in all members of the Archaea and Eukaryote kingdoms. Representatives of the Trm1 family in eukaryotic/archaeal showed similarities to TRM5 that coded for the tRNA modification enzyme synthesizing $m^2{}_2G26$. Interestingly, this modification is not found in bacterial tRNAs, and *TRM1* has no equivalent in bacterial genomes. Finally, some bacterial sequences belonging to the *TrmA* family were identified having limited homology (very low E-values). This enzyme is responsible for synthesis of m^5U54 in bacterial tRNAs. No TrmD homologues were retrieved during any of these searches.

Ultimately, a more informative view will come from comparisons of the crystallographic structures of these two proteins. It is believed that all AdoMet methyltransferases characterized thus far are derived from a common ancestor as judged by the strongly conserved Rossmann fold. Bioinformatic approaches predict TrmD to have a different fold motif (*2*). However, the structure of *E. coli* TrmD was determined and its AdoMet binding domain is very unique, with five beta strands and a distinctive trefoil knot structure (*37*). Ultimately it will be important to compare the crystal

structures of TrmD and TRM5 to determine the degree of structural conservation. Primary structural comparisons are inadequate for this purpose but indicate widely different amino acid sequences and peptide lengths; therefore, a largely different structure can be assumed pending crystallographic results.

Is There a Mitochondrial Form of TRM5? Since only one TRM5 methyltransferase sequence has been identified in BLAST searches of the human genome database, and no bacterial like sequence can be seen, we propose that the TRM5 gene product may serve to methylate substrate tRNA species both in the cytoplasm and in the mitochondria. Brulé et al. (*20*) have already shown that G37 methylated tRNA exists in the human mitochondria. Therefore, we postulate that the TRM5 protein may be imported into the mitochondria. How might this be achieved? It is possible that a putative upstream exon might provide an amino terminal importation peptide signal (see Figure 4), or, as has been recently shown, internal peptide signals may be utilized for importation (*40*). Further experimentation will be required to resolve these important and interesting issues.

ACKNOWLEDGMENT

We thank the Kazusa DNA Research Institute (Kisarazu, Chiba, Japan) for providing the KIAA1393 cDNA clone, Drs. Catherine Florentz and Mark Helm for the gift of numerous plasmids for in vitro transcription of tRNAs, Dr. Magdalena Zalacain (GSK) for constant support and interest in this work, and Dr. Rana Morris with the NCBI/NIH for consultations regarding the bioinformatics approaches herein.

REFERENCES

1. Motorin, Y., and Grosjean, H. (1998) Appendix 1: Chemical structures and classification of post-transcriptionally modified nucleosides in RNA, in *Modification and Editing of RNA* (Grosjean, H., and Benne, R., Eds.) pp 543−549, ASM Press, Inc., Washington, DC.
2. Anantharaman, V., Koonin, E. V., and Aravind, L. (2002) Spout: A class of methyltransferases that includes SpoU and TrmD RNA methylase superfamilies, and novel superfamilies of predicted prokaryotic RNA methylases. *J. Mol. Microbiol. Biotechnol. 4*, 71−75.
3. Sprinzl, M., Horn, C., Brown, M., Ioudovitch, A., and Steinberg, S. (1998) Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res 26*, 148−153.
4. Björk, G. R. (1996) Stable RNA modification, in *Escherichia coli and Salmonella. Cellular and Molecular Biology* (Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Brooks Low, H., Magasanik, B., REznikoff, W. S., Riley, M., Schaechter, M., Umbarger, H. E., Eds.) pp 860−886, ASM Press, Washington, DC.
5. Björk, G. R., Wikstrom, P. M., and Byström, A. S. (1989) Prevention of translational frameshifting by the modified nucleoside 1-methylguanosine. *Science 244*, 986−989.
6. Pütz, J., Florentz, C., Benseler, F., and Giegé, R. (1994) A single methyl group prevents the mischarging of a tRNA. *Nat. Struct. Biol. 1*, 580−582.
7. Byström, A. S., and Björk, G. R. (1982) Chromosomal location and cloning of the gene (trmD) responsible for the synthesis of tRNA (m1G) methyltransferase in *Escherichia coli* K-12. *Mol. Gen. Genet. 188*, 440−446.
8. Björk, G. R., Jacobsson, K., Nilsson, K., Johansson, M. J., Byström, A. S., and Persson, O. P. (2001) A primordial tRNA modification required for the evolution of life? *EMBO J. 20*, 231−239.
9. Thanassi, J. A., Hartman-Neumann, S. L., Dougherty, T. J., Dougherty, B. A., and Pucci, M. J. (2002) Identification of 113 conserved essential genes using a high-throughput gene disruption system in streptococcus pneumoniae. *Nucleic Acids Res. 30*, 3152−3162.
10. Holmes, W. M., Andraos-Selim, C., Roberts, I., and Wahab, S. Z. (1992) Structural requirements for tRNA methylation. Action of *Escherichia coli* tRNA (guanosine-1)methyltransferase on tRNA(1leu) structural variants. *J. Biol. Chem. 267*, 13440−13445.
11. Redlak, M., Andraos-Selim, C., Giegé, R., Florentz, C., and Holmes, W. M. (1997) Interaction of tRNA with tRNA (guanosine-1)methyltransferase: Binding specificity determinants involve the dinucleotide G36pG37 and tertiary structure. *Biochemistry 36*, 8699−8709.
12. Gabryszuk, J., and Holmes, W. M. (1997) TRNA recognition for modification: Solution probing of trna complexed with escherichia coli tRNA (guanosine-1) methyltransferase. *RNA 3*, 1327−1336.
13. Droogmans, L., and Grosjean, H. (1987) Enzymatic conversion of guanosine 3′ adjacent to the anticodon of yeast $tRNA^{phe}$ to n1-methylguanosine and the wye nucleoside: Dependence on the anticodon sequence. *EMBO J. 6*, 477−483.
14. Liu, J., and Straby, K. B. (2000) The human tRNA(m(2)(2)g(26) dimethyltransferase: Functional expression and characterization of a cloned htrm1 gene. *Nucleic Acids Res. 28*, 3445−3451.
15. Maas, S., Gerber, A. P., and Rich, A. (1999) Identification and characterization of a human tRNA-specific adenosine deaminase related to the adar family of pre-mRNA editing enzymes. *Proc. Natl. Acad. Sci. U.S.A. 96*, 8895−8900.
16. Nagase, T., Kikuno, R., Ishikawa, K. I., Hirosawa, M., and Ohara, O. (2000) Prediction of the coding sequences of unidentified human genes xvi. The complete sequences of 150 new cDNA clones from brain which code for large proteins in vitro. *DNA Res. 7*, 65−73.
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. Ball, E. H. (1986) Quantitation of proteins by elution of coomassie brilliant blue r from stained bands after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem. 155*, 23−27.
19. Peterson, G. L. (1977) A simplification of the protein assay method of lowry et al. Which is more generally applicable. *Anal. Biochem. 83*, 346−356.
20. Brulé, H., Holmes, W. M., Keith, G., Giegé, R., and Florentz, C. (1998) Effect of a mutation in the anticodon of human mitochondrial trnapro on its posttranscriptional modification pattern. *Nucleic Acids Res 26*, 537−543.
21. Perret, V., Garcia, A., Grosjean, H., Ebel, J. P., Florentz, C., and Giegé, R. (1990) Relaxation of a transfer RNA specificity by removal of modified nucleotides. *Nature 344*, 787−789.
22. Sampson, J. R., and Uhlenbeck, O. C. (1988) Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed in vitro. *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033−1037.
23. Frugier, M., Florentz, C., and Giegé, R. (1992) Anticodon-independent aminoacylation of an RNA minihelix with valine. *Proc. Natl. Acad. Sci. U.S.A. 89*, 3990−3994.
24. Gemund, C., Ramu, C., Altenberg-Greulich, B., and Gibson, T. J. (2001) Gene2est: A blast2 server for searching expressed sequence tag (est) databases with eukaryotic gene-sized queries. *Nucleic Acids Res 29*, 1272−1277.
25. Grosjean, H., Sprinzl, M., and Steinberg, S. (1995) Posttranscriptionally modified nucleosides in transfer RNA: Their locations and frequencies. *Biochimie 77*, 139−141.
26. Smolar, N., Hellman, U., and Svensson, I. (1975) Two transfer RNA (1-methylguanine) methylases from yeast. *Nucleic Acids Res. 2*, 993−1004.
27. Dong, H., Nilsson, L., and Kurland, C. G. (1996) Co-variation of tRNA abundance and codon usage in *Escherichia coli* at different growth rates. *J. Mol. Biol. 260*, 649−663.
28. Jackman, J. E., Montage, R. K., Malik, H. S., and Phizicky, E. M. (2003) Identification of the yeast gene encoding the tRNA m1G methyltransferase reponsible for modification at position 9. *RNA 9*, 574−585.
29. Grosjean, H., Auxilien, S., Constantinesco, F., Simon, C., Corda, Y., Becker, H. F., Foiret, D., Morin, A., Jin, Y. X., Fournier, M., and Fourrey, J. L. (1996) Enzymatic conversion of adenosine to inosine and to n1-methylinosine in transfer RNA: A review. *Biochimie 78*, 488−501.
30. Segel, I. H. (1976) *Biochemical Calculations*, 2nd ed., John Wiley and Sons, New York.

31. Fauman, E. B., Blumenthal, R. M., and Cheng, X. (1999) Structure and evolution of adomet dependent methyltransferases., in *S-Adenosylmethionine-Dependent Methyltransferases; Structures and Functions* (Cheng, X., Blumenthal, R. M., Eds.) pp 1−38, World Scientific Publishing, Singapore.
32. Thomas, D., and Surdin-Kerjan, Y. (1991) The synthesis of the two S-adenosyl-methionine synthetases is differently regulated in saccharomyces cerevisiae. *Mol. Gen. Genet. 226*, 224−232.
33. Roberts, C. J., and Selker, E. U. (1995) Mutations affecting the biosynthesis of S-adenosylmethionine cause reduction of DNA methylation in neurospora crassa. *Nucleic Acids Res. 23*, 4818−4826.
34. Vidgren, J., Ovaska, M., Tenhunen, C., Lotta, T., and Mannisto, P. T. (1999) Catechol *o*-methyltransferase, in *S-Adenosylmethionine-Dependent Methyltransferases* (Cheng, X., Blumenthal, R. M., Eds.) pp 55−91, World Scientific Publishing, Singapore.
35. Hjalmarsson, K. J., Byström, A. S., and Björk, G. R. (1983) Purification and characterization of transfer RNA (guanine-1)-methyltransferase from *Escherichia coli*. *J. Biol. Chem. 258*, 1343−1351.
36. Chow, C. M., Kang, S., Metzenberg, R. L., and RajBhandary, U. L. (1995) Sequence of the met-10+ locus of *Neurospora crassa*: Homology to a sequence of unknown function in *Saccharomyces cerevisiae* chromosome 8. *Gene 162*, 111−115.
37. Elkins, P. A., Watts, J. M., Zalacain, M., van Thiel, A., Vitazka, P. R., Redlak, M., Andraos-Selim, C., Rastinejad, F., and Holmes, W. M. (2003) Insights into catalysis by a knotted trmD tRNA methyltransferase. *J. Mol. Biol. 333*, 931−949.
38. Gerber, A., Grosjean, H., Melcher, T., and Keller, W. (1998) Tad1p, a yeast tRNA-specific adenosine deaminase, is related to the mammalian pre-mRNA editing enzymes adar1 and adar2. *EMBO J. 17*, 4780−4789.
39. King, M. P., and Attardi, G. (1993) Posttranscriptional regulation of the steady-state levels of mitochondrial tRNAs in HeLa cells. *J. Biol. Chem. 268*, 10228−10237.
40. Bauer, M. F., Hofmann, S., and Neupert, W. (2002). Import of mitochondrial proteins. *Int. Rev. Neurobiol. 53*, 57−90.

BI049671Q