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The tRNA(guanine-26,N^2-N^2) methyltransferase (Trm1) from the hyperthermophilic archaeon *Pyrococcus furiosus*: cloning, sequencing of the gene and its expression in *Escherichia coli*

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ABSTRACT

The structural gene *pfTRM1* (GenBank accession no. AF051912), encoding tRNA(guanine-26,N^2-N^2) methyltransferase (EC 2.1.1.32) of the strictly anaerobic hyperthermophilic archaeon *Pyrococcus furiosus*, has been identified by sequence similarity to the *TRM1* gene of *Saccharomyces cerevisiae* (YDR120c). The *pfTRM1* gene in a 3.0 kb restriction DNA fragment of *P.furiosus* genomic DNA has been cloned by library screening using a PCR probe to the 5′-part of the corresponding ORF. Sequence analysis revealed an entire ORF of 1143 bp encoding a polypeptide of 381 residues (calculated molecular mass 43.3 kDa). The deduced amino acid sequence of this newly identified gene shares significant similarity with the *TRM1*-like genes of three other archaea (*Methanococcus jannaschii, Methanobacterium thermoautotrophicum* and *Archaeoglobus fulgidus*), one eukaryon (*Caenorhabditis elegans*) and one hyperthermophilic eubacterium (*Aquifex aeolicus*). Two short consensus motifs for S-adenosyl-L-methionine binding are detected in the sequence of pfTrm1p. Cloning of the *P.furiosus TRM1* gene in an *Escherichia coli* expression vector allowed expression of the recombinant protein (pfTrm1p) with an apparent molecular mass of 42 kDa. A protein extract from the transformed *E.coli* cells shows enzymatic activity for the quantitative formation of N^2,N^2-dimethylguanosine at position 26 in a transcript of yeast $tRNA^{Phe}$ used as substrate. The recombinant enzyme was also shown to modify bulk *E.coli* tRNAs *in vivo*.

INTRODUCTION

All living organisms can be grouped into three evolutionarily distinct domains: Bacteria, Eukarya and Archaea (1). Recent achievements in genome sequencing projects from organisms of all three domains allowed systematic comparative sequence analysis of archaeal proteins with known or putative functions with their corresponding bacterial and eukaryotic counterparts. It appears that almost all archaeal proteins and enzymes of the central metabolism and of the metabolite uptake systems, as well as enzymes involved in cell wall biosynthesis, show remarkable similarity to bacterial homologues. On the other hand, proteins and enzymes involved in DNA replication and transcription, DNA recombination and repair, RNA translation and also protein secretion are often more closely related to respective eukaryotic homologues (2; reviewed in 3–7).

Because genes that specify functions of genetic information processing are in the main most similar to their eukaryotic counterparts, one could expect that proteins and enzymes involved in RNA processing and modification are also of the eukaryotic type. Indeed, components involved in rRNA processing and tRNA intron excision share much similarity with those of eukaryotes (8,9; reviewed in 10). However, the recently studied archaeal CCA-adding enzyme does not exhibit strong similarity with either the bacterial or the eukaryotic CCA-adding enzymes (11).

A great variety of modified nucleosides is found in tRNAs of all three domains (12). Of 80 different modified nucleosides reported in tRNAs, 36 are found in Archaea (of which nine are unique to this domain), 43 in Bacteria (16 unique) and 47 in Eukarya (21 unique) (reviewed in 13,14). Among those modified nucleosides that are present in archaeal tRNAs, a few are also found in eukaryotic tRNAs but not in any bacterial tRNAs sequenced so far [such as m^2G, $m^2{}_2G$, m^1I, m^5C, s^2U, Am; symbols and common names of modified nucleotides are those of McCloskey and Crain (12) and the universal numbering system for tRNA positions corresponds to that adopted in the tRNA sequence databank (15)]. On the other hand, a few modified nucleotides of bacterial type which have never been detected in eukaryotic tRNAs (such as s^2T, s^4U, m^6A) exist in archaeal tRNAs. Only a few tRNA modifications are exclusively present in archaeal tRNAs (such as $m^1\Psi$, mimG, archaeosine and several 2′-*O*-ribose-methylated derivatives of base-modified nucleosides). The formation of all of these modified nucleosides is catalysed by specific enzymes, for which it would be interesting to know

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whether they exhibit common features within the three domains of life. From the phylogenetic point of view, this knowledge could help to root the universal phylogenetic tree (3,16; see also 17) and to speculate on a stepwise chronology for the early origin of the RNA maturation machinery and the maintenance of modified nucleosides in tRNAs (18).

Recently, the complete genomic sequences of three moderately thermophilic Archaea have been determined (*Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum* and *Archaeoglobus fulgidus*; 19–21). Comparison of the protein sequences deduced from the open reading frames (ORFs) with the known identified proteins of Bacteria and Eukarya (of which the functions have been experimentally tested) led to the identification of numerous putative homologues in Archaea. Among them are the putative modification enzymes responsible for formation of pseudouridine, N^2,N^2-dimethylguanosine (m^2_2G) and archaeosine in tRNA. While these assignments are plausible, they nevertheless remain hypothetical until experimental evidence of the enzyme activity is obtained.

Pyrococcus furiosus is a sulfur metabolizing hyperthermophile of the order Thermococcales which grows optimally at 100°C under strictly anaerobic conditions, as in the deep sea, near hydrothermal vents and in geothermally heated marine sediments (22). Several genes of this microorganism were sequenced and the corresponding protein products studied *in vitro* (23,24), but none of the enzymes involved in tRNA modification have yet been studied. Here we report the identification, cloning and sequencing of the gene corresponding to the tRNA(guanine-26,N^2-N^2) methyltransferase from *P.furiosus*. The recombinant pfTrm1 protein overexpressed in *Escherichia coli* catalyses the *in vivo* formation of m^2_2G at position 26 in *E.coli* tRNAs or *in vitro* using yeast tRNAs as substrates and S-adenosyl-methionine as the methyl group donor.

MATERIALS AND METHODS

Reagents, enzymes and plasmids

^{32}P-Radiolabelled nucleotide triphosphates (400 Ci/mmol) were from Amersham (UK). Reagents and restriction enzymes were from Sigma (St Louis, MO), Boehringer-Mannheim (Mannheim, Germany), MBI Fermentas (Vilnius, Lithuania) or Merck (Germany). Thin layer cellulose plates were from Schleicher & Schuell (Germany). Plasmids p67YF0, p67YF33 and p67YF59 bearing wild-type yeast $tRNA^{Phe}$ and its variants (G_{26}-A_{44}→A_{26}-G_{44}) and (G_{10}-C_{24}→C_{10}-G_{24}) have been described (25); they were a generous gift of Dr O.Uhlenbeck (Boulder, CO).

Strains

Pyrococcus furiosus strain DSM 3638 cells (22) grown under strictly anaerobic conditions were a gift from Prof. P. Forterre (Université d'Orsay, France). *Escherichia coli* strains JM103, *supE thi-1 endA1 hsdR4 sbcB15 str*A Δ(*lac-proAB*) F′[*traD36 proAB*$^+$*lacI lacZ*ΔM15], and BL21(DE3), hsdS gal (λcIts857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene1), were used for library construction and expression of the cloned recombinant protein.

Preparation of *P.furiosus* genomic DNA

Cells of *P.furiosus* (1 g) were resuspended in 15 ml TNE (100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50 mM EDTA), 2 ml 10% *N*-lauryl sarcosyl, 2 ml 10% SDS and 1 ml proteinase K (1 mg/ml) were added, and the mixture was incubated for 3 h at 50°C. Proteins were extracted three times with phenol and once with chloroform/isoamyl alcohol. Nucleic acids were precipitated with ethanol and dissolved in 1 ml TE (100 mM Tris–HCl, pH 7.5, 10 mM EDTA). The remaining RNA was digested with 0.1 mg/ml RNase A for 1 h at 37°C.

Preparation of a radioactive DNA probe for library screening

The DNA fragment for hybridization was prepared by PCR amplification using the deoxyoligonucleotides primer A, 5′-AAAACTCATAGGGAGACTTGTTC, and primer B, 5′-CTG-CAGGAGTCTCTAAGGCAAAC, corresponding respectively to the sequence of *Pyrococcus woesei* putative ribosomal protein L35A (GenBank accession no. M83987) and to the 5′-sequence of ORFz (the first 73 codons of a putative tRNA methyltransferase gene; 26,27). Amplification was performed for 30 cycles: 1 min at 95°C, 2 min at 65°C, 2 min at 72°C. Amplified DNA was extracted with chloroform, ethanol precipitated and purified from 1% low melting point agarose after electrophoresis. The purified fragment was ^{32}P-radiolabelled by random priming using the Megaprime kit (Amersham, Aylesbury, UK) according to the manufacturer's instructions.

Mini-library construction and screening

Pyrococcus furiosus genomic DNA (3 μg) was digested overnight with *Hin*dIII, *Hin*cII, *Xba*I, *Pst*I, *Nci*I or *Mva*I. The digests were loaded onto a 1% agarose gel and run at 80 V for several hours. After electrophoresis the gel was soaked in 0.2 M HCl, followed by a treatment in denaturing solution (1.5 M NaCl, 0.5 M NaOH) and neutralization with 1 M Tris–HCl, pH 7.5, 1.5 M NaCl. Separated DNA fragments were transferred to a GeneScreen hybridization transfer membrane (NEN, Boston, MA) and fixed by a 5 min irradiation under a 254 nm UV light. The membrane was incubated in the presence of 0.2 μg/ml salmon sperm DNA and then hybridized at 65°C with the ^{32}P-radiolabelled probe according to Southern (28).

Cloning of the *P.furiosus TRM1*-like gene was performed as follows. Genomic DNA (100 μg) was digested with *Hin*dIII, loaded on 1% agarose and electrophoresed as described above. After separation, the region of the gel containing fragments of ~3 kb in size was excised and DNA fragments were recovered by electroelution. The extracted fragments were ligated in the cloning vector pUC8 cut with *Hin*dIII. Ligated DNA was introduced into *E.coli* JM103 by electroporation using a BioRad Gene Pulser II (0.2 cm cuvettes, 2500 V, 25 μF). Transformed cells were plated on isopropyl-β-D-thiogalactoside (IPTG)/X-Gal-containing medium and grown overnight at 37°C. The transformants containing recombinant plasmid (white colonies) were selected and screened by Southern hybridization with the ^{32}P-radiolabelled PCR fragment. The resulting plasmid containing the *TRM1*-like gene in a 3 kb insert (pNB5) was used for subcloning.

The *Hin*dIII–*Hin*cII fragment (1.9 kb) of pNB5 containing the *P.furiosus TRM1*-like ORF was purified in a 1% low melting

point agarose gel and ligated into the *Hin*dIII and *Hin*cII sites of pUC8. This construct (pNB6) was used for sequencing the insert.

Nucleotide sequence determination

DNA sequencing was performed using the Taq DNA polymerase-based *fmol*® DNA cycle sequencing system (Promega, Madison, WI) according to the manufacturer's recommendations. First, the 5′-part of the insert (~800 nt altogether) was sequenced using three specific 'walking' primers complementary to regions 182–201, 413–434 and 703–722 of the sequence. In parallel, the 1.9 kb insert in pNB6 was amplified by PCR using pUC universal forward and reverse primers. The amplified DNA fragments were then cut with *Bsu*RI (*Hae*III) or *Rsa*I and the resulting short fragments were inserted into the *Sma*I site of pBluescript II SK(+) vector. As the digestion pattern for both *Bsu*RI and *Rsa*I gave rather long DNA fragments (600–1000 nt), only a part of the entire sequence was determined in this way. To cover the rest of the insert, the sequence was amplified by PCR using a specific 'walking' primer for the 5′-end and a pUC reverse primer for the 3′-end and nested bi-directional deletions using *Exo*III exonuclease/mung bean nuclease were obtained. Fragments (400–1000 nt) were inserted into the *Sma*I site of pBluescript II SK(+) and sequenced. The whole DNA insert (1.9 kb) was thereby sequenced at least three times using independent clones. The sequence described in this paper was deposited in GenBank under accession no. AF051912.

Cloning and expression of *P.furiosus* tRNA(guanine-26,N^2-N^2) methyltransferase in *E.coli*

Cloning of the *P.furiosus TRM1*-like gene in a T7 RNA polymerase promoter-based *E.coli* expression vector (pET28b) was done as follows. The coding sequence together with the 3′-non-coding region (~800 nt) was amplified by PCR using pNB6 as template. The forward primer (5′-AAAACCATGG-GATCTATGGAATTATTTGAAG) contained a *Nco*I (underlined) restriction site at its 5′-end. The *Nco*I site was introduced to provide the translation start (ATG), however, it adds an extra codon (GGA), coding for glycine, following the initiation codon. The reverse primer (5′-TGTTGTGTGGAATTGTGAG) was complementary to the pUC8 sequence in pNB6. The amplified DNA was digested with *Nco*I and *Bam*HI and the 1.7 kb fragment was inserted into the *Nco*I and *Bam*HI sites of plasmid pET28b. The resulting recombinant plasmid (pET28b-*pfTRM1*), recovered from the *E.coli* JM103 transformant, was transferred to *E.coli* BL21(DE3). The bacteria were grown to mid-logarithmic phase (D_{600} ~ 0.7) at 37°C in MM9 minimal medium supplemented with 1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 0.4% casamino acids and 50 µg/ml kanamycin. Expression of the recombinant protein was induced by addition of IPTG to a final concentration of 0.1 mM. After 4 h induction with IPTG at 37°C, the cells were harvested by centrifugation.

Preparation of *E.coli* cell-free extract

The cell-free extract of *E.coli* cells (BL21/DE3) was prepared by sonication in 5 vol 100 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 1 mM EDTA, 300 mM KCl, 1 mM phenylmethylsulphonyl fluoride, 1 mM DFP and 2 mM dithioerythreitol. The homogenate was centrifuged at 10 000 *g* for 15 min at 4°C. Glycerol was added to a final concentration of 20% and S10 extract was stored at –20°C.

Preparation of total *E.coli* tRNA, post-labelling with ^{32}P and analysis of modified nucleotides

Total tRNA from *E.coli* BL21/DE3 cells was prepared by phenol extraction and high molecular mass RNA was removed by precipitation with 2 M NaCl. Further purification was performed by electrophoresis in a 6% denaturing polyacrylamide gel. The location of the large tRNA band in the gel was revealed by UV shadowing. The purified tRNA fraction was then analysed for its modified nucleotide content as described earlier (29). Separation of the ^{32}P-labelled nucleotides was performed by two-dimensional chromatography on thin layer cellulose plates (30). The chromatographic systems and reference maps for identification of the nucleotide spots have been described previously (31).

Preparation of tRNA transcripts

[α-^{32}P]GTP-labelled tRNA transcripts were obtained by *in vitro* transcription with T7 RNA polymerase of linearized plasmids, as previously described (32).

In vitro enzymatic assay

The standard reaction mixture for detection of m^2_2G formation in tRNA transcripts was 100 mM Tris–HCl (pH 7.5 at 20°C), 5 mM $MgSO_4$, 2 mM DTT, 0.1 mM EDTA, 100 mM ammonium acetate, 75 µM S-adenosyl L-methionine (AdoMet) and 50–100 fmol [^{32}P]GTP-labelled tRNA substrate. The reaction was initiated by addition of S10 *E.coli* extract to a final protein concentration of 1 mg/ml in a final volume of 50 µl, overlayed by 10 µl paraffin oil. After incubation at 50°C, proteins were phenol extracted and the nucleic acids ethanol precipitated, collected by centrifugation and hydrolyzed overnight at 37°C with 2 µg nuclease P1 in 50 mM sodium acetate, pH 5.3. The resulting hydrolysate was then analysed by two-dimensional TLC as described above. Radioactivity in the spots of modified nucleoside 5′-phosphates was quantified by scanning in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

RESULTS

Cloning and sequencing of the *pfTRM1* gene encoding tRNA(guanine-26,N^2-N^2) methyltransferase

Sequencing of a 3.1 kb *Pst*I fragment of the hyperthermophilic archaeon *P.woesei* genomic DNA, containing the glyceraldehyde 3-phosphate dehydrogenase gene (ORFgapdh), allowed mapping of the positions of three additional complete neighbouring ORFs (ORFa, ORFb and ORFy) and two truncated ORFs (ORFx and ORFz; see upper part of Fig. 1; 26). The deduced amino acid sequence of the product of ORFz, located downstream of ORFy, showed high similarity (31% identical residues) to the ribosomal protein L35a from *Rattus norvegicus*, while the predicted protein sequence of the product of truncated ORFz (the first 220 bp) was later shown to have a high degree of similarity (up to 50% of identity) to the N-terminal sequence of the tRNA(guanine-26,N^2-N^2) methyltransferase from *Saccharomyces cerevisiae* (27). This yeast enzyme, encoded by the *TRM1* gene (now identified as YDR120c; 33) is known to catalyse formation of $m^2_2G_{26}$ in both cytoplasmic and mitochondrial tRNAs (reviewed

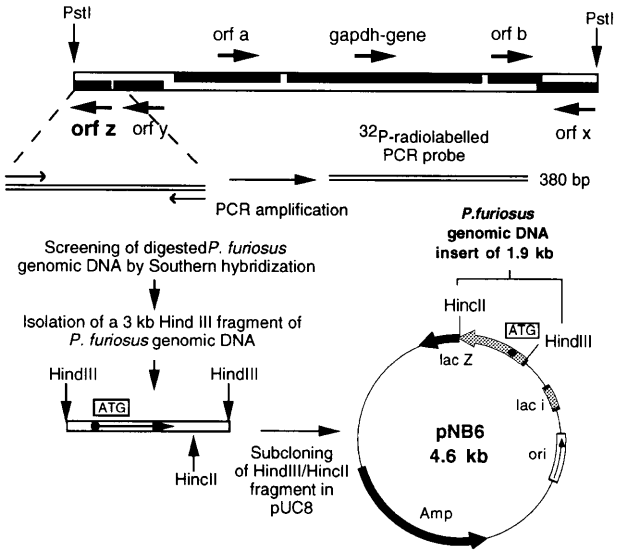

Figure 1. Strategy for identification and cloning of the *P.furiosus TRM1*-like gene. The schematic representation of the 3 kb *Pst*I fragment of *P.woesei* genomic DNA bearing the gene coding for glyceraldehyde 3-phosphate dehydrogenase (gapdh-gene; 26) is given at the top. The orientations of ORFs are indicated by arrows. All details are as described in Materials and Methods.

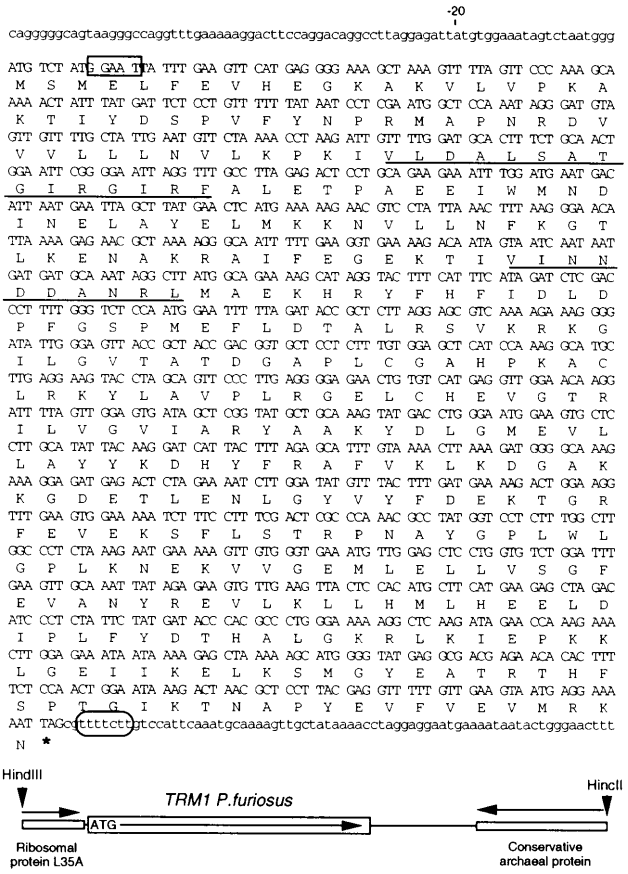

Figure 2. The sequence of the *TRM1*-like ORF from *P.furiosus* and deduced protein sequence using the conventional one letter code for each amino acid (GenBank accession no. AF051912). Non-coding nucleotides are represented by lower case letters. Only 79 of the 170 nt sequenced from the *Hin*dIII restriction site are shown. The putative ribosomal binding site (region +9 to +14) and pyrimidine-rich sequence downstream of the stop codon (putative transcription stop signal) are boxed. Underlined letters correspond to the putative AdoMet bindings motifs (see text). A schematic map of the *Hin*dIII–*Hin*cII fragment of *P.furiosus* genomic DNA is given at the bottom.

in 34). Assuming that the partial ORFz corresponds to a portion of a *P.woesei TRM1*-like gene and that the same locus exists in the rather close archaeon *P.furiosus* (22,35), we used the partial sequence information of the putative *P.woesei TRM1* gene as a basis to clone the homologous gene from *P.furiosus*.

To this end, a DNA sequence coding for the putative N-terminal part of *P.woesei* Trm1-like protein (73 amino acids) and the C-terminal part of ribosomal protein L35a protein (51 amino acids) was used to design oligonucleotides for PCR amplification from genomic DNA from *P.furiosus*. A DNA fragment of the expected size (~380 bp) was obtained, radiolabelled and used as a probe for Southern hybridization with the digested *P.furiosus* genomic DNA (Fig. 1). The results of the probing show only one hybridizing band (data not shown), indicating that the corresponding locus is unique in the genome. An amplified PCR fragment was used to screen a mini-library of *P.furiosus* genomic DNA digested with *Hin*dIII. From 500 recombinants, five positive hybridizing clones were selected and analysed by restriction enzyme mapping and sequencing. Sequencing of the 1.9 kb DNA insert in plasmid pNB6 bearing the *pfTRM1* ORF was performed as described in Materials and Methods. The resulting sequence with neighbouring nucleotides is shown in Figure 2.

Functional expression of the recombinant *pfTRM1* gene product in *E.coli*

In order to remove the non-coding upstream DNA sequence adjacent to the *pfTRM1* gene and to place the sequence under the control of an efficient promoter, a new plasmid, pET-*pfTRM1*, was constructed by inserting the coding sequence into the expression vector pET28b between the *Nco*I and *Bam*HI sites, in-frame with the initiation codon within the *Nco*I site (Materials and Methods). The sequence of this insert was identical to that of the *pfTRM1* ORF in pNB6 (Figs 1 and 2), except that an extra glycine codon was now present at the 5′-end. The recombinant protein was expressed in *E.coli* BL21(DE3) by induction with IPTG. Identically treated *E.coli* BL21(DE3) cells transformed with vector pET28b were used as a control. As shown in Figure 3, IPTG induction leads to the appearance of a new protein band with an apparent molecular mass of 42 kDa as detected by SDS–PAGE of total cellular proteins. The size of this additional protein band corresponds well with the calculated molecular mass of the *P.furiosus* Trm1-like protein (43.3 kDa).

Identification of the activity of the recombinant pfTrm1 protein

Bacterial tRNAs, including *E.coli* tRNAs, do not contain the modified nucleotide $m^2{}_2G_{26}$ (15) due to the absence of the corresponding tRNA(guanine-26,N^2-N^2) methyltransferase activity. Only the recently sequenced genome of the hyperthermophilic eubacteria *Aquifex aeolicus* reveals the presence of a *TRM1*-like gene (36). However, when bulk or individual *E.coli* tRNAs are incubated with protein extracts from eukaryotic cells, such as wheat embryo, yeast, *Tetrahymena pyriformis* or rat liver, $m^2{}_2G_{26}$

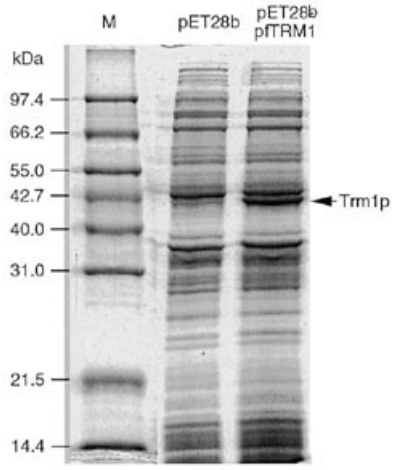

Figure 3. Expression of the Trm1-like protein of *P.furiosus* in *E.coli* BL21(DE3). IPTG-induced cells were harvested by centrifugation, lysed in the presence of 6% SDS and the extract electrophoresed on a 10% denaturing polyacrylamide gel. Control cells bearing empty pET28b vector and pET28b-*pfTRM1*-containing cells were induced for 4 h. Mid-range molecular mass markers (Promega, Madison, WI) were loaded in lane M.

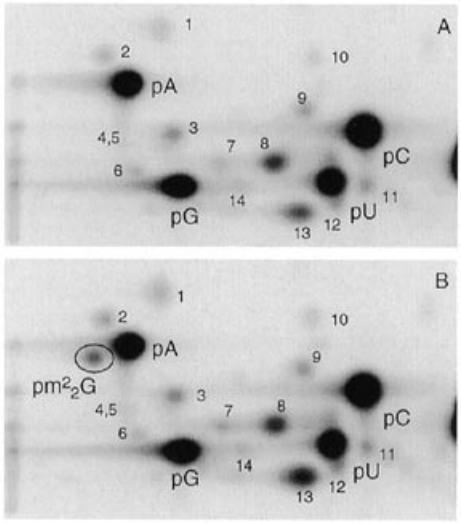

Figure 4. Analysis of modified nucleotides present in the control *E.coli* tRNA sample (**A**) and in tRNA extracted from pET28b-*pfTRM1*-transformed *E.coli* BL21(DE3) (**B**). Analysis was performed by the post-labelling technique as described in Materials and Methods. Two-dimensional separation of radioactive 5′-mononucleotides was achieved with the chromatographic system described in Materials and Methods. Assignment of modified nucleotides on the autoradiogram was by comparison with reference maps (31). The major spots correspond to pA, pC, pU and pG, as indicated. The spot corresponding to pm^2_2G in the tRNA from the induced strain is circled (see B). The other spots on the autoradiograms represent the modified nucleotides normally present in mature *E.coli* tRNA: 1, pAm; 2, pm^6A; 3, pGm; 4 and 5, pm^2G and pt^6A (visible only on the original autoradiogram); 6, pm^1G; 7, probably pQ; 8, pT; 9, pm^7G; 10, pCm; 11, pD; 12, pΨ; 13, most probably ribosyl phosphate; 14, pI (inosine).

is easily formed in an AdoMet-dependent manner (see for example 37–40). Likewise, bulk tRNA isolated from *E.coli* cells transformed with pUC19 bearing the *S.cerevisiae TRM1* gene contains m^2_2G (41). Thus, despite the absence of a naturally modified G_{26} in bacterial tRNAs, their tRNAs can serve as perfect substrates for the eukaryotic N^2,N^2-dimethylguanine transferase. In this study that peculiarity was used to detect the enzyme activity of recombinant pfTrm1p *in vivo*.

Qualitative analysis of the content of modified nucleotides in bulk tRNA isolated from *E.coli* cells transformed either with pET28b containing the insert *pfTRM1* or pET-28b lacking the insert (control experiment) was performed by the post-labelling procedure on tRNA hydrolysate, followed by TLC of the resulting 5′-^{32}P-labelled nucleotide monophosphates. Figure 4 shows the autoradiograms obtained. Only tRNAs extracted from *E.coli* cells transformed with the plasmid pET-*pfTRM1* contain N^2,N^2-dimethylguanine. From these experiments, one can conclude that the *TRM1* gene of *P.furiosus* is the structural gene for a tRNA(guanine,N^2-N^2) methyltransferase and that the *pfTRM1* gene product, although originating from a hyperthermophilic archaeon, is fully active under the physiological conditions of mesophilic *E.coli*. However, in this experiment, we could not determine which guanosine in *E.coli* tRNAs is modified.

Identification of the guanine residue modified in tRNA substrates

To formally identify the site of modification, additional experiments were performed *in vitro* with a protein extract of the induced pET28-*pfTRM1*-containing *E.coli* cells, using synthetic tRNA transcripts as substrates. We used the transcript of wild-type yeast $tRNA^{Phe}$, which was shown to be an excellent substrate for yeast tRNA(guanine,N^2-N^2) methyltransferase when tested in an S100 yeast extract supplemented with AdoMet (42) as well as after microinjection into the cytoplasm of *Xenopus laevis* oocytes (32,43). Our results obtained with cell-free extracts of *P.furiosus* also demonstrate that the transcript of yeast $tRNA^{Phe}$ becomes modified to $m^2_2G_{26}$ and m^2G_{10} upon incubation. The two other substrates correspond to variants of yeast $tRNA^{Phe}$ bearing mutations at positions 26–44 and 10–25 (Fig. 5). This last control was absolutely necessary, since archaeal tRNAs were shown to have m^2_2G not only at position 26, as in most eukaryotic tRNAs, but also at position 10, where a mono m^2G_{10} is found in eukaryotic tRNAs, including yeast $tRNA^{Phe}$ (15).

Each transcript was [^{32}P]GTP-radiolabelled and individually tested with S10 extract of induced transformed *E.coli* in the presence of added AdoMet. The incubation temperature 50°C was chosen, taking into account the results of preliminary experiments demonstrating that maximal enzymatic activity is achieved under these conditions. A further increase in incubation temperature (to 60°C) results in a decreased level of m^2_2G modification in $tRNA^{Phe}$ transcript (data not shown). This temperature optimum (50°C) is related to a limited resistance of the macromolecular RNA substrate (unmodified transcript of yeast $tRNA^{Phe}$) to thermal unfolding. The melting profiles obtained with yeast $tRNA^{Phe}$ and other tRNA transcripts demonstrate that unfolding of the proper three-dimensional structure of tRNA begins between 55 and 60°C, depending on the Mg^{2+} concentration and the nature of the transcript (44). The results of TLC analysis (Fig. 5A–C) show that $m^2_2G_{26}$ was formed in transcripts of wild-type yeast $tRNA^{Phe}$ and its variant C_{10}-G_{25}, but not in the variant A_{26}-G_{44}. Trace amounts of the m^2G_{26} product were also visible on two-dimensional TLC. From determination of the radioactivity level in each spot of the

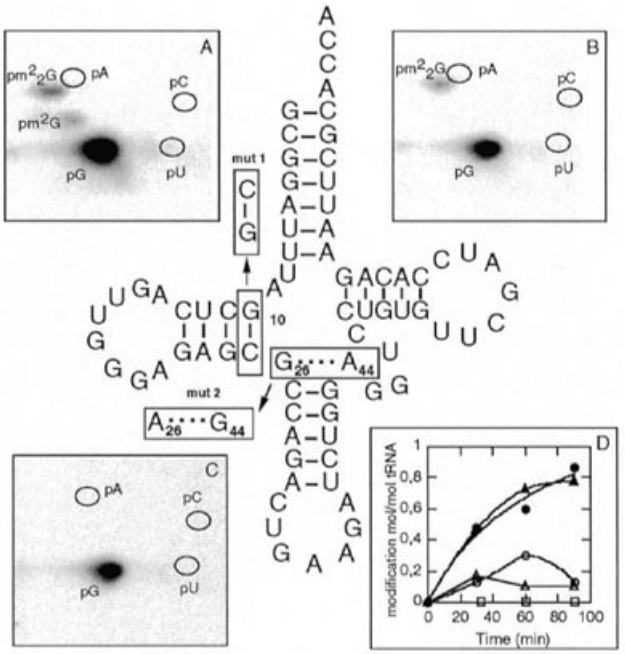

Figure 5. Cloverleaf representation of the nucleotide sequence of wild-type yeast $tRNA^{Phe}$ and two yeast $tRNA^{Phe}$ mutants. mut1 corresponds to reversion of the $G_{10}C_{24}$ base pair to $C_{10}G_{24}$ and mut2 reversion of the $G_{26}A_{44}$ base pair to $A_{26}G_{44}$. T7 transcripts corresponding to these three tRNAs were radiolabelled with [^{32}P]GTP and incubated for 30 min at 50°C with S10 extract from IPTG-induced *E.coli* cells BL21 carrying the plasmid pET28b-*pfTRM1*. The presence of methylated guanines was evaluated by TLC analysis of the P1 nuclease digest of radiolabelled tRNA as described in Materials and Methods. (**A**) Autoradiogram of the TLC plate when yeast $tRNA^{Phe}$-mut1 was used as substrate; (**B**) wild-type yeast $tRNA^{Phe}$; (**C**) $tRNA^{Phe}$-mut2. Open circles indicate migration of markers as visualized by UV shading: AMP (pA); CMP (pC); UMP (pU). (**D**) Time courses for the biosynthesis of m^2G (open symbols) and $m^2{}_2G$ (solid symbols) in *in vitro* transcripts of yeast wild-type $tRNA^{Phe}$ (triangles) and the two yeast $tRNA^{Phe}$ mutants, mut1 (circles) and mut2 (squares). Quantification of the radioactivity in each spot of the TLC plates made at different times of incubation allows determination of the molar ratio of m^2G and $m^2{}_2G$ present in the modified tRNA transcript.

autoradiograms, the molar yield of m^2G and $m^2{}_2G$ can be calculated. The time course of modification (Fig. 5D) shows that ~1 mol $m^2{}_2G_{26}$/mol tRNA is formed after 1 h incubation at 50°C. Also, formation of the intermediate m^2G_{26} product does not accumulate and is efficiently converted into $m^2{}_2G_{26}$ by pfTrm1p.

DISCUSSION

m^2G and $m^2{}_2G$ occur at positions 10 and 26 in fully mature tRNAs, formed by distinct site-specific methyltransferases

In the present paper, we demonstrate that heterologous expression of the *TRM1* gene of *P.furiosus* in *E.coli*, normally devoid of an $m^2{}_2G$-forming enzyme, confers on the host the ability to express a polypeptide having G_{26} methyltransferase activity. Moreover, the protein extract from the induced transformed *E.coli* reveals an enzymatic activity that is able to catalyse the formation of the N^2,N^2-dimethyl derivative of G_{26} but not of G_{10}. Therefore, the *pfTRM1* ORF that we have cloned is the structural gene for the AdoMet-dependent tRNA(guanine-26,N^2-N^2) methyltransferase (pfTrm1p). However, considering that $m^2{}_2G$ is also found at position 10 of several naturally occurring archaeal tRNAs (see below), an unidentified distinct $m^2{}_2G$-forming enzyme in *P.furiosus* has still to be discovered. These observations fit well with what is already known concerning the occurrence and biosynthesis of $m^2{}_2G$ in tRNAs. Indeed, analysis of the nucleotide sequences of 59 mature tRNAs from several Archaea (mostly from *Haloferax volcanii*, but also from *Halobacterium cutirubrum* and *Halococcus morrhuae*, and five tRNA sequences from the thermophilic Archaea *M.thermoautotrophicum*, *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius*) has revealed that methylation of the N^2-amino group of guanine occurs at two distinct sites: at position 10 in the proximal dihydrouridine stem and at position 26 in the bend between the dihydrouridine (D) stem and the anticodon (AC) stem of tRNAs. Depending on the tRNA considered, these guanine modifications involve a single or a double methylation to form m^2G or $m^2{}_2G$, dimethylation being most frequently found at those sites (15). In tRNAs from certain hyperthermophilic Archaea, a 2′-*O*-methyl derivative of $m^2{}_2G$ has also been detected ($m^2{}_2Gm$; 13), however, its exact location, probably at position(s) 10 and/or 26 has not yet been elucidated.

Dimethylation of guanine residues is frequently found in eukaryotic tRNAs (cytoplasmic, mitochondrial and chloroplast), mainly at position 26 and rarely at position 27, while at position 10 m^2G is exclusively present. In higher eukaryotic tRNAs, but not in single cell eukaryotes, m^2G is also frequently found at position 6 (and exceptionally at position 7) of the amino acid stem (15). Previous studies with eukaryotic cell extracts (from rat liver, chicken embryo, *T.pyriformis* and yeast) demonstrated that these two sites are methylated by chromatographically distinct tRNA(guanine) methyltransferases: one specific for monomethylation of N^2-G_{10} with a molecular mass of ~75–85 kDa (45–48) and the other for dimethylation of N^2,N^2-G_{26} with a molecular mass ranging between 180 and 260 kDa (37,38,40,49). In both cases, the molecular masses refer to the native proteins, depending on the origin of the enzymes and on the method used for their evaluation. Eukaryotic tRNA(guanine-26) methyltransferases were shown to be rather unstable under the purification procedure. Also, depending on the nucleotides surrounding G_{26}, they may catalyse only formation of m^2G_{26} (reviewed in 50). Thus, despite the close spatial proximity of G_{10} and G_{26} within the three-dimensional architecture of tRNA (see 51), distinct position-specific tRNA(guanine) methyltransferases exist in archaeal and eukaryotic cells.

A single gene product *pfTrm1p* has both mono- and dimethylating activity at G_{26}

In this work, we have shown that bulk tRNA extracted from an *E.coli* strain transformed with plasmid pET28b-*pfTRM1* contains $m^2{}_2G$. Likewise, transfection of *E.coli* with the *TRM1* gene of *S.cerevisiae* leads to the appearence of $m^2{}_2G$ in the tRNAs of the transformed *E.coli* cells (41). Altogether, these results demonstrate that a single gene product is able to catalyse the transfer of both methyl groups to G_{26} in tRNAs. This conclusion is in accord with our observation that a small amount of m^2G_{26} was also formed as an intermediate when a T7 transcript of yeast $tRNA^{Phe}$ was incubated with the cell extract of transformed *E.coli* BL21(*pET-pfTRM1*). It also agrees with the results obtained for the native tRNA(guanine-26,N^2-N^2) methyltransferase from the protozoan *T.pyriformis* (40). This enzyme was capable of forming *in vitro* both m^2G_{26} and $m^2{}_2G_{26}$ on a single tRNA isoacceptor species, the relative amount of m^2G over $m^2{}_2G$ being dependent on the ratio of enzyme and tRNA substrate. Thus, G_{26} dimethylation is

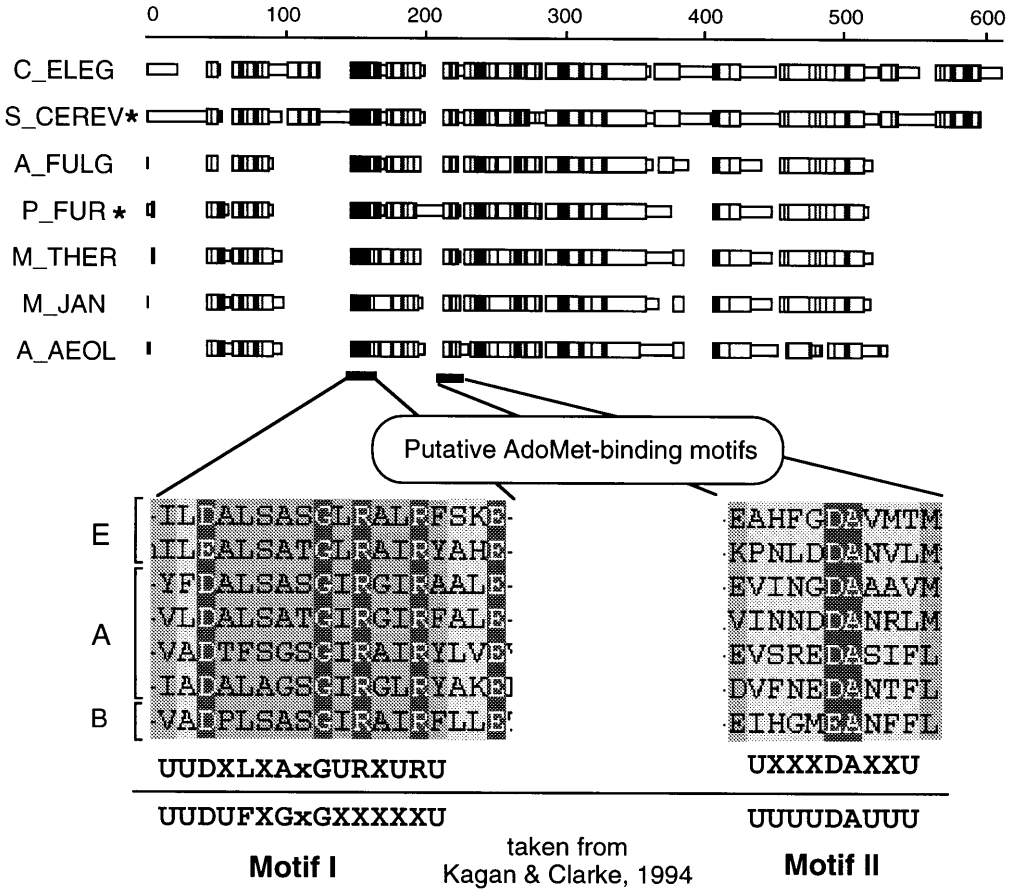

Figure 6. Sequence alignment of *TRM1*-like proteins from *C.elegans* (C_ELEG), *S.cerevisiae* (S_CEREV), *A.fulgidus* (A_FULG), *P.furiosus* (P_FUR), *M.thermoautotrophicum* (M_THER), *M.jannaschii* (M_JAN) and *A.aeolicus* (A_AEOL). The scale in amino acid residues is presented at the top. Identical and conserved residues are shaded. Two putative AdoMet binding motifs and their consensus amino acid sequences are shown at the bottom. The one letter code for each amino acid is used, except for the hydrophobic amino acids I, L, V, M, F and A, which are designated by U. The upper line of the motifs corresponds to the ones defined in this paper, while the second line corresponds to the motifs defined by Kagan and Clarke (57). Asterisks indicate the sequence of proteins for which the activity of the enzymatic reaction was established experimentally. In both cases they correspond to tRNA(guanine-26,N^2-N^2) methyltransferases (EC 2.1.1.32). B, E and A designate Bacteria, Eukarya and Archaea respectively.

a two step process which might require dissociation of the enzyme–monomethylated tRNA intermediate (40). However, taking into account the subunit structure of the enzyme, other alternative scenarios can be envisaged (discussed in 50).

Sequence analysis of the *pfTRM1* ORF

As indicated in Figure 2, the *pfTRM1* ORF starts at the codon (ATG) located at position 170 of the *Hin*dIII–*Hin*cII fragment and covers 1143 nt until an amber TAG stop codon is reached. This ORF encodes a 381 amino acid polypeptide (calculated molecular mass 43.3 kDa), which presents a high similarity to the tRNA(guanine-26,N^2-N^2) methyltransferase (*TRM1* gene) of *S.cerevisiae* (1710 nt coding for 570 amino acids, corresponding to a calculated molecular mass of 63 kDa (41). The sequence 5′-adjacent to the *TRM1*-like ORF has no concensus ribosome binding site (GAGGT). However, the sequence GGAAT, present at position +9 within the *pfTRM1* coding region (boxed in Fig. 2), may serve as an internal ribosome binding site (reviewed in 52; see also 26). The amber stop codon (TAG) of the *pfTRM1* ORF is followed by a stretch of pyrimidines, TTTTCTT (boxed in Fig. 2), which may serve as a transcription termination signal (reviewed in 53).

Analysis of the 3′-UTR of the sequenced *Hin*dIII–*Hin*cII fragment reveals the presence of another ORF in the opposite orientation. A BLAST search using the deduced protein sequence indicates that it is similar to the C-terminal end of a protein of unknown function found in all three archaeal genomes sequenced so far (AE000840, AE000989 and MJ0969).

The G+C content of the *pfTRM1* gene (39.3%) is rather close to the value reported for the total genome of *P.furiosus* (38%) (22). As expected for a gene with a low G+C content, A and T are the preferred bases in the third position of codons (reviewed in 54). In particular, codons NCG (ending with G) for Ser, Pro, Thr and Ala are not or rarely used, whereas for Arg, AGG and AGA codons are strongly preferred (16 out of a total of 20 Arg codons). The same trends have been noticed for many other hyperthermophilic archaeal coding genes (see for example 55,56).

Sequence alignment of several Trm1-like proteins and localization of putative SAM binding motifs

pfTrm1p and scTrm1p are the only two tRNA(guanine-26,N^2-N^2) methyltransferases for which the activity of the corresponding gene products have been experimentally verified. However, based on the similarity of amino acid sequences with scTrm1p, the genes coding for five tentatively assigned $m^2{}_2$G-forming enzymes can be detected (GenBank entries Z77136, U67538, AE000887, AE001048 and AE000710). One of them is from a eukaryon *Caenorhabditis elegans*, three are from Archaea (*M.jannaschii*, *M.thermoautotrophicum* and *A.fulgidus*) and the last is from the thermophilic eubacterium *A.aeolicus*. Most probably these genes do indeed encode for tRNA(guanine-26,N^2-N^2) methyltransferases, yet this plausible hypothesis should be verified experimentally.

Alignment of the amino acid sequences of all seven Trm1-like proteins is schematically illustrated in Figure 6. Despite the differences in length, all seven proteins display a remarkable sequence similarity. Two well-conserved sequences present in all Trm1-like proteins (indicated by bars in Fig. 6) can be tentatively assigned as the AdoMet binding domains in these tRNA methyltransferases. Indeed, extensive analysis of the amino acid sequences of 84 AdoMet binding enzymes acting on various molecules has led to the definition of three characteristic sequence motifs possibly involved in interaction with the methyl donor cofactor (57). However, not all methyltransferases bear the same characteristic motifs (see for example 58–61). In particular, analysis of *S.cerevisiae* Trm1p did not reveal the presence of plausible AdoMet binding motifs (57).

In conclusion, *pfTRM1* is the first gene of an archaeon coding for a tRNA modification enzyme that has been cloned and expressed in an active form in *E.coli*. The amino acid sequence of this newly identified tRNA(guanine-26,N^2-N^2) methyltransferase bears great similarity to those of the corresponding eukaryotic enzymes. The fact that the Trm1p of a hyperthermophilic organism would be functional under the physiological conditions of *E.coli* cells was not *a priori* evident. Indeed, there are cases where recombinant thermophilic enzymes were shown to be inactive at low temperatures or even required a heat treatment after cell lysis in order to recover enzymatic activity *in vitro* (see for example 56,62). Therefore, the possibility of functional expression of the *pfTRM1* gene in mesophilic *E.coli* provides the opportunity for further biochemical, biophysical and possible crystallization studies on this tRNA modification enzyme.

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