Hypermodification of tRNA in Thermophilic Archaea

CLONING, OVEREXPRESSION, AND CHARACTERIZATION OF tRNA-GUANINE TRANSGLYCOSYLASE FROM METHANOCOCCUS JANNA SchI

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The biosynthesis of the 7-deazaguanosines is only partially understood (Fig. 2). GTP is apparently the primary precursor in the biosynthesis of queuosine (14), which in an unknown series of steps is converted to 7-(cyano)-7-deazaguanine (preQ0). PreQ0 is then reduced to PreQ1, and preQ1 is subsequently inserted into the tRNA by the enzyme TGT1 in a transglycosylation reaction in which the genetically encoded base (guanine) is eliminated (15, 16). The remainder of queuosine biosynthesis occurs at the level of the tRNA and involves the unusual utilization of S-adenosylmethionine in the construction of an epoxycyclopentane ring (17), followed by the B12-dependent reduction of the epoxide to give queuosine (18). The presence of a 7-substituted 7-deazaguanine core structure in both queuosine and archaeosine, coupled with the similarity of preQ0 to archaeosine base suggests a parallel biosynthetic pathway in the archaea for archaeosine biosynthesis. In this proposal, the steps leading to preQ0 are presumably identical in both the archaea and bacteria (eukarya are incapable of de novo biosynthesis of queuosine (8)) and diverge at preQ0, with preQ0 (or a related metabolite such as the amide or archaeosine base) serving as the substrate for an archaeal TGT in the key base substitution reaction. This proposal was strengthened with the identification of an open reading frame (MJ0436) with increasing translational efficiency and/or fidelity, and in specific cases, to serve as recognition determinants for aminoacyl-tRNA synthetases (3, 4).

Two of the most remarkable modified nucleosides known to occur in tRNA are archaeosine (G*) (5) and queuosine (Q) (6) (Fig. 1). Although both nucleosides share an unusual 7-deazaguanosine core structure, they are rigorously segregated with respect to phyla, location in the tRNA, and presumed function. Queuosine is ubiquitous throughout the bacteria and eukarya (7), where it occurs specifically at the wobble position (8) in a subset of tRNAs (those coding for Tyr, His, Asp, and Asn). Its location in the anticodon suggests a role in modulating translational fidelity and/or efficiency, and a wealth of physiological studies are consistent with such a role (9–12). In marked contrast, archaeosine is present exclusively in the archaea, where it is found in the majority of known archaeal tRNAs specifically at position 15 of the dihydrouridine loop (13), a site not modified in any tRNA outside of the archaean domain. Its position at a critical interface of the D-loop and T-stem in the tertiary structure of the tRNA is consistent with a role in maintaining the structural integrity of the tRNA.

tRNA is structurally unique among nucleic acids in harboring an astonishing diversity of modified nucleosides. Two structural variants of the hypermodified nucleoside 7-deazaguanosine have been identified in tRNA: queuosine, which is found at the wobble position of the anticodon in bacterial and eukaryotic tRNA, and archaeosine, which is found at position 15 of the D-loop in archaean tRNA. From homology searching of the Methanococcus jannaschii genome, a gene coding for an enzyme in the biosynthesis of archaeosine (tgt) was identified and cloned. The tgt gene was overexpressed in an Escherichia coli expression system, and the recombinant tRNA-guanine transglycosylase enzyme was purified and characterized. The enzyme catalyzes a transglycosylation reaction in which guanine is eliminated from position 15 of the tRNA and an archaeosine precursor (preQ0) is inserted. The enzyme is able to utilize both guanine and the 7-deazaguanine base preQ0 as substrates, but not other 7-deazaguanine bases, and is able to modify tRNA from all three phylogenetic domains. The enzyme shows optimal activity at high temperature and acidic pH, consistent with the optimal growth conditions of M. jannaschii. The nature of the temperature dependence is consistent with a requirement for some degree of tRNA tertiary structure in order for recognition by the enzyme to occur.

A unique feature of the post-transcriptional processing of tRNA is the introduction of extensive chemical modification to the constituent nucleosides, which in some cases can involve up to 25% of the nucleosides in a particular tRNA (1). The nature of nucleoside modification in tRNA is considerably varied, from simple methylation of the base or ribose ring to extensive “hypermodification” of the canonical bases, the latter of which is characterized by radical structural changes and can involve multiple enzymatic steps to complete. There are currently over 80 modified nucleosides that have been identified in tRNA, many of which are highly conserved across broad phylogenetic boundaries. The specific contributions that individual modifications make to tRNA function are well established in only a few cases (2), but it is generally thought that modifications located outside the anticodon region help to maintain the structural integrity of the tRNA, while modifications within and around the anticodon are proposed to play a direct role in
high sequence homology to the Escherichia coli TGT in the genome of the archaeal hyperthermophile Methanococcus jannaschii (19) and the subsequent isolation of an archaeal TGT enzyme from Haloferax volcanii (20).

The TGT enzymes are a particularly intriguing family of enzymes for a number of reasons. First, they are the only enzymes involved in RNA modification that catalyze the re-arrangement of a genetically encoded base with a highly modified base. Second, there is remarkable correlation of their modified base substrate with their segregation within the primary phylogenetic domains; bacterial TGTs utilize preg, eukaryotic enzymes utilize queuine (21), and the archaeal enzymes appear to utilize preQ0. Finally, their origin in the different domains is associated with disparate tRNA recognition elements. In bacterial TGT, a UGU sequence at positions 33–35 of an anticodon-like stem-loop is the only essential structural element required for enzyme recognition (22, 23), while the eukaryotic TGT appears to require higher order structure in the tRNA (24). The presence of archaeosome in a different region of the tRNA necessitates recognition elements that are unique to the archaeal enzyme.

We report here the cloning and overexpression of the putative tgt gene from M. jannaschii, characterization of the recombinant enzyme, and demonstration of its function as a TGT. This is the first report of the cloning and overexpression of an archaeal TGT enzyme, and demonstration of its function as a TGT.

EXPERIMENTAL PROCEDURES

General Methods and Materials—The syntheses of preg, preQ0, archaeosome base, and 7-formamido-7-deazaguanine were carried out as described (25, 26). Protein concentrations were determined with the Coomassie protein reagent (Bio-Rad) according to the method of Bradford (27), using bovine serum albumin as a standard. SDS-PAGE analysis of proteins was performed using 12% gels and visualization with Coomassie Brilliant Blue (28). DNA fragments generated from restriction digestions were purified with the GeneClean® II kit (Bio 101) according to the manufacturer’s instructions. Plasmid minipreparations were carried out as described (28).

Archaeosome G* and Queuosome (Q)

Fig. 1. The structures of queuosome (Q) and archaeosome (G*) nucleosides.

Construction of an E. coli Expression System for M. jannaschii tgt—The M. jannaschii tgt gene was amplified from M. jannaschii genomic DNA via PCR with Pfu polymerase using the primers MJ-US1 (5′-GGCGACTGCCATATAATGGTAGAGG-3′) and MJ-S3 (5′-CCGGCGGATCCTCGTTGATATTCATAATTATC-3′) into the plasmid pYB-I-56. The structure of the plasmid pJL-I-70

Construction of an E. coli Expression System for M. jannaschii tgt—The M. jannaschii tgt gene was amplified from M. jannaschii genomic DNA via PCR with Pfu polymerase using the primers MJ-US1 (5′-GGCGACTGCCATATAATGGTAGAGG-3′) and MJ-S3 (5′-CCGGCGGATCCTCGTTGATATTCATAATTATC-3′) into the plasmid pYB-I-56. The structure of the tgt gene, while the MJ-S3 primer contained a BamHI site (underlined), the later as part of the translation initiation codon (boldface) for the tgt gene, while the MJ-S3 primer contained a BamHI site (underlined) downstream from the translation termination codon. The PCR program included an initial hold for 5 min at 95 °C, followed by 30 cycles of 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 7 min. After amplification, the PCR product was gel-purified (0.8% agarose), subjected to restriction digest with XhoI/BamHI, and ligated into the XhoI/BamHI sites of plasmid pBSK (+) to give the plasmid pYB-1-56. The structure of the tgt gene was confirmed by sequencing and subconed as an NdeI/BamHI fragment into the NdeI/BamHI sites of the E. coli expression vectors pET-11a and pARC306N (29) to give the plasmid pYB-1-120 and pYB-1-93, respectively.

Overexpression and Purification of Recombinant M. jannaschii TGT in E. coli—A single colony of E. coli BL21(DE3)/pYB-I-120 was used to inoculate 3 ml of LB containing 100 μg/ml ampicillin. The cultures were incubated at 37 °C and 250 rpm for approximately 12 h, when a 1-ml aliquot was used to inoculate 100 ml of LB containing 100 μg/ml ampicillin in 500-ml Erlenmeyer flasks, and the cultures were allowed to grow overnight at 37 °C and 250 rpm. A 5-ml portion of the overnight culture was used to inoculate 500 ml of LB containing 100 μg/ml ampicillin in 2.8-liter Erlenmeyer flasks. The cultures were incubated at 37 °C and 250 rpm until they reached an A600 of 0.9, at which time IPTG was added to a final concentration of 1 mM. The cultures were grown an additional 4 h, harvested by centrifugation (5000 × g for 10 min), flash-frozen in liquid N2, and stored at –90 °C.

Frozen cell paste (4.0 g) was suspended in buffer containing 50 mM Tris (pH 7.5), 1 mM DTT, 0.5 mM EDTA, and 1 μM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication, and the solution was clarified by ultracentrifugation (1 h at 100,000 × g). To the supernatant was added solid ammonium sulfate to 65% saturation over 45 min, and after stirring an additional 40 min, the solution was subjected to high speed centrifugation (30 min at 20,000 × g). The protein pellet was dissolved in buffer containing 50 mM sodium phosphate (pH 6.5) and 1 mM DTT and applied to a column of POROS HS, equilibrated with linear gradient of sodium chloride from 0 to 1.5 M. The TGT-containing fractions were pooled; dialyzed against 50 mM Tris (pH 7.5), 1 mM DTT; and applied to a column of POROS Hq, eluting with a linear gradient of sodium chloride from 0 to 1.5 M. The TGT-containing fractions were pooled; concentrated (Centriprep and Centricon); dialyzed against 50 mM potassium succinate (pH 5.5), 1 mM DTT; 50 mM potassium...
Fig. 2. The proposed biogenetic relationship of queuosine and archaeosine.

cloride; and heated at 80 °C for 15 min. The solution was subjected to high speed centrifugation (30 min at 20,000 × g), and the supernatant containing TGT was diluted with an equal volume of 30% glycerol and stored at −90 °C.

Generation of Unmodified H. volcanii tRNA^Gua—A synthetic gene coding for H. volcanii tRNA^Gua containing a T7 promoter site directly adjacent to the transcriptional start was constructed from six oligonucleotides with overlapping complementarity according to the method of Sampson and Uhlenbeck (30). Forty picomoles of each oligomer (Table I) were separately phosphorylated with T7 polynucleotide kinase according to the manufacturer’s instructions. The phosphorylated oligomers were combined in equal stoichiometry and annealed by heating to 90 °C for 2 min followed bycooling to 25 °C over 2 h. The annealed oligomers were then ligated into the BamHI/EcoRI sites of pUC18 with T7 DNA ligase according to the manufacturer’s instructions to give plasmid pDF-II-42. The reaction mixture was transformed directly into competent cells of E. coli DH5α, and positive transformants were identified by blue/white screening and by restriction digests of miniprep plasmid DNA. The structure of the gene was subsequently confirmed by DNA sequencing.

Template DNA for in vitro transcription was prepared by BetNI digestion of pDF-II-42 according to the manufacturer’s instructions, extraction of the reaction mixture with phenol/chloroform/isoamyl alcohol followed by chloroform, and precipitation of the DNA with ethanol. After washing with 70% ethanol, the precipitated DNA was resuspended in Tris/EDTA (pH 8.0). Transcription reactions (1 ml) contained 40 mM Tris (pH 7.9), 20 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 4 mM NTPs, 50 μg of template DNA, anti-RNase (900 units), inorganic pyrophosphatase (2 units), and T7 RNA polymerase (5 units). Reactions were run at 25 °C for 6–10 h, followed by the addition of RNase-free DNase (70 units) and incubation at 37 °C for 2 h. The reaction mixture was extracted with phenol/chloroform/isoamyl alcohol followed by chloroform, and the tRNA transcript was precipitated with ethanol. After washing with 70% ethanol, the RNA was taken up in 3 mM citrate (pH 6.3) and purified on denaturing 15% PAGE gels. To recover the RNA, the gel was frozen at −90 °C, crushed, and soaked in 3 mM citrate (pH 6.3) for 24 h at 4 °C. The gel suspension was then centrifuged, the supernatant was filtered through a sterile 0.22-μm filter, and the RNA was precipitated with ethanol. The RNA was redissolved in 3 mM citrate (pH 6.3), and the concentration was determined spectrophotometrically using the calculated extinction coefficient.

Activity Assays of M. jannaschii TGT—Standard activity assays were carried out in 0.2-ml microcentrifuge tubes in a final volume of 50 μl. The assay buffer contained 100 mM succinate (pH 5.3), 2 mM DTT, 50 mM KCl, 20 mM MgCl₂, and varying concentrations of the RNA and [8-14C]guanine. Assays were preincubated for 2 min at 80 °C and initiated by the addition of enzyme. For investigation of the pH dependence of the enzyme, the assays contained 20 μM [8-14C]guanine, 1.0 μg/μl unfraccionated yeast tRNA (type X), and 200 nM TGT. Assays were carried out at 37 °C for 3 h at the specified pH, using the following buffers: acetate (pH 4.0–5.5), MESS (pH 5.0–6.0), succinate (pH 5.5–6.0), PIPES (pH 6.0–7.0), MOPS (pH 7.0–7.5), HEPES (pH 7.5–8.0), and POPSO (pH 8.0–8.5).

For the temperature dependence studies, assays were carried out at the specified temperature and contained 20 μM [8-14C]guanine, 200 nM TGT, and either 1.0 μg/μl unfractionated yeast tRNA (Type X) or 0.2 μg/μl E. coli tRNA^Val.

All assays were terminated after the specified time by the addition of 3 volumes (150 μl) of 10% trichloroacetic acid (TCA) to each tube, followed by cooling to −20 °C overnight. The [14C]tRNA was pelleted by centrifugation (30 min at 20,000 × g) at 4 °C, washed with 70% ethanol (twice), and dissolved in 3 mM citrate (pH 6.3). The specific activity of the [14C]tRNA was determined by UV spectroscopy and liquid scintillation counting. The concentration of tRNA^Val was estimated from the valine acceptor activity provided by the manufacturer (1600 pmol incorporated per A₅₅₀ unit). The [14C]tRNA was rapidly subjected to ethanol precipitation until the specific activity remained unchanged.

Activity assays contained the standard assay components with the concentration of the relevant base at 50 μM and the [14C]tRNA at 4.8 μM. Assays were initiated with the addition of 50 nM TGT, terminated after the specified time, and worked up as described above.

RNA Specificity of M. jannaschii TGT—Activity assays contained the standard assay components with [14C]guanine at 10 μM and unfraccionated RNA from yeast (type X and X-SA; Sigma) or E. coli (type XX; Sigma), E. coli tRNA^Val (Sigma), or in vitro transcribed H. volcanii tRNA^Gua. The concentration of tRNA was kept at approximately 2 μM for the tRNA^Val and tRNA^Gua and at 0.4 μg/μl for the unfraccionated tRNA. The molar concentrations of the in vitro transcribed tRNA^Val and E. coli tRNA^Val were determined as described above. Reaction assays were initiated with the addition of 50 nM TGT, terminated after the specified time, and worked up as described above.

RESULTS

Identification and Cloning of M. jannaschii tgt in E. coli—The complete genome of the archaeon M. jannaschii has recently been reported (19), and protein homology searching identified an open reading frame (MJ0436) that coded for a protein having significant amino acid homology to bacterial TGT. However, multiple sequence alignment of four bacterial TGTs with the protein product of MJ0436 revealed that a highly conserved N-terminal region present in the bacterial TGTs was absent in the deduced amino acid sequence of the...
putative archaeal TGT. Since this region includes an absolutely conserved aspartate implicated from the crystal structure of the Zymononas mobilis TGT as the catalytic nucleophile in the elimination of guanine (31, 32), it appeared that the translational start had been misassigned. Inclusion of an additional 55 amino acids encoded by the DNA upstream of MJ0436 in the alignment revealed the conserved region 20 amino acids upstream from the designated start. This has also been recently noted by Romier et al. (33) in their sequence analysis of the active sites of TGTs.

Since no in-frame start codons were present upstream of this homologous region, we reasoned that a sequencing error had occurred. To determine the location of the translational start, a region encompassing approximately 320 nucleotides upstream of the start of MJ0436 was amplified from M. jannaschii genomic DNA via PCR and cloned into the SalI/EcoRI sites of pBluescript SK(+), giving the plasmid pJL-I-70. Sequencing of pJL-I-70 revealed that an adenosine 148 nucleotides upstream of MJ0436 had been deleted in the original sequence data, producing a frameshift. When the adenosine was included in the sequence, five in-frame start codons and a stop codon were revealed upstream. When this region was included in the multiple alignment, additional regions of homology were apparent, with the penultimate Met codon aligning well with the start of the bacterial enzymes (Fig. 3).

Having established the putative translational start, the tgt gene was amplified from M. jannaschii genomic DNA via PCR and cloned into the XhoI/BamHI sites of pBluescript II SK(+). After the structure of the tgt gene was confirmed by sequencing (Fig. 4), it was subcloned into the NdeI/BamHI sites of the E. coli expression vector pET11a to give pYB-I-120.

Overexpression and Purification of M. jannaschii TGT—The TGT expression vector pYB-I-120 was transformed into competent cells of BL21(DE3) for overexpression of the tgt gene. Positive transformants of BL21(DE3)/pYB-I-120 were screened for TGT production and activity after induction with IPTG, and the most active transformants were selected for synthesis of TGT. Induction by IPTG was accompanied by the appearance of a new protein band at a molecular mass of approximately 75 kDa on SDS-PAGE (Fig. 5), in good agreement with the calculated mass of 78 kDa obtained from the amino acid sequence. Maximal production of TGT was observed approximately 4 h after induction with 1 mM IPTG as estimated from activity assays and SDS-PAGE, the latter of which indicated that TGT comprised approximately 10% of soluble protein.

The origin of the TGT enzyme from a thermophilic organism prompted us to initially examine heat treatment of the cell-free extract as a preliminary step in the purification. However, significant enzyme activity was lost after incubation for 15 min at either 60 or 80 °C, so we abandoned this approach. Our initial screening of chromatography conditions revealed that TGT eluted in two discreet peaks, one of which contained substantial nucleic acid. When the cell-free extract was subjected to treatment with streptomycin sulfate, the enzyme partitioned between both the pellet and supernatant fractions. In contrast, when the enzyme was precipitated with ammonium

| Oligonucleotide | Sequence |
|----------------|----------|
| T7-ASen        | TATAGTGAAGCTGATTACTGCAGG |
| T7-Sen         | AATTCTGCGATATTACGAC |
| Ser-S1         | TCACATTAGCCGAGGAGTGACCTAGTCAGC |
| Ser-S2         | TCAGAACTACTTGTCTCATTCCGAGACCTGAGTCTCAACTCCCTGCGGCCAG |
| Ser-AS1        | GATCTCGGGGCGAGGAGTTAGATGAAACTACAGTGTCGGAGATGGACAGTA |
| Ser-AS2        | GATCTCGAGAATCTGCCGGCCGGCTGGCGCATGCTCTCGGC |

**Table I**

| Oligonucleotide | Sequence |
|----------------|----------|
| T7-ASen        | TATAGTGAAGCTGATTACTGCAGG |
| T7-Sen         | AATTCTGCGATATTACGAC |
| Ser-S1         | TCACATTAGCCGAGGAGTGACCTAGTCAGC |
| Ser-S2         | TCAGAACTACTTGTCTCATTCCGAGACCTGAGTCTCAACTCCCTGCGGCCAG |
| Ser-AS1        | GATCTCGGGGCGAGGAGTTAGATGAAACTACAGTGTCGGAGATGGACAGTA |
| Ser-AS2        | GATCTCGAGAATCTGCCGGCCGGCTGGCGCATGCTCTCGGC |

**Fig. 3.** Multiple sequence alignment of M. jannaschii TGT and three bacterial TGTs (note the C-terminal 304 amino acids of M. jannaschii TGT are not included in the alignment). S. flex, Shigella flexneri; Z. mobi, Z. mobilis; M. jann, M. jannaschii. Amino acid residues in red represent sequence identity, residues in green represent sequence similarity, and residues in blue show the zinc binding region (CXXCX23–26H) and the active site Asp identified from the crystal structure of the Z. mobilis enzyme (31).
sulfate, it was obtained largely free of nucleic acid. The enzyme was thus purified by ammonium sulfate fractionation followed by cation and anion exchange chromatographies. This provided enzyme that was judged to be 90% homogeneous by SDS-PAGE (Fig. 5). When the temperature and pH optima of the enzyme were subsequently investigated, we discovered that the enzyme was quite stable at high temperatures when the pH was reduced to 5.5 (see below). Thus, the enzyme purified through the HQ step was dialyzed into succinate buffer (pH 5.5) and heated at 80 °C for 15 min as a final purification step, which provided homogeneous TGT as judged by SDS-PAGE. The purification is summarized in Table II. The calculated

**Fig. 4.** Gene sequence of *M. jannaschii tgt* and deduced amino acid sequence of the TGT protein.

**Fig. 5.** SDS-PAGE gel (1%) of fractions from the purification of recombinant *M. jannaschii* TGT from *E. coli*. Lane 1: molecular mass marker; lane 2: S-100 fraction; lane 3: 65% ammonium sulfate fraction; lane 4: POROS HS fraction; lane 5: POROS HQ fraction; lane 6: heat treatment (80 °C for 15 min).
Characterization of M. jannaschii TGT—Activity assays of M. jannaschii TGT were modeled after those developed for the bacterial enzymes, exploiting the ability of the enzymes to carry out a guanine exchange reaction such that radiochemical analysis could be used to quantitate the incorporation of [14C]guanine into the tRNA. Initial activity assays employed a neutral buffering system and mild temperature (37 °C) as described for the bacterial systems. Subsequent analysis of enzyme activity (20) exhibits enzyme does not require high ionic strength for activity and in fact is inhibited at sodium chloride concentrations greater than 400 mM (data not shown).

To obtain preliminary information on the tRNA substrate specificity of M. jannaschii, TGT enzyme activity was investigated with a number of tRNAs, including unfractionated tRNA from E. coli and yeast, E. coli tRNAVal, and unmodified H. volcanii tRNAser prepared through in vitro transcription. Interestingly, while the enzyme exhibited significant activity with both tRNAVal and tRNAser, as well as unfractionated tRNA from E. coli and yeast (type X), no activity was observed with unfractionated type X-SA tRNA from yeast (Fig. 7). The reason for the lack of activity with type X-SA tRNA is unclear; type X-SA differs from type X only in the inclusion of an additional step in the purification. The extra step is apparently similar to ion exchange steps used to isolate specific tRNAs (the nature of the step is proprietary), so it is possible that the type X-SA tRNA has been depleted in substrate tRNA. Importantly, significant activity was observed with tRNA from all three phylogenetic domains.

While the tRNA substrates for bacterial and eukaryotic TGT are identical, their base substrates are markedly different; preQ0 is the substrate for bacterial enzymes, while queuine is the substrate for the eukaryotic enzymes. Given the presence of the amidine functionality at the 7-position of archaeosine and the presumed intermediacy of preQ0 in the biosynthesis, it appeared that the relevant substrate for the archaeal TGT would probably possess the acid oxidation state at the exocyclic carbon. Potential candidates for the physiological substrate were thus archaeosine base itself, preQ0, the 7-carboxamide, and the 7-carboxylate, although the latter was considered the least likely given the absence of a nitrogen. The enzyme exhibits its significant activity when guanine or preQ0 is present as substrate (Fig. 8) but not with either preQ1 or the 7-carboxamide. The enzyme showed low but inconsistent activity with archaeosine, their obvious structural similarity

Table II: Guanine Transglycosylase from Thermophilic Archaea

| Fraction      | Protein Unitsa | Specific Activity | Purification Factor | Recovery |
|---------------|----------------|-------------------|---------------------|----------|
| S-100         | 115            | 920               | 8.0                 | 100      |
| Ammonium sulfate | 103           | 927               | 9.0                 | 100      |
| POROS HS      | 18.2           | 355               | 47                  | 93       |
| POROS HQ      | 7.57           | 712               | 94                  | 77       |
| Heat treatment| 5.01           | 671               | 134                 | 73       |

a 1 unit = 1 pmol/min.

Specific activity of the purified protein is comparable with that reported from the H. volcanii enzyme (20).

FIG. 6. Temperature profile for the recombinant M. jannaschii TGT. Enzyme assays were carried out as described under "Experimental Procedures." □, unfraccionated yeast tRNA (type X); ●, E. coli tRNAVal. Data represent the average of at least three independent experiments, each carried out in duplicate. The S.D. in the data ranged from 0.8 to 13%.

FIG. 7. tRNA substrate specificity of recombinant M. jannaschii TGT. Assays were carried out as described under "Experimental Procedures." □, tRNAVal transcript; ●, unfraccionated yeast tRNA (type X-SA); ○, E. coli tRNAVal, ▲, unfraccionated E. coli tRNA; ■, unfraccionated yeast tRNA (type X). Data represent the average of at least three independent experiments, each carried out in duplicate. The S.D. in the data ranged from 1 to 4%.
has fueled speculation on the possible evolutionary relationship between these modified nucleosides. With the proliferation of full genome sequencing projects, this hypothesis can be addressed directly through the identification of homologous genes and the characterization of their gene products. The initial identification in the *M. jannaschii* genome of a putative TGT with sequence homology to the *E. coli* TGT suggested the existence of this enzyme in the archaea and furthermore that the TGT enzymes from all three domains evolved from a common ancestral protein. The subsequent isolation by Watanabe *et al.* (20) of a TGT from *H. volcanii* confirmed the existence of this activity in archaea. With the identification of the complete tgt gene here and the demonstration of TGT activity with the recombinant protein, it is clear that these enzymes make up a distinct, evolutionarily related family.

The properties of the *M. jannaschii* TGT diverge in a number of ways from its counterpart in *H. volcanii*, but these differences are easily understandable within the context of the ecological niches that the organisms inhabit. Thus, the enzyme from *H. volcanii* is absolutely dependent on high salt concentration and neutral pH for activity, the latter consistent with the bacterial and eukaryotic enzymes, while the *M. jannaschii* TGT is inhibited by high salt concentration and exhibits maximal activity at acidic pH (5.5) and high temperature (80–85 °C).

In considering potential steps for the conversion of preQ₅₀ to archaeosine, two events must take place: the incorporation of the modified base into the polynucleotide and the formal addition of ammonia to give preQ₅₀, and Watanabe *et al.* (20) interpreted the low enzymatic activity observed with archaeosine base in these studies to be due not to direct incorporation of archaeosine base but to the *in situ* formation of preQ₅₀ and its subsequent incorporation into the tRNA. The instability of archaeosine base argues against it as the probable substrate *in vivo*, since the steady-state concentration would be expected to be low and the constant cycling between preQ₅₀ and archaeosine base would be inherently inefficient. Other indirect evidence that discounts archaeosine base as the physiological substrate is the failure of preQ₅₀ to serve as an efficient substrate of the enzyme; were archaeosine base the normal substrate, one would expect preQ₅₀, a closer structural homologue of archaeosine base than preQ₀, to serve as a more efficient substrate. That it does not is compelling evidence that archaeosine base is not the relevant physiologically substrate.

The observation that in bacterial and eukaryotic tRNA queuosine was limited to a subset of tRNA containing a GUN sequence in the anticodon (Tyr, His, Asp, and Asn tRNAs) suggested a putative sequence requirement for the TGT enzyme. Subsequent work unequivocally demonstrated that the minimal sequence requirement was UGU at positions 33–35 of the anticodon stem-loop (22, 23). A survey of archael tRNA sequence data reveals no obvious sequence consensus that is unique to archaeosine-modified tRNA. Unlike queuosine, archaeosine is found extensively in archael tRNA, occurring, for example, in 25 of the 41 tRNAs in *H. volcanii* (35), and is widely distributed across both of the archael divisions (36). However, its presence in specific tRNAs is apparently not universal. For example, archaeosine is present in the initiator tRNA<sub>Met</sub> in both *H. volcanii* and *Halofex cutirubrum* but not in *Sulfolobus acidocaldarius*, *Halococcus morrhuae*, or *Thermoplasma acidophilum* (13, 37), despite the presence of a genetically encoded G nucleotide at position 15 in the later two. And archaeosine occurs in all five of the tRNA<sub>1<sub>isoacceptors in *H. volcanii* but is present in only one of two isoacceptors of tRNA<sub>2<sub> and tRNA<sub>1<sub> (35). Given the fact that no tRNA sequences have been reported for *M. jannaschii* and the obvious paucity of data available on the rules governing modification with archaeosine, the identification of potential archael tRNA substrates of *M. jannaschii* TGT is somewhat problematic. The presence of archaeosine in all of the isoaccepting tRNA<sub>3</sub> in *H. volcanii* and *H. cutirubrum* is currently the most extensively conserved within a group of isoaccepting tRNAs in more than a single species. Thus, the tRNA transcript that we chose to synthesize and explore as a substrate RNA was tRNA<sub>3</sub> (CGA) from *H. volcanii.*

The enzyme shows good activity with the tRNA<sub>3</sub> transcript as well as tRNA from *E. coli* and yeast (Fig. 7), demonstrating that the elements necessary for tRNA recognition by the enzyme are not unique to archael tRNA. This is in contrast to the results reported for the enzyme from *H. volcanii* (20), which did not show significant activity with eukaryotic tRNA (unfractionated yeast or bovine tRNA). This apparent anomaly may be due to the formulation of tRNA used by those authors, since we observed minimal enzyme activity when we investigated enzyme activity with type X-SA tRNA from yeast (<6% of the activity observed with yeast type X tRNA). The basis for this difference has not yet been investigated.

Perhaps most interesting in the behavior of the enzyme with different tRNA substrates is the temperature dependence observed with unfractionated tRNA and tRNA<sub>1</sub> (Fig. 6). While the enzyme exhibits maximal activity with both the unfractionated tRNA and tRNA<sub>1</sub> at temperatures of ≥80 °C, the temperature at which activity peaks and begins to fall is dependent on the identity of the tRNA. Thus, unfractionated yeast tRNA shows optimal activity at ~80 °C and half-maximal activity at ~61 °C, while *E. coli* tRNA<sub>1</sub> exhibits maximal activity at ~86 °C and half-maximal activity at ~73 °C. We interpret the broader activity profile observed with unfractionated yeast
tRNA to be due to the presence of multiple substrate tRNAs, each with somewhat different profiles. This suggests that the decrease in activity is associated with a loss of some essential element of tRNA tertiary structure. Given the postulated role of archaeosine in stabilizing the tertiary structure of archaeal tRNA against thermal denaturation, the possibility that higher order structure in the tRNA would be requisite for TGT activity is unexpected. It should be noted, however, that while the enzyme exhibits remarkable thermostability, our data do not exclude the possibility that a reversible unfolding of the enzyme may be occurring at temperatures above 80 °C, which could contribute to the loss of activity observed when assay temperatures are in this range.

In conclusion, recombinant TGT from M. jannaschii exhibits general behavior consistent with its origin from a hyperthermophile and catalyzes the replacement of guanine 15 with preQ0, but not other 7-substituted 7-deazaguanine compounds, in the tRNA, confirming the results seen previously with the M. jannaschii Illinois for a sample of M. jannaschii. Analysis of enzyme activity with tRNA from all three phylogenetic domains has demonstrated no structural element unique to archaeal tRNA necessary for recognition and catalysis by the enzyme. Quantitative analysis of the enzyme with various base substrates and clarification of the need for archaeal TGTs is the probable physiological substrate for archaeal TGTs. 6. Kasai, H., Ohashi, Z., Harada, F., Nishimura, S., Oppenheimer, N. J., Crain, P. F., Liehr, J. G., von Minden, D. L., and McCloskey, J. A. (1986) Biochemistry 25, 4198–4208 7. Katz, J. R., Baile, B., and McCloskey, J. A. (1982) Science 216, 55–56 8. Kersten, H. (1988) BioFactors 1, 27–29 9. Kersten, H., and Kersten, W. (1990) in Chromatography and Modification of Nucleosides Part B: Biological Roles and Function of Modification (Gehrke, C. W., and Kuo, K. C. T., eds) pp. 69–108, Elsevier, Amsterdam 10. Marks, T., and Farkas, W. R. (1997) Biochem. Biophys. Res. Commun. 230, 233–237 11. Carlson, B. A., Kven, S. Y., Chamorro, M., Oroszlai, S., Hatfield, D. L., and Lee, B. J. (1999) Virology 255, 2–8 12. Durand, J., Okada, N., Tobe, T., Watarai, M., Fukuda, I., Suzuki, T., Naka, N., Komatsu, K., Yoshikawa, M., and Sasakawa, C. (1994) J. Bacteriol. 176, 4627–4634 13. Sprinzl, M., Hartmann, T., Weber, J., Blank, J., and Zeidler, R. (1989) Nucleic Acids Res. 17, (suppl.) 1–67 14. Kuchino, Y., Kasai, H., Nihei, K., and Nishimura, S. (1976) Nucleic Acids Res. 3, 393–398 15. Okada, N., Noguchi, S., Kasai, H., Shindo-Okada, N., Ohgi, T., Goto, T., and Nishimura, S. (1979) J. Biol. Chem. 254, 3067–3073 16. Okada, N., Noguchi, S., Nishimura, S., Ohgi, T., Goto, T., Crain, P. F., and McCloskey, J. A. (1978) Nucleic Acids Res. 5, 2289–2296 17. Slany, R. K., Bosl, M., Crain, P. F., and Kersten, H. (1993) Biochemistry 32, 7811–7817 18. Frey, B., McCloskey, J. A., Kersten, W., and Kersten, H. (1988) J. Bacteriol. 170, 2076–2082 19. Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., Blake, J. A., FitzGerald, L. M., Clayton, R. A., Gocayne, J. D., Kerlavage, A. R., Dougherty, B. A., Tomb, J. F., Adams, M. D., Reich, C. L., Overbeeke, R., Kirkness, E. F., Weinstock, K. G., Merrick, J. M., Glodek, A., Scott, J. L., Geoghegan, N. M., Weidman, J. F., Fuhrmann, J. L., and Venter, J. C. (1996) Science 273, 1058–1073 20. Watanabe, M., Matsuoka, M., Tanaka, S., Akimoto, H., Asahi, S., Nishimura, S., Katz, J. R., Hashizume, T., Crain, P. F., McCloskey, J. A., and Okada, N. (1997) J. Biol. Chem. 272, 20146–20151 21. Shindo-Okada, N., Okada, N., Ohgi, T., Goto, T., and Nishimura, S. (1980) Biochemistry 19, 395–400 22. Nakaniishi, S., Ueda, T., Hori, H., Yamazaki, N., Okada, N., and Watanabe, K. (1994) J. Biol. Chem. 269, 32221–32225 23. Curnow, A. W., and Garcia, G. A. (1995) J. Biol. Chem. 270, 17264–17267 24. Grosgean, H., Edqvist, J., Straby, K. B., and Giege, R. (1996) J. Mol. Biol. 253, 67–85 25. Akimoto, H., Imaniya, E., Hitaka, T., Nomura, H., and Nishimura, S. (1988) J. Chem. Soc. Perkin Trans. I, 1637–1644 26. Migowa, M. T., Hinkley, J. M., Hoops, G. C., and Townsend, L. B. (1996) Synth. Commun. 26, 3317–3322 27. Bradford, M. (1976) Anal. Biochem. 72, 248–254 28. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 29. Street, I. P., and Poulter, C. D. (1990) Biochemistry 29, 7531–7538 30. Sampson, J. R., and Uhlenbeck, O. C. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1033–1037 31. Romier, C., Reuter, K., Suck, D., and Ficner, R. (1996) EMBO J. 15, 2850–2857 32. Romier, C., Reuter, K., Suck, D., and Ficner, R. (1996) Biochemistry 35, 5734–5739 33. Romier, C., Meyer, J. E., and Suck, D. (1997) FEBS Lett. 416, 93–98 34. Robb, F. T., Place, A. R., Sowers, K. R., Schreier, H. J., DasSarma, S., and Fleischmann, E. M. (eds) (1995) Archaea: A Laboratory Manual, p. 4, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 35. Gupta, C. G., Crain, P. F., Gupta, R., Hashizume, T., Hocart, C. H., Kowalcik, J. A., Pomerantz, C. C., Stetter, K. O., and McCloskey, J. A. (1991) J. Bacteriol. 173, 3138–3148 36. Edmonds, C. G., Crain, P. F., Gupta, R., Hashizume, T., Hocart, C. H., Kowalcik, J. A., Pomerantz, C. C., Stetter, K. O., and McCloskey, J. A. (1991) J. Bacteriol. 173, 3138–3148 37. Sprinzl, M., Dank, N., Nock, S., and Schon, A. (1991) Nucleic Acids Res. 19, (suppl.) 2127–2171

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Hypermodification of tRNA in Thermophilic Archaea: CLONING,
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