Archaeosine is a novel derivative of 7-deazaguanosine found in transfer RNAs of most organisms exclusively in the archaeal phylogenetic lineage and is present in the D-loop at position 15. We show that this modification is formed by a posttranscriptional base replacement reaction, catalyzed by a new tRNA-guanine transglycosylase (TGT), which has been isolated from *Haloferax volcanii* and purified nearly to homogeneity. The molecular weight of the enzyme was estimated to be 78 kDa by SDS-gel electrophoresis. The enzyme can insert free 7-cyano-7-deazaguanine (preQ<sub>0</sub> base) *in vitro* at position 15 of an *H. volcanii* tRNA T<sub>T</sub> transcript, replacing the guanine originally located at that position without breakage of the phosphodiester backbone. Since archaeosine base and 7-aminomethyl-7-deazaguanine (preQ<sub>1</sub> base) were not incorporated into tRNA by this enzyme, preQ<sub>0</sub> base appears to be the actual substrate for the TGT of *H. volcanii*, a conclusion supported by characterization of preQ<sub>0</sub> base in an acid-soluble extract of *H. volcanii* cells. Thus, this novel TGT in *H. volcanii* is a key enzyme for the biosynthetic pathway leading to archaeosine in archaeal tRNAs.

A variety of modified nucleosides has been found in tRNA (1, 2), but their functions and, in particular, their biosynthetic pathways are still largely unknown (3). Many modified nucleosides are highly conserved with respect to their sequence locations in tRNA (4), and some are characteristic of the evolutionary origin (2, 5), namely, archaea, bacteria, or eukarya (6). Perhaps the most phylogenetically specific nucleoside in tRNA is archaeosine, which occurs only in archaeal tRNA at position 15, a site that is not modified in tRNAs from the other two primary domains (7). Archaeosine was first discovered by Kilpatrick and Walker (8) during sequencing of tRNA from *Thermoplasma acidophilum*, and it was subsequently shown to be present in many archaeal species (9); in the most extensively studied archaeal tRNA, from *Halobacterium volcanii*, archaeosine occurs in tRNAs specifying more than 15 amino acids (10). Subsequently, the structure of archaeosine was determined to be the non-purine, non-pyrimidine nucleoside 7-formamidino-7-deazaguanosine (Fig. 1A) (11).

The only other known examples of tRNA nucleosides with 7-deazaguanosine structures are the members of the Q<sup>+</sup> nucleoside (12) (Fig. 1E) family (13), which includes precursors in its biosynthesis, such as 7-cyano-7-deazaguanine (preQ<sub>0</sub>; Fig. 1D) (14), 7-aminomethyl-7-deazaguanine (preQ<sub>1</sub>; Fig. 1C) (15), and oQ (16) from bacterial tRNAs, and mannoeyl and galactosyl derivatives of Q (17, 18) from mammalian tRNAs. In contrast to archaeosine, members of the Q nucleoside family are located at the first position of the anticodon (position 34) in bacterial and eukaryotic tRNAs that are specific for only four amino acids (Tyr, His, Asp, and Asn) (19). The key enzyme in the biosynthesis of the Q nucleoside in tRNA is tRNA-guanine transferase (TGT; EC 2.4.2.29), which catalyzes a base-exchange reaction by cleavage of the N=C glycosidic bond at position 34 (20). In bacteria, TGT catalyzes the exchange of guanine at position 34 in tRNA with either guanine base, preQ<sub>1</sub> base, or preQ<sub>0</sub> base (20, 21). preQ<sub>0</sub> base is presumed to be synthesized *de novo* from GTP (1) and was identified as the physiological substrate of *Escherichia coli* TGT (21). After incorporation of preQ<sub>0</sub> into tRNA, it is further modified to oQ by transfer of the ribosyl moiety from S-adenosylmethionine (22), then finally to yield Q in the polynucleotide chain (23). In contrast, in eukarya, TGT can incorporate fully modified Q base into the first position of the anticodon by a base-replacement reaction (24, 25). Animals cannot synthesize Q-related compounds *de novo* and must obtain Q base as a nutrient from their diet or gut flora (26, 27).

Here we report the isolation of a new type of TGT from *H. volcanii*; it catalyzes the incorporation of preQ<sub>0</sub> base into position 15 of tRNA, replacing guanine originally located at that site. Further, we have demonstrated that free preQ<sub>0</sub> base is present in *H. volcanii* cells, implying that TGT utilizes preQ<sub>0</sub> base.

*The abbreviations used are: Q or queosine, 7-[(4, 5-cis-dihydroxy-2-cyclopenten-1-yl)-amino]methyl]-7-deazaguanosine; oQ or epoxy-queosine, 7-[(3, 4-cis-dihydroxy-2-cyclopenten-1-yl)-amino]methyl]-7-deazaguanosine; preQ<sub>0</sub>, 7-aminomethyl-7-deazaguanosine; preQ<sub>1</sub>, 7-cyano-7-deazaguanosine; archaeosine, 2-amino-4,7-dihydroxy-4-oxo-7-beta-ribosofuranosyl-1H-pyrrrolo[2,3-d]pyrimidine-5-carboximidamide, or 7-formamidino-7-deazaguanosine; TGT, tRNA-guanine transglycosylase.*

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 experimential procedures

cells — H. volcanii (ATCC 29605) was grown aerobically at 37 °C on a 500-liter-scale in Gupta’s medium (10), until the absorbance at 600 nm reached 0.8–1.0. About 1.0 kg of cells was collected.

assay of guanine exchange reaction — Exchange between guanine and various 7-deazaguanine analogues, catalyzed by TGT, was assayed as described previously (20) except that the final ionic condition of the reaction mixture was 1.5 m KCl and 1.5 m NaCl. The 7-deazaguanines were synthesized as described previously: preQ1 (29), and archaeosine base (30).

purification of H. volcanii tRNA-Guanine Transglycosylase—Frozen H. volcanii cells (100 g) were suspended in 200 ml of buffer A (50 m M Hepes (pH 7.5), 10% glycerol, 1.0 m M dithiothreitol, and 0.5 m M phenylmethylsulfonyl fluoride) plus DNase I (2.5 µg/ml), and were broken by sonication. The S-100 fraction was obtained by centrifugation at 30,000 × g for 1 h, dialyzed against buffer A, and then adsorbed onto a DEAE-Sepharose FF column (2.5 × 20 cm) (Pharmacia), which was eluted by a linear gradient of NaCl from 0.02 to 0.5 m in buffer A. The eluate containing the active fraction was brought to 40% ammonium sulfate and then applied to a Butyl-Sepharose FF column (2.5 × 20 cm) (Pharmacia), which was eluted with a linear gradient of ammonium sulfate from 40% to 0% in buffer A. The active fraction was next applied to a Butyl-Sepharose 4B column (1.5 × 15 cm) (Pharmacia) and eluted as described above for the Butyl-Sepharose FF column. The active fraction was then applied to a Superdex 200 column (1.6 cm × 60 cm) (Pharmacia), and then eluted with buffer A containing 300 m M NaCl. Finally, the TGT fraction was applied to a Mono Q column (0.5 × 5 cm) (Pharmacia) and eluted with a linear gradient of NaCl from 300 m M at 1 m M. This TGT fraction was stable for at least 1 month when stored at 4 °C. The activity of the enzyme was monitored by incorporation of [8-3H]guanine into unfractionated E. coli tRNA (20). Amino acid sequences of peptide fragments generated by digestion with lysylpeptidase were determined as described previously (31).

construction of a plasmid clone containing the gene for H. volcanii tRNA-Guanine Transglycosylase—Transcription of T7 transcript and TGT in the presence of preQ0 base or an aliquot of acid-soluble extract of H. volcanii was incubated at 37 °C for 1.5 h. After digestion of the T7 transcript by RNase T2, the preQ0 nucleotide was analyzed by post-labeling using T4 polynucleotide kinase and [32P]ATP (21, 35). The enzymes used (RNase T2, T4 polynucleotide kinase, and yeast hexokinase) were inactivated by phenol extraction instead of boiling. After incubation with nuclease P1, the digestion product was applied to a cellulose thin layer plate (20 × 20 cm) and was subjected to two-dimensional chromatography (15).

preparation of an acid extract of H. volcanii cells for detection of preQ0 base—H. volcanii cells were suspended in a solution of 0.2 m M formic acid and shaken for 2 h at 4 °C. After centrifugation, the supernatant was filtered through a Millipore filter. After neutralization with NaOH, soluble substances were extracted with tetrahydrofuran. The organic phase was evaporated, and the material was used for the identification of preQ0 base.

results

purification of tRNA-Guanine Transglycosylase from H. volcanii—E. coli TGT can be assayed by its ability to incorporate [8-3H]guanine into Q-unmodified tRNAs (typically unfractinated yeast tRNA, which constitutively lacks Q, is used) by replacing the guanine base located at the first position of the anticodon (20). By analogy with E. coli TGT, we searched for such an enzymatic activity in a crude extract of H. volcanii using E. coli tRNA as a substrate (see below). H. volcanii TGT was purified to near homogeneity following successive column chromatographies. Table I shows the recovery and the purification factor at each step, and Fig. 2 shows the pattern of SDS-polyacrylamide gel electrophoresis at each step. The molecular mass of the enzyme was deduced to be 78 kDa from a molecular mass of 78 kDa from a profile of the gel (Fig. 2, lane 6). Like E. coli and eukaryotic TGT, H. volcanii TGT does not require ATP for the base replacement reaction. High salt concentration (approximately 2.4

as a substrate leading to the biosynthesis of archaeosine in archaeal tRNAs.

Fig. 1. Structures of derivatives of 7-deazaguanine and 7-deazaguanosine.
Magnesium ion is required for activity with the T7 transcript as a substrate, but activity with unfractionated E. coli tRNA does not require magnesium ion. These results suggest that magnesium ion may be responsible for conformational rigidity of the tRNA, but not for the enzymatic activity itself. Optimum activity occurs near pH 7.5. Purified TGT was digested with lysylpeptidase, and the sequences of several resultant peptide fragments were determined (see below).

Unfractionated E. coli tRNAs and a T7 Transcript of *H. volcanii* tRNA Lys(CUU) Are Substrates for *H. volcanii* tRNA-Guanine Transglycosylase—To examine the specificity for tRNA substrate, we constructed a plasmid clone containing the sequence of *H. volcanii* tRNALys(CUU) and that of T7 promoter upstream of the gene. Its T7 transcript (Fig. 3A) was found to be a good substrate for the enzyme (Fig. 3B). The labeled T7 transcript was isolated, and the site at which [8-14C]guanine had been incorporated was determined by RNA sequencing to be position 15, the exclusive location of archaeosine nucleotide in archaeal tRNA. This result suggested that the enzymatic activity is involved in the biosynthesis of archaeosine nucleotide in tRNA. Unfractionated tRNA from E. coli was also found to be a good TGT substrate, whereas unfractionated *H. volcanii*, yeast, and bovine tRNAs were not (Fig. 3B), although we did not quantitatively measure the efficiency of unfractionated E. coli tRNA and of the T7 lys tran script as substrates. These results further suggest that position 15 of *H. volcanii* tRNAs is fully modified to archaeosine nucleotide.

**preQ<sub>0</sub> Base May Be the Physiological Substrate for *H. volcanii* tRNA-Guanine Transglycosylase**—The ability of various bases to serve as substrates for incorporation into tRNA by *H. volcanii* TGT was examined using the procedure of Okada et al. (21). First, the T7 transcript was labeled with [8-14C]guanine by incubation with TGT. To a reaction mixture that contained 300 pmol of [8-14C]guanine-labeled T7 transcript and the enzyme (15 units) with or without 6 nmol of each base in a final volume of 1,500 μl was prepared. After incubation at 37 °C, an aliquot of 350 μl was taken at the times specified and the radioactivity of the acid-insoluble precipitate was measured. A control experiment was performed without a tRNA substrate.

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*M. Watanabe, M. Matsuo, S. Tanaka, and N. Okada, unpublished observations.*
preQ₀ base, and not archaeosine base, since approximately 20% of archaeosine base is chemically converted to preQ₀ base after incubation of the reaction mixture under the conditions used. Furthermore, the nucleotide at position 15 of the tRNA product after incubation with archaeosine base was found to be preQ₀ nucleotide by RNA sequencing² (see “Discussion”).

preQ₀ Base Is Incorporated at Position 15 of tRNA—To investigate whether preQ₀ base is directly incorporated into tRNA, as well as whether incorporation occurs at position 15 in the D-loop, the sequence of the D-loop region in the T7 transcript after incubation with preQ₀ base was determined by the post-labeling method (33, 34). The RNA was subjected to partial digestion with alkali and the 5′ ends of resultant RNA fragments were labeled by using polynucleotide kinase and [γ-³²P]ATP, followed by separation by electrophoresis in a polyacrylamide gel (Fig. 5A). RNA was extracted from each band in the gel and digested with nuclease P₁. The resultant ³²P-labeled nucleotide 5′-monophosphate was analyzed by thin-layer chromatography. Fig. 5B shows clearly that preQ₀ base was incorporated at position 15 of the tRNA, and also shows that more than 90% of the nucleotide at position 15 is a preQ₀ nucleotide, indicating that the base-replacement reaction by H. volcanii TGT was efficient under the present conditions.

Evidence for the Occurrence of Free preQ₀ Base in H. volcanii Cells—If preQ₀ base is the physiological substrate for H. volcanii TGT, free preQ₀ base could be present in H. volcanii cells. To test this hypothesis, we prepared an acid-soluble extract of H. volcanii and incubated an aliquot of the extract with the T7 transcript of H. volcanii tRNA⁵⁰⁰⁰⁰(CUU) and H. volcanii TGT under the same conditions described in Fig. 4. After the reaction, we analyzed modified nucleotides in the treated tRNA using the post-labeling method (21, 35). As shown in Fig. 6, preQ₀ 5′-monophosphate was detected in the tRNA transcript following incubation in the presence of the acid-soluble extract (Fig. 6B), but it was not detected following incubation with the enzyme alone (Fig. 6C). Further, similar acid treatment of isolated H. volcanii tRNA did not release preQ₀, by the criterion of failure of the T7 transcript to incorporate preQ₀ when incubated with the extract and TGT.² Although archaeosine base is unstable under conditions of high temperature and high salt (see above), archaeosine appears stable when present as a nucleotide in intact tRNA (10). These results suggest that free preQ₀ base is present in H. volcanii cells and that it may serve as the physiological substrate for H. volcanii TGT (see “Discussion”; Fig. 7A).

The normal growth medium for H. volcanii (10) contains Tryptone, which, as a whole meat extract, is a source of Q nucleoside and, therefore, a potential source of preQ₀. To rule out the possibility that H. volcanii may not synthesize archaeosine de novo, tRNA was isolated from cells grown in a chemically defined (Q-free) medium (36) and analyzed for archaeosine; archaeosine content in tRNA from cells grown in the normal growth medium and in chemically defined growth medium was identical.²

H. volcanii and E. coli tRNA-Guanine Transglycosylases Are Evolutionarily Related—Recently, the complete genome sequence of the methanogenic archaeon, Methanococcus jannaschii, has been reported (37). Among 1738 protein-coding genes predicted is a putative M. jannaschii TGT gene (MJ#0436) that exhibits 30% identity to E. coli TGT (38). We determined the amino acid sequences of three peptide fragments, generated from purified H. volcanii TGT by digestion with lysylpeptidase, and compared them with the sequence of the putative M. jannaschii TGT. As shown in Fig. 8, fragments 1 and 2 from H. volcanii TGT appear to be closely related to the M. jannaschii sequence, with identities of 53.5 and 38.5%, respectively, although the C-terminal portion of fragment 3 diverges from that in M. jannaschii. These results suggest that the H. volcanii tRNA-guanine transglycosylase characterized here is the counterpart of the putative TGT whose sequence is present in M. jannaschii (37).

DISCUSSION

tRNA-Guanine Transglycosylase in H. volcanii Has Different Substrate Specificities from That of E. coli—It is well established that TGT is involved in biosynthesis of Q nucleotide in E. coli (Fig. 1E) by exchange of guanine at position 34 by preQ₀ base in tRNAs specific for Tyr, Asp, Asn, and His (20, 21; see Introduction). The resultant preQ₀ nucleotide in tRNA is then modified to the epoxide oQ by the S-adenosylmethionine-requiring enzyme QueA (22), and finally, oQ is converted to Q by an unknown vitamin B₁₂-dependent enzyme (23). These processes are schematically represented in Fig. 7B. In the present study, we provide evidence that, in contrast with the primary substrate of bacterial TGT (preQ₁), preQ₀ base is the normal
substrate for *H. volcanii* TGT. Presumably, the incorporated preQ₀ base then is further converted to archaeosine by (net) addition of ammonia, at the polynucleotide level (Fig. 7A). Therefore, both *E. coli* and *H. volcanii* TGTs catalyze a very similar reaction, namely, the exchange of guanine base in a polynucleotide chain with a free 7-deazaguanine derivative; however, their actual substrates (in terms of base, tRNAs, and the site of replacement in tRNA) are different.

**Functional Implications of 7-Deazaguanosine Nucleosides—** Archaeosine is present at position 15 (D-loop) in most archaeal tRNAs (7), whereas Q and its derivatives are present at position 34 (first position of the anticodon) of four specific tRNAs in bacteria and eukarya (19) (see Introduction). Accordingly, these conserved differences in structure and sequence location suggest differences in function. Q has been proposed to be involved in codon recognition (39) and has been shown to prevent stop codon readthrough in tobacco mosaic virus RNA in a codon context-dependent manner (40). A correlation between the presence of Q-undermodified tRNAs and frameshifts of decodons in certain mutants of *E. coli* (51), the meaning of which has not yet been rationalized (14, 21). The occurrence of these 7-deazaguanine precursor bases in both primary phylogenetic domains, archaea and bacteria, prompts us to speculate a more general role for them in cellular functions. In this respect, more detailed characterization of free preQ₀ base (and possibly free preQ₁ base) in *H. volcanii* cells is required.

**Structural Requirements of Bacterial and Archaeal TGT Enzymes for tRNA Substrates and Their Evolutionary Implications—** tRNA structural requirements for enzyme recognition remain to be identified. Preliminary experiments showed that an 18 nucleotide minihelix containing the D-loop and D-stem of *H. volcanii* tRNA₆⁵(CUU) does not serve as a substrate for the archaeal TGT. By contrast, bacterial TGT recognizes the anticodon loop sequence U₃⁻G⁴⁻U⁵, which is the minimum requirement for recognition by the enzyme, and minihelices containing this triplet sequence are good substrates for the enzyme (52, 53).

By x-ray crystallography, the tRNA-guanine transglycosylase from *Zymomonas mobilis* has been determined to be an irregular (α/β)₈ barrel with a tightly attached C-terminal zinc-containing subdomain (54). Further, the structure of *Z. mobilis* TGT in complex with preQ₁ suggests a binding mode for tRNA where the phosphate backbone interacts with the zinc subdomain and the U₃⁻G⁴⁻U⁵ sequence is recognized by the barrel. The zinc binding motif (CXXC₉CXX₉H) is highly conserved in prokaryotic TGTs known so far (52), and the homologous region in *M. jannaschii* is (CXXC₉CXX₉H). These results demonstrate a structural and functional conservation of the bacterial and archaeal/eukaryotic TGT binding mode with tRNA, despite archaeal modification of the D-loop and bacterial/eukaryotic modification of the anticodon loop. The utilization of 7-deazaguanine derivatives for tRNA processing by interrelated TGT enzymes suggests an evolutionarily fundamental role for 7-deazaguanine.

In contrast to bacterial TGT (52, 55), productive recognition of tRNA by eukaryotic TGT requires not only the U₃⁻G⁴⁻U⁵ sequence of the anticodon loop but also a correctly folded tRNA.
architecture (56). In addition, eukaryotic TGT is believed to be a heterodimer, although this is not conclusive at present (44, 57). More detailed examination of the substrate recognition properties of TGTs from archaea, bacteria, and eukaryotes will elucidate the domain structures of these proteins for the tRNA binding site, as well as further define their evolutionary relationship.

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