Importance of the Anticodon Sequence in the Aminoacylation of tRNAs by Methionyl-tRNA Synthetase and by Valyl-tRNA Synthetase in an Archaeabacterium*

Vaidyanathan Ramesh and Uttam L. RajBhandary‡

From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

The mode of recognition of tRNAs by aminoacyl-tRNA synthetases and translation factors is largely unknown in archaeabacteria. To study this process, we have cloned the wild type initiator tRNA gene from the moderate halophilic archaeabacterium Haloflexas volcanii and mutants derived from it into a plasmid capable of expressing the tRNA in these cells. Analysis of tRNAs in vivo show that the initiator tRNA is aminoacylated but is not formylated in H. volcanii. This result provides direct support for the notion that protein synthesis in archaeabacteria is initiated with methionine and not with formylmethionine. We have analyzed the effect of two different mutations (CAU→CUA and CAU→GAC) in the anticodon sequence of the initiator tRNA on its recognition by the aminoacyl-tRNA synthetases in vivo. The CAU→CUA mutant was not aminoacylated to any significant extent in vivo, suggesting the importance of the anticodon in aminoacylation of tRNA by methionyl-tRNA synthetase. This mutant initiator tRNA can, however, be aminoacylated in vitro by the Escherichia coli glutaminyl-tRNA synthetase, suggesting that the lack of aminoacylation is due to the absence in H. volcanii of a synthetase which recognizes the mutant tRNA. Archaeabacteria lack glutaminyl-tRNA synthetase and utilize a two-step pathway involving glutamyl-tRNA synthetase and glutamine amidotransferase to generate glutaminyl-tRNA. The lack of aminoacylation of the mutant tRNA indicates that this mutant tRNA is not a substrate for the H. volcanii glutamyl-tRNA synthetase. The CAU→GAC anticodon mutant is most likely aminoacylated with valine in vivo. Thus, the anticodon plays an important role in the recognition of tRNA by at least two of the halobacterial aminoacyl-tRNA synthetases.

The sequence and/or structural determinants in the anticodon and in the acceptor stem of tRNAs play an important role in discrimination among tRNAs by aminoacyl-tRNA synthetases. The crystal structure analysis of several aminoacyl-tRNA synthetase-tRNA complexes from eubacteria and eukarya has provided a substantial amount of information on the molecular details of interactions involving these determinants (1–5). However, little is known either at the biochemical level or at the structural level on interaction between aminoacyl-tRNA synthetases and tRNA in archaea. The results of recent work, spurred by a knowledge of the complete genome sequences of several archaea, have highlighted some interesting and surprising differences between three archaeal aminoacyl-tRNA synthetases and their eubacterial and eukaryal counterparts. First, in contrast to lysyl-tRNA synthetases from eubacteria and eukarya, which in general belong to Class II (5), lysyl-tRNA synthetase from Methanococcus jannaschii belongs to Class I (6, 7). Second, a single polypeptide of M. jannaschii has the activity (8, 9) of both a cysteinyl-tRNA synthetase and prolyl-tRNA synthetase. Third, the M. jannaschii tyrosyl-tRNA synthetase has a truncated C-terminal region and lacks most of the tRNA anticodon binding region seen in tyrosyl-tRNA synthetase from eubacteria and eukarya. This finding has led to the suggestion (10, 11) that the anticodon of the M. jannaschii tyrosine tRNA is less important for aminoacylation compared with the anticodon of tRNA3CV from eubacteria and eukarya.

In view of the differences found in the three aminoacyl-tRNA synthetases from M. jannaschii, it is important to study the mode of recognition of tRNAs in general by aminoacyl-tRNA synthetases in these and other archaeal systems. Here, we have studied this process in vivo using the halophilic archaeon Haloferax volcanii. We show that the anticodon sequence in the tRNA plays an important role in the recognition of tRNAs by at least two of the aminoacyl-tRNA synthetases, the methionyl-tRNA synthetase (MetRS)1 and the valyl-tRNA synthetase (ValRS). This finding provides direct support for the commonly held notion that protein synthesis in archaeabacteria does not require formylation of the initiator tRNA (12–14).

EXPERIMENTAL PROCEDURES

Strain, Plasmids, and Extracts—The strain H. volcanii WFD11 and the plasmids pUCspProM and pWl201 (15, 16) were kindly provided by Drs. John R. Palmer and Charles J. Daniels, Department of Microbiology, The Ohio State University, Columbus, OH. The Escherichia coli strains used in this work are E. coli XLI-blue (recA1 endA1 gyrB6 thi-1 hisD17 supE44 relA1 lacZΔ157 F− proAB lacZΔM15 Tn10), E. coli GM2163 (F− ara-14 leuB6 thi-1 thiA31 lacY1 tsc-78 galK2 galT22 supE44 hisG4 rpsL136 (Strr) xyl-5 met-l1 dam14:Thr9 (Camr) dcen-6 merC1 hsdR2 rRs− mK−), E. coli M15 Tn10 (Z− D− recA1 endA1 gyr96 thi-1 hisG4 rpsL136 (Strr) xyl-5 met-l1 dam14:Thr9 (Camr) dcen-6 merC1 hsdR2 rRs− mK−), H. volcanii S40 extract was a kind gift of Dr. Mechthild Pohlschroeder, University of Pennsylvania, Philadelphia, PA.

Isolation of H. volcanii Genomic DNA—H. volcanii WFD11 was grown in a medium containing the following components per liter: 125 g of NaCl, 45 g of MgCl2·6H2O, 10 g of MgSO4·7H2O, 10 g of KCl, 1.34 g of CaCl2·2H2O, 3 g of yeast extract, 5 g of tryptone (17). Cells from a 30-ml culture (grown for 48 h with 4% inoculum at 37 °C with aeration) were harvested and suspended in 9.0 ml of 50 mM Tris-HCl (pH 8.0), 25 mM Na2EDTA by vortexing. Significant lysis was observed during this step.

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‡ To whom correspondence should be addressed. Tel.: 617-253-4702; Fax: 617-252-1556; E-mail: bhandary@mit.edu.

1 The abbreviations used are: MetRS, methionyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; MTF, methionyl-tRNA formyltransferase; GlnRS, glutaminyl-tRNA synthetase.
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step, and lysis was taken to completion with the addition of SDS to a final concentration of 1%. The lysate was extracted with an equal volume of phenol (saturated with 100 mM Tris-HCl (pH 8.0)) by gentle mixing (inversions in a cycle mixer at low rpm for 15 min at room temperature). The aqueous phase was collected and reextracted with phenol/water more and then twice with chloroform/isoamylalcohol (24/1). The aqueous phase was collected, and sodium acetate (pH 5.4) was added to a final concentration of 0.3 M followed by addition of chilled ethanol. The genomic DNA was spooled, as it precipitated, onto a sterile glass rod, rinsed with 70% ethanol, air dried, and gently suspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE) buffer.

Cloning of the Initiator tRNA Gene—Based on the sequence of the tRNA\textsuperscript{Met} locus provided by Dr. C. J. Daniels, the following primers that contained the sites for XbaI and BamHI (italicized) were designed for polymerase chain reaction amplification of the tRNA\textsuperscript{Met} gene: HvMiF1, 5'-ACATCTTGACTGTGTTGATTCCGGCGGGA-3'; HvMiR1, 5'-CTCCGGATCCCGAGTTAGGCTCGGAAACCA-3'. The primers (100 pmol each) were used to amplify the tRNA\textsuperscript{Met} gene using H. volcanii genomic DNA as template under the following conditions: 95 °C/1 min initial denaturation followed by 20 cycles, with each cycle containing the steps 95 °C/1 min, 50 °C/1 min, and 72 °C/1 min. This was followed by incubating the tubes at 72 °C for 5 min. The purified polymerase chain reaction product was digested with XbaI and BamHI and cloned into the plasmid pUCpSProm at these sites to generate pUCpSProm\textsuperscript{Met}. The mutations in the anticodon of the tRNA\textsuperscript{Met} (U35A36 and G34C36) were generated using pUCpSProm\textsuperscript{Met}, as template by the Quik Change mutagenesis procedure. The smaller HindIII + EcoRI fragment containing the initiator tRNA gene and its mutants from the pUCpSProm\textsuperscript{Met}, plasmid was subcloned into the shuttle plasmid pWL201 at these sites to generate pWL201HvMet\textsuperscript{+}, pWL201HvMet\textsuperscript{G34C36}, and pWL201-HvMet\textsuperscript{G34C36}, respectively. All initial cloning was done in E. coli XL-1 blue. After confirming the sequence of the desired wild type and mutant initiator tRNA genes, the respective plasmids were used to transform E. coli GM2163. The plasmid DNA isolated from this strain (lacking adenine methylation) was then used to transform H. volcanii.

Transformation of H. volcanii—The protocol was adapted from Cline et al. (18). All operations were at room temperature. Cells from a 50-mL culture grown to an A\textsubscript{550nm} of ~1.0 (25 h at 37 °C) were harvested and gently suspended in 5 mL of spheroplasting buffer (50 mM Tris-HCl (pH 8.3), 0.5 M NaCl, 30 mM KCl, 15% sucrose, 15% glycerol). To 200 μL of the cell suspension, 20 μL of 0.5 M Na\textsubscript{EDTA} (pH 8.0) was added and gently mixed. Plasmid DNA (1 μg) in 20 μL of spheroplasting buffer was added and mixed gently. An equal volume (240 μL) of polyethylene glycol solution (60% polyethylene glycol-600 w/v in spheroplasting buffer) was then added and the mixture was gently mixed and incubated for 20 min at room temperature. The cells were diluted by adding 9 mL of spheroplast dilution buffer (50 mM Tris-HCl (pH 7.2), 3.4 M NaCl, 175 mM MgSO\textsubscript{4}, 30 mM KCl, 5 mM CaCl\textsubscript{2}, 15% sucrose), harvested by centrifugation, and resuspended in 1 mL of a 1:1 mixture of spheroplast dilution buffer and H. volcanii growth medium. The transformational plasmids were allowed to recover at 37 °C with mild aeration for 12 h. Appropriate aliquots were then treated with molten top agar (55 °C) and poured onto agar plates. The contents of the plates were as follows: bottom agar (per liter), 50 mmol Tris-HCl (pH 7.2), 180 g of NaCl, 43.8 g of MgSO\textsubscript{4}, 2.5 g of KCl, 0.7 g of CaCl\textsubscript{2}·2H\textsubscript{2}O, 3 g of yeast extract, 5 g of tryptone, and 15 g of agar; top agar, same as above except for 5 g/liter agar and 4 mg/liter mevinolin. Mevinolin was converted to the sodium salt as described (19). The plates were incubated at 37 °C for 7 days.

Isolation of Total tRNA under Acidic Conditions—The mevinolin-resistant transformants of H. volcanii were grown in liquid medium in the presence of mevinolin (4 mg/liter) for 3 days. The cells from a 3.0-mL culture were chilled on wet ice. All subsequent steps were carried out in the cold. The cells were harvested, resuspended in 0.3 M 3 NaOAc (pH 4.5) and 10 mM Na\textsubscript{EDTA}, and subjected to two extractions (1 min each) with equal volumes of phenol equilibrated with the same buffer. Total nucleic acids were recovered from the aqueous phase by mixing with 2.5 volumes of ethanol and centrifugation. The pellet was washed with 70% ethanol and dissolved in 20 μL of 10 mM Na\textsubscript{OAc} (pH 4.5), 1 mM Na\textsubscript{EDTA}.

Detection of tRNAs by Northern Blotting—tRNA (0.1 A\textsubscript{260 unit}) was subjected to electrophoresis on acid-urea polyacrylamide gels and electroblotted to a nylon membrane (NiGen Plus membrane; Schleicher & Schuell) as described (20). The membrane was washed with 4× SET (1× SET: 0.05 M NaCl, 0.03 M Tris-HCl, 2 mM Na\textsubscript{EDTA} (pH 8.0) containing 1% SDS), baked at 70 °C for 90 min, and prehybridized at 42 °C in 4× SET containing 250 μg/mL sheared salmon sperm DNA, 1% SDS, and 10× Denhardt's solution (1× Denhardt's solution: 0.02% polyvinylpyrrolidone 40, 0.02% bovine serum albumin, and 0.02% Ficoll). tRNAs were detected by hybridization to 5'-\textsuperscript{32P}labeled oligonucleotide probes complementary to nucleotides 29–47 of the wild type and mutant initiator tRNAs, 26–55 of H. volcanii tRNA\textsuperscript{G43C63}, and 34–47 of H. volcanii tRNA\textsuperscript{G44C64} (21, 22). Hybridization was performed in the same solution at 42 °C for 12 h. The membrane was washed twice (20 min each) with 2× SET and 0.2% SDS at 42 °C. For detecting the mutant tRNAs, the blot was washed with 0.75× SET, 0.05% SDS at 55 °C for 20 min. This washed eliminated the signal from the probe hybridizing to the wild type initiator tRNA\textsuperscript{Met}.

Measurement of Rates of Decacylation of Aminoacyl-tRNAs—Total tRNA was prepared from H. volcanii/pWL201HvMet/G34C36 under acidic conditions as described above. About 2 A\textsubscript{260 unit} units of tRNA in 10 μL were treated with 0.2 M Tris-HCl (pH 9.6). Aliquots (1 μL) were withdrawn after 15, 30, 60, 90, 120, and 180 min, mixed with 2 μL of 0.3 M NaOAc (pH 4.5), and snap-frozen on dry ice (23). At the end of all the time points, the volume was made up to 10 μL with 10 mM NaOAc (pH 4.5). For analyzing the decacylation of Met-tRNA\textsuperscript{Met}, the experimental conditions were the same except that incubation times were shorter: 5, 5.5, 7, 10, and 15 min. An aliquot corresponding to 0.05 A\textsubscript{260 unit} of tRNA from each time point was analyzed by acid-urea polyacrylamide gel electrophoresis, and the tRNAs were detected by Northern blot analysis using appropriate hybridization probes as described above.

Isolation of Total tRNAs—Total tRNAs were prepared from 900 mL of H. volcanii harboring the pWL201 series of plasmids containing the respective tRNA genes. The cells were grown for 60 h in the presence of 4 mg/liter mevinolin. The cells were harvested, resuspended first in 4.5 mL of 3 M NaOAc (pH 5.4), and then made up to 40 mL with TE buffer. The suspension was extracted with an equal volume of phenol saturated with the same buffer by shaking at room temperature for 20 min. The aqueous phase was collected and mixed with 2.5 volumes of ethanol, and the nucleic acids were recovered by centrifugation. The pellet was washed with 70% ethanol, dried, and resuspended in 12 mL of the TE buffer. High molecular weight nucleic acids were precipitated by adding 3 mL of 5 M NaCl. Total tRNA was recovered from the supernatant by ethanol precipitation. The pellet was washed with 70% ethanol, dried, and resuspended in 15 mL of TE buffer containing 0.1 M NaCl. The tRNAs were further purified by DEAE-cellulose chromatography (24).

FIG. 1. Top, schematic diagram of eubacterial and eukaryal initiator tRNAs. The unique features in the eubacterial (left) and eukaryal (right) initiator tRNAs are highlighted by arrows. Bottom, sequence of the H. volcanii initiator tRNA in cloverleaf form. The mutations introduced into the anticodon sequence CAU (boxed) are indicated by arrows emanating from it. Also highlighted (arrows) are features common to archaean initiator tRNAs.
In Vitro Aminoacylation and Formylation of tRNA—Total tRNA (0.6 A260 unit) prepared from *H. volcanii* /pWL201HvMet, cells as described above was aminoacylated with methionine using *E. coli* MetRS or was aminoacylated with methionine and subsequently formylated using *E. coli* methionyl-tRNA formyltransferase (MTF). The reaction mixture was extracted with phenol equilibrated with 10 mM NaOAc (pH 4.5). The tRNA was precipitated and analyzed on acid-urea polyacrylamide gels and detected by Northern blot hybridization with the tRNA sequence-specific DNA probes. Details are given under “Experimental Procedures.”

RESULTS AND DISCUSSION

The *H. volcanii* Initiator tRNA—In the absence of an archaeal *in vitro* protein synthesis system that can translate a natural mRNA or in the absence of any genetic analysis involving components of the translational initiation machinery, identification of the initiator tRNA in halobacteria is based on the ability of the tRNA to be aminoacylated with *E. coli* MetRS and to be formylated by the *E. coli* MTF and the ability of the fMet-tRNA to initiate protein synthesis in an *E. coli* cell-free system (24). These were also the criteria used to identify the initiator tRNA species in eukaryal such as yeast (25), mammalian cells (26), and fungal mitochondria (27) prior to their establishment as initiator tRNAs in eukaryal *in vitro* protein synthesis systems. Based on these criteria, the sequence of initiator tRNA and/or the gene for the initiator tRNA is known from the following nine archaea: *Archaeoglobus fulgidus* (GenBank™ accession number NC000917), *Halococcus morrhuae* (28), *H. volcanii* (21), *Methanobacterium thermoautotrophicum* (GenBank™ accession number NC000916), *M. jannaschii* (GenBank™ accession number NC000909), *Pyrococcus abyssi* (GenBank™ accession number AL096836), *Pyrococcus horikoshii* (GenBank™ accession number NC000961), *Sulfolobus acidocaldarius* (28), and *Thermoplasma acidophilum* (28).

Plasmid-directed Expression in Vivo of the *H. volcanii* Initiator tRNA Gene—To study the effect, *in vivo*, of mutations in the anticodon sequence of initiator tRNA, we cloned the initiator tRNA gene of *H. volcanii* in the plasmid pWL201 (15, 16). This shuttle vector contains a *bla* gene coding for resistance to ampicillin, an *ori* for maintenance of the plasmid in *E. coli*, another *ori* for maintenance of the plasmid in *H. volcanii*, and a gene coding for a mutant 3-hydroxy-3-methylglutaryl-CoA reductase, which confers resistance of *H. volcanii* to mevinolin. The expression of the initiator tRNA in the resulting plasmid pWL201HvMet, is driven by the *H. volcanii* tRNA$^{Met}$ promoter. This plasmid was isolated from *E. coli* XL1-blue strain and used to transform *H. volcanii* WFD11, and the transformants
were selected in the presence of mevinolin. To establish the presence of plasmids in the mevinolin-resistant transformants, an attempt was made to isolate plasmid DNA from these cells. The preparation failed to yield ampicillin-resistant transformants when introduced into the E. coli XL1-blue strain. Plasmid DNA was also not detected in this preparation upon agarose gel electrophoresis and ethidium bromide staining. These results suggest that the mevinolin-resistant transformants of H. volcanii do not contain the plasmids bearing the marker 3-hydroxy-3-methylglutaryl-CoA reductase gene and that the resistance is most likely due to integration of the plasmid by homologous recombination of the mutant 3-hydroxy-3-methylglutaryl-CoA reductase gene with the chromosomal gene. Similar observations were made by Holmes et al. (29), who reported the existence of a restriction barrier between E. coli and H. volcanii. This restriction can be avoided by using DNA lacking the N⁶-methyl adenosine modification within the GATC sequences. For this purpose, the pWL201HvMet, plasmid was used to transform the dam⁻ E. coli GM2163 strain, which is deficient in the N²-adenine methyl transferase. When H. volcanii cells were transformed with the plasmid isolated from the dam⁻ E. coli strain, the efficiency of transformation improved by at least one order of magnitude. The mevinolin-resistant transformants could be maintained and subcultured repeatedly without loss of the plasmid. The plasmid isolated from these transformants also yielded ampicillin-resistant colonies when introduced into E. coli XL1-blue. Based on these results suggesting the stable maintenance of plasmids containing the wild type initiator tRNA gene in H. volcanii, we generated the constructs containing the U35A36 and G34C36 mutations in the GATC sequence of the initiator tRNA.

**Initiator tRNA Is Not Formylated in H. volcanii**—A feature unique to all initiator tRNAs is the presence of three consecutive G:C base pairs in the acceptor stem (Fig. 1; also see Ref. 14 for a review). Other features unique to eubacterial initiator tRNAs include a mismatch between positions 1 and 72 at the end of the acceptor stem and a R¹¹·Y²⁴ base pair in the D-stem (30). Features unique to the eukaryal initiator tRNAs include a A¹·U⁷² base pair at the end of the acceptor stem and A⁵⁴ and A⁶⁰ in the T-loop. The archaeal initiator tRNAs have an A¹·U⁷² base pair at the end of the acceptor stem found in the eukaryal initiator tRNAs and a R¹¹·Y²⁴ base pair in the D-stem found in the eubacterial initiator tRNAs (2, 28).

Initiator tRNA is present predominantly as fMet-tRNA⁰⁰⁻Met in eubacteria and as Met-tRNA⁰⁰⁻Met in eukarya. Two of the unique features of bacterial initiator tRNAs, the 1x72 mismatch and the R¹¹·Y²⁴ base pair, play a role in formylation of the E. coli initiator Met-tRNA by E. coli MTF (31–33). Previous studies showed that extracts of Halobacterium cutirubrum, an extreme halophile, lacks MTF activity. Furthermore, tRNA isolated from [³⁵S]methionine-labeled halobacterial cells contained Met-tRNA but no fMet-tRNA (12). These findings, combined with the more recent finding from the genome sequence of several archaea (GenBank™ accession numbers NC000917, NC000916, NC000909, AL096836, and NC000961) that they lack a protein with homology to eubacterial MTFs, have led to the widespread notion that archaea initiate protein synthesis without formylation of the initiator tRNA. Here, we have investigated directly the in vivo state of the H. volcanii initiator tRNA with a probe specific to this tRNA (Fig. 2). For use as markers, total tRNA isolated from these cells was aminoacylated in vitro with E. coli MetRS (Fig. 2A, lane 2) or aminoacylated with MetRS and subsequently formylated with E. coli MTF (lane 3) and subjected to electrophoresis on the same gel. As for the E. coli initiator tRNA⁰⁰⁻Met (20), all three forms of the H. volcanii initiator tRNA, the tRNA⁰⁰⁻Met, the Met-tRNA⁰⁰⁻Met, and the fMet-tRNA⁰⁰⁻Met, are separated clearly from one another (Fig. 2A, lanes 1, 2, and 3, respectively). The mobility of H. volcanii initiator tRNA isolated from the cells under acidic conditions (Fig. 2A, lane 5) shows that the tRNA⁰⁰⁻Met is quantitatively aminoaacylated; however, there was no detectable accumulation of any fMet-tRNA⁰⁰⁻Met species. This result provides direct support to the notion that protein synthesis in halobacteria is initiated with methionine and not with formylmethionine.

**Fig. 2B, lane 2** shows that the endogenous H. volcanii initiator tRNA was also not formylated in vivo. Thus, the lack of formylation of the H. volcanii initiator tRNA (Fig. 2A, lane 5) is not due to a substantial overproduction of the initiator tRNA in cells carrying the pHW201HvMet plasmid overwhelming the limiting amounts of a putative Met-tRNA, formyltransferase activity in H. volcanii. Further evidence for this is also derived from the results of a Northern blot analysis similar to that in Fig. 2B, lanes 2 and 4, which show that the initiator tRNA was overproduced only to the extent of 50% in H. volcanii cells carrying the pHW201HvMet, plasmid over the endogenous initiator tRNA.

**The CUA Anticodon Mutant Is Aminoaacylated Extremely Poorly in Vivo**—The plasmid pHW201HvMet, U35A36 contain-
ing the U35A36 anticodon sequence mutant initiator tRNA gene (tRNA<sup>Met</sup> (CUA)) was used to transform <i>H. volcanii</i>. The transformants were analyzed for the expression of tRNA by acid-urea polyacrylamide gel electrophoresis followed by Northern blot analysis using a probe specific to the mutant tRNA. As expected, a band corresponding to the U35A36 mutant tRNA<sup>Met</sup> was found only in cells containing the plasmid pWL201HvMet,U35A36 (Fig. 3A, compare lanes 1 and 2 to lanes 3 and 4). However, based on PhosphorImager analysis, only ~8% of the mutant tRNA was aminoacylated in <i>vivo</i> (Fig. 3A, lane 4). In contrast, the serine tRNA, which was used as an internal control, was fully aminoacylated (Fig. 3A, lanes 2 and 4). Thus, a two-nucleotide change in the anticodon sequence of the <i>H. volcanii</i> initiator tRNA results in a tRNA that is aminoacylated extremely poorly. These results suggest that, as in eubacteria and eukarya, the anticodon sequence of a tRNA is important for its aminoacylation by the <i>H. volcanii</i> MetRS. Whether the residual aminoacylation of the mutant tRNA, to the extent of ~8%, is with methionine or another amino acid is not known.

In <i>E. coli</i>, the corresponding <i>E. coli</i> U35A36 mutant initiator tRNA is aminoacylated with glutamine by GlnRS (34, 35). To determine whether the U35A36 mutant tRNA<sup>Met</sup> expressed in <i>H. volcanii</i> can be aminoacylated with glutamine in <i>vivo</i>, we isolated tRNAs from the cells transformed with the plasmid pWL201HvMet,U35A36. The total tRNA preparation was aminoacylated with glutamine using <i>E. coli</i> GlnRS, and the products were analyzed by acid-urea polyacrylamide gel electrophoresis followed by Northern blot analysis (Fig. 3B). The results show that the U35A36 mutant tRNA<sup>Met</sup> can be aminoacylated in <i>vivo</i> by the <i>E. coli</i> GlnRS (Fig. 3B, lanes 2 and 3). Thus, the lack of aminoacylation of the <i>H. volcanii</i> mutant initiator tRNA in <i>vivo</i> is due to the absence of an aminoacyl-tRNA synthetase that recognizes this mutant initiator tRNA.

Archaea lack GlnRS and, like most Gram-positive eubacteria (36), use a two-step pathway to generate Gln-tRNA (21, 37). This pathway (the glutamyl-tRNA synthetase-glutamine amidotransferase pathway) involves aminoacylation of the tRNA with glutamic acid followed by conversion of the glutamic acid on the tRNA to glutamine using the enzyme glutamine amidotransferase (38). The finding that the U35A36 mutant of the <i>H. volcanii</i> initiator tRNA is mostly not aminoacylated in <i>vivo</i> also suggests that the mutant tRNA is a poor substrate for the <i>H. volcanii</i> glutamyl-tRNA synthetase.

The GAC Anticodon Mutant Is Most Likely Aminoacylated with Valine—A second anticodon mutant initiator tRNA gene (G34C36 mutant) containing the GAC anticodon was constructed, and the resulting plasmid pWL201HvMet,G34C36 was used to transform <i>H. volcanii</i>. Expression of the G34C36 mutant initiator tRNA (tRNA<sup>Met</sup> (GAC)) was analyzed by acid-urea Northern blotting using an oligonucleotide probe specific to the mutant tRNA. The G34C36 mutant tRNA was detected only in cells containing the pWL201HvMet,G34C36 plasmid (Fig. 4, compare lanes 1 and 2 to lanes 3 and 4). Approximately 45% of the mutant initiator tRNA was aminoacylated in <i>H. volcanii</i> (Fig. 4, lane 4). The tRNA isolated from the cells was deacylated with 0.25 M Tris-HCl (pH 9.6) for 20 min at 37 °C. Whereas the <i>H. volcanii</i> Ser-tRNA<sup>Met</sup> was completely deacylated under these conditions, up to 30% of the <i>H. volcanii</i> G34C36 mutant tRNA<sup>Met</sup> was still aminoacylated (Fig. 4, lane 3).

The very slow rate of deacylation of the G34C36 mutant aminoacyl-tRNA suggests that the tRNA is not aminoacylated with methionine or most of the other amino acids in <i>H. volcanii</i> (23, 39, 40). It is known that the nature of the amino acid attached to the tRNA determines the rate of deacylation of the aminoacyl-tRNA, with valine and isoleucine having the slowest rates of deacylation (23, 40). The G34C36 mutant initiator tRNA has the anticodon sequence (GAC) corresponding to that of valine tRNA, and it is known that in eubacteria and eukarya, the anticodon sequence is important for aminoacylation of the tRNA with valine (41). Therefore, it is most likely that the G34C36 mutant initiator tRNA is aminoacylated with valine in <i>H. volcanii</i>. To investigate this further, total tRNA isolated from <i>H. volcanii</i>/pWL201HvMet,G34C36 cells under acidic conditions was subjected to deacylation in 0.2 M Tris-HCl (pH 9.6) at 37 °C. The time course of deacylation of the G34C36 mutant aminoacyl-tRNA was compared with those of the <i>H. volcanii</i> Met-tRNA<sup>Met</sup>, Val-tRNA<sup>Val</sup>, and Ile-tRNA<sup>Ile</sup>. Deacylation was followed by withdrawing aliquots of the reaction mixture at intervals and analyzing the samples by acid-urea gel electrophoresis followed by Northern blot analysis using probes specific for the endogenous tRNA<sup>Met</sup>, tRNA<sup>Val</sup>, and the G34C36 mutant tRNA<sup>Met</sup> (23). The results are shown in Fig. 5. A plot of the residual aminoacyl-tRNA <i>versus</i> time (Fig. 6), based on the PhosphorImager analysis of pixels in the tRNA and aminoacyl-tRNA bands, shows that the half-lives of deacylation of the aminoacyl-tRNAs are ~7 min (Met-tRNA<sup>Met</sup>), ~52 min (Val-tRNA<sup>Val</sup>), and ~48 min (the G34C36 mutant aminoacyl-tRNA<sup>Met</sup>). The extreme closeness of the half-life of the G34C36 mutant aminoacyl-tRNA<sup>Met</sup> to that of Val-tRNA<sup>Val</sup> indicates that the amino acid attached to the tRNA is not methionine but, most likely, valine. These results suggest that the anticodon sequence of a tRNA is also important for its aminoacylation by the <i>H. volcanii</i> ValRS.

Further evidence that the G34C36 mutant initiator tRNA is aminoacylated with valine was obtained by a comparison of the valine and isoleucine acceptor activities of total tRNA isolated from cells expressing the G34C36 mutant initiator tRNA. Fig. 7 shows a time course of aminoacylation of tRNAs with valine (left) and with isoleucine (right) using cell-free extracts of <i>H. volcanii</i>. Total RNA isolated from cells expressing the G34C36 mutant initiator tRNA shows an increase in valine acceptor activity compared with the endogenous tRNA (Fig. 7, left) but no increase in isoleucine acceptor activity (Fig. 7, right) or in methionine acceptor activity (data not shown). These
results highlight the importance of the anticodon in aminoaacylation of the tRNA by H. volcanii ValRS. The slower rate of aminoaacylation of the G34C36 mutant initiator tRNA with valine compared with the endogenous tRNA\textsuperscript{Val} suggests that, whereas the anticodon sequence is important for aminoaacylation of a tRNA by the H. volcanii ValRS, there are other determinants that are also important and that are lacking in the G34C36 mutant initiator tRNA. This result is also consistent with the finding that only ~45% of the G34C36 mutant initiator tRNA is aminoaacylated in H. volcanii (Fig. 4, lane 4).

Conclusion—As a first step toward studying the role of the anticodon sequence in aminoaacylation of tRNAs by aminoacyl-tRNA synthetases in an archaebacterium, we have described the expression and analysis in vivo of two anticodon sequence mutants of the H. volcanii initiator tRNA. We show that the anticodon sequence is important for aminoaacylation by at least two of the H. volcanii aminoacyl-tRNA synthetases, the MetRS and the ValRS. These results are similar to those for the corresponding euabacterial and eukaryal enzymes. It will be interesting to extend these studies to other anticodon sequence mutants to see whether the relative importance of the anticodon sequence for aminoaacylation of a tRNA by the archaean aminoacyl-tRNA synthetases is generally similar to those of the corresponding euabacterial and eukaryal aminoacyl-tRNA synthetases.

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REFERENCES

1. Rouxd, M. A., Pernony, J. L., Soll, D., and Steitz, T. A. (1989) Science 246, 1089–1212
2. Ruff, M., Krainnasawamy, S., Boeglin, M., Poterszman, A., Mitschler, A., Podjarny, A., Rees, B., Thierry, J. C., and Moras, D. (1991) Science 252, 1682–1689
3. Bion, V., Yaremchuk, A., Takalo, M., and Cusack, S. (1994) Science 263, 1404–1410
4. Cusack, S., Yaremchuk, A., Krikiliviy, I., and Takalo, M. (1998) Structure 6, 101–108
5. Arnow, J. G., and Moros, D. (1997) Trends Biochem. Sci. 22, 211–216
6. Ibbas, M., Morgan, S., Curnow, A. W., Prindmore, D. R., Vothknecht, U. C., Gardner, W., Lin, W., Woest, C. R., and Soll, D. (1997) Science 276, 1119–1122
7. Ibbas, M., Iose, H. C., Kawarabayasi, Y., Kikuchi, H., bunjun, S., and Soll, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 418–423
8. Stathopoulos, C., Li, T., Longman, R., Vothknecht, U. C., Becker, H. D., Ibbas, M., and Soll, D. (2000) Science 287, 479–482
9. Lipman, R. S. A., Sowers, K. R., and Hou, Y.-M. (2000) Biochemistry 39, 7792–7798
10. Steer, B. A., and Schimmel, P. (1999) J. Biol. Chem. 274, 35601–35606
11. Steer, B. A., and Schimmel, P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13644–13649
12. White, B. N., and Bayley, S. T. (1972) Biochem. Biophys. Acta 272, 583–587
13. Bayley, S. T., and Morton, R. A. (1978), in CRC Critical Reviews in Microbiology, CRC Press, Inc., Boca Raton, FL pp. 151–205
14. RajBhandary, U. L., and Chow, C. M. (1989) in RNA: Structure, Biosynthesis, and Function (Soll, D., and RajBhandary, U. L., eds) pp. 511–528, American Society of Microbiology, Washington, D. C.
15. Lam, W. L., and Doehlert, W. F. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5478–5482
16. Nieuwlandt, D. T., and Daniels, C. J. (1990) J. Bacteriol. 172, 7104–7110
17. Daniels, C. J., Mc Kee, A. H. Z., and Doehlert, W. F. (1990) EMBO J. 3, 745–749
18. Cline, S. W., Lam, W. L., Charlebois, R. L., Schulkwyk, L. C., and Doehlert, W. R. (1998) Can. J. Microbiol. 35, 145–152
19. Kita, T., Brown, M. S., and Goldstein, J. L. (1980) J. Clin. Invest. 66, 1094–1106
20. Varshney, U., Lee, C. P., and RajBhandary, U. L. (1991) J. Biol. Chem. 266, 24712–24718
21. Gupta, R. (1984) J. Biol. Chem. 259, 9461–9471
22. Steinberg, S., Misch, A., and Sprinzl, M. (1993) Nucleic Acids Res. 21, 3011–3015
23. Drabkin, H., and RajBhandary, U. L. (1998) Mol. Cell. Biol. 18, 5140–5147
24. RajBhandary, U. L., and Ghosh, H. P. (1986) J. Biol. Chem. 244, 1104–1113
25. Housman, D., Jacob-Lorena, M., RajBhandary, U. L., and Lodish, H. F. (1970) Nature 227, 913–916
26. Smith, A. E., and Markker, K. (1970) Nature 226, 607–609
27. Heckman, J. E., Hecker, L. I., Schwartzbach, S. D., Barnett, W. E., Baumstark, B., and RajBhandary, U. L. (1978) Cell 1, 183–185
28. Kuchino, Y., Ibara, M., Yabasuki, Y., and Nishimura, S. (1982) Nature 298, 684–685
29. Holmes, M. L., Nuttal, S. D., and Dyall-Smith, M. L. (1991) J. Bacteriol. 173, 3807–3813
30. RajBhandary, U. L. (1994) J. Bacteriol. 176, 547–552
31. Lee, C.-P., Seong, B. L., and RajBhandary, U. L. (1991) J. Biol. Chem. 266, 18012–18017
32. Ramesh, V., Varshsney, U., and RajBhandary, U. L. (1997) RNA (N.Y.) 3, 1220–1232
33. Guilhon, J. M., Meinel, T., Mechulam, Y., Lazennee, C., Blanqulet, S., and Fayat, G. (1992) J. Mol. Biol. 224, 359–367
34. Schulman, L. H., and Pelka, H. (1985) Biochemistry 24, 7309–7314
35. Varshney, U., and RajBhandary, U. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1568–1590
36. Wilcox, M., and Nirenberg, M. W. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 229–236
37. White, B. N., and Bayley, S. T. (1972) Can. J. Biochem. 50, 600–609
38. Curnow, A. W., Hong, K., Yuan, R., Kim, S., Martins, O., Winkler, W., Henkin, T. M., and Soll, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11819–11826
39. Li, Y., Holmes, B. W., Appling, D. R., and RajBhandary, U. L. (2000) J. Bacteriol. 182, 2886–2889
40. Mattheai, J. H., Voigt, H. P., Heller, G., Neth, R., Schoch, G., Kubler, H., Amaelunzeu, G., Sander, G., and Parmeggiani, A. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 25–38
41. Schulman, L. H., and Pelka, H. (1988) Science 242, 765–768
