

An Operational RNA Code for Faithful Assignment of AUG Triplets to Methionine

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SUMMARY

The assignment of AUG codons to methionine remains a central question of the evolution of the genetic code. We have unveiled a strategy for the discrimination among tRNAs containing CAU (AUG-decoding) anticodons. *Mycoplasma penetrans* methionyl-tRNA synthetase can directly differentiate between tRNA^{Ile}_{CAU} and tRNA^{Met}_{CAU} transcripts (a recognition normally achieved through the modification of anticodon bases). This discrimination mechanism is based only on interactions with the acceptor stems of tRNA^{Ile}_{CAU} and tRNA^{Met}_{CAU}. Thus, in certain species, the fidelity of translation of methionine codons requires a discrimination mechanism that is independent of the information contained in the anticodon.

INTRODUCTION

Accurate translation of the extant genetic code requires faithful recognition between mRNA codons and tRNA anticodons, and equally reliable aminoacylation of tRNAs by their cognate aminoacyl-tRNA synthetases (ARSs) (Ibba and Söll, 2000). It is generally accepted that the genetic code evolved from a simpler system through the gradual assignment of triplets to new amino acids (Crick, 1968; Davis, 1999; Schimmel et al., 1993; Wong, 1975). Universal tRNA identity elements in aminoacylation seemingly represent recognition solutions established early during the maturation of the code (Hou and Schimmel, 1989), while those that vary widely across the phylogenetic tree may correspond to amino acid-triplet assignments that were adopted at a later time (Ambrogelly et al., 2002; Ribas de Pouplana et al., 1998).

It has been proposed that ancestral aminoacylation of tRNAs by ARSs depended mainly upon interactions with the acceptor stems of tRNAs, which constituted an operational RNA code (Schimmel et al., 1993). The analysis of ARS structures supports the possibility that contacts between ancestral ARSs and tRNAs were limited to the acceptor stem (Ribas de Pouplana and Schimmel, 2001b). As the complexity of the code and its popu-

lation of tRNAs grew, new contacts with the anticodon region were established to ensure the faithful aminoacylation of tRNAs (Cusack, 1997).

In its current complexity the genetic code requires modified RNA bases for correct decoding. At the ribosome, modifications are utilized at base 34 of many anticodons, corresponding to the third or wobble position of the codon-anticodon interaction (Agris, 1991). Base modifications are also important to prevent misacylation of tRNAs in at least two systems, namely tRNA^{Asp} in *S. cerevisiae* (Pütz et al., 1994), and tRNA^{Ile}_{CAU} in *E. coli* (Muramatsu et al., 1988). Unmodified transcripts of these tRNAs are aminoacylated incorrectly by arginyl- and methionyl-tRNA synthetase (MRS), respectively. Only the modifications of base G37 to 1-methyl-G in tRNA^{Asp}, and of base C34 to 2-lysyl-C (lysidine) in tRNA^{Ile}_{CAU}, prevent misacylation and permit the specific recognition of these substrates by the correct ARS.

In most organisms, AUG and AUA triplets encode methionine and isoleucine, respectively. This is the only case in the standard genetic code where codons that code for two different amino acids are separated only by bases of the same type at the third position. The strategy for the assignment of AUG codons to methionine, and AUA codons to isoleucine, represents a striking case of interspecies variation (Blanquet et al., 2005; Grosjean and Bjork, 2004).

Bacteria utilize the modification enzyme lysidine-tRNA synthetase (TilS) to replace the 2-keto group of C34 with a lysine residue in tRNA^{Ile}_{CAU}, resulting in an anticodon containing lysidine (k²C or L) at the wobble position (Soma et al., 2003). In *E. coli* this modification has a four-fold function: it prevents aminoacylation of tRNA^{Ile}_{CAU} by MRS; it allows aminoacylation of the same tRNA by isoleucyl-tRNA synthetase (IRS); it prevents the pairing of tRNA^{Ile}_{CAU} to AUG codons; and it allows the formation of AUA-LAU codon-anticodon pairs. In addition to modification of tRNA^{Ile}_{CAU}, the C34 residue of *E. coli* tRNA^{Met}_{CAU} is also modified in vivo to N⁴-acetyl-C by an unknown enzyme to prevent it from incorrectly translating isoleucine AUA codons with methionine (Stern and Schulman, 1978).

The situation in archaea has not been well studied, but it is known that C34 of tRNA^{Ile}_{CAU} is also modified in these organisms (Gupta, 1984; Marck and Grosjean, 2002). Archaea do not possess a TilS homolog, and the nature of the tRNA^{Ile}_{CAU} modification has not been determined (Soma et al., 2003). In eukaryotes,

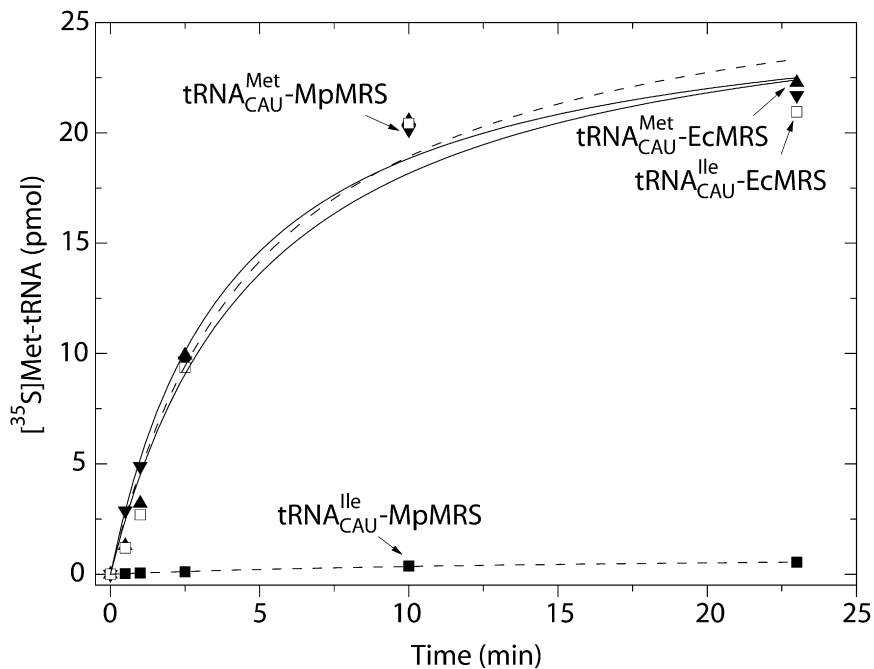


Figure 1. Methionylation of tRNA^{Ile}_{CAU} and tRNA^{Met}_{CAU} by MpMRS and EcMRS

Methionine acceptance of wild-type *M. penetrans* tRNA^{Met}_{CAU} with MpMRS (–▼–) or EcMRS (–▲–) was similar. Methionylation of tRNA^{Ile}_{CAU} by EcMRS (–□–) was similar to tRNA^{Met}_{CAU} while no methionylation by MpMRS (–■–) was seen.

RESULTS

MpMRS Does Not Aminoacylate Unmodified tRNA^{Ile}_{CAU}

Examples of ARSs with idiosyncratic domains abound and are a reflection of the extreme modular nature of these enzymes (Ibba and Söll, 2000). MpMRS has acquired a modular extension not previously described as an ARS attachment. BLAST analysis (Altschul et al., 1997) of its sequence indicates that this extension is formed by two independent domains. The first domain has no significant identity to any known proteins, while the second has over 50% similarity to

tRNA^{Ile} (minor) has a UAU anticodon, which is modified to ΨAU (Ψ being pseudouridine) by pseudouridine synthase (Hellmuth et al., 2000). Thus, eukaryotic genomes avoid the possibility of mischarging tRNA^{Ile} (minor) transcripts with MRS by replacing C34 with U. An additional variation is seen in mitochondria from vertebrates and some fungi, where AUA codes for methionine, clearly separating methionine (YAU) from isoleucine (PAU) triplets (Tomita et al., 1999).

It is of great interest to investigate codon and anticodon discrimination strategies in unexplored regions of the phylogenetic tree, because they may yield information on the development of the genetic code. We have studied this process in *Mycoplasma penetrans*, a human endocellular pathogen that belongs to the bacterial order of Mollicutes (Sasaki et al., 2002). The *M. penetrans* genome codes for 30 identified tRNA genes, including initiator and elongator tRNA^{Met} genes, and one major and one minor isoacceptor tRNA^{Ile} gene (Lowe and Eddy, 1997; Silva et al., 2006). This genome also contains an open reading frame (*metS*) corresponding to an unusually large MRS (*M. penetrans* [MpMRS]—this work), a gene coding for a canonical IRS, and a gene coding for a TiiS homolog (24% identical and 45% similar to *E. coli* TiiS) (Altschul et al., 1997; Sasaki et al., 2002). Thus *M. penetrans* seems to possess all the components utilized by other bacteria for the aminoacylation of tRNA^{Met} and tRNA^{Ile} and the translation of their corresponding codons.

The unusually long *metS* gene in *M. penetrans* encodes two domains that have never before been found associated with ARSs. In order to investigate the unusual structure of MpMRS, we have characterized its biochemical activity. We report here a mechanism for the accurate aminoacylation of tRNA with methionine based on rejection of sequence elements in the tRNA^{Ile} acceptor stem, and we discuss our findings in the context of current knowledge on tRNA^{Met} and tRNA^{Ile} identity elements.

type V aminotransferases (Grishin et al., 1995; see Figure S1 available online).

In order to determine whether this domain plays a role in the recognition of tRNA substrates by MRS, we purified both full-length MpMRS and MpΔMRS (lacking the *M. penetrans*-specific domains), and assayed their ability to aminoacylate tRNA^{Met}_{CAU} and tRNA^{Ile}_{CAU}. The results obtained with MpΔMRS closely mimicked those obtained with full-length MpMRS (Figure S2), indicating that the N-terminal extensions of MpMRS are not involved in the tRNA recognition mechanisms of MpMRS (see below).

Previous studies demonstrated that EcMRS could efficiently aminoacylate a variant of *E. coli* tRNA^{Ile}_{CAU} lacking L34 in addition to its cognate tRNA^{Met}_{CAU}, due to the enzyme's strong dependence on the CAU anticodon as an identity element (Muramatsu et al., 1988). We assayed the ability of MpMRS and EcMRS to aminoacylate in vitro-transcribed *M. penetrans* tRNA^{Met}_{CAU} and tRNA^{Ile}_{CAU}. As expected, EcMRS could aminoacylate these tRNAs with equal efficiency (Figure 1). However, while MpMRS aminoacylates the *M. penetrans* tRNA^{Met}_{CAU} transcript, the tRNA^{Ile}_{CAU} transcript is not an efficient substrate of this enzyme (Figure 1). MpMRS can therefore discriminate between these two tRNAs in vitro, through a mechanism independent of lysidine or any other base modification.

Mutation of tRNA^{Ile}_{CAU} Acceptor Stem Permits Efficient Aminoacylation by MpMRS

To evaluate the mechanism that enables MpMRS to distinguish between tRNA^{Ile}_{CAU} and tRNA^{Met}_{CAU}, we compared the sequences of the three *M. penetrans* tRNAs that contain CAU anticodons (tRNA^{Met}_{CAU}, tRNA^{fMet}_{CAU}, and tRNA^{Ile}_{CAU}). We identified and mutated bases that were uniquely present in tRNA^{Ile}_{CAU} (Figure 2) in an attempt to engineer an efficient substrate for

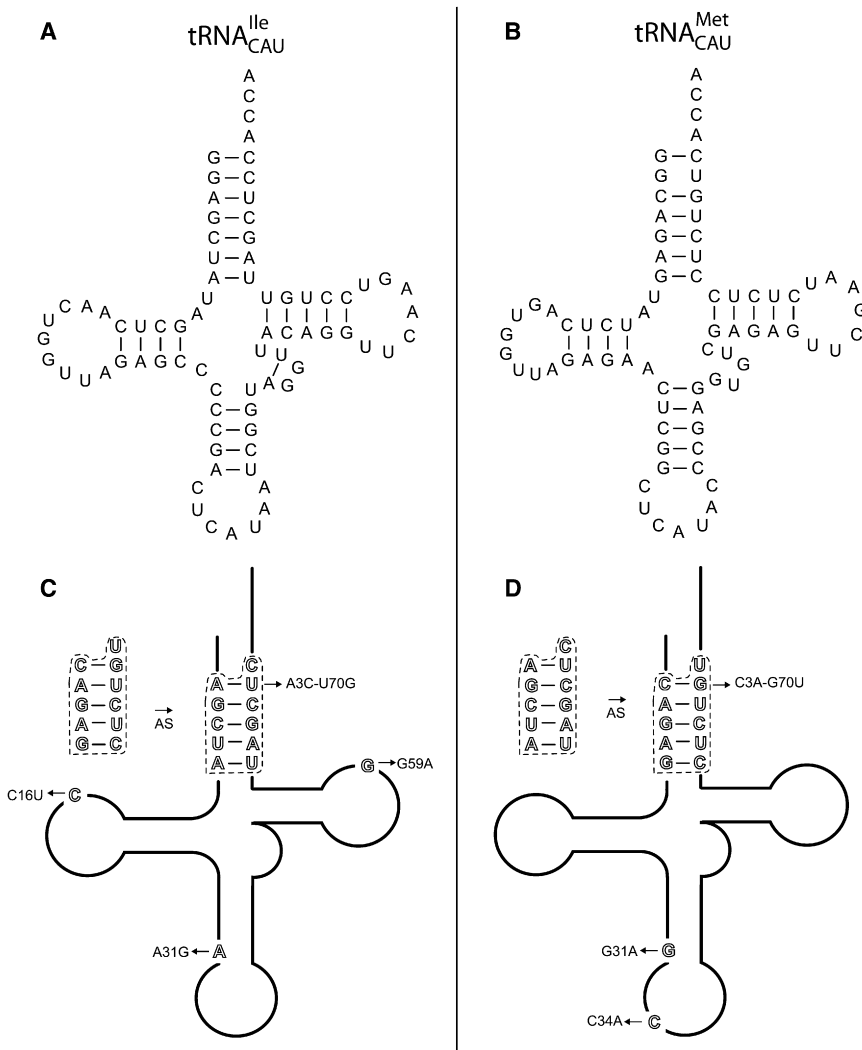


Figure 2. Cloverleaf Representation of the Sequences of *M. penetrans* tRNA^{Ile}_{CAU} and tRNA^{Met}_{CAU}

Wild-type sequences of tRNA^{Ile}_{CAU} (A) and tRNA^{Met}_{CAU} (B) are shown. Mutations described in this work for tRNA^{Ile}_{CAU} (C) and tRNA^{Met}_{CAU} (D) are highlighted over the cloverleaf scaffold. AS, acceptor stem.

acceptor stem residues were modified to the tRNA^{Met}_{CAU} sequence. These substitutions led to a 38-fold increase and 547-fold increase in k_{cat}/K_M for aminoacylation of tRNA^{Ile}_{CAU}(A3C-U70G) and tRNA^{Ile}_{CAU}(AS), respectively (Figure 3A and Table 1).

Comparison of the active tRNA^{Ile}_{CAU} mutants with wild-type tRNA^{Ile}_{CAU} indicates that substitutions in the acceptor stem improve activity primarily through a k_{cat} effect (Table 1), with the 2.8-fold higher K_M of tRNA^{Ile}_{CAU}(AS) responsible for the remaining gap in efficiency relative to tRNA^{Met}_{CAU}. These results point to the importance of the catalytic step in the discrimination of tRNA^{Ile}_{CAU} and tRNA^{Met}_{CAU} by MpMRS.

Identity Elements of tRNA^{Met}_{CAU} Include the Acceptor Stem

We next used mutants of *M. penetrans* tRNA^{Met}_{CAU} to determine the identity elements required for its methionylation (Figure 3B). First, a tRNA^{Met}_{CAU}(C34A) wobble base variant was generated to determine whether the anticodon remains a dominant identity element for

MpMRS (Figure 2C). We also replaced the acceptor stem of tRNA^{Ile}_{CAU} with the acceptor stem of tRNA^{Met}_{CAU} to generate tRNA^{Ile}_{CAU}(AS).

MpMRS efficiently aminoacylated tRNA^{Ile}_{CAU}(A3C-U70G), and an even greater enhancement was seen upon substitution of the full acceptor stem (Figure 3A). No improvement in methionylation was achieved with mutations in the anticodon stem or D and T loops (Figure 3A). Detailed kinetic analyses were performed in order to determine the extent and nature of the effects caused by the acceptor stem substitutions (Table 1). For poorly aminoacylated substrates, for which k_{cat} and K_M could not be determined, relative efficiencies were estimated by using increased quantities of tRNA and enzyme as previously described (Schulman and Pelka, 1988). We estimate that MpMRS aminoacylates tRNA^{Met}_{CAU} with a 1563-fold greater efficiency over tRNA^{Ile}_{CAU}; this corresponds to a $4.35 \text{ kcal}\cdot\text{mol}^{-1}$ difference in the operational free energy of activation. Almost half of this energy difference ($2.15 \text{ kcal}\cdot\text{mol}^{-1}$) was recovered by the single A3C-U70G acceptor stem base pair substitution with an additional $1.58 \text{ kcal}\cdot\text{mol}^{-1}$ being recovered when the remaining

MpMRS as for other bacterial MRSs. Effects of changes to the anticodon stem were assayed with tRNA^{Met}_{CAU}(G31A), which contains the A31 residue of *M. penetrans* tRNA^{Ile}_{CAU}.

As expected, the tRNA^{Met}_{CAU}(C34A) wobble base substitution abolishes aminoacylation by both MpMRS (Figure 3B) and EcMRS (data not shown). This confirms the established critical role of this nucleotide in *E. coli* tRNA^{Met}_{CAU} recognition by EcMRS (Pelka and Schulman, 1986). The tRNA^{Met}_{CAU}(G31A) variant is aminoacylated by MpMRS at a reduced level relative to the wild-type sequence, indicating the importance of the anticodon stem and loop for MpMRS charging efficiency. This marked reduction stands in contrast to the relatively high activity of tRNA^{Ile}_{CAU}(AS) that contains the entire anticodon stem and loop from tRNA^{Ile}_{CAU}, including an A at position 31. Thus, A31 in the context of other anticodon stem and loop residues of tRNA^{Ile}_{CAU}(AS) is not a strong negative determinant. However, this same nucleotide introduced into tRNA^{Met}_{CAU} leads to an A31:C39 mismatch that may interfere with binding by MpMRS.

We then investigated the importance of acceptor stem nucleotides for the recognition of tRNA^{Met}_{CAU} by MpMRS. We

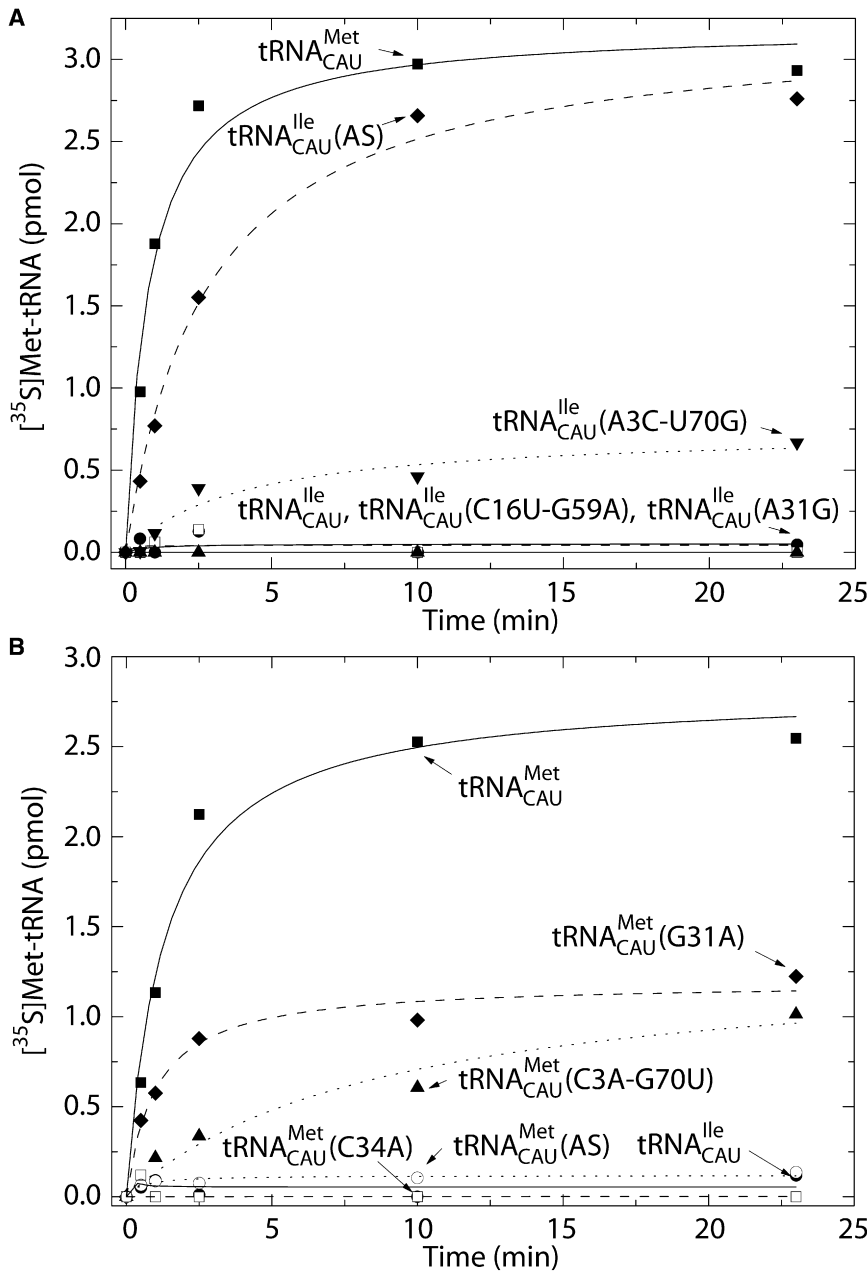


Figure 3. Distribution of Identity Elements for tRNA^{Ile}_{CAU} Discrimination by MpMRS

(A) Methionylation of wild-type and mutant tRNA^{Ile}_{CAU} transcripts by MpMRS. Methionine acceptance of wild-type tRNA^{Met}_{CAU} (■), tRNA^{Ile}_{CAU}(AS) (◆), and tRNA^{Ile}_{CAU}(A3C-U70G) (▼). Lack of methionylation was observed for tRNA^{Ile}_{CAU}(A31G) (▲), tRNA^{Ile}_{CAU}(C16U-G59A) (□), and tRNA^{Ile}_{CAU}(AS) (●). AS, acceptor stem. (B) Methionylation of wild-type and mutant tRNA^{Met}_{CAU} transcripts by MpMRS. Methionine acceptance of wild-type tRNA^{Met}_{CAU} (■), tRNA^{Met}_{CAU}(G31A) (◆), and tRNA^{Met}_{CAU}(C3A-G70U) (▲). No methionylation was seen for tRNA^{Met}_{CAU}(C34A) (□), tRNA^{Met}_{CAU}(AS) (●), and tRNA^{Ile}_{CAU} (○). AS, acceptor stem.

aminoacylation of tRNA^{Met}_{CAU}(AS) over tRNA^{Met}_{CAU}(C3A-G70U), pointing to the existence of a critical interaction during the transition state between the acceptor stem and the MpMRS core in the discrimination of tRNA^{Ile}_{CAU} and tRNA^{Met}_{CAU} (Table 1).

DISCUSSION

The discriminator base and first five base pairs of the acceptor stem uniquely define all the tRNAs of *E. coli* (McClain, 1995) and may constitute the remnant of a recognition system used by ancestral ARSs (Ribas de Pouplana and Schimmel, 2001a; Schimmel et al., 1993). This ancestral set of interactions evolved with the tRNA population, as the genetic code grew, possibly driven by additional parameters such as the evolution of amino acid biosynthetic pathways (Cusack, 1997; Davis, 1999; Moras, 1992; Wong, 1975). However, in extant organisms the anticodon is a major identity element for most ARSs, including MRS (Ghosh et al., 1990; Pallanck and Schulman, 1991; Schulman and Pelka, 1988).

mutated the C3-G70 base pair of the acceptor stem of tRNA^{Met}_{CAU} to the corresponding A3-U70 base pair present in tRNA^{Ile}_{CAU} and assayed the effect of this mutation on aminoacylation by MpMRS. The C3A-G70U mutation in tRNA^{Met}_{CAU} causes a 15-fold reduction in k_{cat}/K_M for aminoacylation, which corresponds to a 1.59 kcal·mol⁻¹ difference in the operational free energy of activation (Table 1). This effect indicates the importance of the C3-G70 base pair of tRNA^{Met}_{CAU} as a recognition element for MpMRS, and correlates with the values calculated for the converse mutation in tRNA^{Ile}_{CAU}(A3C-U70G) (vide supra).

When the entire acceptor stem of tRNA^{Met}_{CAU} was changed to the tRNA^{Ile}_{CAU} sequence, the energy of activation was increased by 3.07 kcal·mol⁻¹. This represents a 12-fold drop in k_{cat}/K_M for

An ancestral tRNA recognition mechanism primarily based on interactions between the ancestral domains of ARSs and the acceptor stem of primordial tRNAs can be clearly deduced from the analysis of sequences and structures of both molecules (Ribas de Pouplana and Schimmel, 2001b; Rodin et al., 1996; Rodin and Ohno, 1995). By definition, the extant recognition of anticodon sets by synthetases must have been established after each tRNA acquired its final identity. In the cases where this identity was defined close to the eventual separation of the main life taxons, recognition mechanisms may have evolved differently in each domain of life.

Bacterial MRSs were thought to recognize tRNAs mainly through anticodon interactions. Substitutions at acceptor stem

Table 1. Methionylation of tRNA^{Ile}_{CAU} and tRNA^{Met}_{CAU} and Their Derivatives by MpMRS

Transcripts	K _M (μM)	k _{cat} (s ⁻¹)	-ΔΔG (kcal/mol)	k _{cat} /K _M	rel k _{cat} /K _M
tRNA ^{Met} _{CAU}	2.3	0.50	0	0.22	1
tRNA ^{Met} _{CAU} (C3A-G70U)	6.2	0.093	1.59	0.015	0.068
tRNA ^{Met} _{CAU} (AS)	NM	NM	3.07	<0.0012	0.0055
tRNA ^{Ile} _{CAU}	NM	NM	4.35	<0.00014	0.00064
tRNA ^{Ile} _{CAU} (A3C-U70G)	9.6	0.050	2.20	0.0052	0.024
tRNA ^{Ile} _{CAU} (AS)	6.4	0.50	0.62	0.078	0.35

Relative aminoacylation efficiencies (rel k_{cat}/K_M) were calculated using k_{cat}/K_M value for tRNA^{Met}_{CAU} as a reference. AS, acceptor stem. NM, not measurable. -ΔΔG is defined as RT ln([k_{cat}/K_M]^{variant} / [k_{cat}/K_M]^{wild-type}), where R = 1.98272 cal/mol•K and T = 298 K.

nucleotides of tRNA^{Met}_{CAU} have no effect on its aminoacylation by EcMRS due to the strong binding of the anticodon by the enzyme (Martinis and Schimmel, 1993). Thus, in the case of EcMRS, the ancestral discrimination based on acceptor stem interactions may have been “overpowered” by anticodon recognition.

In contrast, we have demonstrated that modification of the tRNA^{Ile}_{CAU} anticodon is not a requirement for accurate discrimination of tRNAs by *M. penetrans* MRS. Instead, MpMRS rejects tRNA^{Ile}_{CAU} through the recognition of negative identity elements in its acceptor stem. Bacterial MRSs therefore have at least two different ways of discriminating between tRNAs with CAU anticodons.

To our knowledge, the acceptor stem-based discrimination mechanism described in this work is the first reported differentiation of tRNA^{Met}_{CAU} and tRNA^{Ile}_{CAU} by an ARS by a means other than base 34 modification. Modifications at base 34 are also essential for accurate codon-anticodon interactions at the ribosome. For example, *E. coli* tRNA^{Met}_{CAU} lacking its C34 N⁴-acetyl modification was unable to accurately discriminate between AUA and AUG codons (Stern and Schulman, 1978). Our results reinforce the importance of anticodon stem and loop recognition by MpMRS but also point to the acceptor stem as the primary site of tRNA^{Met}_{CAU} and tRNA^{Ile}_{CAU} discrimination by the enzyme. Indeed, the discrimination of tRNA^{Met}_{CAU} and tRNA^{Ile}_{CAU} by MpMRS does not require base modifications, suggesting that, in this organism, a simpler mechanism exists for aminoacylating tRNA^{Met}_{CAU} than for ensuring the correct translation of methionine codons at the ribosome.

Whether recognition of either acceptor stem or anticodon nucleotides represents the ancestral tRNA discrimination mode is not clear. A mechanism centered on interactions with the acceptor stem of cognate tRNA would be reminiscent of an ancestral operational RNA code (Schimmel et al., 1993). In this context, it could be argued that MpMRS uses an ancestral mode of recognition, and that EcMRS may have lost the ability to discriminate the acceptor stems of tRNA^{Met}_{CAU} from tRNA^{Ile}_{CAU} a posteriori.

This interpretation of our data would indicate that, during the course of bacterial evolution, some species transferred the core of their recognition strategy to the anticodon, taking advantage of the modified base at position 34, which had evolved to allow the correct pairing to methionine and isoleucine codons. In support of this possibility, it should be noted that the structures of bacterial MRSs display strong variability in a region of

their active site potentially involved in tRNA acceptor stem recognition (Mechulam et al., 1999). Moreover, the region of MpMRS responsible for the rejection of tRNA^{Ile}_{CAU} is the core structure of the enzyme, and not the idiosyncratic N-terminal domains that are unique to this species. These observations argue against the possibility that MpMRS’s ability to discriminate against tRNA^{Ile}_{CAU} is the result of a recent evolutionary process linked to the addition of new functional modules to this enzyme.

Alternatively, the ability of MpMRS to reject tRNA^{Ile}_{CAU} could be a late adaptation that results from the highly evolved nature of *M. penetrans* (Peterson et al., 2001; Sasaki et al., 2002). It is interesting to note that the only known exception to the requirement of the lysidine modification in bacteria occurs in a related organism. *Mycoplasma mobile* lacks both a tRNA^{Ile}_{CAU} and the lysidine-modifying enzyme TilS (Silva et al., 2006), and contains a unique bacterial tRNA^{Ile}_{UAU} with a U34 residue that is presumed to decode AUA codons (Silva et al., 2006). The fact that the only known bacteria that lacks a lysidine modification machinery also lacks a tRNA^{Ile}_{CAU} points again to the critical role of lysidine modification in decoding at the ribosome.

If the recognition strategy utilized by MpMRS is a new adaptation, an explanation for its limited distribution may be linked to the simplified tRNA recognition problem faced by *Mycoplasma* ARSs. Indeed, *M. penetrans* contains only 30 tRNAs compared to 86 in *E. coli*. It has been suggested that the complexity of the tRNA recognition problem is one of the forces driving conservation of discrimination mechanisms for tRNAs between species (Ribas de Pouplana, 2005). The reduced set of tRNAs used by *M. penetrans* may have permitted the evolution of new specificity determinants in *Mycoplasma* tRNAs that remain inaccessible to organisms with larger tRNA sets.

Regardless of the evolutionary order that gave rise to the two tRNA^{Ile}_{CAU} recognition mechanisms, our data strengthen the hypothesis that assignment of the Ile-Met codons is a late event during the maturation of the code. This possibility is in good agreement with theories of coevolution between the triplet structure of the code and the metabolic pathways for the synthesis of amino acids (Davis, 1999; Di Giulio and Medugno, 1999; Wong, 1988).

EXPERIMENTAL PROCEDURES

Materials

DNA oligonucleotides were synthesized by Sigma-Genosys. L-[³⁵S]methionine, MonoQ, and HisTrap nickel columns were from Amersham Biosciences.

Restriction enzymes were from New England Biolabs, and vector pCR2.1-TOPO was from Invitrogen. Expression vector pQE-70 was from QIAGEN. Pfu Ultra DNA polymerase, XL-10 Gold cells and QuikChange multi-site-directed mutagenesis kit were from Stratagene. *M. penetrans* genomic DNA was a gift from Dr. Y. Sasaki of the National Institute of Infectious Diseases, Japan.

Transfer RNA Substrate Preparation

Wild-type or mutated tRNAs were assembled using six DNA oligonucleotides that were first annealed, and then ligated, between HindIII and BamHI restriction sites of plasmid pUC19. In vitro transcription using T7 RNA polymerase was performed according to standard protocols (Sampson and Uhlenbeck, 1988). *E. coli* tRNA^{Met}_{CAU} was transcribed from BstNI-linearized plasmid pMet (Schulman and Pelka, 1988). Transcripts were separated on denaturing PAGE; full-length tRNAs were eluted from gel using an EluTrap electroelution apparatus (Schleicher & Schüll; Keene, NH) and refolded (80°C followed by gradual reduction of temperature in presence of 1 mM MgCl₂). Finally, aminoacylation plateaus were used to calculate the concentration of active molecules for each tRNA preparation.

Enzyme Cloning and Mutagenesis of TGA Codons

The 3264 base pair gene coding for *M. penetrans* MRS (GenBank MYPE9380) was amplified by PCR from *M. penetrans* HF-2 genomic DNA and cloned into the pCR2.1-TOPO vector. Seven rounds of mutagenesis were used to mutate 12 TGA codons to TGG tryptophan codons and to remove an internal SphI restriction site. The gene was subcloned into the SphI site of the pQE-70 vector (QIAGEN), and the resulting plasmid pQE-70-MpMRS was verified by sequencing.

The portion of the *E. coli metS* gene corresponding to the N-terminal 547 amino acids of *E. coli* MRS was cloned into pET28 (Novagen; Madison, WI) to generate pSW101, which encodes an N-terminally His₆-tagged *E. coli* MRS monomer (EcMRS) (Alexander et al., 1998).

Enzyme Overexpression and Purification

XL-10 Gold cells (Stratagene) transformed with pQE-70-MpMRS were grown at 37°C for 12 hr to an optical density A₆₀₀ = 1.2. Protein expression, and purification on nickel affinity columns, were performed according to the manufacturer's protocol. Initial elutions yielded a major 128 kDa band corresponding to the full-length protein and a 63 kDa band. Edman sequencing of the smaller band demonstrated that the N-terminal five residues were MKKFY, corresponding to the start of the canonical 63 kDa MRS domain; this truncated protein is designated MpΔMRS. Similar internal initiation events have been observed for the overexpression of other *Mycoplasma* proteins in *E. coli* (Notarnicola et al., 1990).

MpΔMRS was separated from MpMRS by ion exchange chromatography using a MonoQ column. The protein preparations were each ~95% pure as determined by SDS-PAGE (Figure S3). Enzyme concentrations were determined by active-site titration (Fersht et al., 1975). N-terminally His₆-tagged EcMRS was expressed and purified as reported (Alexander et al., 1998).

Aminoacylation Assays

Aminoacylation of tRNA was performed at 25°C in 20 mM HEPES•KOH (pH 7.6), 100 μM methionine, 10 mM MgCl₂, 5 mM DTT, 4 mM ATP, 150 mM NH₄Cl, 100 μM Na₂EDTA, 0.5 μCi/ml L-[³⁵S]methionine, and varying concentrations of tRNA transcripts (0.5–20 μM) as reported (Schulman and Pelka, 1988).

Supplemental Data

Supplemental Data include three figures and can be found with this article online at <http://www.molecule.org/cgi/content/full/29/3/401/DC1/>.

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