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Unconventional decoding of the AUA codon as methionine by mitochondrial tRNA^{Met} with the anticodon f^{5}CAU as revealed with a mitochondrial *in vitro* translation system

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ABSTRACT

Mitochondrial (mt) tRNA^{Met} has the unusual modified nucleotide 5-formylcytidine (f^{5}C) in the first position of the anticodon. This tRNA must translate both AUG and AUA as methionine. By constructing an *in vitro* translation system from bovine liver mitochondria, we examined the decoding properties of the native mt tRNA^{Met} carrying f^{5}C in the anticodon compared to a transcript that lacks the modification. The native mt Met-tRNA could recognize both AUA and AUG codons as Met, but the corresponding synthetic tRNA^{Met} lacking f^{5}C (anticodon CAU), recognized only the AUG codon in both the codon-dependent ribosomal binding and *in vitro* translation assays. Furthermore, the *Escherichia coli* elongator tRNA$^{Met}_m$ with the anticodon ac^{4}CAU (ac^{4}C = 4-acetylcytidine) and the bovine cytoplasmic initiator tRNA^{Met} (anticodon CAU) translated only the AUG codon for Met on mt ribosome. The codon recognition patterns of these tRNAs were the same on *E. coli* ribosomes. These results demonstrate that the f^{5}C modification in mt tRNA^{Met} plays a crucial role in decoding the nonuniversal AUA codon as Met, and that the genetic code variation is compensated by a change in the tRNA anticodon, not by a change in the ribosome. Base pairing models of f^{5}C-G and f^{5}C-A based on the chemical properties of f^{5}C are presented.

INTRODUCTION

In 1981, the complete nucleotide sequence of the human mitochondrial (mt) genome was reported (1). Variations from the universal genetic code were suggested to exist in this organelle, based on comparisons of the DNA sequences of several mt protein genes and the actual amino acid sequences of the corresponding protein products. For example, a termination codon, UGA, and the AUA Ile codon in the universal genetic code are read as Trp and Met, respectively, and the AGA and AGG Arg codons become termination codons in human mitochondria. These 'nonuniversal' codons have also been observed in the mt genomes of many other organisms (2–5), and species-specific variations also exist in some codons (6,7).

The genetic code variation for methionine, first found in human mitochondria, is also observed in other mammalian mitochondria in which the genomes possess only a single tRNA^{Met} gene with the CAT anticodon. In addition, there had been no reports suggesting the import of cytoplasmic tRNAs into mammalian mitochondria (8), unlike the import observed in many lower eukaryotic and plant mitochondria. Therefore, the single tRNA^{Met}

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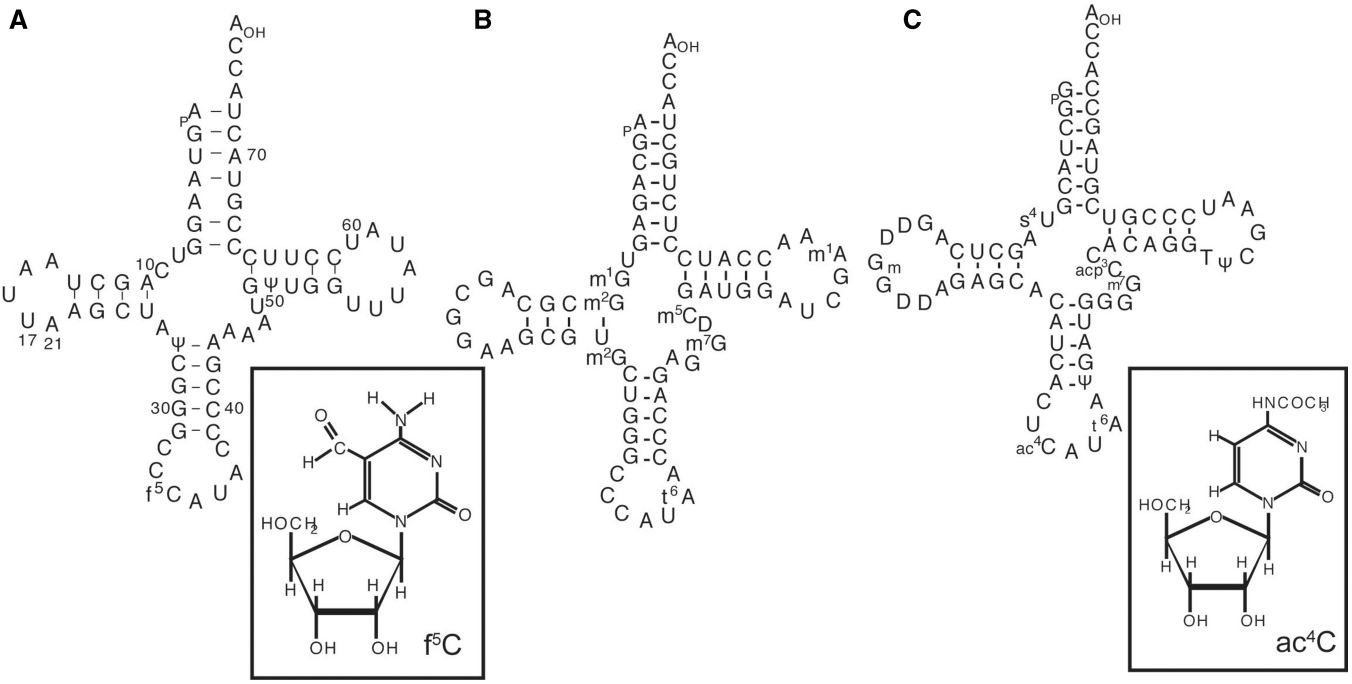

Figure 1. Nucleotide sequences of tRNAs used in this study and chemical structures of the modified nucleotide in the wobble position. (**A**) Bovine mt $tRNA^{Met}$ and 5-formylcytidine, (**B**) bovine cytoplasmic initiator $tRNA^{Met}$ and (**C**) *E. coli* elongator $tRNA^{Met}$ and 4-acetylcytidine. The numbering of the residues in the cloverleaf structure of the mt tRNA conforms to that in the previous report (71).

in mammalian mitochondria should recognize both the AUA and AUG codons as Met, serving as both the elongator and initiator tRNA. Although the anticodon CAU of $tRNA^{Met}$ cannot base pair with the AUA codon, according to the conventional Watson–Crick pairing (9), there have been several observations that the C–A pair at the wobble position can be formed *in vivo* as well as *in vitro* under certain limited conditions (10,11). Since the AUA codon occurs much more frequently than the AUG codon in mammalian mt genomes, it had been assumed that some special mechanism must exist in the mitochondrial translation system to allow the AUA codon to be translated as Met. In 1994, we found a novel modified nucleoside, 5-formylcytidine (f^5C), in the first position of the anticodon of mt $tRNA^{Met}$ from bovine liver (12) and the nematode, *Ascaris suum* (13) (Figure 1A). This modified nucleoside was a promising candidate to permit $tRNA^{Met}$ to translate both the AUA and AUG codons through its ability to pair with not only G but also with A in the third position of the codon. The $tRNAs^{Met}$ from squid, fruit fly (14,15), frog, rat and chicken mitochondria (16) also possess f^5C in the wobble position.

The simplest and clearest way to determine whether f^5C in the wobble position of mt $tRNA^{Met}$ actually recognizes A at the third position of the codon, would be to demonstrate that mt $tRNA^{Met}$ with the anticodon f^5CAU decodes the AUA codon while the tRNA without this modification is restricted to decoding AUG in an efficient mitochondrial *in vitro* translation system. For these experiments, it is not feasible to simply substitute the well-characterized *Escherichia coli in vitro* translation system, because some factors necessary for translation cannot be exchanged between mitochondria and *E. coli* protein biosynthetic systems. For example, the binding of mt aminoacyl-tRNA to the A-site of ribosomes is efficiently carried out only by the mt elongation factor Tu and Ts complex (mt EF-Tu/Ts) and not by bacterial EF-Tu (17). Although mt elongation factor G (EF-G) can work with both mt and *E. coli* ribosomes, *E. coli* EF-G works only on *E. coli* ribosomes (18). A mitochondrial translation system was reported previously, but it was not useful for estimating the codon recognition, because its activity was much lower than that of the *E. coli* system (17,19).

In the current work, we have constructed an improved *in vitro* mitochondrial translation system with enough activity to test codon recognition activity. This system consists of mt aminoacyl-tRNAs, partially purified mt EF-Tu/Ts complex, mt EF-G, and mt ribosomes. Using this system we demonstrate that the modified nucleoside f^5C of $tRNAs^{Met}$ is crucial for the recognition of the non-universal AUA codon as Met.

MATERALS AND METHODS

Chemicals

[^{14}C]-Phenylalanine (16.6 GBq/mmol), [^{14}C]-isoleucine (11 GBq/mmol) and [^{35}S]-methionine (37 TBq/mmol) were purchased from Amersham. The [^{35}S]-methionine used here was diluted to 200 GBq/mmol with nonradioactive methionine. Poly(U), pyruvate kinase (PK), spermine and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Sigma.

The oligoribonucleotides, $(AUN)_6$, $(AUN)_{11}$ (N = A, C, G) and half fragments of $tRNA^{Met}$, were synthesized with an automatic DNA synthesizer, ABI 381A, and were deprotected as described previously (20). T4 polynucleotide kinase (PNK) was obtained from TOYOBO, T4 RNA ligase from TAKARA, RNase T_1 from Sankyo, RNase U_2 from Sigma, RNase PhyM, and RNase CL_3 from Boehringer Mannheim.

Partial purification of PheRS, MetRS and IleRS

Mitochondria were prepared from fresh bovine liver as reported (18,21). Crude mitochondrial extracts were prepared and fractionated by chromatography on DEAE-Sepharose fast flow (Pharmacia), with a 5–300 mM KCl gradient as described (22). The fractions containing the desired aminoacyl-tRNA synthetase (aaRS) activities were pooled and dialyzed against 60 volumes of Buffer A [10 mM potassium phosphate (pH 7.2), 50 mM KCl, 10% glycerol, 6 mM 2-mercaptoethanol and 0.2% CHAPS] with three buffer exchanges. Further purification was performed on a ceramic hydroxyapatite column (Bio-RAD) with a gradient of 10–300 mM potassium phosphate in Buffer A. The mt aaRS activities were defined by the amino acid acceptance of partially purified *E. coli* $tRNA^{Met}$ or $tRNA^{Ile}$.

The extract of *E. coli* strain A19, harvested at the mid-log phase ($0.6A_{600}$), was prepared as described (23) and applied to a DEAE-Sepharose column at 50 mM NH4Cl. Fractions eluted with 400 mM NH_4Cl were pooled and used as *E. coli* aaRS after dialysis against the initial buffer (23).

Preparation of tRNA and aminoacyl-tRNA

Amino acid-specific mt tRNAs were purified by a hybridization method, using DNA probes complementary to the 3′-end 30 bases of the tRNA genes, as previously reported (24,25). *Escerichia coli* $tRNA^{Phe}$, $tRNAs^{Met}$ and $tRNAs^{Ile}$ were purified by DEAE-Sepharose chromatography under two different pH conditions and by reverse phase chromatography (RPC-5), as described (26,27). Unlike $tRNA^{Met}{}_f$ or $tRNA^{Ile}{}_1$, $tRNA^{Met}{}_m$ and $tRNA^{Ile}{}_2$ could not be purified completely after RPC-5, so these tRNAs were isolated from the enriched fraction of RPC-5 by 8% native polyacrylamide gel electrophoresis (data not shown). Aminoacylation was performed as described (19). The charged mt tRNA was recovered from the reaction mixture by the acid–guanidine–phenol–chloroform method to improve the yield slightly (28). Sequences of the all tRNAs prepared in this study were confirmed by Donis–Keller's method (29–32) and the modified nucleotides were identified by the method of Kuchino *et al*. (31).

Synthetic $tRNA^{Met}$ variants were prepared by four steps (Figure 3A), as previously reported (33). The process consists of 5′-phosphorylation of the 3′-half fragment by T4 PNK (step 1), removal of the 3′-teminal nucleotide of the 3′-half fragment by $NaIO_4$ treatment (step 2), ligation of the 5′-half and 3′-half fragments by T4 RNA ligase (step 3), and 5′-phosphorylation and 3′-dephosphorylation of the ligated tRNA by T4 PNK (step 4). The 5′-half and 3′-half fragments have sequences identical to the respective sequences of native $tRNA^{Met}$, except for f^5C and pseudouridine (Ψ). Two species of synthetic $tRNA^{Met}$ were prepared. One does not possess any modification. The other possesses only Ψ at the positions 27 and 50 (Figure 3B). The sequences of the fragments were confirmed by Donis–Keller's method before and after ligation (29,30,32).

The modified nucleotide f^5C on mt $tRNA^{Met}$ was reduced to 5-hydroxyl-cytidine by $NaBH_4$. Purified mt $tRNA^{Met}$ ($0.5A_{260}$ unit), was dissolved in 0.1 ml of 0.5 M Tris–HCl buffer containing 5 mM $MgCl_2$ and 100 mM $NaBH_4$ (pH 7.6), and incubated on ice for 30 min in the dark. After the addition of 0.2 ml of 0.5 M sodium acetate (pH 5.0), the tRNA was recovered by ethanol precipitation. The location and identification of the modified nucleotides were determined according to by the method of Kuchino *et al*. (31,34). The reduced mt $tRNA^{Met}$ partially digested with formamide was labeled with [γ-^{32}P]ATP at the 5′-end, and the resulting [^{32}P]-labeled oligonucleotides were separated by polyacrylamide gel electrophoresis. They were eluted from the gel and digested with nuclease P_1 to produce 5′-[^{32}P]-labeled mononucleotides, which were analyzed by two-dimensional thin layer chromatography (2D-TLC) on Avicel SF plate (Funakoshi Co.). The standard 5′-mononucleotides (pA, pG, pU and pC) were prepared by digestion of crude tRNA mixture with nuclease P_1 and detected by UV absorption.

General components used in the translation assay

Mitochondrial EF-G and EF-Tu/Ts were prepared from bovine liver mitochondria, as described (22). *Escherichia coli* EF-Tu/Ts was prepared by standard methods (23). Ribosomes were prepared from *E. coli* strain A19 and mitochondria as reported (35,36), and were stored in the following ribosome buffers. *E. coli* 70S ribosome buffer: 20 mM Hepes-KOH (pH 7.6), 6 mM $Mg(OAc)_2$, 30 mM NH_4Cl and 6 mM 2-mercaptoethanol; mt 55S ribosome buffer: 20 mM Tris-HCl (pH 7.6), 20 mM $MgCl_2$, 80 mM KCl and 6 mM 2-mercaptoethanol.

To check the purity of the mitochondrial ribosomes, samples were analyzed by sucrose density gradient centrifugation using linear sucrose gradients (38 ml, 6–38% in the mt ribosome buffer). Gradients were prepared using the Gradient Mate model 117 (BioComp), according to the user's manual. The ribosomes (80 A_{260} units), dissolved in 0.6 ml of the ribosome buffer without sucrose, were layered onto the gradients. The centrifugation was carried out at 20 000 r.p.m. for 16 h in an SW28 rotor using an XL-7 rotor (Beckman) at 4°C. Fractions (0.45 ml) were collected from top to bottom, using the Piston Gradient Fractionator (BioComp).

Ribosome binding assay

Reaction mixtures (40 µl) contained 50 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol (DTT), 1 mM spermine, 8 mM $MgCl_2$, 40 mM KCl, 2.5 mM phosphoenolpyruvic acid (PEP), 0.5 mM GTP, 1 mM methionine, 2.5 U/ml PK, 14 U of mt EF-Tu/Ts, 0.1 A_{260} (3.2 pmol, 0.08 µM)

mt ribosomes and 2.6 pmol [^{35}S]-Met-tRNA (22 000 c.p.m./pmol). The units are defined as described previously (37). The amount of the $(AUN)_6$ oligonucleotide (N means A, C or G) is indicated in the figures. Incubation was carried out at 30°C for 15 min. The reaction was stopped by dilution with chilled wash buffer (5 ml), containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 40 mM KCl. The amount of [^{35}S]Met-tRNA bound to the ribosomes was determined essentially as reported previously (19).

In vitro translation system

For optimization of mt poly(U)-dependent poly(Phe) synthesis, reaction mixtures (20 µl) contained 50 mM Tris–HCl (pH 8.5), 1 mM DTT, 1 mM spermine, 7 mM $MgCl_2$, 30 mM KCl, 0.5 mM GTP, 2.5 mM PEP, 2 U/ml PK, 0.125 mg/ml poly(U), 11 U of mt EF-Tu/Ts, 12 U of mt EF-G, 0.04 A_{260} (1.2 pmol, 0.1 µM) mt ribosomes and 7 pmol [^{14}C]Phe-tRNA (928 c.p.m./pmol). In the case of *E. coli* ribosomes (4 U, 0.05 A_{260}), the concentration of $MgCl_2$ was reduced to 6 mM, the 30 mM KCl was replaced by 60 mM NH_4Cl, and *E. coli* EF-G was used. The concentration of spermine was reduced to 0.2 mM for *E. coli* tRNA. The polymerization reactions were carried out at 37°C, and were stopped by the addition of 4 ml of 5% trichloroacetic acid (TCA), followed by deacylation of the peptidyl-tRNAs by heating at 95°C for 10 min. After the solutions were poured onto nitrocellulose membranes (Advantec, A045B025A), the membranes were washed with 20 ml of 1% TCA and dried. The amount of label remaining on the membrane was measured with a liquid scintillation counter (Packard Co. TRI-CARB).

The reaction mixtures (12 µl) for the *in vitro* (AUN)-translation system contained 50 mM Tris–HCl (pH 8.5), 1 mM DTT, 0.6 mM spermine, 7 mM $MgCl_2$, 30 mM KCl, 0.5 mM GTP, 2.5 mM PEP, 2 U/ml PK, 1 mM methionine, 1 mM isoleucine, 0.1 mg/ml mRNA [oligo $(AUN)_{11}$], 14 U of mt EF-Tu/Ts, 12 U of mt EF-G, 0.04A_{260} (1.2 pmol, 0.1 µM) mt ribosomes and 1.2 pmol aminoacyl-tRNA ([^{35}S]Met-tRNA or [^{14}C]Ile-tRNA).

RESULTS

Construction and optimization of an *in vitro* mitochondrial translation system using *E. coli* tRNA

An efficient mitochondrial (mt) translation system was required to characterize the universal and nonuniversal codon recognition by mitochondrial tRNA. Mitochondrial ribosomes and translational factors were prepared as described previously (18). As indicated in Figure 2A, both *E. coli* and mt ribosomes catalyze poly(Phe) synthesis using *E. coli* Phe-tRNA and the mt EF-Tu/Ts. The optimum concentration of spermine depended on the tRNA species: the mt tRNA preferred a higher concentration (0.8–1.5 mM) of spermine than *E. coli* tRNA did (0.2 mM). The poly(U)-dependent poly(Phe) synthesis activity in the complete mt system using mt ribosomes was equal to that obtained using *E. coli* Phe-tRNA, under the respective optimized conditions described in the Materials and methods section (Figure 2B).

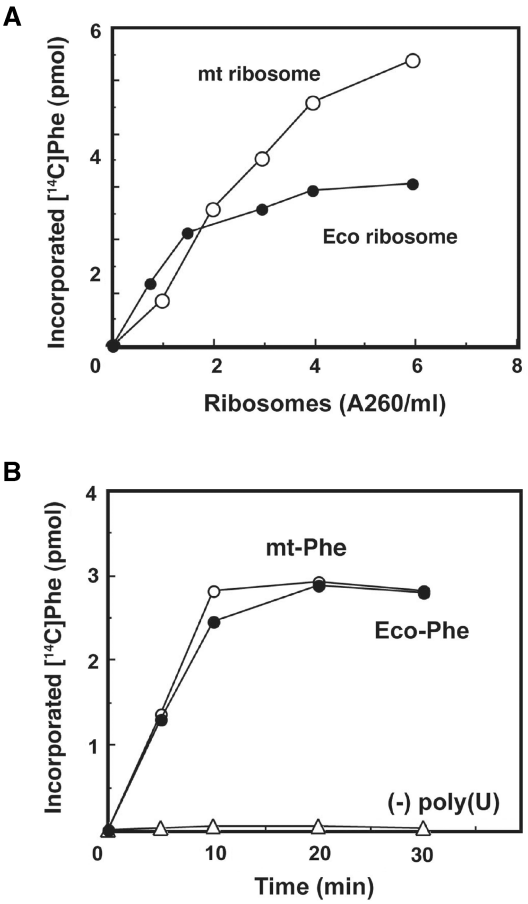

Figure 2. *In vitro* translation system prepared from bovine liver mitochondria. (**A**) Dose–response of poly(Phe) synthesis, as a function of the amount of mitochondrial (open circle) or *E. coli* (filled circle) ribosomes, using *E. coli* Phe-tRNA and mt EF-Tu/Ts. The reactions were carried out at 37°C for 8 min. (**B**) Poly(U)-dependent poly(Phe) synthesis activity of the complete mitochondrial system under the optimized conditions for Phe-tRNA from mitochondria (open circle) and *E. coli* (filled circle), respectively, as described in the Materials and methods section.

Preparation of mitochondrial $tRNA^{Met}$ with or without f^5C, and other nonmitochondrial $tRNAs^{Met}$

To elucidate whether f^5C is necessary for mt $tRNA^{Met}$ to decode the AUA codon, three species of mt $tRNA^{Met}$ were prepared. The native $tRNA^{Met}$, possessing two pseudouridines (Ψ) at positions 27 and 50 as well as f^5C at position 34 (Figure 1A), was prepared from bovine liver mitochondria as reported (12). A synthetic tRNA without any modified nucleoside, and a synthetic tRNA with two Ψ's but no f^5C were obtained by enzymatic ligation of the 3′-half and 5′-half fragments with or without Ψ's (Figure 3A), which were both chemically synthesized (33). The sequences of the native and synthetic $tRNAs^{Met}$ were confirmed by Donis–Keller's method (Figure 3B) (29,30). The methionine acceptance activities of these synthetic tRNAs, catalyzed by the partially purified mt MetRS, gave values comparable to those of the native $tRNA^{Met}$ (native $tRNA^{Met}$, K_m = 0.31 µM; synthetic $tRNA^{Met}$ with and without Ψ, K_m = 0.37 µM). The kinetic parameters were comparable to those of human mt MetRS (38).

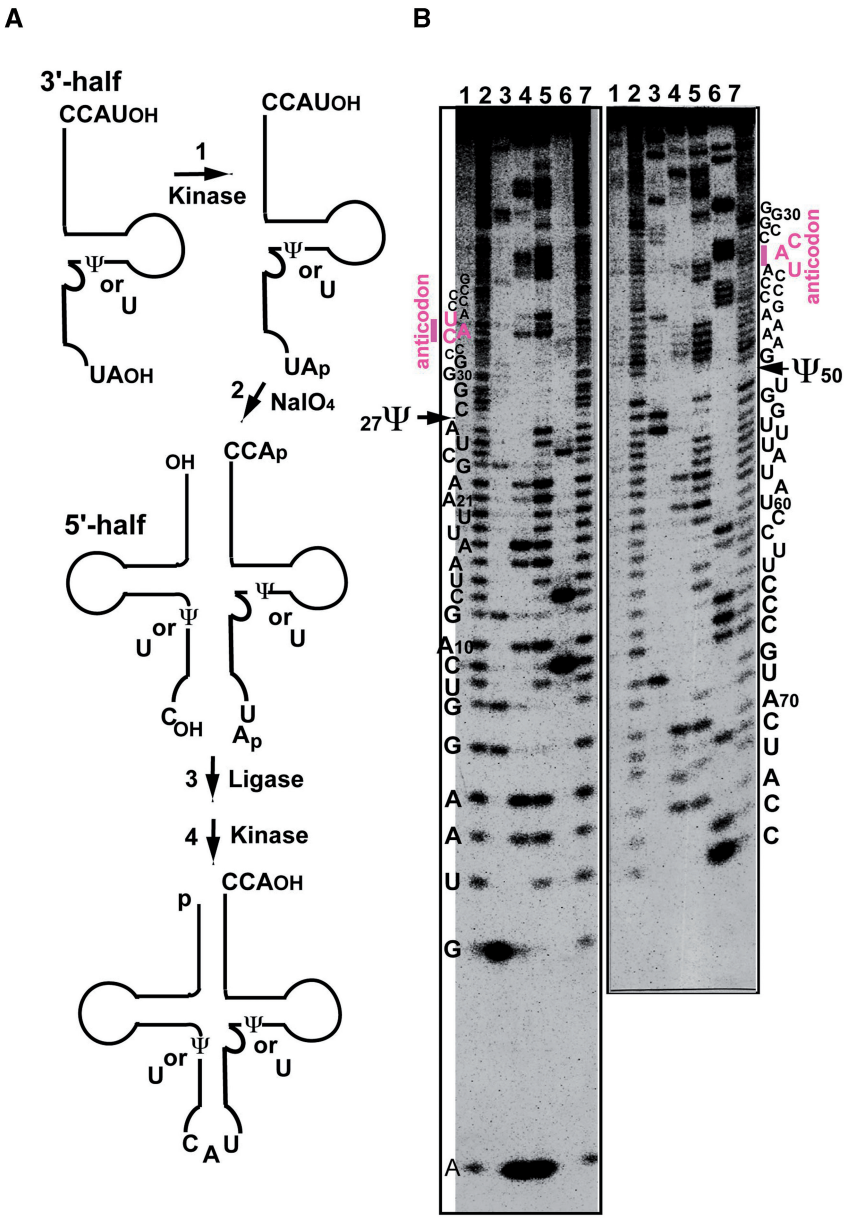

Figure 3. Preparation of synthetic $tRNA^{Met}$ variants. (**A**) Process of synthesizing mt $tRNA^{Met}$ by a combination of chemical synthesis and ligation (33). The synthetic tRNAs have sequences identical to the respective sequences of native $tRNA^{Met}$, except for f^5C and pseudouridine (Ψ). (**B**) Sequencing analysis using the Donis–Keller′s method of the synthetic mt $tRNA^{Met}$ labeled with [^{32}P] at the 5′-end (left) and the 3′-end (right), respectively (30,32). Electrophoresis was performed on a 15% polyacrylamide–7 M urea–10% glycerol gel. Lanes: control without ribonuclease (RNase) (lane 1); limited alkaline hydrolysis (lanes 2 and 7); digestion by RNase T_1 (specific for G: lane 3), RNase U_2 (specific for A: lane 4), RNase PhyM (specific for A and U: lane 5) and RNase CL_3 (specific for C: lane 6). As indicated by the arrows, Ψ was not digested by RNase PhyM (12). The numbering of the residues in the cloverleaf structure of the mt tRNA conforms to that in the previous report (71).

We also purified *E. coli* $tRNA^{Met}_m$ (anticodon ac^4CAU) and bovine cytoplasmic initiator tRNA (anticodon CAU) from *E. coli* cells and bovine liver, respectively, as reported (12,26), which were both used as references for examining the decoding properties of mt $tRNA^{Met}$.

Codon-dependent ribosomal binding of mitochondrial $tRNA^{Met}$ analogs

Ribosome binding experiments were carried out using these mt tRNAs and chemically synthesized mRNAs. Synthetic polyribonucleotides comprising six repeats of AUN codon triplets (N is A, C or G) were used to provide efficient binding. As shown in Figure 4, ~50% of the input native Met-tRNA (1.6 pmol) bound to ribosomes in the presence of the AUG codon. Similar binding was obtained with the Met-$tRNAs^{Met}$ lacking f^5C, regardless of the presence or absence of the two Ψ residues. This observation is expected since the anticodon of the synthetic tRNA (CAU) is the complement for the AUG codon. These data also argue that the formylation of C34 does not interfere with pairing with the G residue in the third position

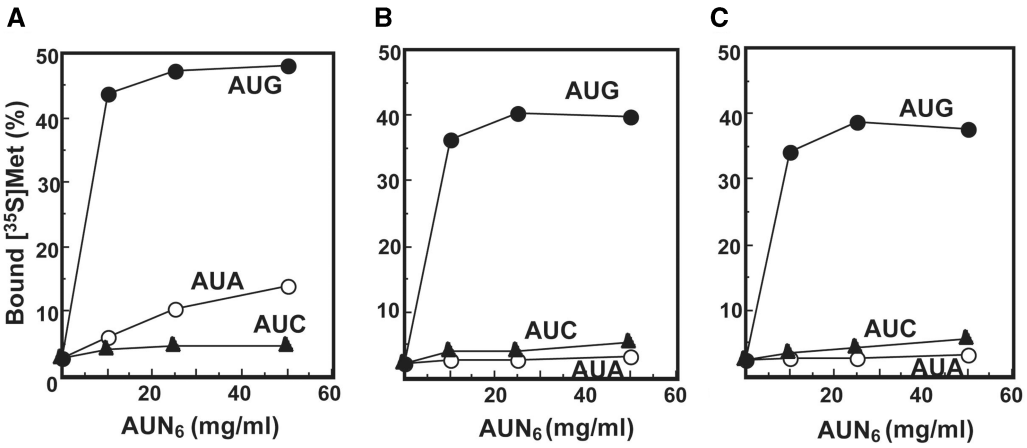

Figure 4. Codon-dependent ribosome binding assays of bovine mt $tRNA^{Met}$ variants. (**A**) Native mt $tRNA^{Met}$ purified from bovine liver mitochondria. (**B**) Synthetic mt $tRNA^{Met}$ with no modified bases. (**C**) Synthetic mt $tRNA^{Met}$ with two Ψ's. Oligonucleotides AUG_6 (filled circle), AUA_6 (open circle) and AUC_6 (filled triangle) were used as mRNA. The presented values are the averages of three independent experiments and were reproducible within ± 1.2%.

of the codon. The native Met-tRNA was also capable of reading the AUA codon although it was less efficiently recognized than the AUG codon. The synthetic Met-$tRNA^{Met}$ that did not carry the f^5C modification could read the AUG codon effectively (Figure 4B and C). However, this tRNA could not read the AUA codon at all (Figure 4B and C). These data strongly argue that the recognition of AUA as a methionine codon requires the modification of C34 by the formylation of C5. Comparison of the two synthetic Met-$tRNAs^{Met}$ indicates that the conversion of U to Ψ at positions 27 and 50 is not important for recognition of the AUG codon (Figure 4B and C). These results are in line with the recent report that a chemically synthesized RNA anticodon stem-loop fragment with modified bases f^5C and Ψ confers affinity for the AUA and AUG codons on *E. coli* 70S ribosomes (39).

In vitro translation assay using $tRNAs^{Met}$ with various anticodons

To further examine the role of the f^5C modification in the translation of AUA codons, the *in vitro* mitochondrial translation system was used with a message carrying eleven repeats of the AUN codon triplets $(AUN)_{11}$, to allow the translation products to be efficiently precipitated with TCA. Unlike poly(Phe) synthesis, the background level of Met incorporation in the absence of mRNA increased with concentrations of spermine >2 mM. Therefore, 0.6 mM spermine was included in the reaction mixture to reduce the background. Mitochondrial EF-Tu was used in all of the assay systems shown in Figure 5, except for Figure 5F. Thus, we adjusted all the conditions of the translation assay so that the AUA codon could be recognized by mt $tRNA^{Met}$ efficiently.

Native Met-$tRNA^{Met}$ was active in oligo(Met) synthesis with both AUG and AUA codons (Figure 5A), which is in agreement with the ability of this Met-tRNA to bind both codons in the ribosome binding assays. As expected, the native Met-tRNA was not able to read the near-cognate AUC codon. In contrast, the synthetic Met-tRNA carrying the two Ψ residues is active with the $(AUG)_{11}$ template but not with the $(AUA)_{11}$ template (Figure 5B). These data are in agreement with the ribosome binding experiments that indicate that the f^5C modification is required to read the AUA codon.

Two control Met-tRNAs were also used in these experiments. First, the bovine cytoplasmic initiator Met-$tRNA^{Met}_i$ with the CAU anticodon (Figure 5C) and secondly the *E. coli* elongator Met-$tRNA^{Met}_m$ with the ac^4CAU anticodon (Figure 5D) were tested in $(AUN)_{11}$-dependent oligo(Met) synthesis with mt ribosomes. Both of these Met-tRNAs translated AUG codons as Met. However, neither of them could read the AUA codon. The failure of the *E. coli* elongator $tRNA^{Met}$ ($tRNA^{Met}_m$) to translate the AUA codon (Figure 4D) is reasonable, because AUA is an isoleucine codon in *E. coli*, and ac^4C at position 34 of $tRNA^{Met}_m$ is known to serve in preventing the misreading of the AUA codon (40). The mitochondrial ribosomes and mt EF-Tu/Ts used in these experiments work more rapidly with the more canonical cytoplasmic initiator and bacterial elongator than they do with the mitochondrial Met-tRNA. This difference may reflect the somewhat unusual structures of the mitochondrial tRNAs that are generally shorter than canonical tRNAs and that lack some of the common features of these tRNAs (Figure 1).

We noticed that the bovine cytoplasmic initiator $tRNA^{Met}_i$ could read the AUG codon in this system (Figure 5C). This observation suggests that mt EF-Tu can recognize bovine cytoplasmic initiator $tRNA^{Met}$, unlike the case of yeast initiator $tRNA^{Met}$. This is probably because the bovine cytoplasmic initiator $tRNA^{Met}_i$ has no 2′-phosphoribosyl residue in the T-stem (Figure 1B), which is known as the antideterminant of yeast initiator tRNA toward the cytoplasmic EF-1α (41,42). Further, the single $tRNA^{Met}$ gene in mammalian mitochondria provides both the initiator and elongator tRNAs for methionine and mt EF-Tu has presumably evolved to be somewhat flexible in its ability to accommodate

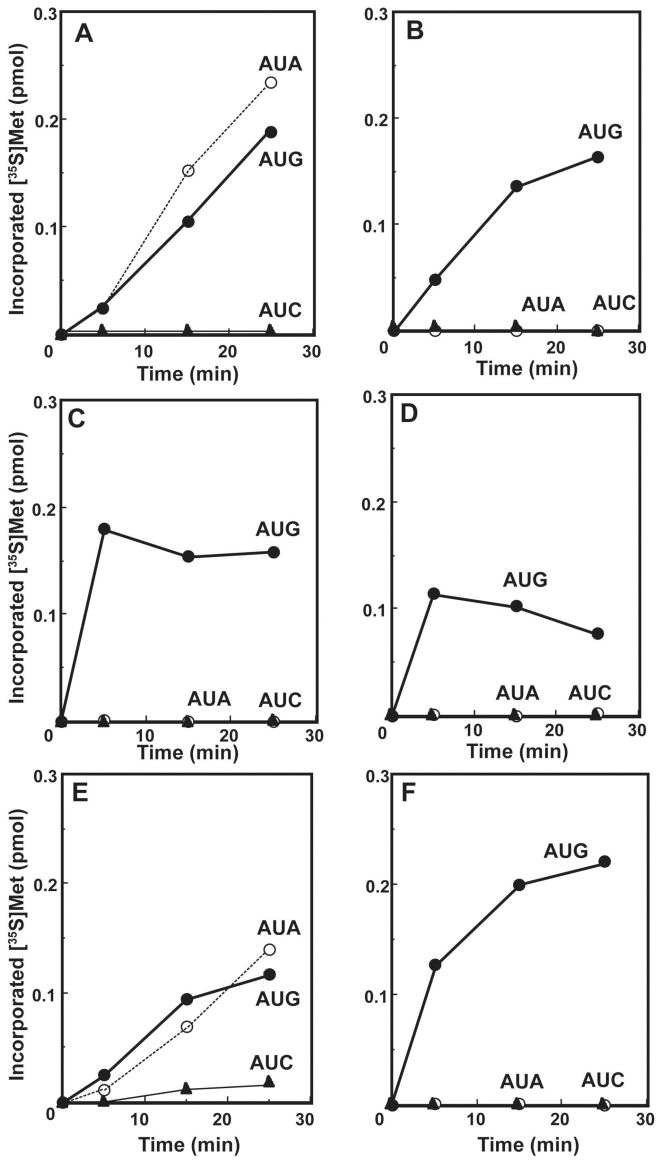

Figure 5. The codon recognition abilities of $tRNAs^{Met}$ possessing different anticodons. Time courses of oligo(AUN)-dependent oligo(Met) synthesis in an *in vitro* translation system with mitochondrial (**A–D**) or *E. coli* (**E** and **F**) ribosomes. (A and E) Native bovine mt $tRNA^{Met}$ purified from bovine liver mitochondria. (B) Synthetic mt $tRNA^{Met}$ with two Ψ's. (C) Bovine cytoplasmic initiator $tRNA^{Met}$. (D and F) *E. coli* elongator $tRNA^{Met}$. Oligonucleotides AUA_{11} (open circle), AUC_{11} (filled triangle) and AUG_{11} (filled circle) were used as mRNA. The incorporation of [^{35}S]Met was normalized by subtracting the value without mRNA, which was <2% of the input c.p.m. (about 0.03 pmol).

Met-tRNAs with some features generally observed in initiator tRNAs.

Both bovine cytoplasmic initiator $tRNA^{Met}$ and mt $tRNA^{Met}$ have C at position 33, just 5′-adjacent to the anticodon. This position is usually occupied by U, to form the U-turn structure in the anticodon loop (43). However, this unusual base replacement does not appear to play a role in the recognition of the AUA codon (Figure 5C). In the first position of the anticodon, the bovine cytoplasmic initiator $tRNA^{Met}$ has an unmodified C, which is unable to pair with A at the third letter of the AUA codon, and thus it cannot be a candidate for recognizing AUA codons in the mt system.

Codon recognition ability of mitochondrial tRNA on *E. coli* ribosomes

To examine whether the ability of the native Met-$tRNA^{Met}$ to read the AUA codon was a property of the tRNA alone or was dependent also on the use of mitochondrial ribosomes, the decoding of $(AUG)_{11}$ and $(AUA)_{11}$ was examined on *E. coli* ribosomes (Figure 5E). These data clearly show that the native mt Met-$tRNA^{Met}$ decodes AUA on bacterial ribosomes indicating that this decoding is a property of the tRNA and not of the ribosome. Thus, it is evident that the translation of the AUA codon for Met is achieved by the strict recognition of the AUA codon by the tRNA anticodon containing f^5C34, irrespective of the origin of the ribosomes. Mitochondrial Met-$tRNA^{Met}$ with *E. coli* EF-Tu/Ts did not work on either mt or *E. coli* ribosomes using any oligo $(AUN)_{11}$ (data not shown). This observation is consistent with previous results obtained with mt Phe-$tRNA^{Phe}$, which was not effectively delivered to the ribosomal A-site with *E. coli* EF-Tu (17). In a control experiment (Figure 5F), the clear discrimination of the *E. coli* elongator Met-$tRNA_m^{Met}$ against the AUA codon on *E. coli* ribosomes was confirmed.

To verify the accurate discrimination of the AUN codon box, the codon recognition abilities of mt $tRNA^{Ile}$, *E. coli* $tRNA^{Ile}_1$ and *E. coli* $tRNA^{Ile}_2$ were tested using both mt and *E. coli* ribosomes (see Supplementary Material). As expected, all of the $tRNAs^{Ile}$ recognized the AUC codon, but not the AUG codon. In addition, $tRNA^{Ile}_2$, possessing the anticodon k^2CAU (k^2C = 2-lysylcytidine), recognized both AUA and AUC codons. This modified base is known to switch both the amino acid acceptance (from Met to Ile) and codon recognition (from AUG to AUA codons) of the tRNA (44). Again the recognition profile was independent of the source of the ribosomes used in agreement with the previous data.

Investigation of a role of the modified nucleotide f^5C in mitochondrial $tRNAs^{Met}$

All of the tRNAs used in this study, except for mt $tRNA^{Met}$, have N^6-threonylcarbamoyladenosine (t^6A37) adjacent to U36, the first position of the anticodon (45). It has been verified that t^6A37 provides additional stability to codon–anticodon interactions through a cross-strand stack above the first base pair in the ribosomal A-site (46). Bacterial initiator $tRNAs^{Met}$ possess an unmodified A37 because the initiator tRNA has to enter only to the ribosomal P-site during translation initiation. Although mt $tRNA^{Met}$ also possesses an unmodified A37, it plays a dual role as an initiator and an elongator tRNA in mitochondria. Therefore, the unique modification such as f^5C may have a significant advantage in stabilizing codon–anticodon base pairs.

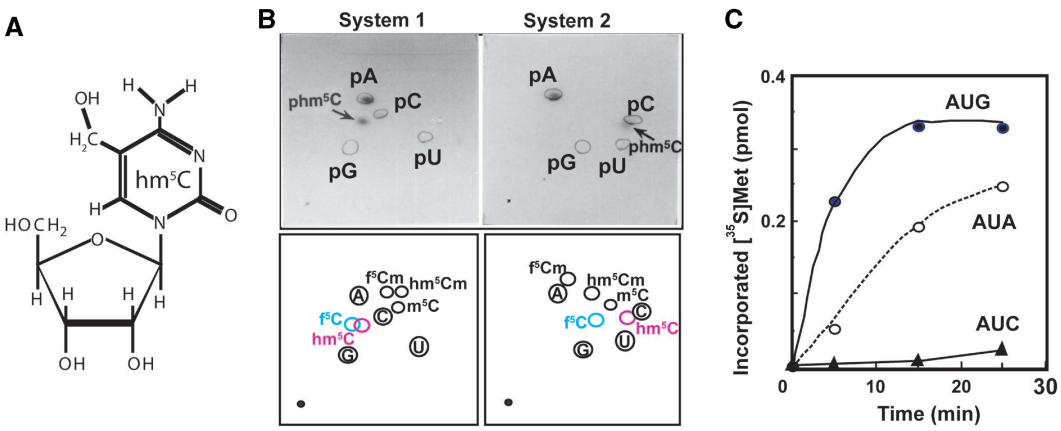

Figure 6. Reduction of 5-formylcytidine to 5-hydroxylcytidine (hm^5C) of mt $tRNA^{Met}$. (**A**) The chemical structure of hm^5C. (**B**) 2D-TLC analysis of the nucleotide at the anticodon wobble position of mt $tRNA^{Met}$ after reduction (upper panels), and diagrams of chromatographic mobility of modified cytosine 5′-monophospates (lower panels) (49,50). The detailed procedure is described in Materials and Methods section. The solvent used for the first dimension in both systems was isobutyric acid/concentrated ammonia/water (66:1:33 v/v/v). For the second dimension, 2-propanol/HCl/water (70:15:15 v/v/v) and ammonium sulfate/0.1 M sodium phosphate (pH 6.8)/1-propanol (60 g:100 ml:2 ml) was used in the systems 1 and 2, respectively. (**C**) The codon recognition ability of the reduced mt $tRNA^{Met}$ (anticodon hm^5CAU) on mt ribosomes. The incorporation of [^{35}S]-Met was normalized by subtracting the value without mRNA.

Proton and nitrogen nuclear magnetic resonance (NMR) studies of deoxycytosine derivatives, f^5C, 5-hydroxymethylcytosine (hm^5C), and 5-hydroxycytosine, show that the 4-amino group and the oxygen group of the substituent at C-5 form an intrabase hydrogen bond indicated by significant changes in both ^1H and ^{15}N chemical shifts (47). Further, it was reported that the Klenow fragment incorporates dAMP against f^5C more frequently than cytidine (48). These results suggest that the intrabase hydrogen-bonding of C5-substituent derivatives may introduce electronic effects on cytosine, enabling the modified cytidine to base pair with A. To examine the codon recognition ability of another C5-substiuent, f^5C of mt $tRNA^{Met}$ was reduced by sodium borohydride to form hm^5C (Figure 6A). The sequence of the reduced mt $tRNA^{Met}$ was confirmed by the Donis–Keller's method (29,30,32) and reduction of f^5C to hm^5C34 was confirmed by the two-dimensional thin-layer chromatography (2D-TLC) in the two solvent systems (34,49,50). As shown in Figure 6B, f^5C at the position 34 was clearly reduced to hm^5C. The reduced $tRNA^{Met}$ was efficiently aminoacylated by mt MetRS (K_m = 0.46 μM), indicating that the reduced $tRNA^{Met}$ retained the normal ternary structure required for the biochemical activity. The hydroxymethyl group was not an anti-determinant for recognition by mt MetRS. The codon recognition ability of Met-tRNA with the anticodon hm^5CAU was examined in the mt homologous system described above. As shown in Figure 6C, Met-tRNA with the anticodon hm^5CAU recognized both the AUG and AUA codons. This observation suggests that a common chemical property of f^5C and hm^5C is responsible for base pairing with A.

DISCUSSION

Mitochondrial translation system using various tRNA species

We reconstituted an *in vitro* mitochondrial translation system, which had sufficient efficiency to test the codon recognition ability of Met-$tRNA^{Met}$. The addition of spermine significantly improved translational efficiency. The optimum concentration of spermine was different with the mitochondrial and *E. coli* tRNA species, suggesting that spermine contributes to the structural stability of mt tRNA. Spermine has been observed in tRNA crystal structures (PDB ID: 1evv, 1tn2 and 2tra), which is assumed to contribute to structural stability (51–53). Spermine may play a more important role in stabilizing the structures of mt tRNAs since these tRNAs lack may of the stabilizing features observed with canonical tRNAs. After optimization of mt poly(U)-dependent poly(Phe) synthesis, AUN-dependent oligo(Met) synthesis was improved by decrease of mRNA concentration and addition of nonradioactive Met. The former was effective on the AUA-dependent Met-incorporation but had no effect on the AUG-dependent incorporation. On the other hand, the latter was useful for decrease of the background level.

The codon recognition pattern of each tRNA on mt ribosomes was similar to that on *E. coli* ribosomes. However, the efficiency of peptide synthesis was not always the same at least with $tRNA^{Phe}$, $tRNA^{Met}$ and $tRNA^{Ile}$. The efficiency of poly(Phe) synthesis by *E. coli* $tRNA^{Phe}$ in the mt system was the same as that in the *E. coli* system (Figure 2B), while that of oligo(Met) or oligo(Ile) by *E. coli* $tRNA^{Met}$ or $tRNA^{Ile}$ in the mt system was lower than that in the *E. coli* system (Figure 5D and F, and Supplementary Figure 3). Mitochondrial $tRNA^{Met}$ or $tRNA^{Ile}$ could work on mt ribosomes better than on *E. coli* ribosomes (Figure 5A and E, and Supplementary Figure 2). We could not find conditions under which *E. coli* $tRNA^{Met}_m$ worked efficiently with mt EF-Tu/Ts on 70S ribosomes (data not shown). In contrast, *E. coli* $tRNA^{Phe}$ could work well with any combination of EF-Tu and ribosomes (Figure 2). These results suggested that the combination of tRNA, EF-Tu and ribosomes from heterogeneous sources would cause subtle differences in their interactions with one another when the ternary complex of

aminoacyl-tRNA, EF-Tu and GTP associates with ribosomes. Although mt EF-Tu shares high homology and similar structural domains with those of *E. coli* (54,55), a slightly different affinity between EF-Tu and rRNA or ribosomal proteins would probably affect the peptide synthesis efficiency. This heterogeneous system might be useful to clarify what happens in the ribosome through the entry of the ternary complex. Although the efficiency differs, according to the tRNA species or the combination of EF-Tu/Ts and ribosomes, this assay system would be useful to verify the codon recognition ability of mt tRNAs that have unusual, unstable structures.

The role of the modified nucleotide f^{5}C in the expanded codon recognition

Mammalian mitochondria face the challenge of decoding both AUG and AUA in both the P-site and the A-site using a single tRNA^{Met}. The work described here indicates that a critical feature of tRNA^{Met} in the decoding process is the modified residue f^{5}C in the first position of the anticodon. This work represents the first experimental evidence in an organelle that a modified nucleotide in the anticodon expands the codon recognition ability of a tRNA. Our results clearly demonstrate that native mt Met-tRNA^{Met} with the anticodon f^{5}CAU, can read the AUA codon in addition to the AUG codon. The putative interactions in the f^{5}C-G and f^{5}C-A base pairs can be modeled by base replacements of C34 with f^{5}C in the anticodon of tRNA$^{Met}_f$ (PDB: 2J00), and, by the replacement of G3 of the methionine codon with adenine, respectively (Figure 7). The f^{5}C-G pair clearly forms similar interactions to those observed with a normal C–G pair (Figure 7A and B).

It is more difficult to visualize how f^{5}C base pairs with A. Two views of an f^{5}C-A base pair can be proposed. In the first view, a hydrogen bond forms between N1 of A and N3 of f^{5}C (Figure 7C, black line). In the second view, N1 of A is hydrogen bonded to N4 of f^{5}C (Figure 7C, magenta line). Although the distance between N1 of A and N3 of f^{5}C is adequate to form a hydrogen bond (2.95 Å), N1 of A must be protonated for this interaction to occur. On the other hand, hydrogen bonding between N4 of f^{5}C and N1 of A does not require protonation of the A residue.

There have been several observations of the C–A$^+$ base pair (A$^+$ means adenosine protonated at N-1) by NMR and crystallographic studies. The protonation of A (A$^+$) in an A–C pair has been shown in double-stranded (ds) DNA (56) and in tandem C–A base pairs in dsRNA (57). NMR studies demonstrated that pK_a values of A residues closed to the cleavage site of ribozymes were shifted to 6.2 and 6.5 (58,59). In the anticodon loop of *E. coli* tRNA$^{Lys}_3$ where an A residue is observed with a pKa of about 6 (60). In the case of mt tRNA^{Met}, there is no direct evidence for the protonation of N1 of A3. However, our NMR analyses have revealed that the f^{5}C nucleoside adopts the most rigid C3′-*endo* conformation in all of the modified cytidine nucleosides studied to date and that the pK_a value of f^{5}C is very low (61,62). It suggests that the approach of f^{5}C could bring about the protonation of A to form the f^{5}C-A$^+$ base pair.

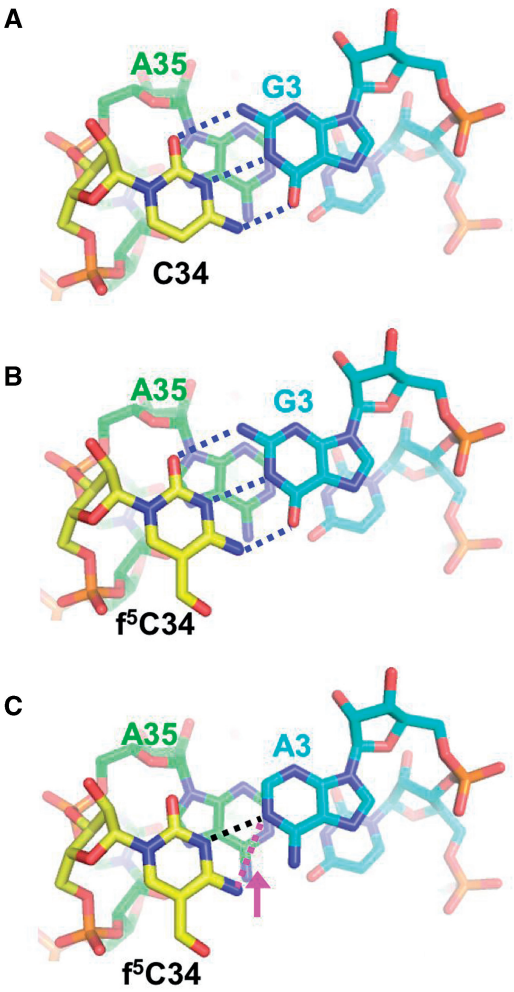

Figure 7. Base pairing model of f^{5}C-G and f^{5}C-A. (**A**) C34-G1 base pair of the initiator tRNA^{Met} and the AUG codon (PDB: 2J00). The first letter of the anticodon C34 (yellow) base pairs with the third letter of the codon G3 (cyan) and stacks on the 3′-adjacent nucleotide A35 (green). Hydrogen bonds between C34 and G3 are shown as blue dotted lines. (**B**) f^{5}C-G base pair model. The coordinates of f^{5}C were generated with the PRODRG server (http://davapc1.bioch.dundee.ac.uk/prodrg) (72) and superposed to the C34 in (A). Putative hydrogen bonds between f^{5}C34 and G3 are shown as blue dotted lines. (**C**) The f^{5}C-A base pair model. The coordinates for adenine were also generated with the PRODRG server and superposed on G3 in (B). Putative hydrogen bonds between f^{5}C34 and A3 are shown as dotted lines. The distance between N3 of f^{5}C34 and N1 of A3 or N4 of f^{5}C34 and N1 of A3 is 2.95 Å (black dotted line) or 3.44 Å (magenta dotted line), respectively. An arrow (magenta) represents an expected shift to form a hydrogen bond between N4 of f^{5}C34 and N1 of A3. These figures were prepared with PyMOL, from DeLano Scientific (http://www.pymol.sourceforge.net/).

Alternatively, in the second model above f^{5}C shifts slightly from the position taken in the base pair with G to form a hydrogen bond with A (magenta arrow in Figure 7C). In our previous NMR study, using the transferred nuclear Overhauser effect (TRNOE) method, we found that the conformation of the f^{5}C-A pair was different from that of the f^{5}C-G pair, and that the AUA codon

adopted an extended A-form RNA conformation (63–65). During the decoding process the codon should be fixed in the A-form (66,67), so that the anticodon loop of mt $tRNA^{Met}$ would adapt its own conformation to keep the mRNA in the A-form *in vivo*. This conformational change in the sugar–phosphate backbone would be tolerant at the wobble position like the inosine–adenosine base pair (68). In addition, NMR studies of 2′-deoxycytosine derivatives, f^5C and hm^5C show that the 4-amino group (N4) and the oxygen group of 5-substituent (O5) are fixed in the planar configuration against the cytosine base through intrabase hydrogen-bonding between N4 and O5 (47). The reduction of f^5C to hm^5C maintains the expanded codon recognition ability (Figure 6C), suggesting that common characteristics between f^5C and hm^5C enable the modified cytidine to base pair with A3 (the third letter of the codon). The tautomeric form of these 5-substituents of C34 would have the advantage of enhanced stacking against A35, compensating for the single hydrogen bond. This idea is supported by recent crystallographic studies of an RNA anticodon stem-loop fragment with modified bases in the ribosomal A-site. This structure clearly shows that the anticodon nucleotides exhibit a C3′-*endo* conformation and stack against the 3′-adjacent base (46,68–70). Because of the 3′ stack of the anticodon, the modified U34 prefers to base pair with G3 in the standard Watson–Crick base pairing geometry, not in the G–U wobble geometry (46,69). Moreover, even a single hydrogen bonding in the wobble base pair is compensated through increased stacking of the modified base and additional hydrogen bonding among the modification moiety and proximate groups in the adjacent nucleotide (46,69). Considering these steric interactions, it seems more probable that f^5C acquires an altered conformation, perhaps the tautomeric form, due to the presence of the 5-substituent group, and forms a 3′-stack with A35, which would compensate for a single hydrogen bonding in the f^5C-A base pair. Further investigations are required to clarify which nitrogen group of f^5C, N3 or N4, interacts with N1 of A3.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Anderson,S., Bankier,A.T., Barrell,B.G., de Bruijn,M.H.L., Coulson,A.R., Drouin,J., Eperon,I.C., Nierlich,D.P., Roe,B.A., Sanger,F. *et al.* (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457–465.
2. Himeno,H., Masaki,H., Kawai,T., Ohta,T., Kumagai,I., Miura,K. and Watanabe,K. (1987) Unusual genetic codes and a novel gene structure for tRNA(AGYSer) in starfish mitochondrial DNA. *Gene*, **56**, 219–230.
3. Wolstenholme,D.R. (1992) Animal mitochondrial DNA: structure and evolution. *Int. Rev. Cytol.*, **141**, 173–216.
4. Xu,X. and Arnason,U. (1994) The complete mitochondrial DNA sequence of the horse, *Equus caballus*: extensive heteroplasmy of the control region. *Gene*, **148**, 357–362.
5. Krettek,A., Gullberg,A. and Arnason,U. (1995) Sequence analysis of the complete mitochondrial DNA molecule of the hedgehog, Ernaceus europaeus, and the phylogenic position of the Lipotyphla. *J. Mol. Evol.*, **41**, 952–957.
6. Osawa,S., Juckes,T.H., Watanabe,K. and Muto,A. (1992) Recent evidence for evolution of the genetic code. *Microbiol. Rev.*, **56**, 229–264.
7. Watanabe,K. and Osawa,S. (1995) In Soll,D. and RajBhandary,U. (eds), *tRNA: Structure, Biosynthesis, and Function*, American Society for Microbiology, Washington DC, pp. 225–250.
8. Roe,B.A., Wong,J.F.H. and Chen,E.Y. (1981) In Walton,A. G. (ed.), *Proceedings of the Third Cleveland Symposium on Macromolecules*, Elsevier, Amsterdam, pp. 167–176.
9. Crick,F.H.C. (1966) Codon-anticodon pairing: the wobble hypothesis. *J. Mol. Biol.*, **19**, 548–555.
10. Mikelsaar,R. (1981) C-A base pairs in transfer ribonucleic acids and codon-anticodon complexes. *J. Theoritical. Biol.*, **92**, 163–180.
11. Samuelsson,T., Axberg,T., Boren,T. and Lagerkvist,U. (1983) Unconventional reading of glycine codons. *J. Biol. Chem.*, **258**, 13178–13184.
12. Moriya,J., Yokogawa,T., Wakita,K., Ueda,T., Nishikawa,K., Crain,P.F., Hashizume,T., Pomerantz,S.C., McClosky,J.A., Kawai,G. *et al.* (1994) A novel modified nucleoside found at the first position of the anticodon of methionine trna from bovine liver mitochondria. *Biochem.*, **33**, 2234–2239.
13. Watanabe,Y., Tsurui,H., Ueda,T., Furushima,R., Takamiya,S., Kita,K., Nishikawa,K. and Watanabe,K. (1994) Primary and higher order structures of nematode (*Ascaris suum*) mitochondrial tRNAs lacking either the T or D stem. *J. Biol. Chem.*, **269**, 22902–22906.
14. Tomita,K., Ueda,T. and Watanabe,K. (1997) 5-formylcytidine (f^5C) found at the wobble position of the anticodon of squid mitochondrial $tRNA^{Met}CAU$. *Nucleic Acids Symp. Ser.*, **37**, 197–198.
15. Tomita,K., Ueda,T., Ishiwa,S., Crain,P.F., McClosky,J.A. and Watanabe,K. (1999) Codon reading patterns in *Drosophila melanogaster* mitochondria based on their tRNA sequences: a unique wobble rule in animal mitochondria. *Nucleic Acids Res.*, **27**, 4291–4297.
16. Takemoto,C., Ueda,T., Miura,K. and Watanabe,K. (1999) Nucleotide sequences of animal mitochondrial $tRNAs^{Met}$ possibly recognizing both AUG and AUA codons. *Nucleic Acids Symp. Ser.*, **42**, 77–78.
17. Kumazawa,Y., Schwartzbach,C.J., Liao,H.-X., Mizumoto,K., Kaziro,Y., Miura,K., Watanabe,K. and Spremulli,L.L. (1991) Interaction of bovine mitochondrial phenylalanyl-tRNA with ribosomes and elongation factors from mitochondria and bacteria. *Biochem. Biophys. Acta*, **1090**, 167–172.
18. Eberly,S.L., Locklear,V. and Supremulli,L.L. (1985) Bovine mitochondrial ribosomes, elongation factor specificity. *J. Biol. Chem.*, **260**, 8721–8725.
19. Takemoto,C., Koike,T., Yokogawa,T., Benkowski,L., Spremulli,L.L., Ueda,T., Nishikawa,K. and Watanabe,K. (1995)

The ability of bovine mitochondrial transfer RNA^{Met} to decode AUG and AUA codons. *Biochimie*, **77**, 104–108.
20. Scaringe,S.A., Franklyn,C. and Usman,N. (1990) Chemical synthesis of biologically active oligoribonucleotides using beta-cyanoethyl protected ribonucleoside phosphoramidites. *Nucleic Acids Res.*, **18**, 5433–5441.
21. Matthews,D.E., Hessles,R.A., Denslow,N.D., Edwards,J.S. and O'Brien,T.W. (1982) Protein composition of the bovine mitochondrial ribosome. *J. Biol. Chem.*, **257**, 8788–8794.
22. Schwartzbach,C.J., Farwell,M., Liao,H.-X. and Spremulli,L.L. (1996) Bovine mitochondrial initiation and elongation factors. *Methods Enzymol.*, **264**, 248–261.
23. Ravel,J., Shorey,R., Foehner,S. and Shive,W. (1986) A study of the enzymatic transfer of aminoacyl-tRNA to *E. coli* ribosomes. *Arch. Biochem. Biophys.*, **125**, 514–526.
24. Tsurui,H., Kumazawa,Y., Sanakawa,R., Watanabe,Y., Kuroda,T., Wada,A., Watanabe,K. and Shurai,T. (1994) Batchwise purification of specific tRNAs by a solid-phase DNA probe. *Anal. Biochem.*, **221**, 166–172.
25. Wakita,K., Watanabe,Y., Yokogawa,T., Kumazawa,Y., Nakamura,S., Ueda,T., Watanabe,K. and Nishikawa,K. (1994) Higher-order structure of bovine mitochondrial tRNAPhe lacking the 'conserved' GG and TYCG sequences as inferred by enzymatic and chemical probing. *Nucleic Acids Res.*, **22**, 347–353.
26. Seno,T., Kobayashi,M. and Nishimura,S. (1968) Purification of Escherichia coli methionine tRNAf and tRNAm and studies on their biophysical and biochemical properties. *Biochem. Biophys. Acta*, **169**, 80.
27. Nishimura,S. (1971) *Proceedings in Nucleic Acid Research*, Harper & Row, New York.
28. Chomcznski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
29. Donis-Keller,H., Maxam,A.M. and Gilbert,W. (1977) Mapping adenosines, guanosines, pyrimidines in RNA. *Nucleic Acids Res.*, **4**, 2527–2538.
30. Donis-Keller,H. (1980) PhyM: an RNase activity specific for U and A residues useful in RNA sequence analysis. *Nucleic Acids Res.*, **8**, 3133–3142.
31. Kuchino,Y., Hanyu,N. and Nishimura,S. (1987) Analysis of modified nucleosides and nucleotide sequence of tRNA. *Methods Enzymol.*, **155**, 379–396.
32. Kuchino,Y. and Nishimura,S. (1989) Enzymatic RNA sequencing. *Methods Enzymol.*, **180**, 154–163.
33. Ohtsuki,T., Kawai,G., Watanabe,Y., Kita,K., Nishikawa,K. and Watanabe,K. (1996) Preparation of biologically active *Ascaris suum* mitochondrial tRNA^{Met} with a TV-replacement loop by ligation of chemically synthesized RNA fragments. *Nucleic Acids Res.*, **24**, 662–667.
34. Yokogawa,T., Watanabe,Y., Kumazawa,Y., Ueda,T., Hirao,I., Miura,K. and Watanabe,K. (1991) A novel cloverleaf structure found in mammalian mitochondrial tRNA(Ser) (UCN). *Nucleic Acids Res.*, **19**, 6101–6105.
35. Nierhaus,K.H. (1990) In Spedding,G. (ed.), *Ribosomes and Protein Synthesis*, Oxford University Press, New York, pp. 161–189.
36. O'Brien,T.W. and Denslow,N.D. (1996) Bovine mitochondrial ribosomes. *Methods Enzymol.*, **264**, 237–248.
37. Schwartzbach,C.J. and Spremulli,L.L. (1989) Bovine mitochondrial protein synthesis elongation factors identification and initial characterization of an elongation factor Tu- elongation factor Ts complex. *J. Biol. Chem.*, **264**, 19125–19131.
38. Spencer,A., Heck,A., Takeuchi,N., Watanabe,K. and Spremulli,L. (2004) Characterization of the human mitochondrial methionyl-tRNA synthetase. *Biochem.*, **43**, 9743–9754.
39. Lusic,H., Gustilo,E.M., Vendeix,F.A., Kaiser,R, Delaney,M.O., Graham,W.D., Moye,V.A., Cantara,W.A., Agris,P.F. and Deiters,A. (2008) Synthesis and investigation of the 5-formylcytidine modified, anticodon stem and loop of the human mitochondrial tRNA^{Met}. *Nucleic Acids Res.*, **36**, 6548–6557.
40. Stern,L. and Schulman,L.H. (1978) The role of the minor base N^4-acetylcytidine in the function of the *Escherichia coli* noninitiator methionine transfer RNA. *J. Biol. Chem.*, **253**, 6132–6139.
41. Forster,C., Chakraburtty,K. and Sprinzl,M. (1993) Discrimination between initiation and elongation of protein biosynthesis in yeast: identity assured by a nucleotide modification in the initiator tRNA. *Nucleic Acids Res.*, **21**, 5679–5683.
42. von Pawel-Rammingen,U., Astrom,S. and Bystrom,A.S. (1992) Mutation analysis of conserved position potentially important for initiator tRNA function in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **12**, 1432–1442.
43. Quigley,G.J. and Rich,A. (1976) Structural domains of transfer RNA molecules. *Science*, **194**, 796–806.
44. Muramatsu,T., Nishikawa,K., Nemoto,F., Kuchino,Y., Nishimura,S., Miyazawa,T. and Yokoyama,S. (1988) Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. *Nature*, **336**, 179–181.
45. Rozenski,J., Crain,P.F. and McCloskey,J.A. (1999) The RNA modification database: 1999 update. *Nucleic Acids Res.*, **27**, 196–197.
46. Murphy,F.V., Ramakrishnan,V., Malkiewicz,A. and Agris,P.F. (2004) The role of modifications in codon discrimination by tRNA(Lys)UUU. *Nat. Struct. Mol. Biol.*, **11**, 1186–1191.
47. LaFrancois,C.J., Fujimoto,J. and Sowers,L.C. (1998) Synthesis and characterization of isotopically enriched pyrimidine deoxynucleoside oxidation damage products. *Chem. Res. Toxicol.*, **11**, 75–83.
48. Karino,N., Ueno,Y. and Matsuda,A. (2001) Synthesis and properties of oligonucleotides containing 5-formyl-2′-deoxycytidine: in vitro DNA polymerase reactions on DNA templates containing 5-formyl-2'-deoxycytidine. *Nucleic Acids Res.*, **29**, 2456–2463.
49. Keith,G. (1995) Mobilities of modified ribonucleotides on two-demensional cellulose thin-layer chromatography. *Biochimie*, **77**, 142–144.
50. Grosjean,H., Droogmans,L., Roovers,M. and Keith,G. (2007) Detection of enzymatic activity of transfer RNA modification enzymes using radiolabeled tRNA substrates. *Methods Enzymol.*, **425**, 55–101.
51. Quigley,G.J., Teeter,M.M. and Rich,A. (1978) Structural analysis of spermine and magnesium ion binding to yeast phenylalanine transfer RNA. *Proc. Natl Acad. Sci. USA*, **75**, 64–68.
52. Brown,R.S., Dewan,J.C. and Klug,A. (1985) Crystallographic and biochemical investigation of the lead(II)-catalyzed hydrolysis of yeast phenylalanine tRNA. *Biochemistry*, **24**, 4785–4801.
53. Jovine,L., Djordjevic,S. and Rhodes,D. (2000) The crystal structure of yeast phenylalanine tRNA at 2.0 Å resolution: cleavage by Mg(2+) in 15-year old crystals. *J. Mol. Biol.*, **301**, 401–414.
54. Woriax,V.L., Burkhart,W. and Spremulli,L.L. (1995) Cloning, sequence analysis and expression of mammalian mitochondrial protein synthesis elongation factor Tu. *Biochim. Biophys. Acta*, **1264**, 347–356.
55. Andersen,G., Thirup,S., Spremulli,L. and Nyborg,J. (2000) High resolution crystal structure of bovine mitochondrial EF-Tu in complex with GDP. *J. Mol. Biol.*, **297**, 421–436.
56. Hunter,W., Brown,T., Anand,N. and Kennard,O. (1986) Structure of an adenine-cytidine base pair in DNA and its implications for mismatch repair. *Nature*, **320**, 552–555.
57. Jang,S.B., Hung,L.W., Chi,Y.I., Holbrook,E.L., Carter,R.J. and Holbrook,S.R. (1998) Structure of an RNA internal loop consisting of tandem C-A$^+$ base pairs. *Biochemistry*, **37**, 11726–11731.
58. Cai,Z. and Tinoco,I. (1996) Solution structure of loop A from the hairpin ribozyme from tobacco ringspot virus satellite. *Biochemistry*, **35**, 6026–6036.
59. Legault,P. and Pardi,A. (1994) In situ probing of adenine protonation in RNA by ^{13}C NMR. *J. Am. Chem. Soc.*, **116**, 8390–8391.
60. Durant,P.C. and Davis,D.R. (1999) Stabilization of the anticodon stem-loop of tRNA$^{Lys}_3$ by an A$^+$-C base-pair and by pseudouridine. *J. Mol. Biol.*, **285**, 115–131.
61. Kawai,G., Yokogawa,T., Nishikawa,K., Ueda,T., Hashizume,T., McCloskey,J.A., Yokoyama,S. and Watanabe,K. (1994) Conformational properties of a novel modified nucleoside, 5-formylcytidine, found at the first position of the anticodon of bovine mitochondrial tRNA^{Met}. *Nucleosides Nucleotides*, **13**, 1189–1199.
62. Kawai,G., Hashizume,T., Yasuda,M., Mayazawa,T., McCloskey,J.A. and Yokoyama,S. (1992) Conformational rigidity of N^4-acetyl-2′-O-metylcytidine found in tRNA of extremely

thermophilic Archaebacteria (Archaea). *Nucleosides Nucleotides*, **11**, 759–771.
63. Kashiwai,T., Takemoto,C., Ohtsuki,T., Kawai,G. and Watanabe,K. (1998) NMR study on non-universal codon recognition by mitochondrial tRNA possessing a modified nucleoside 5-formylcytidine. *Nucleic Acids Symp. Ser.*, **39**, 155–156.
64. Rosevear,P. and Mildvan,A. (1989) Ligand conformations and ligand-enzyme interactions as studied by the nuclear Overhauser effect. *Methods Enzymol.*, **177**, 333–358.
65. Clore,G., Gronenborn,A. and Mclaughlin,L. (1984) Structure of the ribonucleoside diphosphate codon UpUpC bound to $tRNA^{Phe}$ from yeast. *J. Mol. Biol.*, **174**, 163–173.
66. Topal,M.D. and Fresco,J.R. (1976) Base pairing and fidelity in codon-anticodon interaction. *Nature*, **263**, 289–293.
67. Lim,V.I. and Curran,J.F. (2001) Analysis of codon:anticodon interactions within the ribosome provides new insights into codon reading and the genetic code structure. *RNA*, **7**, 942–957.
68. Murphy,F.V. and Ramakrishnan,V. (2004) Structure of a purine-purine wobble base pair in the decoding center of the ribosome. *Nat. Struct. Mol. Biol.*, **11**, 1251–1252.
69. Weixlbaumer,A., Murphy,F.V., Dziergowska,A., Malkiewicz,A., Vendeix,F.A., Agris,P.F. and Ramakrishnan,V. (2007) Mechanism for expanding the decoding capacity of transfer RNAs by modification of uridines. *Nat. Struct. Mol. Biol.*, **14**, 498–502.
70. Kurata,S, Weixlbaumer,A., Ohtsuki,T., Shimazaki,T., Wada,T., Kirino,Y., Takai,K., Watanabe,K., Ramakrishnan,V. and Suzuki,T. (2008) Modified uridines with C5-methylene substituents at the first position of the tRNA anticodon stabilize U.G wobble pairing during decoding. *J. Biol. Chem.*, **283**, 18801–18811.
71. Sprinzl,M., Hartmann,T., Weber,J., Blank,J. and Zeidler,R. (1989) Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.*, **17**, r1–r171.
72. Schüttelkopf,A.W. and von Aalten,D.M. (2004) PRODRG: a tool for high-throughput crystallography of protein-ligand complexes. *Acta Crystallogr. D*, **60** (Pt 8), 1355–1363.