A Novel Modified Nucleoside Found at the First Position of the Anticodon of Methionine tRNA from Bovine Liver Mitochondria†

Jiro Moriya,§ Takashi Yokogawa,§ Kawori Wakita,§ Takuya Ueda,‡ Kazuya Nishikawa,§ Pamela F. Crain,⊥ Takeshi Hashizume,⊥ Steven C. Pomerantz,⊥ James A. McCloskey,⊥ Gota Kawai,‡ Nobuhiro Hayashi,‡ Shigeyuki Yokoyama,∥ and Kimitsuna Watanabe*,‡

Department of Industrial Chemistry, Faculty of Engineering, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan, Department of Biological Sciences, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midoriku, Yokohama 227, Japan, Department of Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112, and Department of Biochemistry and Biophysics, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

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ABSTRACT: Methionine tRNA was purified from bovine liver mitochondria, and its nucleotide sequence was determined. The tRNA possesses only three posttranscriptionally modified nucleosides, two pseudouridines in the anticodon and T stems and a previously unknown nucleoside specified by the gene sequence as cytidine, in the first position of the anticodon. Structure analysis of the anticodon nucleoside by mass spectrometry revealed a molecular mass 28 Da greater than that of cytidine, and unmodified ribose, with substitution at C-5 implied by hydrogen–deuterium exchange experiments. Proton NMR of the intact tRNA showed presence of a formyl moiety, thus leading to the candidate structure 5-formylcytidine (f^5C), not a previously known compound. The structure assignment was confirmed by chemical synthesis and comparison of data from combined HPLC/mass spectrometry and proton NMR for the natural and synthetic nucleosides. The potential function of f^5C in the $tRNA^{Met}$ anticodon is discussed with regard to codon–anticodon interactions.

Mitochondria possess their own genetic systems, which are semiindependent of the nuclear genome [reviewed recently by Wolstenholme (1992)]. Bovine mitochondrial (mt) DNA has been sequenced and contains genes for 13 protein open reading frames, two rRNAs, and 22 tRNAs (Anderson et al., 1982). Translation of the genetic code with such a small number of tRNAs is achieved in mitochondria by an altered pattern of codon recognition (Heckman et al., 1980; Barrell et al., 1980; Bonitz et al., 1980). One such variation is reassignment of AUA (in addition to AUG) as a codon for methionine (Anderson et al., 1980; Osawa et al., 1992) instead of for isoleucine.

Twenty-two tRNAs were isolated from bovine mitochondria, and their sequences reportedly correspond to the 22 mt tRNA genes (Roe et al., 1981); there was no evidence for tRNAs imported from the cytoplasm. There is a single gene for mt $tRNA^{Met}$, and the sequence of the corresponding mt $tRNA^{Met}$ (Roe, 1983) contains a CAU anticodon. The issue then arises how the single $tRNA^{Met}$ can recognize both AUG and AUA codons, because according to the "wobble" hypothesis (Crick, 1966) C in the first anticodon position of the tRNA is not expected to base-pair with A in the third position of the AUA Met codon. Furthermore, this single $tRNA^{Met}$ must participate both in initiation and in elongation processes, that is, it must be recognized and utilized by both the mitochondrial initiation factor (IF-2-mt) and the elongation factor (EF-Tu-mt), as required. To address these questions, we reanalyzed the nucleotide sequence of the $tRNA^{Met}$ from bovine liver mitochondria and unexpectedly found a novel and previously unknown modified cytidine in the first position of the anticodon. We report here the structure determination of this nucleoside and discuss its potential role(s) in base-pairing with A and also in interaction with IF-2-mt and EF-Tu-mt.

MATERIALS AND METHODS

Purification of $tRNA^{Met}$ from Bovine Liver Mitochondria

Total mitochondrial tRNA was prepared from bovine liver mitoplasts as described by Yokogawa et al. (1989). Mitochondrial methionine tRNA was purified by successive column chromatographies on DEAE–cellulose or DEAE–Sephacel FF (Pharmacia), BD–cellulose (Boehringer) or hydroxyapatite (Bio-Rad), and RPC-5 as previously reported (Nishimura, 1971; Yokogawa et al., 1989; Kumazawa et al., 1989; Yokogawa et al., 1991). The $tRNA^{Met}$ was assayed by aminoacylation using [^{14}C]L-methionine (Amersham) and a mt S-100 fraction from bovine liver and by hybridization (Yokogawa et al., 1989) using as a probe 5′-TAGTACGG-GAAGGATATA-3′, which is complementary to the 3′ end of mt $tRNA^{Met}$ (Anderson et al., 1982; Roe, 1983). A total of 1.5 mg of $tRNA^{Met}$ was thus purified from 100 kg of bovine liver for analysis by NMR spectroscopy and mass spectrometry. A final purification by gel electrophoresis (Yokogawa et al., 1989) was used to prepare tRNAs for sequence determination.

Sequencing of $tRNA^{Met}$

RNase T_1, RNase PhyM, and T_4 RNA ligase were purchased from Pharmacia, RNase T_2 and RNase CL3 from Seikagaku Kogyo, RNase U_2 from Sigma, and *Escherichia coli* alkaline phosphatase from Takara Shuzo. T_4 polynu-

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* To whom correspondence should be addressed. TEL & FAX: 81-3-5800-6950.

‡ Department of Industrial Chemistry, University of Tokyo.

§ Tokyo Institute of Technology.

⊥ University of Utah.

∥ Department of Biochemistry and Biophysics, University of Tokyo.

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cleotide kinase and other chemical reagents were purchased from Wako Pure Chemical Industries. 5′-[^{32}P]pCp and [γ-^{32}P]ATP were purchased from Amersham.

The nucleotide sequence of tRNA^{Met} was determined mainly by the method of Donis-Keller (1980). The location and identification of the modified nucleotides were carried out according to Kuchino et al. (1987). The mt tRNA^{Met} partially digested with formamide was labeled with [γ-^{32}P]ATP at the 5′-ends 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 detected by two-dimensional thin-layer chromatography (2D-TLC) on Avicel SF plates (Funakoshi Pharmaceutical Co.) (Yokogawa et al., 1991).

Directly-Combined High-Performance Liquid Chromatography/Mass Spectrometry (LC/MS) of Digested Bovine mt tRNA^{Met}

Bovine mt tRNA^{met} (20 μg) was digested to nucleosides with 1 unit of nuclease P_1 (Boehringer-Mannheim), 1 milliunit of phosphodiesterase I (Sigma type VII), and 0.5 unit of bacterial alkaline phosphatase (Sigma type III-N) (Crain, 1990). The digests were fractionated by reversed-phase HPLC (Supelco LC-18S column; 4.6 mm × 250 mm) using a 0.25 M ammonium acetate (pH 6.0)/40% (aq) acetonitrile buffer and analyzed by LC/MS. The instrument, procedures, and interpretation of LC/MS data have been described (Pomerantz & McCloskey, 1990). A tRNA^{Met} hydrolysate (5 μg) was analyzed by LC/MS using deuterated HPLC solvents (Edmonds et al., 1988) to determine the number of exchangeable hydrogens by mass shifts resulting from hydrogen–deuterium exchange.

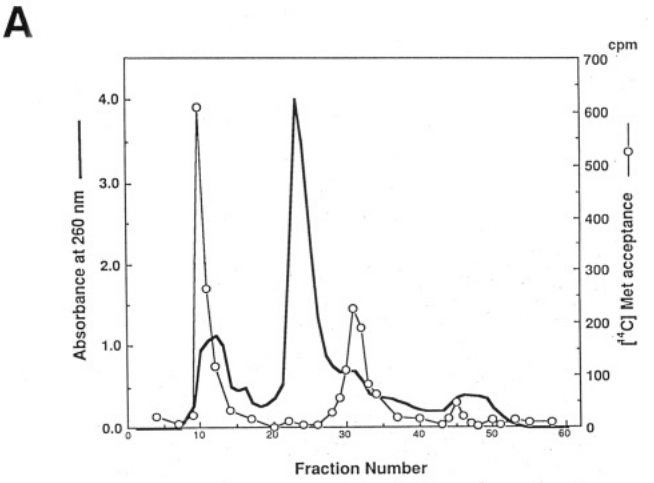
NMR Spectroscopy of Bovine mt tRNA^{Met}

The tRNA sample was dissolved in 1 mL of phosphate buffer (5 mM sodium phosphate (pH 7.5), 10 mM $MgCl_2$, and 150 mM NaCl) and concentrated by ultrafiltration with a Centricon 10 (Amicon). This dilution and concentration step was repeated five times to wash the sample, and the sample volume was finally adjusted to 0.2 mL with phosphate buffer. The sample solution was evaporated to dryness and redissolved in 0.2 mL of ^2H_2O (99.9 atom % ^{2}H; ISOTEC Inc.). Finally, the sample was redried and dissolved in 0.2 mL of ^2H_2O (99.98 atom % ^{2}H; ISOTEC Inc).

^{1}H NMR spectra (500 MHz) were recorded on a Bruker AMX-500 spectrometer at a probe temperature of 37 °C. Free induction decays were accumulated with 16K data points and spectra of 16K real data points (spectral width of 8064 Hz) were obtained. For two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiments (Ernst et al., 1987), 512 free induction decays of 2K data points were accumulated with a mixing time of 300 ms, and spectra of 1K × 2K real data points were obtained. Before two-dimensional Fourier transformation, a $2/\pi$-shifted sine-bell window function was applied for each dimension.

Synthesis of 5-Formylcytidine

5-Formylcytosine (**1**). To a suspension of 5-(hydroxymethyl)cytosine (**2**, Sigma; 35.3 mg, 0.25 mmol) in 1 mL of water was added 1 M ceric(IV) ammonium nitrate (0.8 mL; 0.8 mmol) (Trahanovski et al., 1967) in one portion with vigorous stirring at room temperature. The suspension immediately became a clear dark red; stirring was continued



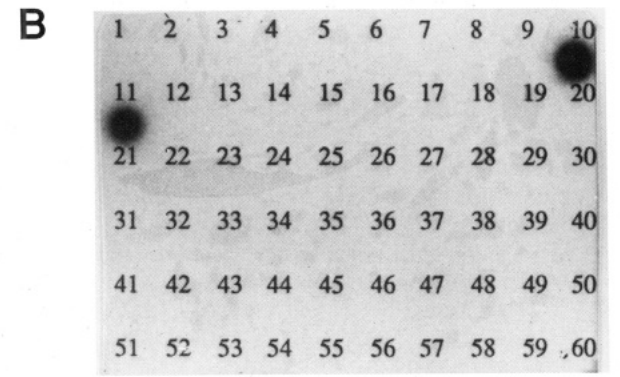


FIGURE 1: (A) RPC column chromatography of bovine mt tRNA^{Met}. One-thousand A_{260} units of tRNA^{Met}-rich fractions obtained by BD-cellulose column chromatography were injected onto the column (1 cm × 100 cm). Elution was performed with a linear gradient of NaCl from 0.4 M to 1 M (500 mL each) in 10 mM Tris-HCl (pH 7.5), 10 mM $Mg(OAc)_2$ and 15 mM 2-mercaptoethanol. About 10-mL fractions were collected. Panel B shows hybridization assay using a probe complementary to the 3′ end of mt tRNA^{Met}. The numerals correspond to RPC column chromatography fraction numbers.

at room temperature for 15 h, while monitoring the quantitative formation of **1** (t_R 9.0 min) from **2** (t_R 6.0 min) by reversed-phase HPLC (Supelcosil LC-18-DB column; 10 mm × 250 mm; elution buffer 50 mM ammonium bicarbonate/acetonitrile (98:2), pH 6.5 at a flow rate of 3 mL/min). The resulting pale yellow solution was evaporated to dryness, and the residue was washed with acetone. **1** was collected as an acetone-insoluble solid by filtration (33 mg; 94.8% yield). HPLC analysis showed a single peak eluting at 9.0 min together with a minor peak from the cerium oxidant. The themospray mass spectrum (Pomerantz & McCloskey, 1990) showed an MH^+ ion at m/z 140. This product was used for subsequent reaction without further purification.

5-Formylcytosine Dimethyl Acetal (**3**). A mixture of **1** (100 mg; 0.72 mmol) and *p*-toluenesulfonic acid monohydrate (5 mg; 27 mmol) in freshly distilled methanol (5.5 mL) was refluxed for 6.5 h and then cooled to room temperature to give 27 mg (20.3% yield) of the acetal **3**. HPLC (conditions described above) showed that this preparation contained approximately 10% of **1** and was used for the next reaction without further purification. Evaporation of the mother liquor gave an additional 28 mg (21%) of crystals consisting of **1** and **2** (1:1).

5-Formylcytidine. Compound **3** (24.2 mg; 0.13 mmol) was suspended in a mixture of hexamethyldisilazane (0.7 mL) and trimethylchlorosilane (0.1 mL) and refluxed for 2 h with

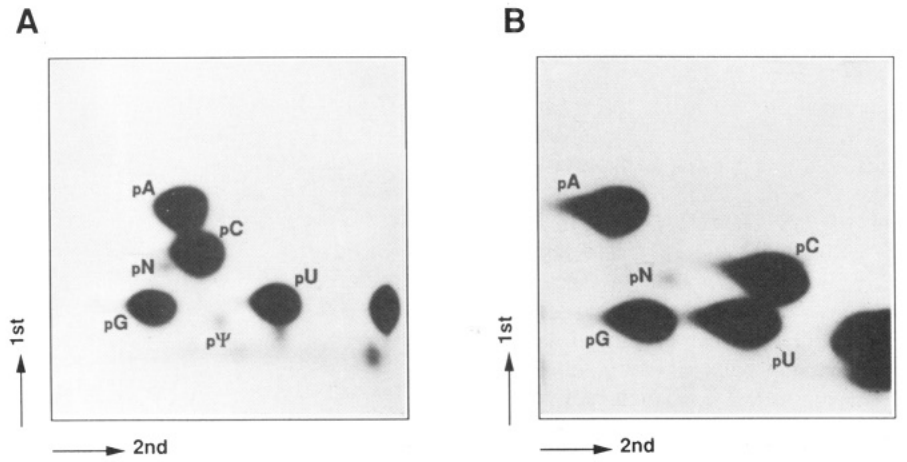


FIGURE 2: Two-dimensional TLC with Avicel SF plates of 5′-[^{32}P]-labeled nucleotides derived from RNase T_2-digestion of mt tRNA^{Met} (see text). The solvents used were isobutyric acid/concentrated ammonia/water (66:1:33 v/v/v) for the first dimension for both plates, and 2-propanol/HCl/water (70:15:15 v/v/v) (left plate) and 0.1 M sodium phosphate (pH 6.8)/ammonium sulfate/*n*-propanol (100 mL:60 g:2 mL) (right plate) for the second dimension. pN indicates a modified nucleoside 5′-phosphate (pC*).

exclusion of atmospheric moisture. The resulting clear solution was dried under reduced pressure, and the residue was dissolved in dry benzene (1 mL). The resulting clear solution was reevaporated to give a gummy residue (approximately 43 mg) which was dissolved in freshly distilled (over P_2O_5) acetonitrile (0.5 mL). This solution, consisting principally of O^2,N^4-bis-(trimethylsilyl)-5-dimethoxymethylcytosine, was mixed with 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (41.3 mg; 0.13 mmol) in anhydrous acetonitrile (0.5 mL). The solution was cooled to −10 °C, and freshly distilled $SnCl_4$ (33.9 mg; 0.13 mmol) in anhydrous acetonitrile (200 mL) was added slowly, with stirring and cooling for 15 min, and then it was stirred at room temperature for 3 h. Dichloromethane (3 mL) was added to the solution which was washed in turn with saturated sodium hydrogen carbonate and water. Additional desired product was recovered from the washings. Removal of the solvent under reduced pressure gave a viscous clear residue, to which 2.5 mL of methanolic ammonia (saturated in a dry ice/2-propanol bath) was added. The solution was maintained at −4 °C overnight. The reaction mixture was dried under reduced pressure to give a pale-yellow solid which was washed with a small quantity of methanol to yield 5-formylcytidine (19 mg; 54% yield). For an analytical sample, this product was purified by reversed-phase semipreparative HPLC (conditions described above except that the solvent was 50 mM ammonium hydrogen carbonate/acetonitrile (91.5:8.5)).

5-Formylcytidine eluted at 6.2 min and was collected and evaporated to dryness; no α-anomer was detected. UV: $\lambda_{max}^{H_2O}$ 222 nm; 247 nm (s); 281 nm. The thermospray mass spectrum contained the ions *m*/*z* 272, 140, and 150, assigned as MH^+, BH_2^+, and $(SH)NH_4^+$ respectively (Pomerantz & McCloskey, 1990). f^5C eluted 1.2 min after guanosine, equivalent to 16.9 min in a standardized gradient system (Pomerantz & McCloskey, 1990); the UV absorbance ratio (254 nm:280 nm) was 1.1. The chemical shifts from ^{1}H NMR were (δ, ppm in ^2H_2O) 9.153 (1H, CHO), 8.925 (1H, H-6), 5.904 (1H, H-1′, $J_{1'2'}$ = 1.91 Hz), 4.354 (1H, H-2′), 4.251–4.190 (2H, H-3′ and H-4′), 4.074 (1H, H-5′), 3.895 (1H, H-5′).

RESULTS

Purification of mt tRNA^{Met}. Mitochondria were isolated from bovine liver and treated with digitonin to reduce contamination by cytoplasmic tRNAs (Ueda et al., 1985). The unfractionated mt tRNA was first fractionated by BD-cellulose column chromatography. The fraction eluting at a NaCl concentration of 0.6–0.7 M showed Met acceptor activity and also hybridized to the DNA probe, so it was further fractionated on an RPC-5 column. As shown by Met acceptor activity, two peaks are observed (Figure 1A). The DNA probe complementary to mt tRNA^{Met} only hybridized to the earlier eluting peak, which is thereby identified as the mt tRNA^{Met} (Figure 1B). The later eluting peak was sequenced and thereby identified as the cytoplasmic initiator tRNA^{Met} (Sprinzl et al., 1989).

Nucleotide Sequence of Bovine mt tRNA^{Met}. The nucleotide sequence of the mt tRNA^{Met} was determined and found to be identical to that reported by Roe (1983) except for one nucleotide. As shown in Figure 2, two modified nucleotides were detected by 2D-TLC of 5′-[^{32}P]-labeled nucleotides. One of them was identified as pseudouridine 5′-phosphate which occurs twice, in positions 27 and 50 in the anticodon and T stems, respectively [numbering of residues conforms to the proposal of Sprinzl et al. (1989)]. The position of another modified nucleotide did not correspond to that of any nucleotide so far identified in tRNA, by 2D-TLC in two different solvent systems. Its location was determined to be position 34, corresponding to the first letter of the anticodon (Figure 3). This position is occupied by C in the gene sequence (Anderson et al., 1982) and unmodified C is reported in the tRNA sequence (Roe, 1983). This modified nucleoside was sensitive toward RNase CL3 in the Donis-Keller sequencing method (data not shown), and to RNase T_2, so that presumably it is a derivative of cytidine (designated C*) with a modified base moiety.

Identification of C by LC/MS.* A sample of nucleosides resulting from nuclease P_1 digestion of mt tRNA^{Met} was analyzed by LC/MS to identify C*. The chromatogram from the LC/MS analysis (Figure 4A) revealed the presence of two minor nucleoside species, pseudouridine and a nucleoside eluting at 17.0 min, which corresponds to C*. The ratio of UV absorbance for C* (254 nm/280 nm; latter channel not shown in Figure 4A) was 1.08. The thermospray mass spectrum of this nucleoside (Figure 4B) contained the ions *m*/*z* 272 and *m*/*z* 140, assigned as MH^+ and BH_2^+ ions, respectively (Edmonds et al., 1985), corresponding to a M_r of 271.

NMR Analysis of Bovine mt tRNA^{Met}. Figure 5 shows the ^{1}H NMR spectrum of the tRNA^{Met} (upper panel; A) and the NOESY spectrum (lower panel; B). A strong NOE cross

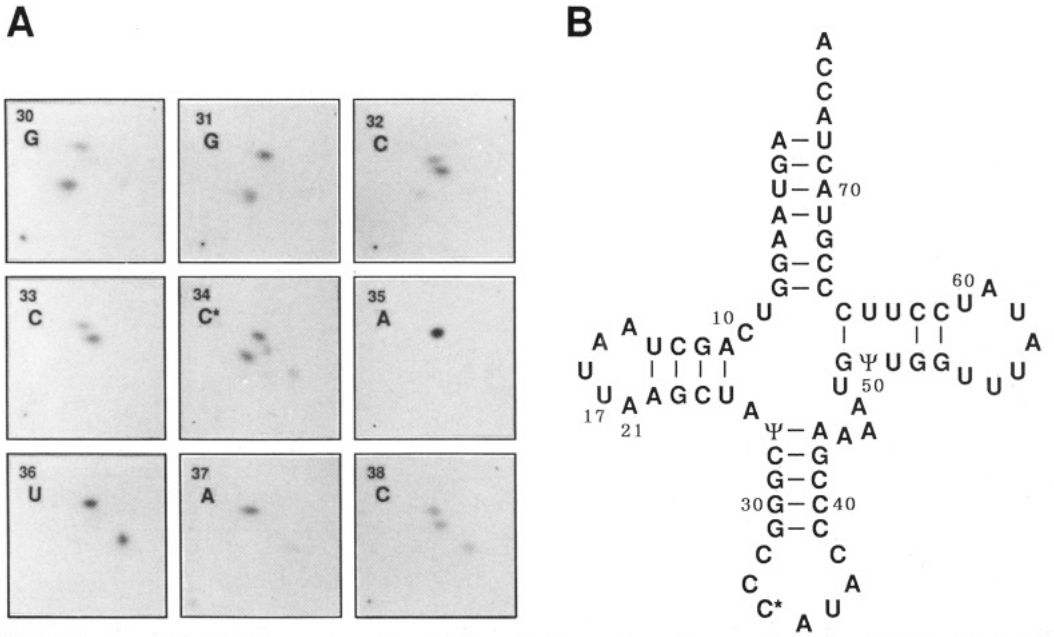


FIGURE 3: (A) Identification of nucleotide sequence around the anticodon region using two-dimensional TLC with Avicel SF plates, as described in Materials and Methods (Kuchino et al., 1987). The solvents used are the same as those in Figure 2A. The numbering of residues in the cloverleaf structure of tRNA^{Met} conforms to the system of Sprinzl et al. (1989). Panel B shows the cloverleaf structure of bovine mt tRNA^{Met} deduced from the sequencing. C* is a new modified nucleoside found in this study.

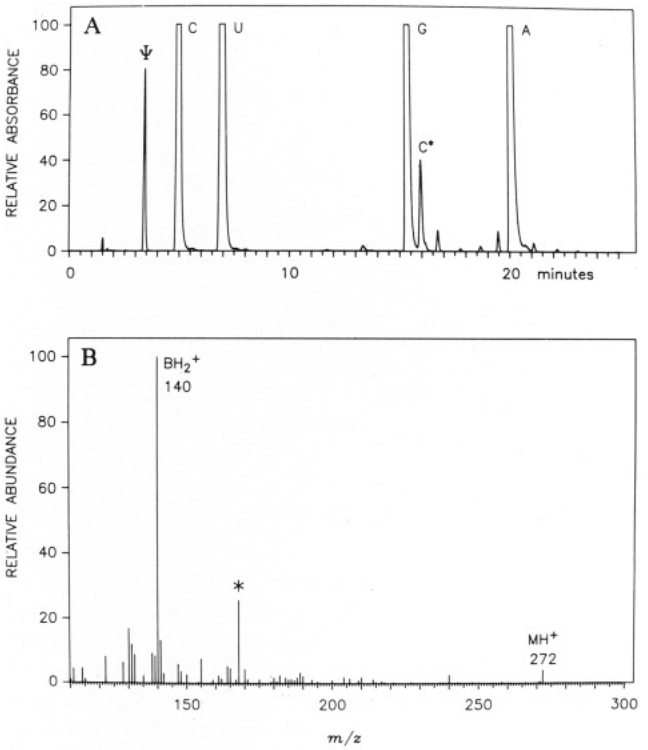


FIGURE 4: LC/MS analysis of 15 μg of digested mt tRNA^{Met}: A, chromatographic separation of nucleosides (UV detection at 254 nm); B, thermospray mass spectrum of nucleoside C*. The asterisk denotes a background ion whose time *vs* intensity profile does not track with ions from C*.

peak is present between resonances at 9.55 and 8.76 ppm. Because it is unlikely that such an NOE peak in the low-field region is due to a pair of proton resonances of usual nucleosides or the pseudouridine residue, the two resonances are presumably derived from C*. Furthermore, a plausible candidate for a proton giving rise to a signal resonating at such a low field (9.55 ppm) is that in an aldehyde group. In addition, it should be noted that no signals from methyl resonances are present.

Characterization of C- as 5-Formylcytidine and Confirmation by Chemical Synthesis.* The molecular weight of C* is 271, which requires that the base moiety contain an odd number of nitrogen atoms and is consistent with the mass of cytidine + 28 Da. The mass difference between the MH^+ and BH_2^+ ions (132 Da) indicates that the sugar is normal ribose, so the 28-Da modification is located on the cytosine moiety. This mass increment corresponds in principle to net addition of either CO or two methyl groups. The number of exchangeable hydrogen atoms in the base moiety was determined from a separate LC/MS analysis in which deuterated HPLC mobile phase was used (Edmonds et al., 1988). The mass of the BH_2^+ ion shifted by 4 Da from 140 Da to 144 Da, indicating that the base moiety in the neutral nucleoside contains two exchangeable hydrogens (the same as for cytidine), implying the absence of substitution on oxygen or nitrogen. The site of the modifying substituent, therefore, was predicted to be C-5. The 28-Da substituent in C* was assumed to be a formyl group based on this mass spectral data and the NMR data described above (Figure 5).

Thus, 5-formylcytidine (f^5C) (Figure 6) was selected as a structure candidate for synthesis and comparison with C*. 5-Formylcytidine was prepared by Ce(IV) oxidation of 5-hydroxymethylcytosine to give 5-formylcytosine, which was then converted to the dimethyl acetal and ribosylated using Sn(IV) catalyst. The UV absorbance ratio (254 nm/280 nm) of synthetic f^5C was measured as 1.10 (*vs* 1.08 for C*) and its thermospray mass spectrum contained the same ions as C* (m/z 272; m/z 140). A small amount of f^5C was mixed with 5 μg of digested mt tRNA^{Met} for comparison by HPLC, and as shown in Figure 7, its retention time is indistinguishable from that of C*.

DISCUSSION

The mt tRNA^{Met} whose sequence corresponds to the single mt tRNA^{Met} gene has been isolated from bovine mitochondria. A second tRNA^{Met} was also recovered, but after sequencing it was found to correspond to the cytoplasmic initiator tRNA^{Met}. We have yet to clarify whether this cytoplasmic

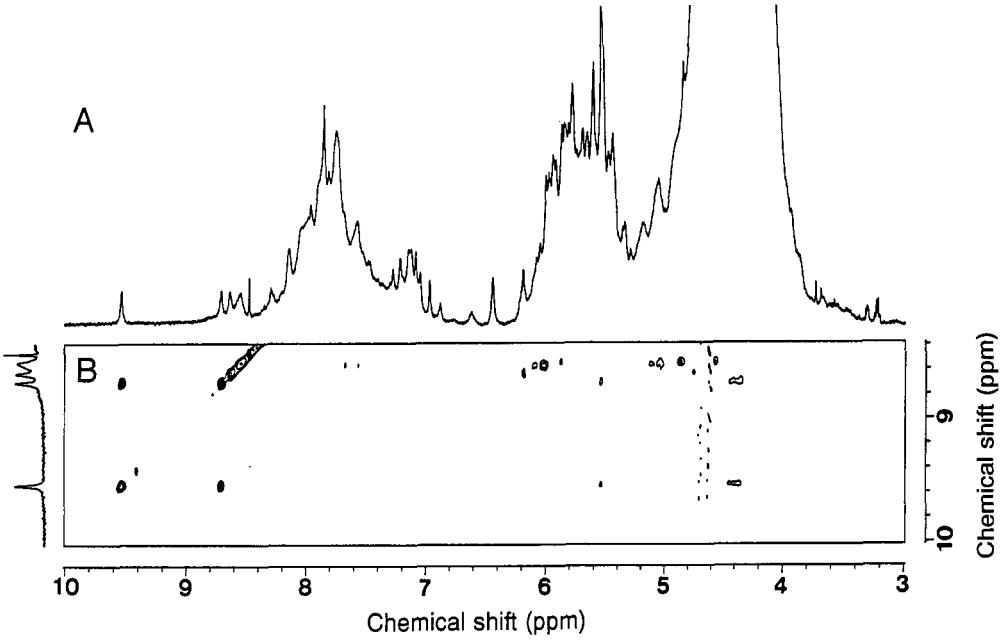


FIGURE 5: ^1H NMR spectra (500 MHz) of mt $tRNA^{Met}$: A, one-dimensional spectrum; B, a portion of the NOESY spectrum.

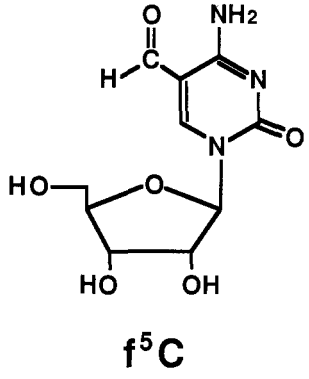


FIGURE 6: Chemical structure of 5-formylcytidine.

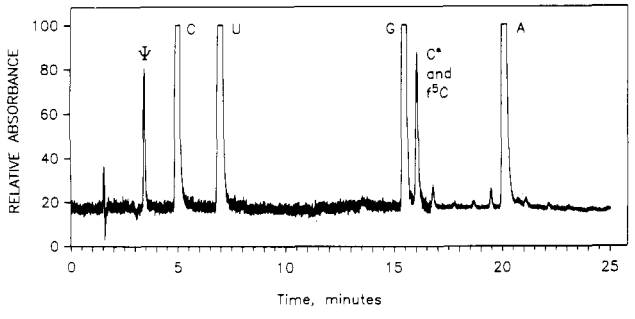


FIGURE 7: Comparison of C* nucleoside from digested mt $tRNA^{Met}$ (5 μg) and synthetic f^5C by coinjection and reversed-phase HPLC; UV detection at 254 nm.

$tRNA^{Met}$ is merely a contaminant, or a true mitochondrial component. We consider the latter possibility unlikely because no cytoplasmic tRNAs are imported into bovine mitochondria (Roe et al., 1981) and the mt genome (Anderson et al., 1982) does not contain a DNA sequence corresponding to that of the cytoplasmic $tRNA^{Met}$.

The single mt $tRNA^{Met}$ characterized in the present study presumably serves as either initiator or elongator, depending on its interaction with the corresponding mt translational factors. Preliminary quantification of f^5C content in mt $tRNA^{Met}$ suggests that about 1 mol of f^5C is present in 1 mol of $tRNA^{Met}$, so that partial modification of C-34 does not

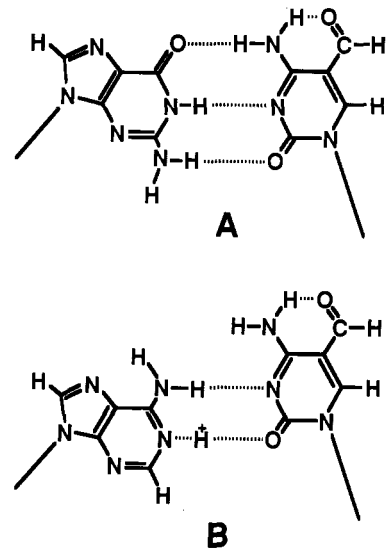


FIGURE 8: Possible base-pairing schemes of f^5C with G (A) and protonated A (B).

appear to act to double the number of molecular species of this tRNA. Therefore, this sole mt $tRNA^{Met}$, which contains f^5C in the "wobble" position, must decode the AUA codon.

Conformation analysis of f^5C by 500-MHz NMR showed that the nucleoside takes a very rigid C3′-endo-anti form (Kawai et al., 1994) the same as that of unmodified cytidine. This feature may be advantageous for the decoding properties of mt $tRNA^{Met}$, because a very rigid pyrimidine in the first position of the anticodon cannot form base pairs with U and C (Yokoyama et al., 1985; Kawai et al., 1992), so that the mt $tRNA^{Met}$ would not recognize the AUU and AUC isoleucine codons. In addition, it is to be anticipated that the stability conferred by a rigid ribose moiety in the first anticodon nucleotide will to some extent be propagated to the second and third anticodon residues. This would result in greater overall stability of the stacked anticodon bases, and thus of codon–anticodon pairings.

It is apparent that f^5C can interact in the expected manner with G of the Met AUG codon, as shown in Figure 8A, reflecting the finding that the conformation of f^5C is similar

to that of cytidine (Kawai et al., 1994). In order to read the Met AUA codon, the intriguing possibility exists that f^{5}C pairs with A in the Met AUA codon by protonation as illustrated in Figure 8B. The protonation of A at N-1 in an A–C pair at pH values above the pK of the monomer has been demonstrated in oligodeoxyribonucleotide duplexes (Wang et al., 1991). More recently, A was found to adopt a pK of 6.5 in the active site of a Pb-dependent ribozyme (A. Pardi, personal communication, 1993).

Another possibility of decoding of AUA codon by mt tRNA^{Met} may lie in the "two out of three" hypothesis proposed by Lagerkvist (1978). However, the hypothesis requires G or C in the first and/or second letter(s) of the codon, so that the hypothesis would not be applied to the present case in which the first and second letters are occupied by A and U, respectively.

We have already confirmed that mt tRNA^{Met} binds to bovine mt ribosomes in the presence of the AUG triplet using the ribosomal binding system of Nirenberg and Leder (1964), but not to AUA in the same system (unpublished experiments with L. L. Spremulli). We are presently examining the translational activity of mt tRNA^{Met} using synthetic mRNAs containing AUG or AUA codons in an *in vitro* mt translation system (Kumazawa et al., 1991).

The means by which IF-2-mt and EF-Tu-mt utilize a single tRNA^{Met} for both initiation and elongation remains to be determined. As it is known that IF-2-mt only binds to fMet-tRNA^{Met}, and EF-Tu-mt only binds to unformylated Met-tRNA^{Met} (in the system of mt ribosomes and yeast tRNA^{Met}; L. L. Spremulli, et al., unpublished information), presence or absence of f-Met is the determinant of initiator *vs* elongator function of tRNA^{Met}. We are presently purifying and characterizing the mt formyltransferase from bovine liver mitochondria to investigate its interaction with tRNA^{Met}.

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