

Synthesis and investigation of the 5-formylcytidine modified, anticodon stem and loop of the human mitochondrial tRNA^{Met}

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ABSTRACT

Human mitochondrial methionine transfer RNA (hmtRNA^{Met}_{CAU}) has a unique post-transcriptional modification, 5-formylcytidine, at the wobble position-34 (f⁵C₃₄). The role of this modification in hmtRNA^{Met}_{CAU} for the decoding of AUA, as well as AUG, in both the peptidyl- and aminoacyl-sites of the ribosome in either chain initiation or chain elongation is still unknown. We report the first synthesis and analyses of the tRNA's anticodon stem and loop domain containing the 5-formylcytidine modification. The modification contributes to the tRNA's anticodon domain structure, thermodynamic properties and its ability to bind codons AUA and AUG in translational initiation and elongation.

INTRODUCTION

Mitochondria generate over 90% of the energy used by mammalian cells through oxidative phosphorylation. Thirteen proteins, components of the electron transfer chain and the ATP synthase, are the products of mitochondrial DNA. The synthesis of these proteins is carried out by a specific protein synthesizing machinery within this organelle. During the almost-three decades since the sequencing of the human mitochondrial genome (1), the mitochondrial genetic code has been found to differ significantly from the universal code. The human mitochondrial gene for the one methionine specific tRNA (hmtRNA^{Met}_{CAU}, where CAU is the anticodon) plays a unique role since it must provide the tRNA used for both the initiation of protein synthesis and the elongation of the protein chain by responding to the codon AUA,

normally an isoleucine codon in the cytoplasm, as well as the universal methionine code, AUG. This is highly unusual since all cytoplasmic protein biosynthetic systems employ two different tRNA^{Met} species, one for initiation and one for elongation, and both respond to the single methionine codon, AUG. Maternally inherited mutations in the gene of this tRNA, including an A₃₇ to G₃₇ mutation adjacent to the anticodon nucleosides that read the two codons (Figure 1), are responsible for some devastating diseases (2–5). Moreover, the hmtRNA^{Met}_{CAU} has a unique modification, 5-formylcytidine (Figure 1), at the wobble position-34 (f⁵C₃₄) seen only in one other tRNA, a bovine liver, cytoplasmic tRNA^{Leu} with a f⁵C₃₄ further modified with a 2'-O-methyl (6). Nothing, however, is known about the decoding characteristics of tRNA^{Leu}_{f⁵C₃₄}. Since its discovery in bovine and nematode mitochondrial tRNA^{Met} in 1994 (7,8), f⁵C₃₄ also has been found in the mitochondrial tRNA^{Met} of squids, frogs, chickens, rats and fruit flies (9–11). The contribution of the f⁵C₃₄ modification to the structure of the hmtRNA^{Met}_{CAU}, its role in the decoding of AUG and AUA and its possible participation in either chain initiation or chain elongation by this unique tRNA^{Met} is still unknown.

We speculate that mitochondria have a unique mechanism to partition this single hmtRNA^{Met}_{CAU} species between initiation and elongation. While a tRNA^{Met}_{CAU} unmodified at the wobble position-34 can read AUG, we hypothesize that the 5-formyl modification allows one tRNA^{Met} to expand codon reading to include recognition of the AUA codon in mitochondrial mRNAs. Toward proving the hypothesis that this wobble modification affords the single tRNA^{Met} the ability to decode AUG and AUA, we are reporting the first synthesis of a 5-formylcytidine-modified RNA and the initial structural and biological

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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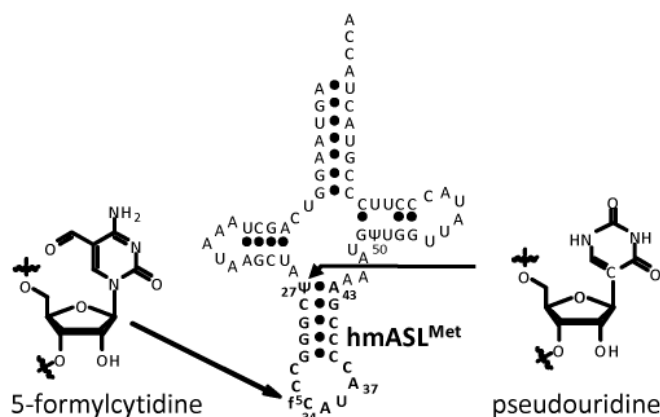


Figure 1. Human mitochondrial tRNA^{Met}_{CAU}. The sequence and secondary structure of hmtRNA^{Met}_{CAU} are shown with the heptadecamer, anticodon stem and loop domain in bold. Nucleosides 27 and 50 are modified to pseudouridine, ψ. The wobble position-34 modification is 5-formylcytidine, f⁵C₃₄.

investigations. Previously, bovine mitochondrial tRNA^{Met}_{CAU} had been shown to translate AUG and AUA in an *Escherichia coli* translational system *in vitro*, where AUG coded for methionine and AUA for isoleucine (12). However, site-specific binding was not investigated. Here we compare the codon binding affinities of the f⁵C₃₄-modified anticodon stem and loop of human mitochondrial tRNA^{Met} (hmtASL^{Met}-f⁵C₃₄) with that of the unmodified ASL. Our results for both AUA and AUG codons at both the A and P-site of *E. coli* ribosomes increase our understanding of the modification's contributions to decoding and are consistent with previous results from the translation of poly(AUA) by bovine mtRNA^{Met}_{CAU} on *E. coli* ribosomes (12).

MATERIALS AND METHODS

Experimental procedures and analytical data for the synthesis of the f⁵C phosphoramidite (9)

All reagents used in the following experiments are of the highest purity and dryness possible. Before use, glassware was thoroughly cleaned and dried (oven at 110°C for 30 min). NMR analysis of intermediates was conducted in the appropriate deuterated solvent (referenced accordingly for CDCl₃: ¹H 7.24 p.p.m., ¹³C 77.23 p.p.m.; and CD₃CN: ¹H 1.94 p.p.m.) using a Bruker Avance UltraShield 300 MHz spectrometer. Phosphorus, ³¹P, NMR experiments were referenced according to an external H₃PO₄ standard (0.00 p.p.m.). Mass-spec analysis of the samples was performed on a Micromass LCT ESI-TOF or an Agilent LC-TOF. Analytes were dissolved in acetonitrile and flown against a Leucine Enkephalin lock mass standard. Chemical and physical properties of the intermediates were those of the expected compounds (Supplementary Data).

2',3'-O-isopropylidencytidine (2). To a suspension of cytidine (1, Figure 2, 1 g, 4.1 mmol) in 50 ml of acetone was added 2,2-dimethoxypropane (6 ml, 5.1 g, 49 mmol).

HClO₄ was then added dropwise until the solution turned clear. The reaction mixture was stirred at room temperature for 12 h, after which it was neutralized by addition of Ca(OH)₂, filtered and evaporated. Purification by silica gel chromatography using CHCl₃:MeOH (80:20) with 2% TEA, afforded 966 mg of 2 as a white foam (83% yield). The analytical data obtained matched known literature data for 2 (13).

5-(Hydroxymethyl)-2',3'-O-isopropylidencytidine (3). To a solution of 2 (Figure 2, 1 g, 3.5 mmol) in 15 ml of 0.5 M KOH was added paraformaldehyde (1.05 g, 35 mmol). The reaction was stirred at 55°C for 36 h, after which it was cooled to room temperature and neutralized with 6 M HCl. The solution was filtered and evaporated. The oily residue was dissolved in MeOH:DCM (40:60), filtered and evaporated again. Purification by silica gel chromatography using MeOH:DCM (gradient 3:97, 8:92, 12:88) with 2% TEA, afforded 416 mg of 5-(hydroxymethyl)-2',3'-O-isopropylidencytidine (3) as a white foam (38% yield, 60% yield based on 357 mg of recovered starting material 2',3'-O-isopropylidencytidine (2)).

5-Formyl-2',3'-O-isopropylidencytidine (4). To 3 (Figure 2, 100 mg, 0.32 mmol) in 3 ml dioxane was added 500 mg ruthenium dioxide hydrate (five weight equivalents). The reaction mixture was refluxed for 12 h and filtered. Purification by silica gel chromatography MeOH:DCM (5:95) with 2% TEA, afforded 81 mg of the 5-formyl-2',3'-O-isopropylidencytidine (4) as a white solid (82% yield).

5-Formylcytidine (5). The acetonide, 5-formyl-2',3'-O-isopropylidencytidine (4, Figure 2, 500 mg, 1.61 mmol) was suspended in 1 M HCl (15 ml) at room temperature. The reaction progress was monitored by TLC. Upon disappearance of the starting material, the solution was neutralized with TEA. Water was subsequently evaporated. Recrystallization from MeOH afforded 414 mg of f⁵C (5) as a white solid (95% yield).

N⁴-[(Diisobutylamino)methylidene]-3',5'-O-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-5-formylcytidine (6). 5-Formylcytidine (5, Figure 2, 0.47 g, 1.72 mmol) was dissolved in a mixture of 20 ml of pyridine and 2 ml of DMF. The solution was cooled to 0°C and TIPDSCl₂ (0.59 g, 1.89 mmol) in 2 ml of pyridine was added dropwise over a period of 1 h. The reaction was allowed to gradually warm to room temperature overnight. The following morning the reaction was quenched with 5 ml of MeOH and evaporated to dryness. The resulting paste was co-evaporated twice with 20 ml of toluene and the crude material was purified by flash chromatography on 30 ml of silica gel using a gradient of MeOH in DCM (3–4%). Product fractions were pooled and evaporated to afford the TIPDS protected intermediate (0.88 g, 100%) as light yellow oil that is contaminated with residual pyridinium salts. The above compound was used as is without further purification to remove the residual pyridinium salts. TIPDS protected f⁵C (5) (0.88 g, 1.72 mmol) was dissolved

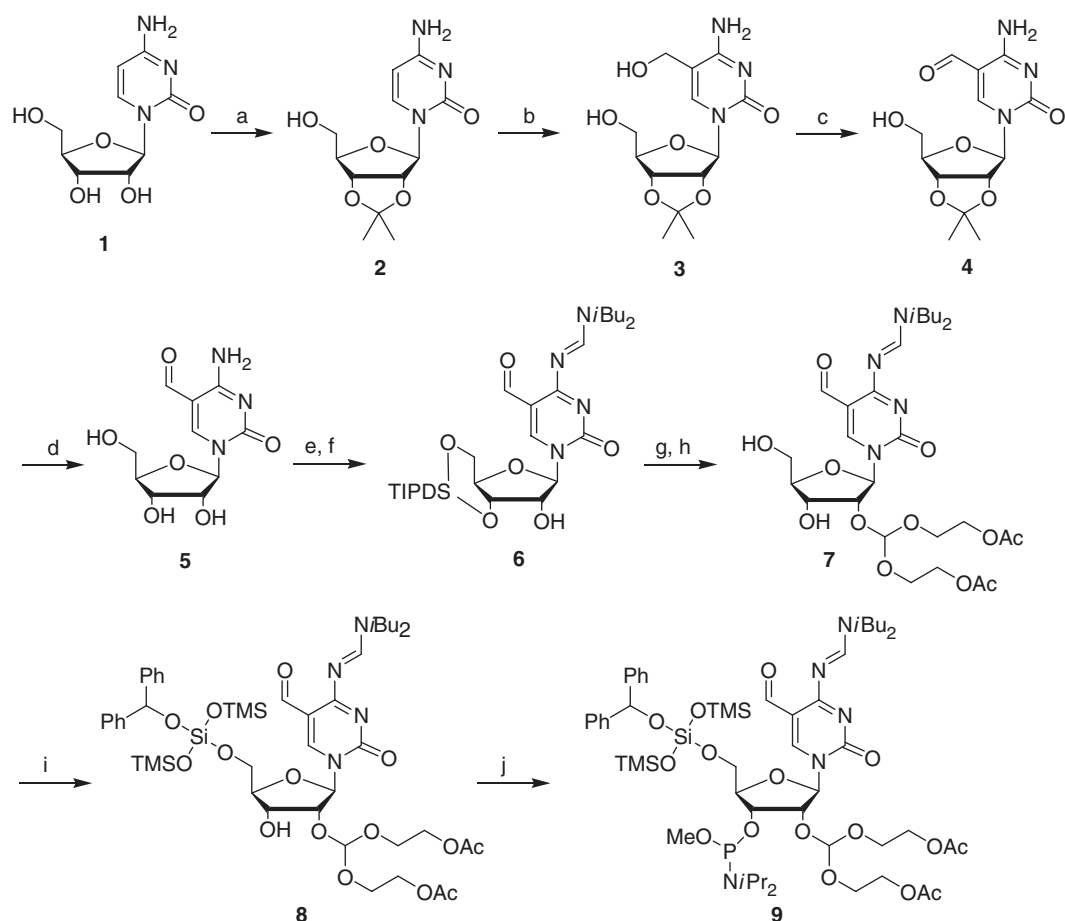


Figure 2. Synthesis of the 5-formylcytidine phosphoramidite. The starting compound cytidine is numbered compound **1**, intermediates are numbered **2–8** and the protected ^5C phosphoramidite is compound **9**. The synthetic transformations are: (a) acetone, dimethoxypropane, cat HClO_4 (83%); (b) paraformaldehyde, 0.5M KOH, 55°C (38%, 60% brsm); (c) $\text{RuO}_2 \cdot x\text{H}_2\text{O}$, dioxane, reflux (82%); (d) 1M HCl (95%); (e) TIPDSCl₂, Pyr, DMF; (f) DBF-CH(OMe)₂, DMF (71% over 2 steps); (g) ACE-orthoester, PPTS, TBDMS-pentanedione, DCM; (h) HF-TEMED, CH₃CN (53% over 2 steps); (i) BZH-Cl, DIA, DCM (84%); (j) P(OMe)(DIA)₂, DIA, S-Et Tetrazole, DCM (93%).

in 20 ml DMF and *N,N*-diisobutylformamide dimethyl acetal (**14**) (0.70 g, 3.44 mmol) was added. The reaction was stirred for 16 h and evaporated under high vacuum. The resulting loose oil was coevaporated twice with 20 ml of toluene and the crude material was purified by flash chromatography on 30 ml of silica gel using a gradient of MeOH in DCM (1–2%). Product fractions were pooled and evaporated to afford 0.80 g of **6** as light yellow oil in 71% overall yield from ^5C (**5**).

2'-O-[Bis(2-acetoxyethoxy)methyl]-N⁴-[(diisobutylamino)methylidene]-5-formylcytidine (7). A mixture of **6** (Figure 2, 0.80 g, 1.23 mmol), pyridinium para-toluenesulfonate (0.31 g, 1.23 mmol), and Tris(2-acetoxyethoxy)methyl orthoformate (1.98 g, 6.15 mmol) was dissolved in 5 ml of DCM and stirred at room temperature. After 2 days, TBDMS-pentanedione (0.53 g, 2.46 mmol) was added and the reaction was stirred at ambient temperature. After stirring for an additional day, the reaction was quenched with 1 ml of TEMED. The crude material was separated from excess reagents by flash chromatography on 50 ml silica gel using a gradient of 25% ethyl acetate in hexanes with 0.1% TEMED to 50% ethyl acetate in

hexanes with 0.1% TEMED. This material was concentrated to near dryness and taken directly onto the desilylation reaction.

A freshly made solution of TEMED (0.71 g, 6.15 mmol) in 10 ml of acetonitrile at 0°C was added 48% HF (0.15 ml, 4.30 mmol). This solution was allowed to stir for 5 min and added to the foregoing material from above at room temperature. The reaction was stirred for 2 h and concentrated to dryness. The crude material was purified by flash chromatography on 50 ml silica gel using a gradient of 20% hexanes in ethyl acetate with 0.1% TEMED to 1% methanol in ethyl acetate with 0.1% TEMED. Product fractions were pooled and evaporated to leave the *2'-O*-protected compound [**7**] as a light yellow oil (0.41 g) in 53% yield from the nucleobase protected compound **6**.

5'-O-[Benzydryloxy-bis(trimethylsilyloxy)silyl]-2'-O-[bis(2-acetoxyethoxy)-methyl]-N⁴-[(diisobutylamino)methylidene]-5-formylcytidine (8). Diisopropylamine (0.07 g, 0.65 mmol) was added to a solution of the *2'-O*- and *N⁴*-protected nucleoside, **7** (Figure 2, 0.41 g, 0.65 mmol) in 7 ml of DCM and the solution was cooled

to 0°C. In a separate flask BZHCl (0.34 g, 0.81 mmol) was diluted in 5 ml of DCM. Diisopropylamine (0.10 g, 0.98 mmol) was added to the silylating solution and the solution was allowed to stir for 2 min before being added dropwise to the nucleoside solution. The addition was completed within 30 min and the reaction was allowed to stir for 3 h and the reaction was quenched with 1 ml of MeOH and evaporated to dryness. The crude material was purified by flash chromatography on 30 ml silica gel using a gradient of 10% acetone in hexanes containing 0.1% (v/v) TEA to 20% acetone in hexanes containing 0.1% (v/v) TEA. Product fractions were pooled and evaporated to afford the 5'-O-protected compound **8** as a colorless oil. The yield was 0.56 g (84%).

5'-O-[Benzyldryloxy-bis(trimethylsilyloxy)silyl]-2'-O-[bis(2-acetoxyethoxy)-methyl]-N⁴-[(diisobutyl-amino)methylidene]-5-formylcytidine-3'-(methyl-N,N-diisopropyl) phosphoramidite (9). Bis(diisopropyl-amino) methoxy phosphine (0.21 g, 0.82 mmol) was dissolved in 3 ml of DCM and a 0.5-M solution of 5-ethylthio-1-H-tetrazole in anhydrous acetonitrile (0.08 ml, 0.55 mmol) was added. Diisopropylamine (0.06 g, 0.55 mmol) was then added and the phosphine solution was allowed to stir for 5 min at ambient temperature. In a separate flask, the 2'-O-, 5'-O- and N⁴-protected f⁵C, compound **8** (Figure 2, 0.56 g, 0.55 mmol) and diisopropylamine (0.06 g, 0.55 mmol) were dissolved in 5 ml of DCM. The activated phosphine solution was added into the nucleoside solution and the reaction was stirred at room temperature. After 16 h the reaction was quenched with 2 ml of absolute ethanol and concentrated to dryness. The resulting white paste was purified by flash chromatography on 30 ml of silica gel using a mixture of DCM in hexanes [5:95 (v/v)] containing 2% (v/v) TEA followed by acetone in hexanes [1:9 (v/v)] to 2:8 (v/v) containing 0.5% (v/v) TEA. Product fractions were pooled and evaporated to afford the protected f⁵C phosphoramidite **9** (Figure 2), as a colorless oil.

Polyribonucleotide synthesis of 5'-ΨCGGGCC-f⁵C-AUA CCCCCGA-3'

The above sequence was synthesized on a 1-μmol scale using a ABI 394 DNA synthesizer using previously published procedures (15,16). The f⁵C phosphoramidite (**9**, 0.067 M in anhydrous acetonitrile) was coupled to the growing polyribonucleotide chain for 3.5 min using 5-ethylthio-1H-tetrazole (0.5 M in anhydrous acetonitrile) as the activator. Once the synthesis of the polyribonucleotide chain was completed, the phosphate protecting groups were removed from the immobilized polyribonucleotide by treatment with disodium 2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate in DMF for 10 minutes. The support was washed excessively with water for 5 min and then flushed with Argon gas for 5 min to dry the support. The support was then transferred to a 2-ml Eppendorf tube and the polyribonucleotide was cleaved from the support and the exocyclic amine protecting groups were removed with 1:3 (v/v) tert-butylamine:water for 6 h at 60°C. The sample was cooled to room temperature, filtered and lyophilized to obtain the crude

polyribonucleotide. The hmASL^{Met}_{CAU}-Ψ₂₇;f⁵C₃₄ was deprotected with acetate/TEMED according to standard Dharmacon protocols, purified by ion exchange HPLC (17), and dialyzed extensively against H₂O. The hmASL^{Met}_{CAU}-Ψ₂₇ was synthesized and deprotected under standard conditions (18).

Confirmation of nucleoside composition by nucleoside HPLC and NMR of the hmASL^{Met}_{CAU} constructs

Incorporation of f⁵C modification within the hmASL^{Met} was confirmed by NMR, including the two-dimensional NOESY (Figures 3 and 4). The nucleoside composition of the hmASL^{Met} products was confirmed by enzymatic hydrolysis of the RNA to its constituent nucleosides (17) and then subjected to HPLC monitored by diode array UV spectrometry, the peaks identified, integrated and quantified (19) (Figure 4).

Analysis of thermodynamic stability, circular dichroism and molecular dynamics simulations

The ASL samples were dissolved to obtain a concentration of ~4 μM in 20 mM Na-K phosphate buffer (pH 6.8). UV-monitored, thermal denaturations and renaturations were replicated five times and monitored by measuring UV absorbance (260 nm) using a Cary 3 spectrophotometer as published (20,21). The data points were averaged over 20 s and collected four times a minute with a temperature change of 0.5°C/min from 4 to 90°C. The data were analyzed (22), and the thermodynamic parameters were determined (Origin software, Microcal, Inc.) (Figure 5A). CD spectral ellipticity data were collected using a Jasco 600 spectropolarimeter and an interfaced computer (Jasco, Inc.). hmASL^{Met}_{CAU}-Ψ₂₇ or the hmASL^{Met}_{CAU}-Ψ₂₇;f⁵C₃₄ (0.2 A₂₆₀/ml, 20 mM Na-K phosphate buffer, pH 6.8) was placed in a temperature-regulated, 1-cm path-length quartz cell. Each sample was scanned 10 times at 25°C. The final data are an average of the 10 scans (Figure 5B). The molecular dynamics simulation (MDS) were performed by following standard published protocol (23) with the exception of using a truncated octahedral TIP3P water box (24).

Ribosomal binding assay

The 27-mer mRNA oligos used in codon binding assays were designed from that of T4 gp32 mRNA (25) and purchased (Dharmacon RNA Technologies). They were chemically deprotected and HPLC-purified in our lab. Each mRNA sequence was entered into the program RNA Structure 4.2 (26) and was found to have a low probability of folding into any stable conformation. The mRNA sequences are as follows (mitochondrial methionine codons AUA and AUG are in bold):

- (i) 5'-GGCAAGGAGGUAAAAUAGUAGCACG U-3';
- (ii) 5'-GGCAAGGAGGUAAAAUGGUAGCACGU-3';
- (iii) 5'-GGCAAGGAGGUAAAAGUAAUAGCACG U-3';

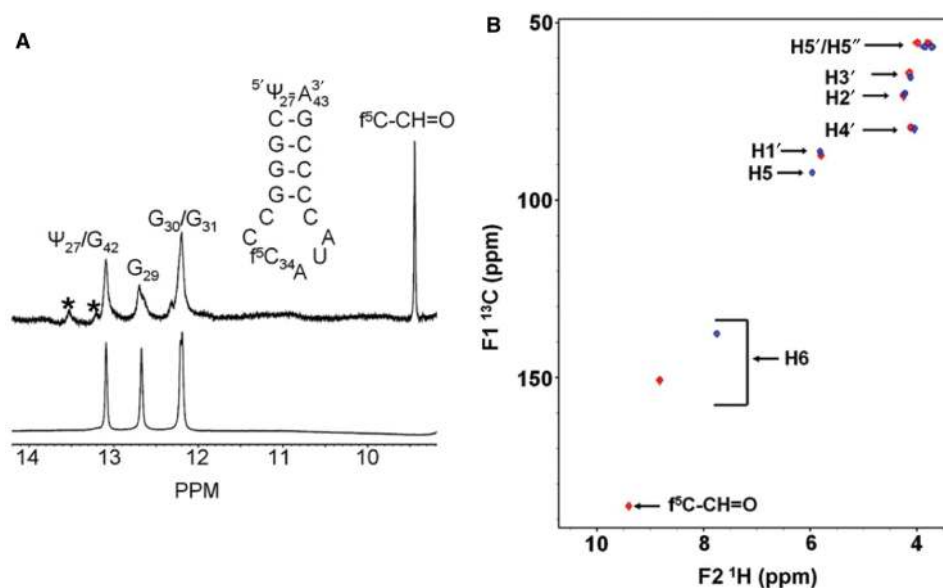


Figure 3. NMR spectra of the hmASL_{CAU}^{Met}. (A) One-dimensional ¹H-NMR spectrum (in H₂O) of hmASL_{CAU}^{Met}-Ψ₂₇:f⁵C₃₄ (top) is compared to that of the unmodified hmASL_{CAU}^{Met}-Ψ₂₇ (bottom). The formyl proton's chemical shift in the RNA is almost identical to that of the mononucleoside f⁵C. *Denotes impurities. (B) Superimposed ¹H-¹³C HMQC spectra of cytidine (blue) and 5-formylcytidine (red).

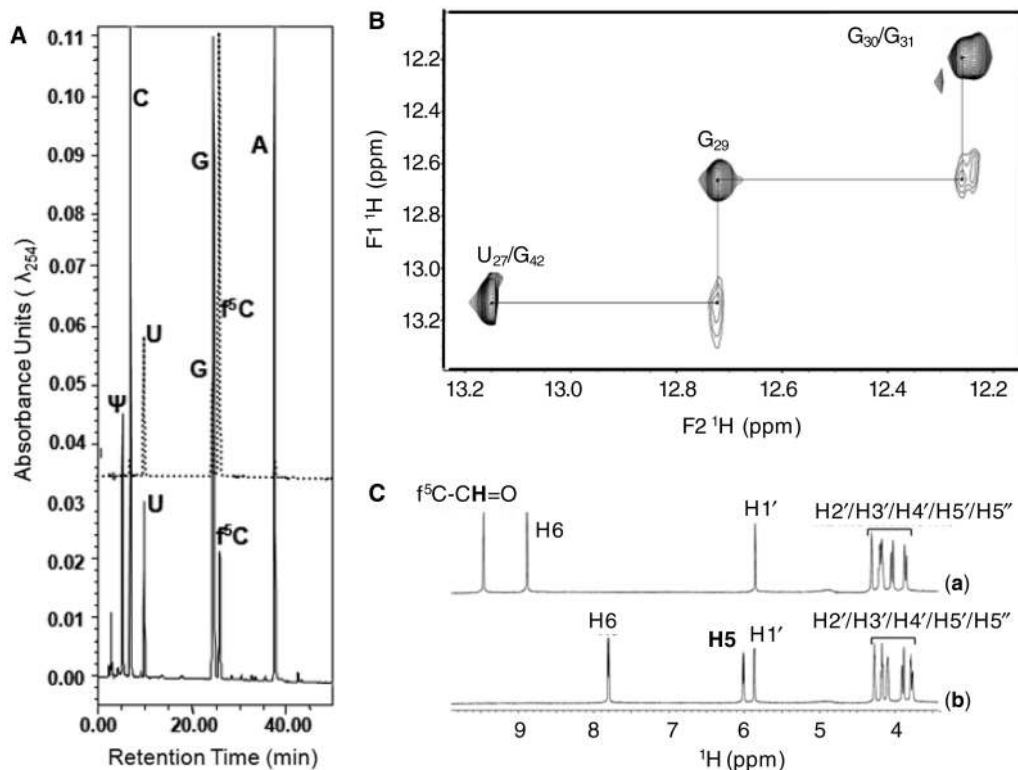


Figure 4. HPLC nucleoside composition and NMR analyses of hmASL_{CAU}^{Met} and f⁵C. (A) The upper section of the figure depicts the HPLC separation of standard ribonucleosides where C, G and A (solid line) had been injected together, and U, G and f⁵C (dotted line) had been injected in a separate control experiment. The lower section depicts the chromatography of nucleoside composing the hmASL_{CAU}^{Met} to include Ψ, as well as f⁵C. (B) The NOESY connectivities between imino protons at 500 MHz in a 2D ¹H NOESY NMR spectrum (mix = 250 ms) of hmASL_{CAU}^{Met} (90% ¹H₂O + 10% ²H₂O; 20 mM PO₄³⁻, 50 mM NaCl, pH 6.2; 2°C) with water suppression using the WATERGATE sequence. (C) 1D ¹H NMR spectra of (a) cytidine (1) and (b) 5-formylcytidine (5) (500 MHz; 100% ²H₂O; 20 mM PO₄³⁻, 50 mM Na⁺, 50 mM K⁺; pH = 6.2; 25°C) using the presaturation NMR sequence to suppress the water peak. The spectral regions and peaks corresponding to the various proton types in the molecules are labeled.

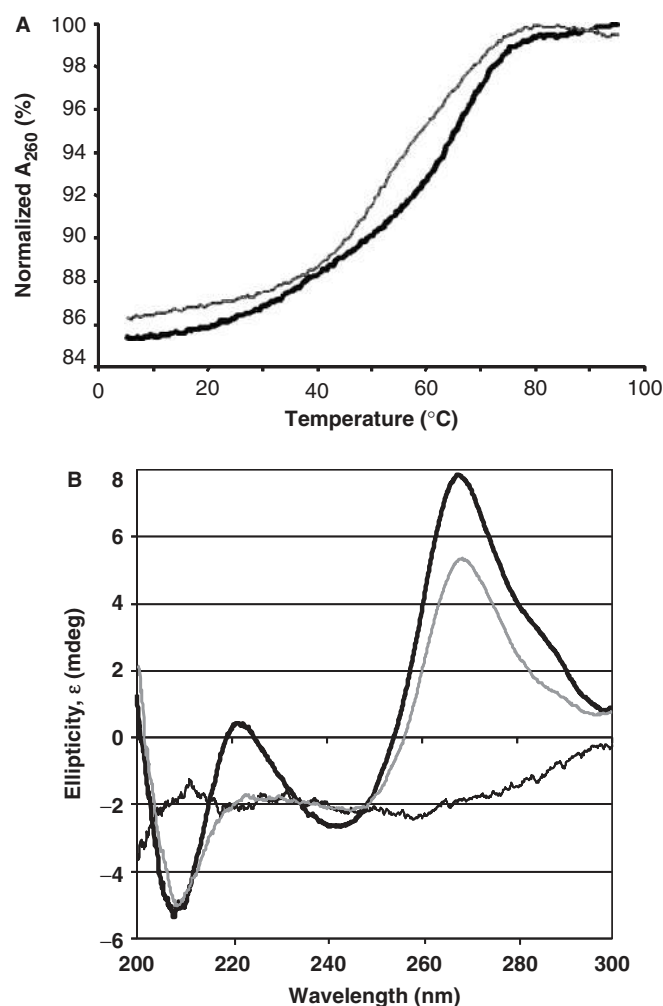


Figure 5. Thermal denaturations and circular dichroism spectra of the hmASL_{CAU}^{Met}-Ψ₂₇ and the hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄. (A) Thermodynamic stability of the ASLs. UV-monitored, thermal data were averaged from three denaturations and two renaturations for the hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ (thin gray line) and for the hmASL_{CAU}^{Met}-Ψ₂₇ (thick black line). (B) Circular dichroism spectra. Spectra of the hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ (thin gray line) and that of the hmASL_{CAU}^{Met}-Ψ₂₇ (thick black line) at the approximately equal concentrations of 2 μM were collected over the wavelength range of 200 to 300 nm.

(iv) 5'-GGCAAGGAGGUAAAAGUAAUGGCACGU-3'.

The 70S ribosomes were prepared from *E. coli* MRE600 (27). The ASLs were 5'-end ³²P-labeled using [γ-³²P] ATP (MP Biomedicals). Unlabeled ASLs (5 μM) were mixed with 10 000 CPM of 5'-end ³²P-labeled ASL. The assay was performed in ribosomal binding buffer (50 mM HEPES, pH 7.0; 30 mM KCl; 70 mM NH₄Cl; 1 mM DTT; 100 μM EDTA; 20 mM MgCl₂). The ribosomes, activated at 42°C for 10 min and then slowly cooled to 37°C, were then programmed with 2.5 μM mRNA for 15 min at 37°C. The ribosomal site not in observation was saturated with ASL_{UAC}^{Val3} (unmodified) for 15 min at 37°C. P-site binding was performed prior to A-site binding. ASL_{UAC}^{Val3} binds to the Val codon GUA; see underlined codons of the mRNA sequences above. Binding of

ASL_{CAU}^{Met} in either A- or P-site was allowed to proceed for 30 min at 37°C. The reaction mixtures (20 μl each) were then placed on ice for 20 min and filtered through nitrocellulose in a modified Whatman Schleicher and Schuell (Brentford, UK) 96-well filtration apparatus (28). Prior to filtration of experimental samples, the nitrocellulose filter was equilibrated in binding buffer at 4°C for at least 20 min and each well of the filtration apparatus was washed with 100 μl of cold binding buffer. Cold binding buffer (100 μl) was added to each sample, and the entire 120-μl volume was quickly filtered. Each well was then washed twice with 100 μl of cold binding buffer. The nitrocellulose was dried out on 'kim' wipes, and the radioactivity was measured using a phosphorimager (Molecular Dynamics, GE Healthcare). Data were measured for radioactive intensity using ImageQuant (Amersham). Nonspecific binding was determined by the binding of ASLs to ribosomes without mRNA and subtracted from the experimental data. The final data are a result of at least three separate experiments, each done with samples in triplicate, i.e. minimally nine determinations for each binding (Figure 6).

Analysis of the f⁵C pKa

UV spectra were compiled (220–320 nm) using a Varian Cary3 Spectrophotometer at different pH values for cytidine and 5-formylcytidine. The spectra were normalized to 0.2 OD at 260 nm. Entire spectra were collected to ensure that they all intersected at 260 nm at an OD of ~0.2. However, the absorbance maximum at 280 nm was plotted against the pH, a previously published method of assessing the pKa of nucleosides (29). A pH range of 2.2–7.0 (citrate-phosphate buffer) was used for cytidine, and a pH range of 1.1–5.0 was used for f⁵C (KCl-HCl buffer for pH values between 1.1 and 2.0 and citrate-phosphate buffer for pH values between 2.2 and 5.0). The line fitting and data analysis was conducted with Prism v3.00 (Graphpad Software, Inc.) (Figure 7).

RESULTS AND DISCUSSION

A 5-formylcytidine (f⁵C) has previously been synthesized from 5-(hydroxymethyl)cytosine (13) and from 5-methyluridine (30), but not incorporated into an RNA sequence. First, we developed a short (four steps) and facile synthesis of f⁵C (compound **5**, Figure 2) from commercially available cytidine (**1**, Figure 2), starting by protecting cytidine as the acetonide **2** under standard acid catalysis with an 83% yield. Installation of the hydroxymethylene unit occurred through an assisted Baylis-Hillman-type reaction with formaldehyde (**3**, 38% yield, 60% yield based on recovered starting material; Supplementary Data). Selective oxidation of the allylic alcohol with RuO₂ to the aldehyde **4** proceeded with an 82% yield. The acetonide protecting group was subsequently removed to deliver f⁵C (**5**) in 95% yield. The comparison between the NMR signals of C and those of f⁵C clearly demonstrated that the C-5 position of f⁵C was substituted (Figures 3B and 4C). This substitution was further confirmed to be the formyl group by the presence of a low field shifted signal at (F1 = 185 p.p.m.;

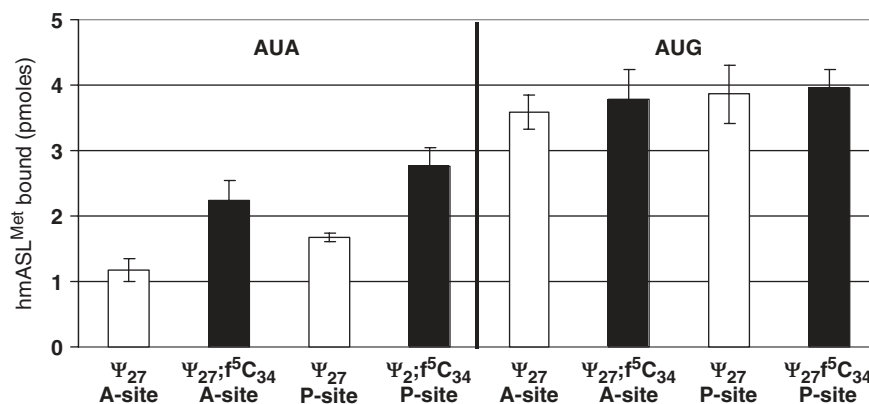


Figure 6. Codon binding by hmASL^{Met}_{CAU}-Ψ₂₇ and the hmASL^{Met}_{CAU}-Ψ₂₇;f⁵C₃₄. The equilibrium binding of the two ASLs to the cognate and noncognate codons, AUG and AUA respectively, was assessed using programmed *E. coli* ribosomes. (The ASL unmodified and the wobble position is designated 'Ψ₂₇'; ASL modified at the wobble position is designated as 'Ψ₂₇;f⁵C₃₄'.) The ASLs were bound to the A-site with *E. coli* ASL^{Val3}_{UAC} bound to its cognate codon in the P-site. The ASLs were bound to the P-site with the ASL^{Val3}_{UAC} bound to its cognate codon in the A-site.

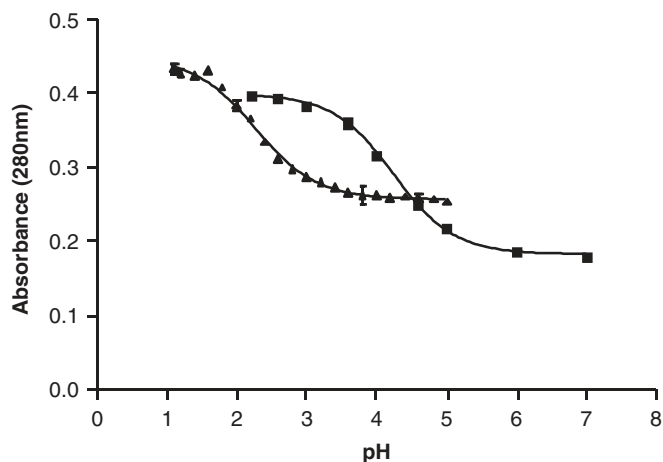


Figure 7. Analysis of the pK_a of (filled squares) cytidine in comparison to that of (filled triangles) 5-formylcytidine. UV spectra were collected for the two nucleosides over a range of pH values, pH 2.2–7.0 for cytidine, and a pH 1.1–5.0 for f⁵C. The absorbance at 280 nm was normalized and plotted against the pH.

F2 = 9.40 p.p.m.) corresponding to the CH group of the f⁵C modification. A complete and unambiguous assignment of the non-exchangeable protons was achieved by using the two dimensional (2D) ¹H-¹³C Heteronuclear Multiple Quantum Correlation (HMQC) method (31,32). As expected, the NMR peaks observed between (F1 = 50.00–85.00 p.p.m.; F2 = 4.50–6.00 p.p.m.) corresponded to the protons (H1'-H5'/H5'') of the ribose moiety (Figure 3B) (33). Conversion of f⁵C into the 2',5'-protected f⁵C phosphoramidite (**9**, Figure 2) commenced with the protection of the 3' and 5' hydroxyl groups as a disiloxane followed by protection of the 4-NH₂ group as the formamidine **6** (71% yield). Installation of a 2'-ACE orthoester [2'-O-bis(acetoxyethoxy)methyl-] followed by fluoride treatment delivered the diol **7** in 53% yield over two steps (15). The synthesis of the f⁵C phosphoramidite **9** was completed through 5'-BZH (5'-O-benzhydroxy-bis(trimethylsiloxy)silyl-) protection (**8**, 84% yield) and

phosphor-amidite formation (93% yield). A major concern for the incorporation of f⁵C phosphoramidite into synthetic RNA oligomers was the formation of imine adducts with the formyl group under resin cleavage and deprotection conditions. However, we decided not to protect the formyl group since the final deprotection of the 2'-ACE groups under mildly acidic conditions would potentially hydrolyze any imine formation that results during base-deprotection back to the formyl group.

In order to investigate the contribution of f⁵C₃₄ to the structure of the anticodon loop and to the decoding of both the AUG and AUA codons at both the A- and P-sites, f⁵C was incorporated into the anticodon stem and loop domain of hmRNA^{Met}_{CAU} (hmASL^{Met}_{CAU}) at the wobble position 34, along with pseudouridine, Ψ₂₇. The oligonucleotide was synthesized with Ψ₂₇ and with and without f⁵C₃₄ (hmASL^{Met}_{CAU}-Ψ₂₇;f⁵C₃₄, and hmASL^{Met}_{CAU}-Ψ₂₇) using care not to oxidize the formyl group. Incorporation of f⁵C into RNA was accomplished by activating with *S*-ethyl tetrazole and coupling of the activated species for 3.5 min to the growing polyribonucleotide on the solid-support. Cleavage from the support and deprotection of the exocyclic amines was tested using NH₄OH at room temperature for 24 h, methylamine at room temperature for 6 h, and *t*-butyl amine in water (1:3, v/v) at 60°C for 6 h. Only the *t*-butyl amine conditions resulted in the correct mass upon MALDI-TOF analysis of the crude products. There was no indication of any *t*-butyl-amine adducts present from the MALDI-TOF results. Successful incorporation of f⁵C was confirmed by NMR measurements (Figures 3 and 4) and HPLC of constituent nucleosides (Figure 4). The proton resonance of the formyl group is observed in the low field region of the 1D spectrum of hmASL^{Met}_{CAU}-Ψ₂₇;f⁵C₃₄ and absent from that of the hmASL^{Met}_{CAU}-Ψ₂₇ (Figure 3). The formyl proton in hmASL^{Met}_{CAU}-Ψ₂₇;f⁵C₃₄ resonates at the same chemical shift as that of the mononucleoside f⁵C₃₄, as observed in the superimposed ¹H-¹³C HMQC spectra of cytidine and 5-formylcytidine (Figure 3B). The HPLC

Table 1. Thermodynamic contributions of f⁵C₃₄

hmASL _{CAU} ^{Met}	T _m (°C)	ΔG ₃₇ ^o (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/K·mol)	Hyperchromicity (%)
Ψ ₂₇	67.7 ± 1.8	-2.8 ± 0.1	-31.1 ± 2.0	-91.3 ± 6.3	16 ± 1
Ψ ₂₇ ;f ⁵ C ₃₄	60.1 ± 0.8	-1.6 ± 0.1	-22.8 ± 1.2	-68.4 ± 3.4	16 ± 1

nucleoside composition analysis confirms the presence of the f⁵C (Figure 4A).

The structure of the resulting hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ was characterized by 1D ¹H and 2D ¹H NOESY NMR experiments conducted in H₂O at 2°C (34), and by determining the thermodynamic contributions of f⁵C to the RNA. The formyl proton resonance was found at 9.45 p.p.m. corresponding almost exactly to that of the nucleoside alone (Figure 3A). The imino protons of the stem of hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ were found to resonate between 12 and 13.5 p.p.m. on the ¹H 1D NMR spectrum (Figure 3A). The NMR spin systems that involve the exchangeable imino protons of hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ were identified by conducting NMR experiments in H₂O at 2°C (Figures 3A and Figure 4B). The identification and assignment of the exchangeable protons were indicative of the overall stability the hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ in solution, and the comparison with hmASL_{CAU}^{Met}-Ψ₂₇ (Figure 3A) demonstrated the successful incorporation of f⁵C₃₄ into the sequence of hmASL_{CAU}^{Met}.

The modified RNA synthesis has allowed us to begin examining the role of f⁵C₃₄ in thermal stability and decoding activity of hmtRNA^{Met}. Thermodynamic parameters were extracted from the repeated denaturations and renaturations of both hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ and hmASL_{CAU}^{Met}-Ψ₂₇ (Table 1 and Figure 5A). Introduction of f⁵C₃₄ lowered the melting temperature and standard free energy (ΔG₃₇^o) considerably, but did not alter the ASL's hyperchromicity. The circular dichroism spectrum of the hmASL_{CAU}^{Met}-Ψ₂₇ exhibited a greater ellipticity at 270 nm than that of the hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄. The lower degree of ellipticity of hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ is indicative of a decrease in base stacking. The decreased base stacking must be attributed to the anticodon loop nucleosides because of the location of the modification. These differences in thermodynamics and circular dichroism ellipticity between hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ and hmASL_{CAU}^{Met}-Ψ₂₇ indicated that f⁵C₃₄ may enhance the motional dynamics of the loop. This difference in motional dynamics was observed by a molecular dynamics simulation (MDS) performed on the hmASL_{CAU}^{Met}-Ψ₂₇ and the hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ using AMBER 9 (35). The hmASL_{CAU}^{Met}-Ψ₂₇ displayed an average root mean square deviation from the starting structure of 2.18 ± 0.23 as opposed to hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ for which higher fluctuations of 2.60 ± 0.60 were detected (Supplementary Data). The enhanced motional dynamics may be important for the decoding of AUA, as well as AUG.

The tRNA^{Met} anticodon CAU is a cognate pair for the Met codon AUG. According to Crick's Wobble Hypothesis (36), the binding of anticodon CAU to

codon AUA would be unlikely due to the C-A mismatch at the wobble position (wobble pair nucleosides in bold). However, the mitochondrial ribosome decodes both AUG and AUA using one tRNA with the anticodon CAU. This one tRNA consists of the modification f⁵C₃₄. In contrast, two tRNAs decode the one Met codon AUG in the cytoplasm (37). One of the tRNAs is an initiator tRNA that decodes AUG in the ribosome's peptidyl- or P-site at the initiation of translation, where AUG is the first codon to be translated on the mRNA. This initiator tRNA_{CAU}^{Met} consists of an unmodified CAU anticodon. The second cytoplasmic tRNA_{CAU}^{Met} is responsible for elongation and recognizes AUG located within the mRNA, and thus responds only to the aminoacyl- or A-site codon. In *E. coli*, this elongator tRNA^{Met} is modified with N4-acetylcytidine at the wobble position (ac⁴C₃₄) (38). Thus, at the anticodon, one of the main distinguishing factors between the cytoplasmic initiator and elongator tRNA_{CAU}^{Met} is the modification at the wobble position. We used a codon-binding assay to observe the affinity of the hmASL_{CAU}^{Met}-Ψ₂₇; f⁵C₃₄ and hmASL_{CAU}^{Met}-Ψ₂₇ for the codons AUA and AUG at either A-site or P-site of *E. coli* 70S ribosomes. To ensure binding of the two ASL^{Met}s to the A- or the P-site, the ribosomal site not in observation (P- or A-site, respectively) was saturated with the unmodified *E. coli* ASL_{UAC}^{Val3} in response to its cognate codon GUA. The unmodified ASL_{UAC}^{Val3} binds its cognate codon with high affinity and specificity (39).

The hmASL_{CAU}^{Met}-Ψ₂₇ bound AUG in the A-site and the P-site with an affinity comparable to what have observed previously for certain ASLs with unmodified wobble positions responding to cognate codons (Figure 6) (21,39). In contrast, the hmASL_{CAU}^{Met}-Ψ₂₇ bound poorly to AUA in both the A- and P-sites. Surprisingly, introduction of f⁵C₃₄ enhanced binding to AUA by 2-fold (Figure 6). Our results indicated that of the two codons at either of the two ribosomal sites, the f⁵C₃₄ modification appears to be most important for reading AUA.

Both the hmASL_{CAU}^{Met}-Ψ₂₇ and the fully modified hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ exhibited considerable affinity for AUG, and at both the A-site and the P-site. However, only the hmASL_{CAU}^{Met}-Ψ₂₇;f⁵C₃₄ exhibited significant affinity for AUA. There was a doubling in the affinity of ASL^{Met} for the AUA codon when f⁵C₃₄ was present. This increase in affinity of the f⁵C₃₄-modified ASL in comparison to that of the hmASL_{CAU}^{Met}-Ψ₂₇, unmodified at the wobble position, was not observed on AUG and may therefore be the sole contributor to the efficient translation of AUA codons. A 2-fold increase in affinity of tRNA toward a codon has been shown to be significant in translation (40). Although some ASL modifications cause small increases in codon-binding affinity, others can dramatically increase affinity to codons (39).

Of particular interest is the chemical and conformational mechanisms by which a stable, but noncanonical base pair occurs between f^5C_{34} and the third base of the AUA codon, an adenosine, on the ribosome. C-A base pairs are extremely unusual. Although the C-A pairing has been found in the folded structure of some RNAs such as ribosomal RNAs (rRNAs), it is rarely found in anticodon:codon pairs. An anticodon:codon C-A mismatch has been detected when C_{34} of tRNA^{Leu}_{CAU}, modified with lysidine (k^2C_{34}) at the wobble position, is paired to the cytoplasmic isoleucine codon AUA. The lysine moiety of C_{34} on the anticodon provides an amino group which hydrogen bonds to A of the codon, thus allowing the wobble position C-A mismatch to occur. One could imagine that the 5-formyl modification raises the pK_a of cytidine's N3 to the physiological range where an additional hydrogen bond could be formed to AUA. However, the pK_a of f^5C determined by UV spectral analysis was lower than that of C (2.3 and 4.2, respectively; Figure 7), corresponding well with previous determinations (41) including those for df^5C (42) and for f^5U (43). Thus, f^5C must contribute to the decoding of the mitochondrial genome through a different mechanism. Another C-A anticodon:codon mismatch may occur at the wobble position when tRNA^{Leu}_{f⁵CmAA} pairs with the leucine codon UUA. Similar to hmtRNA^{Met}, this tRNA^{Leu} isoacceptor has a 5-formylated, 2'-O-methylated C at the wobble position, f^5Cm_{34} . The wobble modifications are thought to be a general characteristic of mammalian cytoplasmic tRNA^{Leu} that may aid in the decoding of leucine codons UUG and UUA and prevent the miscoding of the similar codons of phenylalanine, UUU and UUC. However, there is a lack of information on the decoding properties of tRNA^{Leu}_{f⁵CmAA} and therefore, there is the possibility that an isoacceptor other than tRNA^{Leu}_{f⁵CmAA} is responsible for specifically reading UUA (6,38).

Eighty percent of the methionine codons internal to mitochondrial mRNA are the AUA codon. Thus, the enhanced affinity of hmASL^{Met}_{CAU-Ψ₂₇;f⁵C₃₄} for AUA in the A-site of the *E. coli* ribosome has important implications for the affinity and kinetics of decoding AUA during elongation. The enhanced A-site binding of AUA by the f^5C -modified, hmASL^{Met}_{CAU} may be even more evident on the mitochondrial ribosome, a concept not studied here. Also, the disease-related A37-G37 (A4435G) mutation, associated with an increased penetrance and expression of the primary Leber hereditary optic neuropathy mutation (G11778A), LHON (44), may critically alter the anticodon architecture such that either or both decoding events do not occur. This has yet to be examined. The synthesis of the wild-type modified and unmodified anticodon stem and loops of the hmASL^{Met}_{CAU} and their physical, chemical characterizations will be important in understanding the contributions of the modification to biological function and in characterization of the human disease-relevant mutant tRNA.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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