A complete landscape of post-transcriptional modifications in mammalian mitochondrial tRNAs

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ABSTRACT

In mammalian mitochondria, 22 species of tRNAs encoded in mitochondrial DNA play crucial roles in the translation of 13 essential subunits of the respiratory chain complexes involved in oxidative phosphorylation. Following transcription, mitochondrial tRNAs are modified by nuclear-encoded tRNA-modifying enzymes. These modifications are required for the proper functioning of mitochondrial tRNAs (mt tRNAs), and the absence of these modifications can cause pathological consequences. To date, however, the information available about these modifications has been incomplete. To address this issue, we isolated all 22 species of mt tRNAs from bovine liver and comprehensively determined the post-transcriptional modifications in each tRNA by mass spectrometry. Here, we describe the primary structures with post-transcriptional modifications of seven species of mt tRNAs which were previously uncharacterized, and provide revised information regarding base modifications in five other mt tRNAs. In the complete set of bovine mt tRNAs, we found 15 species of modified nucleosides at 118 positions (7.48% of total bases). This result provides insight into the molecular mechanisms underlying the decoding system in mammalian mitochondria and enables prediction of candidate tRNA-modifying enzymes responsible for each modification of mt tRNAs.

INTRODUCTION

Mitochondria, organelles present in most eukaryotic cells, provide the chemical energy required by living cells in the form of adenosine triphosphate (ATP), which is synthesized by the electron transport chain and oxidative phosphorylation (1). Mitochondria contain their own genomic DNA, called mitochondrial (mt)DNA, and unique transcription and translation machinery that converts their genetic information into proteins. In mammals, the mtDNA is a circular double-stranded DNA, ~16 kilobase pairs (kb) in length, which contains 13 genes encoding essential subunits of the respiratory chain complexes and 24 RNA genes (2 ribosomal RNAs and 22 tRNAs) required for mitochondrial protein synthesis.

The mammalian mitochondrial decoding system differs from the canonical decoding system by its use of four non-universal codons (Table 1) (2): AUA for Met, UGA for Trp and AGR (R = A or G) for Stop. The 60-sense codons are deciphered by 22 species of mitochondrial tRNAs, which constitute the smallest set of tRNAs necessary to translate all sense codons among all kingdoms of life, including other organelle decoding systems. Post-transcriptional modifications at the first letters of tRNA anticodons play a critical role in establishing this minimal decoding system. To reduce the number of tRNA species, each of eight family boxes in mitochondria is decoded by only a single tRNA. The tRNAs responsible for the family boxes frequently have unmodified uridines (U34) at the first (wobble) position of an anticodon. According to Crick’s wobble rule (3), U34 can recognize only A and G at the third position of a codon. In the decoding systems of some bacteria and most organelles, U34 can base-pair with any of the four bases by the so-called ‘four-way wobble rule’ (2) or ‘super wobbling’ (4). The conformational flexibility of U34 is thought to enable U:U and U:C pairing. On the other hand, tRNAs responsible for two-codon sets ending in purines (NNR; N = any four nucleotides) have modified uridines (xm5s2U-type) at their wobble positions (5). In general, xm5s2U-type modification restricts conformational flexibility of the wobble base, thereby strengthening recognition of NNR codons and preventing misrecognition of codons ending in pyrimidines (NNY; Y = U and C). We previously discovered that taurine-containing modified uridines in mammalian mt tRNAs are responsible for NNR codon sets: 5-taurinomethyluridine (tMUA) at the wobble position of human and bovine mt tRNA5453 (5), and 5-taurinomethyl-2-thiouridine (tMS2U) at the wobble position of human and bovine mt tRNA5456 (6). Subsequently, tMS2U was also found at the wobble position of the bovine mt tRNA for Glu and Gln (7).

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age of unmodified or modified U34 in tRNAs would reveal a fundamental principle of the minimal decoding system in mammalian mitochondria. nullnull

5-formylcytidine (f5C), another unique modification in mammalian mitochondria (8), is present at the wobble position of mt tRNA<sub>Lys</sub>. Biochemical studies show that f5C is required for recognition of the non-universal AUG codon, in addition to the canonical AUG codon (9,10). Possi-
ble base pairing between f5C and A was demonstrated by a crystallographic study using an f5C-containing anti-
codon stem-loop bound to the 30S subunit of Thermus ther-
mophilus (11).

Defective mitochondrial translation results in mito-
chondrial dysfunction, ultimately causing pathological
consequences (12). Numerous pathogenic mutations
associated with mitochondrial diseases have been found
in mtDNA (http://www.mitomap.org/MITOMAP) (13). These pathogenic mutations are maternally inherited.
Among over 400 pathogenic mutations compiled to date,
~200 have been mapped to mt tRNA genes; these muta-
tions diminish biogenesis, stability and function of tRN-
As (2,12,14–15). Two major subgroups of mitochondrial
cenchal in mitochondria: mitochondrial encephalopathy, lact-
acidosis and stroke-like syndrome (MELAS), caused by
a mutation in mt tRNA<sub>Lys</sub>, and myoclonus epilepsy
with ragged-red fibers (MERRF), caused by a mutation
in mt tRNA<sub>Met</sub>. Biochemical studies show that f5C is
present in mutant mt tRNA<sub>Leu(UUR)</sub> harboring the A3243G
mutation, and another 10% have an A-to-G mutation at
position 3271 (17). MERRF patients have an A8344G
mutation in the mt tRNA<sub>Lys</sub> gene (18). We previously reported that τ<sup>m2</sup>U and τ<sup>m3</sup>s<sup>2</sup>U were not
present in mutant mt tRNA<sub>Leu(UUR)</sub> harboring the A3243G
or T3271C mutation (19), or in mt tRNA<sub>Lys</sub> harboring the
A8344G mutation (20). We also confirmed the absence of
taurine modifications in MELAS patient tissues harboring
A3243G, G3244A, T3258C, T3271C or T3291C mutations
(21), as well as in MERRF patients harboring the A8344G
mutation (22). These pathogenic point mutations are
assumed to act as negative determinants of τ<sup>m3</sup>(s<sup>2</sup>)U biogen-
esis. In MELAS, the absence of τ<sup>m3</sup>U in mt tRNA<sub>Leu(UUR)</sub>
results in a defect in decoding the UUG codon, leading to
lower expression of the UUG-rich protein ND6 (23). Simi-
larly, in MERRF the absence of both 5-taurinomethyl and
2-thio groups of τ<sup>m3</sup>(s<sup>2</sup>)U in mt tRNA<sub>Lys</sub> leads to severe
translation failure of both types of AAR codon (24). These
observations imply that deficiency in modification of mt
tRNA plays a key role in molecular pathogenesis. As men-
tioned earlier, unmodified U34 can read any of four bases
at the third letter of codons in a family box by the four-way
wobbling. However, unmodified U34 is only used in tRNAs
responsible for family box codons in which at least one G
or C is present at the first or second letter of codons. If the
codon–anticodon interaction is stabilized by one or two GC
pairing at the first two base pairings, U34 is considered to
read any of four bases in the family boxes. In the case of
mt tRNA<sub>Leu(UUR)</sub> and mt tRNA<sub>Lys</sub>, there is no G or C at
the first or second letter of their cognate codons. This is the
reason why unmodified U34 in the mutant tRNAs cannot
decipher cognate codons efficiently, not by expanding their
decoding capacity.

Mitochondrial diseases are also caused by pathogenic
mutations in nuclear-encoded genes (2), including genes en-
coding translation factors, aminocycl-tRNA synthetases,
tRNA processing enzymes and tRNA-modifying enzymes.
Loss-of-function mutations in these genes hamper the biogen-
esis and function of mt tRNAs. Several instances of
pathogenic point mutants in tRNA-modifying enzymes
have been reported to date: mitochondrial myopathy and
sideroblastic anemia (MLASA), acute infantile liver failure
and hypertrophic cardiomyopathy with lactic acidosis are
associated with pathogenic mutations in <i>PUSI</i> (25), <i>MTU1</i>
(26) and <i>MTOS</i> (27,28), respectively.

To gain more insight into the molecular basis of the
mitochondrial decoding system, as well as the molecular
pathogenesis of human diseases caused by deficiencies in
mt tRNA modifications, it is necessary to obtain a com-
plete picture of post-transcriptional modifications in all 22
species of mammalian mt tRNAs. To date, 11 species of
human or bovine mt tRNAs have been sequenced and their
post-transcriptional modifications determined (2). In pre-
vious work, we isolated all 22 species of bovine mt tRNAs
(29). By analyzing these tRNAs by mass spectrometry, we
determined the post-transcriptional modifications of seven
species of mitochondrial tRNAs that have never been re-
ported, and provide some revised information regarding the
modified bases in five other mitochondrial tRNAs. In to-
tal, we identified 15 species of modified nucleosides at 118
positions in 22 species of bovine mt tRNAs. We discuss
the basic principles of the mitochondrial decoding system
in mammals, and propose candidate tRNA-modifying en-
zymes whose roles remain to be confirmed experimentally.

MATERIALS AND METHODS

Isolation of individual mitochondrial tRNAs from bovine liver

Bovine liver RNA was prepared as described previously
(6,29). Briefly, crude RNA was extracted from buffer-
homogenized bovine liver by phenol extraction, and the
tRNA fraction was roughly concentrated by anion ex-
change chromatography. Individual tRNAs were isolated
by chaplet column chromatography (29). Seventeen of them
were isolated homogeneously based on the polyacrylamide
gel electrophoresis analysis. Five mt tRNAs for Leu(UUR),
Asn, Thr and Pro were isolated as major bands
but had some minor bands. However, the qualities of
the isolated tRNAs are sufficient enough to analyze post-
transcriptional modifications.

Cyanoethylation of pseudouridine in tRNA

Cyanoethylation of tRNA was performed basically as de-
scribed (30). Eight micrograms of isolated tRNA dissolved
in less than 4 μl of Milli-Q water was added to 30 μl of 41%
(v/v) ethanol/1.1 M trimethylammonium acetate (pH 8.6).
After addition of 4 μl of acrylonitrile (Wako Pure Chemi-
cal Industries), the mixture was incubated at 70°C for 2 h,
lyophilized and dissolved in Milli-Q water. The solution
was then subjected to RNase digestion and analyzed by RNA
mass spectrometry, as described below.
RNA mass spectrometry

RNA fragments digested by RNases were analyzed by mass spectrometry as described previously (31,32). In brief, 2–5 ng of isolated tRNA was digested with RNase T1 (Epicentre) or RNase A (Ambion) and analyzed by an LTQ Orbitrap mass spectrometer (Thermo Scientific) with a nano-electrospray connected with a splitless nanoflow high pressure liquid chromatography system (DiNa, KYA Technologies). Alternatively, 0.4–2 μg isolated tRNA was digested with RNase T1 and analyzed on a LCQ DUO ion-trap mass spectrometer with an ESI (electrospray ionization) source (Thermo Finnigan) and HP1100 liquid chromatography system (Agilent Technologies) in negative ion detection mode. Nucleoside analysis was performed as described previously (31). In brief, about 4 μg of isolated tRNA was digested to nucleosides with nuclease P1 (Wako Pure Chemical Industries) and bacterial alkaline phosphatase C75 (Takara bio), and then analyzed on the LCQ DUO ion-trap mass spectrometer with an ESI-MS (electrospray ionization-mass spectrometry) source (Thermo Scientific) with a nano ESI-MS to analyze RNA fragments generated with RNase T1 (Figure 1C) or RNase A (Figure 1D). ProMass (Novatia) was used to obtain the multiply charged mass spectra.

RESULTS

Isolation of 22 species of bovine mt tRNAs

To date, 11 species of bovine mt tRNAs have been described in published work, along with the details of their post-transcriptional modifications: Ser(UCN) (33), Ser(AGY) (34,35), Phe (36), Arg (35), Gly (35), Ile (35), Met (8,37), Val (35), Trp (35), Glu (7) and Gln (7). In addition, sequences of four species of bovine mt tRNAs for Thr, Leu(UUR), Leu(CUN) and Lys have recently been deposited in the tRNA databases as personal communications (38,39). However, some tRNA sequences deposited in the tRNA databases contain unidentified modified bases and mis-annotation of modifications determined by outmoded methods. To accurately define the complete landscape of post-transcriptional modifications of mammalian mt tRNAs, we analyzed all 22 species of mt tRNAs, which we previously isolated from bovine liver by chaplet column chromatography (29).

Mass spectrometric analysis of post-transcriptional modifications in bovine mt tRNAs

We performed a series of mass spectrometric analyses of each individual mt tRNA to determine its post-transcriptional modifications. As an example, determination of post-transcriptional modifications in mt tRNAAla is depicted in Figure 1A. First, nucleoside analysis by LC/ESI-MS was carried out to determine the composition of modifications, revealing that 1-methyladenosine (m1A), N2-methylguanosine (m2G) and pseudouridine (ψ) were present in mt tRNAAla (Figure 1B). In parallel, we used capillary LC/nano ESI-MS to analyze RNA fragments generated by digestion with RNase T1 (Figure 1C) or RNase A (Figure 1D). Unmodified fragments were identified by comparing the observed m/z values with the calculated m/z values deduced from the mt tRNAAla gene encoded in bovine mtDNA (GenBank accession number V00654). Nucleotide compositions of internal fragments were calculated as 5′-hydroxyl (5′OH) and 3′-phosphate (3′p). The sequence of each fragment was further analyzed by collision-induced dissociation (CID) (Figure 1E). Because tRNA has 5′-phosphate and 3′-hydroxyl groups, the 5′-terminal fragment pGAGGAUp (MW 2097.2) and the 3′ terminal fragment OH-CAAUCCUUACCA-OH (MW 3697.5) were detected as species with unique molecular masses distinct from those of the internal fragments. Furthermore, RNA fragments containing modified nucleosides could be identified from the deduced molecular mass calculated from the RNA sequences deposited in the databases as personal communications (38,39).

Table 1. Codon–anticodon pairing in the bovine mitochondrial genetic code

| Codon   | Amino acid (anticodon) | Codon   | Amino acid (anticodon) | Codon   | Amino acid (anticodon) | Codon   | Amino acid (anticodon) |
|---------|------------------------|---------|------------------------|---------|------------------------|---------|------------------------|
| UUU     | Phe                    | UCU     | Ser                    | UAU     | Tyr                    | UGU     | Cys                    |
| UUC     | (GAA)                  | UCC     | (UGA)                  | UAC     | (QUA)                  | UGC     | (GCA)                  |
| UUA     | Leu                    | UCA     | (UGA)                  | UAA     | stop                   | UGA     | Trp                    |
| UUG     | (m3UAA)                | UCG     |                        | UAG     |                        | UGG     | (m3UCA)                |
| CUU     |                        | CUC     | Pro                    | CAU     | His                    | CGU     | Arg                    |
| CUC     | Leu                    | CCC     |                        | CAU     |                        | CGG     | (UCG)                  |
| CAU     | (UAG)                  | CCA     | (UGG)                  | CAA     | Gin                    | CGA     |                        |
| CUG     | CCG                    | CAG     | (m3s2UG)               | CAG     |                        | CGG     |                        |
| AUU     | Ile                    | ACU     |                        | AAU     | Asn                    | AGU     | Ser                    |
| AUC     | (GAU)                  | ACC     | Thr                    | AAC     | (QUU)                  | AGC     | (GCU)                  |
| AUA     | Met                    | ACA     | (UGU)                  | AAA     | Lys                    | AGA     | stop                   |
| AUG     | (5CAU)                 | ACG     |                        | AAG     |                        | AGG     |                        |
| GUU     | Val                    | GCU     | Ala                    | GAU     | Asp                    | GGU     |                        |
| GUC     | Val                    | GCC     | (UGC)                  | GAC     | (UCU)                  | GCC     | Gly                    |
| GUA     | (UAC)                  | GCA     | (UGC)                  | GAA     | Glu                    | GGA     | (UCC)                  |
| GUG     | GCG                    | GAG     | (m3s2UUC)              | GAG     |                        | GGG     |                        |

Non-universal genetic codes are denoted in bold type. AUA: Ile (universal), Met (mitochondria); UGA: stop (universal), Trp (mitochondria); AGA/G: Arg (universal), stop (mitochondria).
Figure 1. Mass spectrometric analysis of bovine mt tRNA\textsubscript{Ala} for assignment of post-transcriptional modifications. (A) Secondary structure of bovine mitochondrial tRNA\textsubscript{Ala} with post-transcriptional modifications determined in this study. The position numbers of the modifications are displayed according to the nucleotide numbering system from the tRNA compilation (40). Symbols for modified nucleosides are as follows: m\textsubscript{1}A, 1-methyladenosine; m\textsubscript{2}G, N\textsuperscript{2}-methylguanosine and \Psi, pseudouridine. Watson–Crick base pairs are indicated by solid lines, whereas G–U pairs are indicated by asterisks. (B) Nucleoside analysis of bovine mitochondrial tRNA\textsubscript{Ala}. Left, top panel: UV chromatogram at 254 nm of the four major nucleosides (C, U, G and A). Left, lower panels: extracted-ion chromatograms (XIC) for the protonated ion of m\textsubscript{1}A nucleoside (m/z 282, black line) with its base ion (m/z 150, gray line) (second panel), m\textsubscript{2}G nucleoside (m/z 298, black line) with its base ion (m/z 166, gray line) (third panel) and \Psi nucleoside (m/z 245, black line) (bottom panel). The XIC for the base ion (20% upper offset) is overlaid on the XIC for the nucleoside ion. Right: mass spectra of m\textsubscript{1}A and m\textsubscript{2}G. Cleavage positions for the base-related ions are indicated on the chemical structures. (C) RNA fragment analysis of RNase T\textsubscript{1} digests of bovine mitochondrial tRNA\textsubscript{Ala}. Assigned fragments are indicated on the base peak chromatogram (BPC) in the first panel. ‘p’ stands for the terminal phosphate group. The XIC for the doubly charged negative ion of a modification-containing fragment (AUUUm\textsubscript{1}Am\textsubscript{2}Gp, m/z 982.6) is indicated in the second panel. Because m\textsubscript{2}G at position 10 is a partial modification, both AUUUm\textsubscript{1}Am\textsubscript{2}Gp and AUUUm\textsubscript{1}AGp were detected. (D) RNA fragment analysis of RNase A digests of bovine mitochondrial tRNA\textsubscript{Ala}. Assigned fragments are indicated on the BPC. The XIC for the doubly charged negative ion of the 5’-terminal fragment (pGAGGAUp, m/z 1047.6) is indicated in the second panel. (E) A CID spectrum of a cyanoethylated RNA fragment to determine the location of a \Psi site. The doubly charged negative ion of the RNA fragment (m/z 1617.7) shown in the inset was used as the precursor ion for CID. The product ions were assigned according to McLuckey \textit{et al.} (41). The asterisks in the spectrum denote product ions containing ce\textsubscript{\Psi}. (F) Whole mass analysis of intact bovine mt tRNA\textsubscript{Ala}. A series of multiply charged negative ions is shown in the mass spectrum. The charge values are indicated in parentheses. The observed mass obtained by deconvoluting the mass spectra is shown in the inset.
the combinations of unmodified and expected modified nucleotides. For example, in the RNase T1 digest of mt tRNA\text{Ala}, we found a species with m/z 982.627, corresponding to a doubly charged negative ion of dimethylated AUU-UAGp. The positions of modifications were determined by CID analysis, which indicated the modified sequence AUU-UAU(\text{dimethyl})G(\text{dimethyl}). According to the nucleoside analysis, this species was assigned as AUUUm\text{1}Am\text{2}Gp, with m\text{1}A and m\text{2}G at positions 9 and 10, respectively. Similarly, m\text{2}G was also found at position 26.

To identify \(\Psi\), a mass-silent modification, each tRNA was treated with acrylonitrile to cyanoethylate \(\Psi\) (1-cyanoethyl \(\Psi\); ce\(\Psi\)). The derivatized tRNA was then digested by RNase T1 and subjected to mass spectrometry to detect the cyanoethylated fragments with molecular mass increased by 53 Da. Three cyanoethylated fragments were detected in the RNase T1 digest, and each fragment was further analyzed by CID. As shown in Figure 1E, \(\Psi\) at position 39 was identified from the assignment of product ions of CAUce\(\Psi\)CAAUUAGp. Accordingly, we identified three \(\Psi\)s at positions 28, 39 and 57. Finally, to confirm the assignment of post-transcriptional modifications, the total molecular mass of mt tRNA\text{Ala} was measured by deconvoluting the multiply charged negative ions produced by ESI (Figure 1F). The observed mass (23178.5 Da) was fairly close to the calculated mass (23180.6 Da), which corresponds to the base composition of mt tRNA\text{Ala} (pU27C9A20G16) with three methyl groups. Other tRNAs were basically analyzed using the same procedure as for mt tRNA\text{Ala} (Supplementary Information and Table S1).

**Post-transcriptional modifications in seven bovine mt tRNAs**

For 10 of the species of mt tRNAs described in the previous literature, our determinations of modifications yielded results consistent with published findings: Phe, Gly, Leu(CUN), Met, Arg, Ser(UCN), Ser(AGY), Val, Glu and Gln (Supplementary Figure S1).

Several of the tRNAs we characterized have not been previously reported or deposited in any databases. We determined the primary structures and modifications for seven such mt tRNAs: Cys, Asp, His, Asn, Pro and Tyr (Figure 2 and Supplementary Table S1) plus the aforementioned mt tRNA\text{Ala} (Figure 1). We identified queuosine (Q) at the wobble positions of mt tRNAs for Asp, His, Asn and Tyr. In cytosolic tRNA\text{Asp} and tRNA\text{His} by LC-MS/MS, Q was glycosylated to mannosyl-Q and galactosyl-Q, respectively; however, Q was not glycosylated in the mt tRNAs. The mt tRNAs for Ala and Pro correspond to family boxes; as expected, unmodified U was present at the wobble position. In mt tRNA\text{His}, we identified a G at position -1 (G\(_{-1}\)) (Supplementary Figure S2). This base is likely to be added enzymatically after transcription and 5′-end processing. In mt tRNA\text{Asp}, m\text{3}G was present at position 6, the first such case to be reported in mammalian mitochondria. At position 37, which is 3′-adjacent to the anticodon, N\text{6}-isopentenyladenosine (i\text{6}A) and 2-methylthio-N\text{6}-isopentenyladenosine (m\text{i2}i\text{6}A) were present in mt tRNA\text{Asp} and mt tRNA\text{His}, respectively. N\text{6}-threonylcarbamoyladenosine (t\text{6}A) and m\text{1}G were also present in the mt tRNAs for Asn and Pro, respectively. During our analysis, we found two polymorphisms in the sequences of mt tRNAs for Asp and Tyr: in mt tRNA\text{Asp}, a mixture of A and G were present at position 57; similarly, A and G were mixed at position 5 in mt tRNA\text{Tyr} (Figure 2). Both sites are encoded as A7356 (H-chain) and A5681 (L-chain) in the bovine mtDNA sequence (GenBank V00654) used as a reference. We speculate that these polymorphisms are the result of mtDNA heteroplasmy in the bovine liver we analyzed. In fact, both sites are encoded as guanosines in the mtDNA sequence of Bos taurus isolate FC3 (GenBank accession number DQ124389.1), suggesting that these sites are polymorphic in populations of healthy individuals.

**Full complement of modifications in five bovine mt tRNAs**

Although sequences and modifications of the remaining five mt tRNAs [Ile, Leu(UUR), Lys, Thr and Trp] have been published or deposited in the database (38,39), we discovered eight previously unreported modifications in these tRNAs (Figure 3): N\text{2}-, N\text{2}-dimethylguanosine (m\text{2}G\text{2}) and m\text{1}A at positions 26 and 58 in mt tRNA\text{Ile}; m\text{3}U at position 34 in mt tRNAs for Leu(UUR) and Trp; m\text{3}s\text{2}U and t\text{6}A at positions 34 and 37 in mt tRNA\text{Leu} and 5-methylcytidine (m\text{C}) at position 72 in mt tRNA\text{Thr} and position 48 in mt tRNA\text{Trp}. The wobble modifications of bovine mt tRNAs for Leu(UUR), Lys and Trp remained unidentified in tRNA\text{Leu}(UUR) and mt tRNA\text{Lys}, respectively. To date, however, their full sequences had not been reported. The m\text{3}C72 in mt tRNA\text{Thr} is the first reported instance of this modification in mammalian mitochondria.

**DISCUSSION**

Here, we report the post-transcriptional modifications in seven species of bovine mt tRNAs not previously characterized, and eight previously unidentified modified bases in five mt tRNAs whose sequences and modifications were determined in earlier studies. In total, we identified 15 species of modified nucleosides at 118 positions in the complete set of bovine mt tRNAs (Supplementary Table S2), i.e. 7.48% of the bases in these tRNAs are modified. The sites and species of all modifications are summarized in a schematic cloverleaf structure (Figure 4). Notably, all modifications are base modifications; the absence of 2′-O-methylation is a characteristic feature of mt tRNAs.

Eight mt tRNAs responsible for family boxes had unmodified U at their wobble positions (Table 1), suggesting that the family boxes in mitochondria are decoded by single tRNAs via the four-way wobble rule. For NNR codons, all six mt tRNAs had wobble modifications (Table 1): t\text{C} in mt tRNA\text{Met}, m\text{3}U in mt tRNAs for Leu(UUR) and Trp and m\text{3}s\text{2}U in mt tRNAs for Lys, Gln and Gln. We consider that these modifications are required for efficient recognition of the cognate codons, as well as to prevent misreading of near-cognate codons. Q was present at the wobble position in four mt tRNAs (Tyr, His, Asn and Asp) responsible for NAY codons (Table 1). Q34 is known to restrict the conformational flexibility of the anticodon loop by making hydrogen bonds between the side chain amine...
of Q-base and 2' OH of U33 (42). Q34 enables tRNA to decode NAU efficiently (43). Although the functional role of Q still remains obscure, it is associated with various physiological events in cytoplasmic tRNAs, including cell development and proliferation, neoplastic transformation and translational read-through or frameshift essential for retroviral production (44–46). These facts suggest that the presence of Q in the four mt tRNAs plays a modulatory role in deciphering NAY codons.

Other mt tRNAs responsible for NNY codons [Phe, Ile, Cys, Ser(AGY)] had unmodified G at the wobble position (Table 1). The results clearly reveal a general principle to decipher the minimal decoding system by the base modifications at the wobble positions.

Hypermodifications are frequently found at position 37 of tRNAs. These modifications play important roles in maintaining translational efficiency and integrity (47,48). Four species of base modifications were present at position 37 in 13 mt tRNAs (Figure 4 and Supplementary Figure S1). Among six mt tRNAs responsible for UNN codons, five had \(^t\)A or ms\(^2\)tA at position 37. t\(^6\)A37 was present in the mt tRNAs for Ile, Thr, Asn, Lys and Ser(AGY), whereas m\(^2\)G37 was present in the mt tRNAs for Leu(CUN), Pro and Gln. Last year, we discovered cyclic t\(^6\)A, which is formed by ATP-dependent dehydration of t\(^6\)A catalyzed by TcdA, as a bonafide modification at position 37 of tRNAs from bacteria, yeast, plants and protists (49). However, we did not detect any cyclic form of t\(^6\)A in bovine mt tRNAs, and there is no homolog of TcdA in mammalian genomes.

Loss-of-function mutations in tRNA-modifying enzymes can cause human diseases. Consistent with this idea, large-scale disease-associated exome analyses have identified a number of genes that encode tRNA-modifying enzymes (50,51). Identification of all genes involved in mt tRNA modifications will help us to identify genes and mutations associated with various diseases, especially those linked to mitochondrial dysfunction. Basically, the structure and sequence of human mt tRNAs are similar to those of bovine mt tRNAs. In fact, 81% of total bases in 22 mt tRNAs are conserved between these two mammals. That is why human mt tRNA-modifying enzymes can be predicted based on information of bovine mt tRNAs. So far, nine human genes have been confirmed to be responsible for base modifications in mammalian mt tRNAs, and others have been predicted based on studies of tRNA modifications in model organisms (Table 2). Several of these modifications, and the enzymes that may catalyze their formation, are described in the following paragraphs.

On the basis of studies of bacterial and yeast mitochondria (63,78), GTPBP3 and MTO1 are predicted to be enzymes involved in \(^m\(^3\)U\) formation; however, to date, no direct evidence in support of this prediction has been published. The GTPBP3–MTO1 complex has been proposed to recognize mt tRNAs for Leu(UUR), Trp, Lys, Glu and Gln.
### Table 2. List of confirmed and predicted genes responsible for post-transcriptional modifications in mammalian mt tRNAs

| Position<sup>a</sup> | tRNA species | Modification<sup>b</sup> | Confirmed gene(s) in human or mammals | Predicted gene(s) in human |
|----------------------|--------------|--------------------------|--------------------------------------|----------------------------|
| 6                    | Asp          | m<sup>2</sup>G           | **THUMPD3 or THUMPD2 (52,53)**       |                            |
| 9                    | Ala, Asp, Glu, Phe, Gly, His, Lys, Leu(UUR), Leu(CUN), Asn, Pro, Arg, Thr, Val, Trp, Cys, Ile, Leu(UUR), Gin, Tyr | m<sup>1</sup>A | **TRMT10C and SDR5C1 (54)** |                            |
| 10                   | Ala, Phe, Gly, His, Lys, Leu(UUR), Leu(CUN), Asn, Pro, Val, Trp, Tyr | m<sup>2</sup>G | **TRMT10C and SDR5C1 (54)** | **TRMT1I and TRMT1I2 (55)** |
| 20                   | D            |                          |                                      | **DUS2 (56)**             |
| 26                   | Ala, Glu, Leu(UUR) Ile | m<sup>2</sup>G |                          | **TRMT1**                 |
| 27<sup>a</sup>       | Ser(UCN)     | ψ                        |                                      | **PUS1 (25)**             |
| 28                   | Ala, Cys, Glu, Lys, Leu(UUR), Leu(CUN), Asn, Pro, Val, Met, Ser(UCN), Tyr | ψ | **PUS1 (25)** |                            |
| 29                   | Ser(UCN)     | ψ                        |                                      |                            |
| 31                   | Asp, Leu(CUN) | ψ                        |                                      |                            |
| 32                   | Cys, Val     | ψ                        |                                      |                            |
| 34                   | Ser(UCN), Thr Leu(UUR), Trp, Glu, Lys, Gin, Glu, Lys, Gin | τm<sup>3</sup>U | **MTU1<sup>c</sup> (63) and NFS1<sup>e</sup> (64)** |                            |
|                      | Met          | t<sup>6</sup>C          |                                      | Unidentified               |
|                      | Asp, His, Asn, Tyr | Q             |                                      | **hQTRT1 and hQTRTD1 (65,66)** |
|                      | Ile, Lys, Asn, Ser(AGY), Thr | t<sup>8</sup>A | **YRD<sub>C</sub> and QRI7 (OSGEPL1) (67)** |                            |
|                      | Cys, Phe, Ser(UCN), Trp, Tyr | i<sup>8</sup>A | **TRIT1 (68)** |                            |
|                      | Phe, Ser(UCN), Trp, Tyr Leu(UUR), Pro, Gin | ms<sup>1</sup><sup>b</sup>A<sup>f</sup> | CDK5RAP<sub>1</sub><sup>f</sup> (69) |                            |
| 39                   | Ala, Cys, Phe, Gly, His, Leu(UUR), Gin, Arg, Tyr, Glu, Gin | ψ | **PUS3 (71)** |                            |
| 40                   | Glu, Gin     | ψ                        |                                      |                            |
| 48                   | Leu(UUR), Asn, Trp | m<sup>2</sup>C |                                      | **NSUN2 (72,73)**         |
| 49                   | Glu, Ser(AGY) | m<sup>3</sup>C |                                      | **NSUN2 (72,73)**         |
| 50                   | Met          | ψ                        |                                      | **TRUB2 (74)**            |
| 55                   | Glu, Gin, Ser(UCN), Tyr | ψ |                                      | Unidentified               |
| 57                   | Ala          | ψ                        |                                      |                            |
| 58                   | Cys, Glu, Ile, Lys, Leu(UUR), Ser(UCN) | m<sup>1</sup>A | **TRMT61B (75)** |                            |
| 67                   | Thr          | ψ                        |                                      | **PUS1<sup>d</sup> (76)** |
| 72                   | Thr          | m<sup>2</sup>C          |                                      | Unidentified               |

<sup>a</sup>Numbering system for tRNA comes from the tRNAdb compilation (40).

<sup>b</sup>Symbols for modifications originate from MODOMICS (http://modomics.genesilico.pl/) (39).

<sup>c</sup>This position number is unique to mt tRNAs<sub>Ser(UCN)</sub> (77).

<sup>d</sup>These predictions were altered from our previous prediction (2).

<sup>e</sup>MTU1 and NFS1 are involved in 2-thiolation of τm<sup>3</sup>U34. MTU1 (Mitochondrial tRNA-specific 2-thiouridylase 1) is known as TRMU which originated from bacterial trmU (tRNA methyltransferase U). However, trmU (renamed as numA) was found to be a mis-annotation because it is not a tRNA methyltransferase. CDK5RAP1 is required for 2-methylthiolation of ms<sup>1</sup><sup>b</sup>A37.

<sup>f</sup>CDK5RAP<sub>1</sub> is required for 2-methylthiolation of ms<sup>1</sup><sup>b</sup>A37.
Figure 3. Revised information regarding post-transcriptional modifications of five bovine mt tRNAs. The updated modified bases are represented in bold type. Symbols for modified nucleoside are as follows: m$^2$G, N$^2$,N$^2$-dimethylguanosine; D, dihydrouridine; m$^5$U, 5-taurinomethyluridine; m$^5$s$^2$U, 5-taurinomethyl-2-thiouridine; t6A, N$^6$-threonylcarbamoyladenosine; m$^3$C, 3-methylcytidine.

However, these five mt tRNAs share little sequence similarity and no consensus motif. Nonetheless, m$^5$U formation is sensitive to a single pathogenic point mutation associated with MELAS or MERRF (12), implying strict substrate specificity of the enzyme. To resolve this issue, it will be necessary to perform in vitro reconstitution of m$^5$U on mt tRNAs using recombinant proteins.

We previously identified MTU1, a thiouridylase responsible for 2-thiolation of m$^5$s$^2$U in mt tRNAs (63). The sulfur atom comes from Cys, a process mediated by a cysteine desulfurase, NFS1 (64). During bacterial 2-thiouridine formation, several sulfur mediators transfer persulfide sulfur from cysteine desulfurase to thiouridylase (79). To date, however, there is no information available regarding the involvement of such sulfur mediators in the formation of 2-thiouridine in mitochondria.

In mammals, the Q-base is obtained either from the diet or through intestinal microflora (45), because no homologous genes responsible for Q-base biogenesis de novo are encoded in mammalian genome. Mammals possess two tRNA-guanine transglycosylases (TGTases), QTRT1 and QTRTD1, which may transglycosylate the wobble base with the dietary Q-base. According to its subcellular localization, QTRTD1 appears to be the mitochondrial TGTase (65,66). Further studies will be necessary to confirm the identity of the mitochondrial TGTase, as well as the transport pathway by which dietary Q enters the mitochondria. Q is not present in mt tRNA$^{Asp}$ from Morris hepatoma cells (80).

In yeast, t$^6$A37 is synthesized by two enzymes, Sua5p and Qri7p (67). YRDC and OSGEPL1, the human homologs of yeast Sua5p and Qri7p, are presumably involved in t$^6$A formation in mammalian mt tRNAs.

TRIT1 is a tumor suppressor gene, mutations in which are associated with cancer progression. Human TRIT1 was identified as a tRNA isopentenyltransferase (IPTase) homologous to bacterial MiaA and yeast Mod5; the latter is responsible for i$^6$A37 formation in both cytoplasmic and mt tRNAs. Knockdown of TRIT1 in human cells reduced the abundance of i$^6$A in mt tRNA$^{Ser(UCN)}$ (68). In the present study, we determined that bovine mt tRNAs for Tyr and Cys have i$^6$A37; in total, five mt tRNAs have been identified as potential substrates for TRIT1 in mitochondria.

The human protein CDK5RAP1 is a 2-methylthiolase that is homologous to bacterial MiaB. A CDK5RAP1 variant localizes in mitochondria, and knockdown of CDK5RAP1 results in a reduction of ms$^2$i$^6$A levels (69). Thus, four mt tRNAs [Phe, Ser(UCN), Tyr and Trp] are likely to be modified by CDK5RAP1.

TRMT5, a methyltransferase responsible for m$^1$G37 formation, is predicted to be localized to the mitochondria (70,81), and recombinant TRMT5 can modify in vitro transcribed mt tRNA (70), suggesting that TRMT5 methylates three mt tRNAs: Leu(CUN), Pro and Gln.

Base methylation at position 9 is frequently observed among bovine mt tRNAs. Indeed, 19 mt tRNAs have m$^1$A9 or m$^1$G9 (Figure 4). m$^1$A9 stabilizes the canonical clover-
According to the studies of yeast and Escherichia coli tRNAs, Ψ39 and Ψ40 are likely to be introduced by PUS3 (71), whereas Ψ55 is probably modified by TRUB2 (74). In yeast mitochondria, Ψ31 and Ψ32 are introduced by PUS6 and PUS9, respectively (85,86). Although we previously predicted that either RPUSD2 or RPUSD4 was responsible for Ψ31 and Ψ32 (2), two other homologs, RPUSD1 and RPUSD3, should also be regarded as candidates for these modifications. Regarding Ψ50 and Ψ57, no candidate enzymes can be predicted at present. Biochemical and genetic analyses of Ψ synthase genes will be necessary to determine the enzyme responsible for each Ψ site.

In contrast to cytoplasmic tRNAs, most mammalian mt tRNAs do not have consensus sequences of D- and T-loops, which are conserved among tRNAs in general; consequently, they have lost the canonical D-loop/T-loop interaction. This feature is characteristic of mt tRNAs in mammals (2). In the D-loop, dihydrouridines (D) at positions 16 and 17, which are frequently observed in canonical tRNAs from various sources, are not present in bovine mt tRNAs. Instead, D20 was present in three species of bovine mt tRNAs. Based on a study on yeast tRNA, it is reasonable to predict that human tRNA-dihydrouridine synthase 2 (DUS2) introduces D20 (56). Human DUS2 has been implicated in pulmonary carcinogenesis (87). In the T-loop, Ψ55 and m1A58, both of which are typical modifications in canonical tRNAs, are present in four and six mt tRNAs, respectively. We previously identified human TRMT61B as the methyltransferase responsible for m1A58 in mt tRNAs for Leu(UUR), Ser(UCN) and Lys (75). In addition to these three tRNAs, we also found m1A58 in mt tRNAs for Cys, Glu and Ile. 5-methyluridine (m5U) at position 54 is one of the most common T-loop modifications in canonical tRNAs. m5U54 is present in the human mt tRNAs for Leu(UUR) (19) and Ser(UCN) (88); however, we did not detect m5U54 in any bovine mt tRNAs.

m2G6 is present in bovine mt tRNAAsp. Human mt tRNAAsp does not have m2G6 (89), because this position is replaced by A6. The methyltransferase responsible for m2G6 was identified as Trm14 in Methanocaldococcus jannaschii (52) and TTH1157 in Thermus thermophilus (53). A similarity search using the Trm14 sequence retrieved two human homologs, THUMPD2 and THUMPD3. According to the Wolf-P-sort (90) prediction of subcellular localization, the former is likely localized in the cytoplasm and the latter in mitochondria. Although further investigation is necessary, THUMPD3 is the most plausible candidate for the enzyme that introduces m2G6 in mt tRNAAsp, whereas THUMPD2 probably acts on cytoplasmic tRNAs. Although both genes are encoded in the human genome, no m2G6 is present in human mt tRNAAsp, implying the presence of other substrates for this enzyme in human mitochondria.

In this study, we identified G1 at the 5’ terminus of bovine mt tRNAHis. In general, G1 is an identity element for aminoclylation by histidyl-tRNA synthetase (91). This is the first reported instance of this base in mammalian mitochondria, although G1 is present in both yeast and starfish mt tRNAHis (92,93). Judging from the fact that the 5’-adjacent nucleotide of the mt tRNAHis gene in bovine mt DNA is T rather than G (e.g. GenBank V00654), it is
likely that G₁₃ is added enzymatically after 5′-terminal processing by mt RNase P. THG1L is a mammalian guanylyltransferase that adds G₁₃ at the 5′ terminus of cytoplasmic tRNA\(^{\text{His}}\). Because THG1L is predicted to have a mitochondria-targeting sequence at the N-terminus (94), THG1L is likely to be responsible for G₁₃ addition of mt tRNA\(^{\text{His}}\).

In summary, we have compiled the first complete picture of post-transcriptional modifications in mammalian mt tRNAs. The results of this study enable a deeper understanding of the molecular mechanisms underpinning the minimal decoding system in mammalian mitochondria, and should help predict the human tRNA-modifying enzymes responsible for each modification in mt tRNAs. The list of tRNA-modifying enzymes serves as a practical landmark that encourages us to identify all genes responsible for tRNA modifications in mammalian mitochondria, and to further investigate human diseases caused by tRNA modification disorders in mitochondria. An important goal for future efforts is the identification of all modifications in human mt tRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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