Decoding Mechanism of Non-universal Genetic Codes in Loligo bleekeri Mitochondria

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Background: Non-universal genetic codes are frequently found in animal mitochondrial decoding systems. In squid mitochondria, four codons deviate from the universal genetic code, namely AUA, UGA, and AGA/AGG (AGR) for Met, Trp, and Ser, respectively. To understand the molecular basis for establishing the non-universal genetic code, we isolated and analyzed five mitochondrial tRNAs from a squid, Loligo bleekeri. Primary structures of the isolated tRNAs, including their post-transcriptional modifications, were analyzed by mass spectrometry. tRNAMet(AUR) possessed an unmodified cytidine at the first position of the anticodon, suggesting that the AUA codon is deciphered by CAU anticodon via non-canonical A-C pairing. We identified 5-taurinomethyluridine (m5U) at the first position of the anticodon in tRNA^Trp(UGR). m5U enables tRNA^Trp to decipher UGR codons as Trp. In addition, 5-taurinomethyl-2-thiouridine (m5s2U) was found in mitochondrial tRNAs for Leu(UUR) and Lys in L. bleekeri. This is the first discovery of m5U and m5s2U in molluscan mitochondrial tRNAs. Variation in the genetic code is a characteristic feature of mitochondrial decoding systems. In animal mitochondria, six codons deviate from the universal genetic code (1). The AUA codon (for Ile) specifies Met in most metazoan mitochondria. The AAA codon (for Lys) changes to assign Asn in echinoderm and some platyhelmint mitochondria. The AGA and AGG (AGR) codons (for Arg) are used for Ser in most invertebrate mitochondria, for Gly in tunicate mitochondria, and as a stop codon in vertebrate mitochondria. The UGA stop codon is used for Trp in all animal mitochondria, and the UAA stop codon is used for Tyr in nematode mitochondria.

The abbreviations used are: mt, mitochondrial; cmnm5U, 5-carboxymethylaminomethyluridine; CID, collision-induced dissociation; f5C, 5-formylcytidine; m7G, 7-methylguanosine; m2G, 2-dimethylguanosine; RCC, reciprocal circulating chromatography; t6A, 6-threonylcarbamoyladenosine; m3U, 5-taurinomethyluridine; m3s2U, 5-taurinomethyl-2-thiouridine; tRNA^AUR, tRNA responsible for AUR codons; R, A or G; Y, U or C; N, A, G, U or C; U+me, monomethylated uridine. The significance is that the AUA codon is deciphered by unmodified CAU anticodon through non-canonical A-C pairing.

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TABLE 1
Codon table of the squid mitochondrial genetic code

| Codon | No. of universal codons | Codon | No. of universal codons | Codon | No. of universal codons | Codon | No. of universal codons |
|-------|------------------------|-------|------------------------|-------|------------------------|-------|------------------------|
| UUU   | Phe (GAA)              | UUC   | Ser (TGA)              | UAU   | Tyr (GUA)              | UGU   | Gys (GCA)             |
| UUC   | UCC (TGA)              | Leu   | UCA (UGC)             | UAA   | UAG (stop)            | UGA   | Trp (TCG)             |
| UGA   | UGG (TAG)              | Leu   | CCA (TGC)             | CAA   | His (GGG)            | CGU   | Arg (UGC)            |
| GCA   | GCG (TGG)              | CUA   | TAG (CCG)             | CAG   | Gln (CGG)             | CGA   | Arg (UGC)            |
| AUG   | Ile (AUG)              | Acu   | ACC (TGG)             | AUA   | Asn (AAU)            | AGU   | Ser (GCT)            |
| AUA   | Met (CAT)              | ACA   | ATG (TGG)             | AAG   | Lys (TTG)             | AGG   | Gly (GGG)            |
| AUA   | Met (CAT)              | ACG   | GCT (TGG)             | AAA   | GGC (CTG)             | GUG   | Arg (UGC)            |
| GUG   | Val (TAC)              | GCA   | GTA (TGT)             | GAU   | Asp (GCA)             | GGU   | Ser (GGC)            |
| GUA   | GCG (TGG)              | Ala   | GCT (TGG)             | GAC   | Glu (GGU)             | GAG   | Gly (GGG)            |
| GUG   | GCG (TGG)              | Ala   | GCT (TGG)             | GAG   | Gln (GGG)             | GAG   | Gly (GGG)            |

EXPERIMENTAL PROCEDURES

Isolation of mt tRNAs—The procedure for isolation of mt tRNAs from edible muscle of L. bleekeri was largely the same as that used for ascidian mt tRNAs reported previously (10). After separation by DEAE-cellulose column chromatography, 2400 A260 units of the crude tRNA fraction were isolated from 280 g of squid muscle. Total tRNA (700 A260 units) was obtained from 1200 A260 units of the crude tRNA fraction by removing contaminating polysaccharides with TRizol-LS (Invitrogen) followed by rinsing with chloroform. For the isolation of individual mt tRNAs, the following 5’-EC amino-modified DNA probes (Sigma-Aldrich) were designed using Raccess software (17) and used for reciprocal circulating chromatography (RCC) (15): 5’-GGGGATATGAAAAACACGTTATTTTTTAGCTAC-3’ for tRNAMet(AUR), 5’-TTGAAAGCCCTCAGTTAATCTTTATT-3’ for tRNAAla(CUG), 5’-AAAAGTTATATGATGCATGCTAATCTT-3’ for tRNAThr(GCU), 5’-AAACCTAACTGCAAATCTCTTATT-3’ for tRNAVal(UCA), and 5’-CTTGTGCCTACCTCATTGCCCATGCTT-3’ for tRNAAsp(AAS). The tDNA sequences were obtained from the NCBI database of L. bleekeri mtDNA (accession number NC_002507) (18, 19). The DNA probes were covalently immobilized on NHS-activated Sepharose 4 Fast Flow (GE Healthcare) and packed into respective tip columns for the RCC instrument. The five tRNA species were simultaneously isolated from 700 A260 units of total tRNA by RCC as described (15).

Mass Spectrometric Analysis of mt tRNAs—Each purified tRNA was digested with RNase T1, and then analyzed by capillary liquid chromatography (LC)/nanoelectrospray ionization mass spectrometry (MS) as described (16). A linear ion trap-orbitrap hybrid mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific) equipped with a custom-made nanospray ion source and a stainless steel HPLC system (DiNa; Kaya Technologies) was employed in this study. Modified bases were assigned by comparing observed m/z values of RNase T1-digested fragments to the calculated values of these fragments. The bases were allocated on the basis of the sequences of the fragments, which were inferred from the collision-induced dissociation (CID) spectra and assisted by referring to evolutionary conservation of each modified base in tRNAs. N2-methylguanosine (m2G) can be distinguished from 1-methylguanosine (m1G) by examining the 3’-terminal structure generated by RNase T1, and 7-methylguanosine (m7G) at the wobble position of the mt tRNA isolated from L. bleekeri liver (12), indicating that all four AGN codons are deciphered by the f5C modification at the wobble position of squid mt tRNAMet (14), implying that the AUA codon is deciphered by the f5CAU anticodon, as observed in other cases of the AUA codon, our group used a postlabeling method to identify a partial f5C modification at the wobble position of the mt tRNA encoding the AUA codon in animal mitochondria. Moreover, we discuss our results in the wider context of the evolutionary reorganization of decoding systems in animal mitochondria.
**RESULTS**

Five species of squid mt tRNAs were isolated from 700 A260 units of total tRNA by RCC, namely, tRNA^{Met}(AUR), tRNA^{Trp}(UGR), tRNA^{Ser}(AGN), tRNA^{Leu}(UUR), and tRNA^{Lys}(AAR). Each individual tRNA was faintly detectable on the polyacrylamide gel of the total eluted fraction from the RCC column (Fig. 1). Each band was excised, and tRNA was eluted from the gel pieces for mass spectrometric analysis. Although the final yield of each tRNA could not be precisely measured, we estimated the yield to be within the range of 10–300 fmol.

Isolated tRNAs were digested by RNase T1 and subjected to capillary LC/nanoelectrospray ionization MS to analyze the chemical structures of the modifications. RNA fragments from each tRNA were efficiently separated by capillary LC on the basis of their lengths, base compositions, and sequences (Fig. 2A). Singly and multiply charged anions of RNA fragments were detected with high mass resolution (30,000 units). Modified bases in the tRNA sequence (Fig. 2). The modifications within the tRNA sequence (Fig. 2A and supplemental Table 1). Moreover, we detected monomethylated uridines (U+me) with unidentified structures at positions 12 and 13 (Fig. 2A and supplemental Table 1).

*L. bleekeri* mt tRNA^{Ser}(AGN) contains m7G at the wobble position and t6A at position 37 (12). The presence of both modifications was confirmed using MS analysis (Figs. 2A and 3A, and supplemental Table 1). m7G is distinguishable from the other methylguanosines because RNase T1 does not cleave the 3’ end of m7G. In addition, because the N-glycosyl bond of m7G is unstable, the m7G base dissociates easily from the RNA fragment upon CID, generating product ions lacking m7G base (Fig. 2B). The mass chromatogram showed that the wobble base was completely modified to m7G (Fig. 3A).

Having detected m7G in mt tRNA^{Trp}(UGR), we further analyzed mt tRNA^{Leu}(UUR) and mt tRNA^{Lys}(AAR) to identify additional instances of m5U and its derivative in squid mt tRNAs. As expected, the wobble bases of both tRNAs were modified to m5U at position 48 (Fig. 2B). The mass chromatograms of the anticond-containing fragments with different modification statuses (Fig. 3B) showed that 88% of mt tRNA^{Leu}(UUR) and 90% of mt tRNA^{Lys}(AAR) contained m5U, whereas only 7% of both tRNAs contained m7U. The remaining molecules had a 2-thiouridine or an unmodified U at the wobble position. Other modifications were found in mt tRNA^{Leu}(UUR) at positions 6 and 10 (m2G) and at positions 9 and 37 (m1G) (Fig. 3B) and in mt tRNA^{Lys}(AAR) at position 9 (m2G), position 10 (m3G), and position 37 (t6A) (Fig. 3B). Monomethylated uridines (U+me) with unidentified structures were also found at positions 16, 48, and 52 (Fig. 3B and supplemental Table 1).

**DISCUSSION**

We have shown here that *L. bleekeri* mt tRNA^{Met}(AUR) possesses an unmodified C at the wobble position (Fig. 3A). Our group previously reported that the wobble position was par-
Squid Mitochondrial tRNAs

A

\begin{align*}
\text{tRNA}^{\text{Met}(AUR)} & \\
\text{BPC} & \quad \text{Relative abundance (§)} \\
\text{m/z} 1392.69 & \quad \psi\text{UAU}^\delta\text{ACC}C\text{AAA}\text{Am}^\delta\text{UGp} \\
\text{tRNA}^{\text{Trp}(UGR)} & \\
\text{BPC} & \quad \text{Relative abundance (§)} \\
\text{m/z} 1397.19 & \quad \text{CUU}^\text{m}^\delta\text{UCAm}^\delta\text{Am}^\gamma\text{G}=\text{p} \\
\text{tRNA}^{\text{Ser}(AGN)} & \\
\text{BPC} & \quad \text{Relative abundance (§)} \\
\text{m/z} 1840.22 & \quad \text{CUm}^\gamma\text{GCU}^\delta\text{AAC}\psi\text{UUAUUUGp} \\
\text{tRNA}^{\text{Leu}(UUR)} & \\
\text{BPC} & \quad \text{Relative abundance (§)} \\
\text{m/z} 1372.16 & \quad \text{AAU}\text{U}^\text{m}^\delta\text{m}^\delta\text{UAAm}^\gamma\text{G}=\text{p} \\
\text{tRNA}^{\text{Lys}(AAR)} & \\
\text{BPC} & \quad \text{Relative abundance (§)} \\
\text{m/z} 1473.50 & \quad \text{ACU}^\text{m}^\delta\text{m}^\delta\text{UUU}^\delta\text{AUCUAGp} \\
\end{align*}

B

\begin{align*}
\psi\text{UCA}U^\delta\text{ACC}C\text{AAA}\text{Am}^\delta\text{UGp} & \\
\text{CUU}^\text{m}^\delta\text{UCAm}^\delta\text{Am}^\gamma\text{G}=\text{p} & \\
\text{CUm}^\gamma\text{GCU}^\delta\text{AAC}\psi\text{UUAUUUGp} & \\
\text{AAU}\text{U}^\text{m}^\delta\text{m}^\delta\text{UAAm}^\gamma\text{G}=\text{p} & \\
\text{ACU}^\text{m}^\delta\text{m}^\delta\text{UUU}^\delta\text{AUCUAGp} & \\
\end{align*}
tially modified to f5C in the same tRNA (14). In the study, a weak f5C spot along with a strong C spot was detected on the two-dimensional TLC by the postlabeling method, suggesting a false positive. Otherwise, we might use a different strain of L. bleekeri for this study. There might be some individual differences of squid variably expressing a putative tRNA-modifying enzyme.

**FIGURE 2.** Mass spectrometric analysis of L. bleekeri mt tRNAs. A, base peak chromatograms (BPC) of RNase T1-digested fragments (upper panels) and mass chromatograms of the anticodon-containing fragments (lower panels) for tRNA^{Met}(AUR), tRNA^{Trp}(UGR), tRNA^{Ser}(AGN), tRNA^{Leu}(UUR), and tRNA^{Lys}(AAR). The numbering system of tRNAs is based on the tRNA database (tRNA db 2009) (36). Pseudouridines were determined previously (12, 14). Frequency of modifications at the wobble position and position 37 is indicated in parentheses. B, CID spectra of the anticodon-containing fragments from L. bleekeri mt tRNAs. The precursor ions for CID were m/z 1392.69, 1397.17, 1380.41, 1372.16, and 1473.50, respectively. Product ions of the c- and y-series are indicated on the spectra, and the corresponding sequences are indicated. In tRNA^{Ser}(AGN), a3-B, c3-B, and c4-B represent product ions lacking m7G base.
enormous for F^C formation. Judging from the sensitivity of our MS analysis, if there is only 1% of F^C in the anticodon-containing fragment, we can surely detect it. Thus, if a partial F^C modification does exist, its frequency must be <1%. In any case, we demonstrated here that a large majority of mt tRNAMet(AUR) is occupied by unmodified C at the wobble position, indicating that AUA codon is deciphered by CAU anticodon via non-canonical A-C pairing in L. bleekeri mitochondria. This may be a remnant of a primitive decoding system 0 that has been used early in evolution, because the domain-specific wobble modifications might have been acquired late during evolution, as suggested by the phylogenetic study on tRNA-modifying enzymes (22).

Some instances of the AUA decoding by a CAU anticodon have been reported in other mitochondrial systems. In D. melanogaster (6), there are two species of mt tRNAsMet; one has a CAU anticodon with t^A at position 37, the other has an F^CAU anticodon with an unmodified A at position 37. This indicates that the AUA codon can be read by a CAU anticodon with the assistance of t^A37 because t^A37 stabilizes tRNA binding to the A site codon (23, 24), or by F^CAU without the assistance of t^A37. In this study, we detected t^A37 in mt tRNAMet(AUR) (Fig. 3A). The frequency of the t^A modification in mt tRNAMet(AUR) was estimated to be 93% according to the MS data (Fig. 3A). The high modification efficiency indicates the importance of t^A37 for the decoding of AUA as Met in L. bleekeri mitochondria. In addition to the anticodon and 3'-adjacent modifications, decoding efficiency can be affected by other regions in tRNAs (25, 26). In the case of Hirsh suppressor tRNA^Trp with G24A and A9C mutations, UGA codon is efficiently deciphered by the CCA anticodon, in which C34 of anticodon pairs with A3 of codon by a non-standard geometry with a single hydrogen bond between N4 imino group of C34 and N1 of A3 (26). These suppressor mutations are considered to enable C34-A3 pairing by facilitating the distortion of tRNA body at A/T state during decoding (26). Thus, there might be some elements in mt tRNAMet(AUR) that enable CAU anticodon to recognize AUA codon efficiently. In Saccharomyces cerevisiae (27, 28) and mosquito (Aedes albopictus) (29), mt tRNAMet are known to have an unmodified CAU anticodon. In S. cerevisiae, the initiator and elongator mt tRNAMet have CAU anticodons with m^1G37 and t^A37, respectively (27, 28). Collectively, the AUA decoding by a CAU anticodon with the assistance of the modified bases at position 37 might be a general rule.

In animal mitochondria, three strategies are used to decipher AUA codon as Met. The first strategy involves the F^CAU anticodon in mt tRNA^Met from mammals, nematode, and Drosophila. Indeed, AUA decoding by F^CAU anticodon has been confirmed biochemically (30, 31). The second strategy involves the m^3UAAU anticodon in ascidian (Halocynthia roretzi) mitochondria. In general, the m^3U modification prevents mis-reading of near cognate codons ending in pyrimidines (NNY) (9). m^3U would enable tRNA to efficiently decode AUR codons as Met. The third strategy involves the unmodified CAU anticodon described in this study. Non-canonical A-C pairing might be involved in AUA decoding by the CAU anticodon, in which t^A37 would stabilize the codon-anticodon pairing. Biochemical and structural studies will be required to provide mechanistic insights into this type of decoding. The distribution of different types of AUA decoding in animal mitochondria was assumed to be associated with AUR codon usage. In both human and L. bleekeri mitochondria, AUA codons are used about four times more frequently than AUG codons, indicating that AUA is an abundant codon, whose usage is independent of the type of decoding. Therefore, we were unable to draw any meaningful correlation between AUR codon usage and the types of AUA decoding. The three types of AUA decoding mentioned above rely heavily on the specificities of tRNA-modifying enzymes. In the first type, mt tRNAMet, containing a CAU anticodon, may be recognized by a putative F^C-modifying enzyme that has not been identified yet. In the second type, a m^3U-modifying enzyme, probably GTPBP3/MT01 (4), recognizes mt tRNA^Met with a UAU anticodon. In the third type, although there is no enzyme for F^C formation, t^A-modifying enzymes (4) recognize mt tRNA^Met with a CAU anticodon and introduce t^A at position 37. Further studies directed toward determining the evolutionary distribution of these three types of AUA decoding and characterizing tRNA-modifying enzymes will deepen our understanding of AUA decoding in animal mitochondria.

In L. bleekeri, we identified m^3U in mt tRNA^Trp(UGR), and m^3s^U in mt tRNA^Lys(UUR) and mt tRNA^Lys(AAR). This is the first reported instance of m^3U and m^3s^U in molluscan mt tRNAs. m^3(s)^U was first identified in mammalian mt tRNAs responsible for Leu(UUR), Trp, Lys, Glu, and Gln (4, 7). Moreover, we also found m^3(s)^U in fish, suggesting that m^3(s)^U may be conserved in vertebrate mt tRNAs. In addition, m^3(s)^U was found in three mt tRNAs responsible for non-universal genetic codes in ascidian (H. roretzi) mitochondria (10). On the other hand, m^3(s)^U was not found in yeast or nematode mt tRNAs, which use 5-carboxymethylaminomethy- luridine (cmnm^3U) instead (32–34). Due to the chemical similarity between cmnm^3U and m^3U, both nucleotides are thought to be synthesized by a similar biosynthetic pathway, which, in the case of m^3U, results in the replacement of glycine with taurine (7, 33). To identify the phylogenetic junction at which m^3U replaced cmnm^3U in animal phyla, it will be necessary to analyze additional invertebrate mt tRNAs. Specifically, m^3U seems to have emerged in cephalopoda mt tRNAs.

It is intriguing to note that m^3s^U is present in L. bleekeri mt tRNA Leu(UUR). In general, 2-thiouridine derivatives occur in three tRNA species for Lys, Glu, and Gln. In mammalian mitochondria, m^3s^U is found in mt tRNAs for Lys, Glu, and Gln, but not in mt tRNAs for Leu(UUR) or Trp (4). This is because MnmA, a 2-thiouridylase, strictly discriminates the anticodons of tRNAs (35). In human mitochondria, MTU1, a homolog of MnmA, recognizes mt tRNAs for Lys, Glu, and Gln (33). A MTU1 homolog in L. bleekeri may have evolved to recognize mt tRNA Leu(UUR).

Another intriguing finding is that m^3U is present at position 48 in the extra loop of squid mt tRNA^Met(AUR), and probably in mt tRNA^Lys(AAR) as well. In many tRNAs, m^3U can be found at position 54 in the T-loop, and m^3U54-methyltransferase is
widely distributed across domains of life. Although various modifications occur in the extra loop of many tRNAs, this is the first instance of tRNAs containing m^5U48. We speculate that a specific methyltransferase for m^5U48 exists in L. bleekeri and that m^5U48 is widely distributed in cephalopod and molluscan mt tRNAs. m^5C is often observed at positions 48 and 49 in the extra loop of tRNAs (36), and m^5C49 contributes to the structural stabilization of tRNA (37, 38). We speculate that m^5U48 might have a similar function for the stabilization of tRNAs. In addition, m^1G38 in squid mt tRNA^{Trp(UGR)} is a unique modification that has never been detected in any other tRNAs to our knowledge. m^1G38 is widely distributed in cephalopod and molluscan mt tRNAs. m^7GCU anticodon of mt tRNAs. m^5C is often observed at positions 48 and 49 in the extra loop of tRNAs (36), and m^5C49 contributes to the structural stabilization of tRNA (37, 38). We speculate that m^5U48 might have a similar function for the stabilization of tRNAs. In addition, m^1G38 in squid mt tRNA^{Trp(UGR)} is a unique modification that has never been detected in any other tRNAs.

FIGURE 4. Decoding system of the non-universal codons in L. bleekeri mitochondria. Non-universal AUA, UGA, and AGR codons are decoded by mt tRNAs with anticodons CAU, m^5UCA, and m^6GCU, respectively. Non-universal codons are boxed. aa represents amino acid.

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MARCH 15, 2013 • VOLUME 288 • NUMBER 11 JOURNAL OF BIOLOGICAL CHEMISTRY 7651
Squid Mitochondrial tRNAs

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