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*Review Article*

# tRNA Modification and Genetic Code Variations in Animal Mitochondria

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In animal mitochondria, six codons have been known as nonuniversal genetic codes, which vary in the course of animal evolution. They are UGA (termination codon in the universal genetic code changes to Trp codon in all animal mitochondria), AUA (Ile to Met in most metazoan mitochondria), AAA (Lys to Asn in echinoderm and some platyhelminth mitochondria), AGA/AGG (Arg to Ser in most invertebrate, Arg to Gly in tunicate, and Arg to termination in vertebrate mitochondria), and UAA (termination to Tyr in a planaria and a nematode mitochondria, but conclusive evidence is lacking in this case). We have elucidated that the anticodons of tRNAs deciphering these nonuniversal codons ($tRNA^{Trp}$ for UGA, $tRNA^{Met}$ for AUA, $tRNA^{Asn}$ for AAA, and $tRNA^{Ser}$ and $tRNA^{Gly}$ for AGA/AGG) are all modified; $tRNA^{Trp}$ has 5-carboxymethylaminomethyluridine or 5-taurinomethyluridine, $tRNA^{Met}$ has 5-formylcytidine or 5-taurinomethyluridine, $tRNA^{Ser}$ has 7-methylguanosine and $tRNA^{Gly}$ has 5-taurinomethyluridine in their anticodon wobble position, and $tRNA^{Asn}$ has pseudouridine in the anticodon second position. This review aims to clarify the structural relationship between these nonuniversal codons and the corresponding tRNA anticodons including modified nucleosides and to speculate on the possible mechanisms for explaining the evolutional changes of these nonuniversal codons in the course of animal evolution.

## 1. Introduction

Up to now six codons have been known which are deciphered by the corresponding tRNAs as amino acids different from those assigned by the universal genetic code in animal mitochondria (Figure 1) [1]. UGA termination codon in the universal genetic code is deciphered to Trp in all animal mitochondria, AUA Ile to Met in most metazoan except echinoderm, planarian, cnidarian, placozoan and poriferan mitochondria, AAA Lys to Asn in echinoderm and some platyhelminth mitochondria, and AGA/AGG Arg to Ser in most invertebrate mitochondria, Gly in tunicate (urochordata) mitochondria, and termination codon in vertebrate mitochondria. UAA termination codon was assumed to be a Tyr codon in a planaria [2] and a nematode mitochondria [3], but there is neither structural information on mt $tRNA^{Tyr}$ that decodes the UAA codon, nor information about the mitochondrial (mt) release factor relevant to this phenomenon. Thus, this issue is no more discussed here.

The codon-amino acid correspondence was first deduced by comparison of mt DNA sequence containing the codon with amino acid sequence of the corresponding protein [4]. Since mt proteins exist in a small number (in most cases, 13) and which are encoded by a small sized mt DNA (16,500 bp in the case of human mitochondria) [5], the correspondence can be unambiguously accomplished. In the next step, in order to analyze the molecular mechanism of the codon change, the corresponding tRNA sequence was analyzed especially focused on the anticodon sequence [6]. It is an advantageous point for analysis of mt tRNAs that in metazoan mitochondria, tRNA genes are restricted to 22~24 species on the mt genomes, and that tRNAs are not imported from the cytoplasm in almost all metazoan mitochondria [7] except for a few cases such as in Cnidaria. As the results,

several modified nucleosides such as 5-carboxymethylaminomethyl(2-thio)uridine($cmnm^5(s^2)U$), 5-taurinomethyl(2-thio)uridine ($\tau m^5(s^2)U$), 5-formylcytidine($f^5C$), 7-methylguanosine($m^7G$) in the anticodon first position, and pseudouridine ($\Psi$) in the anticodon second position were found to be involved in the genetic code variations (Figure 2), in which $\tau m^5(s^2)U$ [8] and $f^5C$ [9] are novel modified nucleosides found by our group. Thus, an expanded wobble rule was established in which $cmnm^5(s^2)U$, $\tau m^5U$, and $f^5C$ at the anticodon first position pair with A or G in the codon third position, and $m^7G$ pairs with all nucleotides in the codon third position [10, 11] (Figure 3). It was speculated that unmodified G in the anticodon first position should pair with C, U, and A in the codon third position in the case of fruit fly, *Drosophila melanogaster* $tRNA^{Ser}_{GCU}$ for decoding AGU/AGC/AGA codons [11], and $\Psi$ in the anticodon second position strengthens the pairing interaction with A in the codon second position in the case of echinoderm $tRNA^{Asn}_{G\Psi U}$ for decoding AAA codon [12]. In the third step, the wobble pairings as inferred from the above-expanded wobble rule were confirmed by an *in vitro* translation system of animal mitochondria, in which *in vitro* translation was performed by *E. coli* or bovine mt translation system using synthetic polyribonucleotide made of a series of nonuniversal codon as a messenger, and incorporation of a certain amino acid corresponding to the nonuniversal codon was identified [13, 14]. In the forth step, the wobble pairings were confirmed by an *in vitro* experiment, in which natural mRNA including a specific codon which was replaced with a certain nonuniversal codon was translated *in vitro,* and the mRNA activity was detected by enzymatic activity if the mRNA encodes a certain enzyme such as dihydrofolate reductase (DHFR) (Hanada, T., Suzuki, T. and Watanabe, K., unpublished results).

In this review, we summarize mostly the results obtained in the second step, together with a few cases in the third step, and inquire into the nature of codon-anticodon interaction in the mt translation process and the relationship between nonuniversal genetic code and modified nucleoside in the tRNA anticodon deciphering the codon, during the course of animal evolution. These studies may lead to the understanding as to how the genetic code evolves and how mt tRNAs keep up with the genetic code variations by providing modification at the anticodon of tRNAs.

It is also important to consider the involvement of aminoacyl-tRNA synthetase (aaRS) in the recognition of tRNA, especially in the case when aaRS recognizes the anticodon region of tRNA as the identity determinant. Since a few cases have been known about the animal mt aaRSs, some discussions will be added in the applicable sections, about the recognition mechanisms of aaRSs toward the corresponding tRNAs involved in the genetic code variations.

## 2. Genetic Code Variations and the Anticodon Structure of the Corresponding tRNA

*2.1. $cmnm^5(s^2)U$ for Decoding UGA/UGG Codons.* In mitochondria of all animal phyla, UGA is read as Trp instead of termination codon in the universal genetic code [4, 6]. In the protostome (nematode and probably platyhelminth) mitochondria, the anticodon first position (wobble position) is modified to 5-carboxymethylaminomethyluridine ($cmnm^5U$) or 5-carboxymethylaminomethyl-2-thiouridine ($cmnm^5s^2U$) [15, 16]. Therefore, the modification of U to $cmnm^5(s^2)U$ in the anticodon first position in $tRNA^{Trp}_{UCA}$ may restrict the base pairing only with purine nucleosides (A/G, symbolized by R) at the codon third position (Figures 3(a) and 3(b)), which would overcome the competition with mitochondrial release factor. Thus, UGA codon is read as Trp. It is known that unmodified uridine (U) at the wobble position recognizes all four nucleosides (A/G/U/C, symbolized by N) at the codon third position [17]; thus, in mitochondria all four codon boxes are mostly read by the respective single tRNAs with unmodified U at the anticodon wobble position (see Figure 1).

$cmnm^5U$ was first found in yeast mitochondria [18]. This modified uridine is similar in the chemical structure with $cmnm^5Um$ (5-carboxymethylaminomethyl-O-2′-methyluridine) in *Escherichia coli* $tRNA^{Leu}_4$ [19] and $mnm^5U$ in *E. coli* $tRNA^{Arg}$ and is considered to fix its conformation by interresidual hydrogen bonding. The $mnm^5U$ possesses the same side chain at position 5 of uracil base as $mnm^5s^2U$ in $tRNA^{Glu}$, which enables to take "rigid" conformation for the construction of the C3′-endo form.

In the *in vitro* translation system using MS2 RNA as a messenger, $tRNA^{Leu}_4$ having $cmnm^5Um$ and $tRNA^{Leu}_5$ having 2′-O-methylcytidine (Cm) at the anticodon first position, both recognized UUA and UUG codons, but not UUU and UUC codons at all [20]. It was clarified by NMR analysis that the orthodox C3′-endo-$G^-$ form of both $cmnm^5Um$ and Cm are very stable. It is considered that the posttranscriptional modification to form $cmnm^5Um$ and Cm fixes their conformations very rigid, which regulates not to recognize the UUU/UUC codons [21].

In summary, in the tRNAs recognizing A- or G-ending codons by dividing 2:2 in the codon box, U and C (in the case of $f^5C$, see Section 2.3) at the anticodon first position are modified by introducing side chains to position 5 of the nucleobase, 2-thiolation at position 2 of the nucleobase, or methylation at position 2 of the ribose moiety. By combining these modifications, the conformation of the nucleoside becomes more rigid and which guarantees the precise recognition toward NNA/NNG codons (Figures 3(a) and 3(b)) [22].

*2.2. $\tau m^5U$ for Decoding UGA/UGG and AGA/AGG Codons.* In the tunicate (ascidian) and vertebrate mitochondria, the anticodon first letter of $tRNA^{Trp}$ is modified to 5-taurinomethyuridine ($\tau m^5U$) ([8, 23]). The same position of ascidian *Halocynthia roretzi* mt $tRNA^{Gly}$ is also occupied by $\tau m^5U$ [23]. That $\tau m^5U$ recognizes only A- and G-ending codons (Figures 3(a) and 3(b)) were verified in two ways. Yasukawa et al. examined the translation activity of human $tRNA^{Leu}_{UAA}$ possessing $\tau m^5U$ (at that time it was an unknown modified uridine (U*) [24], and later it was elucidated to be $\tau m^5U$ [8]) in a bovine mitochondrial *in vitro* translation system using synthetic mRNAs possessing 30

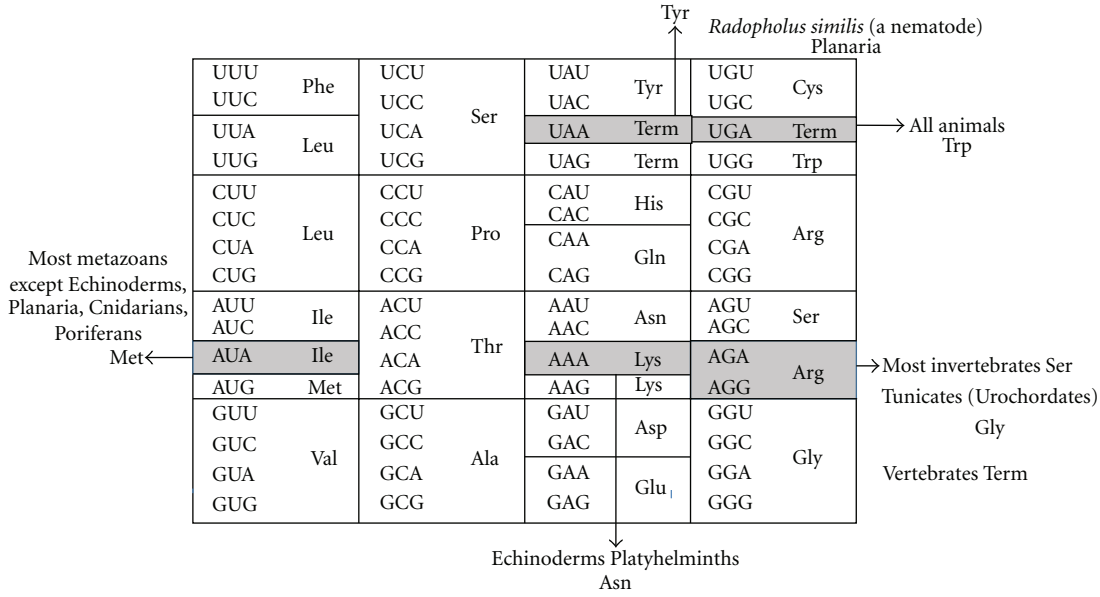


Figure 1: Universal genetic code (inside the box) and variations in animal mt genetic code (outside). Term: termination codon.

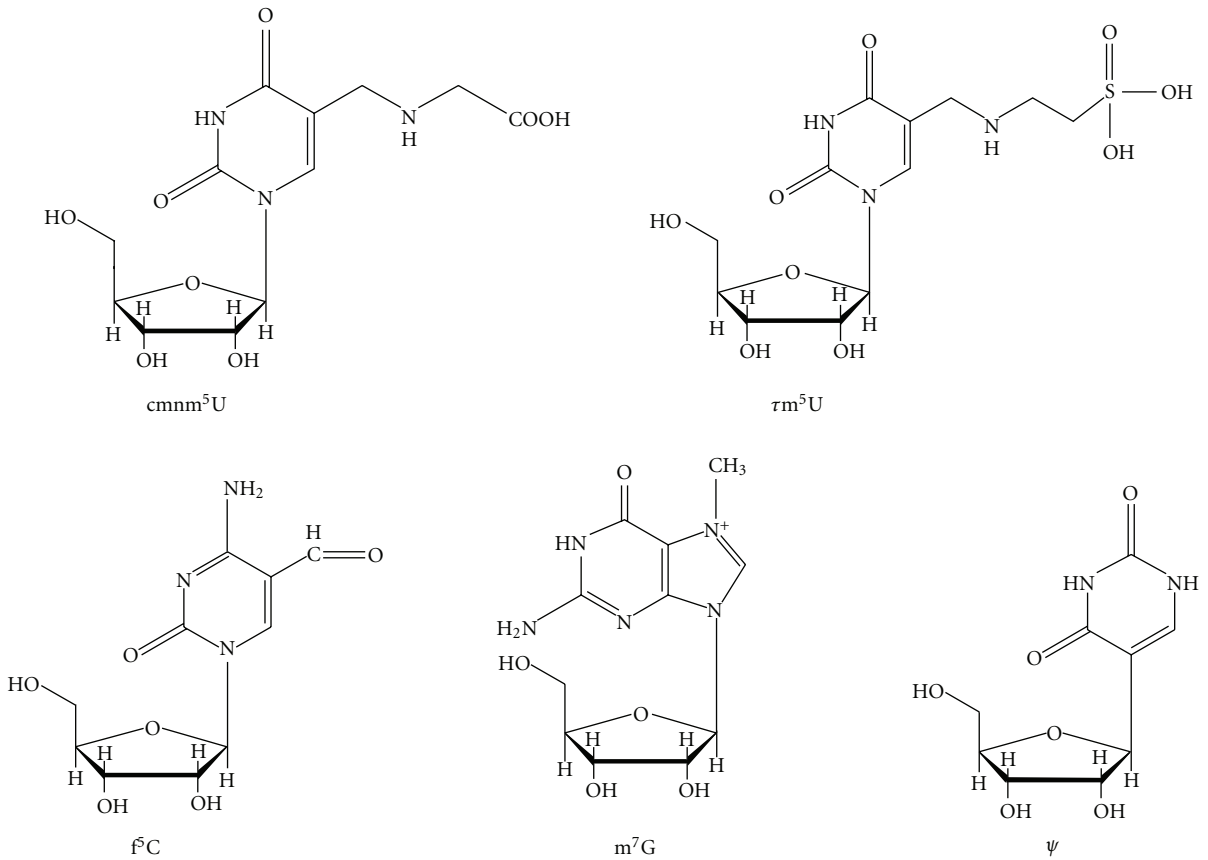


Figure 2: Chemical structures of modified nucleosides located at the anticodon wobble position (cmnm$^5$U, $\tau$m$^5$U, f$^5$C, and m$^7$G) and the second position ($\Psi$) of mt tRNAs.

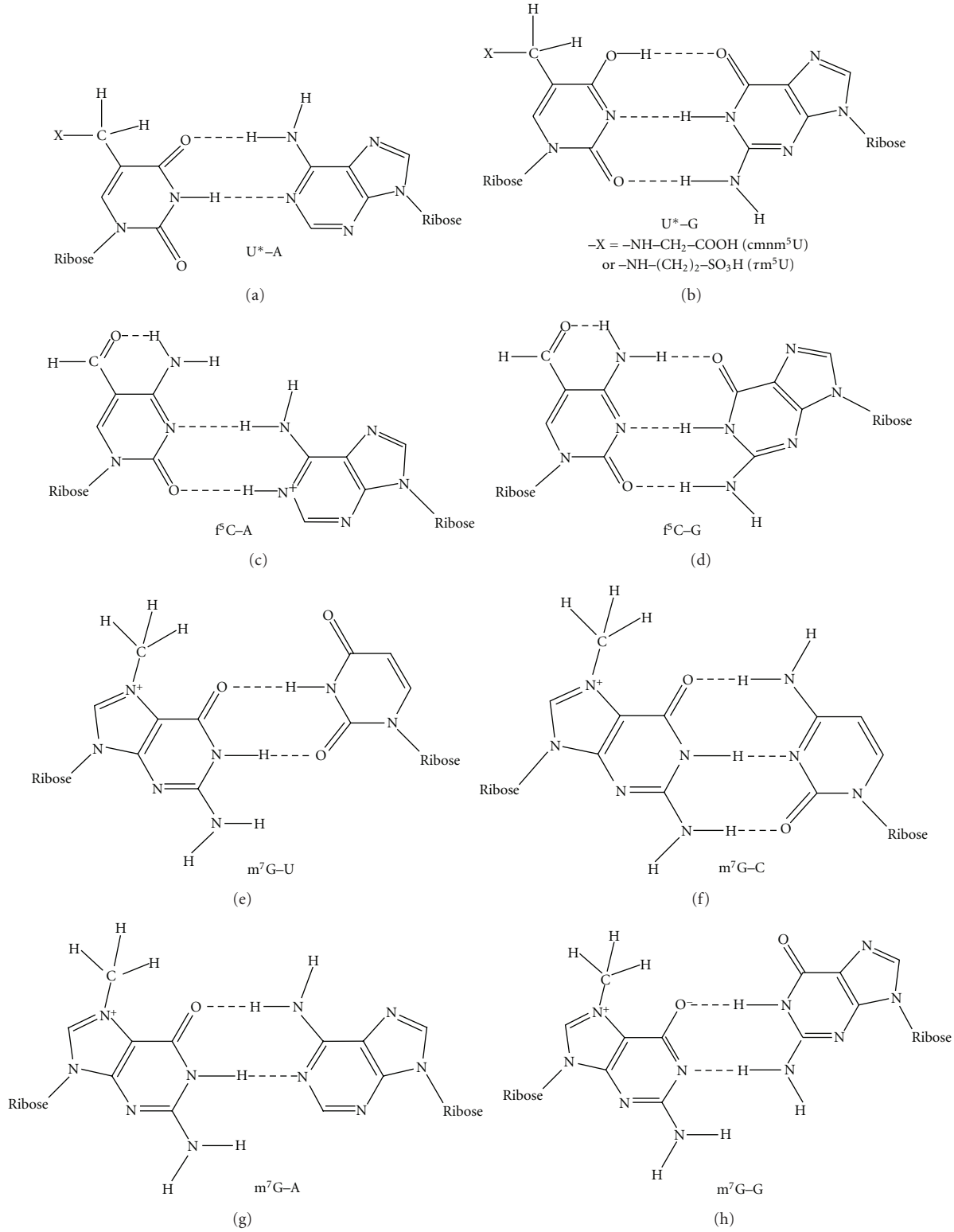


Figure 3: Possible scheme of base pairing between modified nucleosides at the anticodon wobble position and nucleosides at the codon third position in animal mitochondrial translation systems. These structures are only schematic drawing of base-pairing and do not show precise dimension of each nucleoside.

triplet repeats for the Leu codons UUA and UUG, as well as UUC as a negative control [24, 25]. They showed clearly that wild-type $tRNA^{Leu}_{UAA}$ translated both $poly(UUA)_{30}$ and $poly(UUG)_{30}$ to poly(Leu) efficiently, but scarcely translated $poly(UUU)_{30}$ or $poly(UUC)_{30}$.

Kurata et al. measured the decoding activity of *E. coli* $tRNA^{Leu}_{UAA}$ possessing $\tau m^5U$, $cmnm^5U$, or unmodified U (negative control) in the anticodon wobble position which was constructed by using molecular surgery technique [26] in *E. coli* S30 *in vitro* cell-free system with synthetic oligoribonucleotides including UUN codon as messengers [27]. They clearly demonstrated that $tRNA^{Leu}_{UAA}$ possessing either $\tau m^5U$ or $cmnm^5U$ in the anticodon wobble position could translate UUA/UUG-containing messengers efficiently but could translate neither UUU- nor UUC-containing messenger. The $tRNA^{Leu}_{UAA}$ with unmodified U34 efficiently translated the UUA codon, but decoding of the UUG codon was approximately one third the activity of decoding UUA. However, this $tRNA^{Leu}_{UAA}$ could not translate either UUU or UUC in consistent with the results obtained for mitochondrial translation [28]. Thus, either $\tau m^5U$- or $cmnm^5U$-modification was proved to be essential for restricting the base-pairing to the purine-ending codons (Figures 3(a) and 3(b)).

The conformation of $\tau m^5U$ has not been analyzed, but it can be speculated that it is very similar to that of $cmnm^5U$, because only their side chains at position 5 of the uridine base are different; glycine and taurine are linked to 5-methyluridine in $cmnm^5U$ and $\tau m^5U$, respectively (Figure 2). As described above, the modified uridine possessing the side chain at position 5 of uridine base which is linked through a methylene group takes very rigid conformation, which enables base pair with G as well as A in the codon third position.

Mitochondrial glycyl-tRNA synthetase (GlyRS) has scarcely been studied at the molecular level. It is suggested that a tunicate (*Ciona intestinalis*) genome encodes single GlyRS gene responsible for synthesis of both cytoplasmic and mt GlyRSs (Yokobori et al., unpublished results). Kondow et al. elucidated that a tunicate (*H. roretzi*) mt $tRNA^{Gly}_{\tau m^5UCU}$ is glycylated *in vivo* [29]. These results strongly suggest that tunicate mt GlyRS is possible to recognize $tRNA^{Gly}_{\tau m^5UCU}$ possessing $\tau m^5U34$.

*2.3. $f^5C$ for Decoding AUA Codon.* 5-formylcytidine ($f^5C$) occurs at the anticodon wobble position of $tRNA^{Met}$ of most invertebrate (fruit fly, *Drosophila melanogaster* [12], squid, *Loligo bleekeri* [30], and nematode, *Ascaris suum* [31]), and vertebrate (bovine, *Bos taurus* [9]) mitochondria, where AUA codon is read as Met instead of Ile [1, 5]. However, in echinoderm [32], some platyhelminth (such as planaria), cnidarian, placozoan, and poriferan mitochondria, AUA codon is read as Ile, like universal genetic code. In the former case, it is considered that $f^5C34$ of $tRNA^{Met}$ could restrict the base pair with A and G in the codon third letter (Figures 3(c) and 3(d)), but in the latter case, the wobble nucleoside of $tRNA^{Ile}$ is G34, so that this tRNA would translate AUA codon as not Met, but Ile.

By constructing an *in vitro* translation system from bovine liver mitochondria, Takemoto et al. examined the decoding properties of the native mt $tRNA^{Met}$ carrying $f^5C$ in the anticodon compared to a transcript that lacks the modification [14]. The native mt Met-$tRNA^{Met}$ could recognize both AUG and AUA codons as Met, but the corresponding synthetic $tRNA^{Met}$ lacking $f^5C$ (anticodon CAU) recognized only the AUG codon in both the codon-dependent ribosomal binding and *in vitro* translation assays. Furthermore, the *E. coli* elongator $tRNA^{Met}_m$ with the anticodon $ac^4CAU$ ($ac^4C$=4-acetylcytidine) and the bovine cytoplasmic initiator $tRNA^{Met}$ (anticodon CAU) translated only the AUG codon for Met on mt ribosome. The codon recognition patterns of these tRNAs were the same on *E. coli* ribosomes. These results demonstrate that the $f^5C$ modification in mt $tRNA^{Met}$ plays a crucial role in decoding the nonuniversal AUA codon as Met, and that the genetic code variation is compensated by a change in the tRNA anticodon, not by a change in the ribosome.

Conformation analysis of $f^5C$ by 500-MHz NMR showed that the nucleoside takes a very rigid C3'-endo-anti form [33]. This feature may be advantageous for the decoding properties of $tRNA^{Met}$, because a very rigid pyrimidine in the first position of the anticodon cannot form base pairs with U and C in the codon third position [34, 35], so that the $tRNA^{Met}$ cannot decode the AUU and AUC Ile codons. In addition, it is to be anticipated that the stability conferred by a rigid ribose moiety in the first anticodon nucleotide will to some extent be propagated to the second and third anticodon residues. This would result in greater overall stability of the stacked anticodon bases, and thus of codon-anticodon pairings.

It is apparent that $f^5C$ can interact in the expected manner with G of the AUG Met codon (Figure 3(d)), reflecting the finding that the conformation of $f^5C$ is similar to that of cytidine [33]. In order to read the AUA Met codon, the intriguing possibility exists that $f^5C$ pairs with A in the AUA Met codon by protonation (Figure 3(c)). The protonation of A at N-1 in an A-C pair at pH values above the pK of the monomer has been demonstrated in oligoribonucleotide duplexes [36]. In addition, A was found to adopt a pK of 6.5 in the active site of a Pb-dependent ribozyme [37].

Human mt MetRS has been studied by Spremulli's group [38]. They found that the enzyme recognizes the anticodon region of $tRNA^{Met}$ as an identity determinant and aminoacylates both human mt $tRNA^{Met}_{CAU}$ transcript and bovine $tRNA^{Met}_{f^5CAU}$ with similar $K_M$ (0.15~0.16 $\mu$M) and $k_{cat}$ (0.02 $s^{-1}$) values. As the nucleotide sequences of human and bovine mt $tRNAs^{Met}_{CAU}$ are almost identical to each other with 5 nucleotide differences at positions 16, 27, 50, 56, and 60, and it has turned out that human mt $tRNAs^{Met}_{CAU}$ also possesses $f^5C$ at the anticodon wobble position [39] these kinetic data clearly demonstrate that human mt MetRS recognizes the substrate $tRNA^{Met}_{CAU}$ irrespective of the presence or absence of $f^5C34$.

*2.4. $m^7G$ and Unmodified G Which Decodes AGA or AGG Codon in Most Invertebrates.* AGA and AGG codons are read as Ser in most metazoan mitochondria [32]. Matsuyama et al.

found that 7-methylguanosine ($m^7G$) is present at the wobble position of $tRNA^{Ser}_{GCU}$ in most invertebrate mitochondria [10]. Therefore, the anticodon $m^7GCU$ of $tRNA^{Ser}_{GCU}$ is most likely responsible for reading all four AGN codons as Ser (Figures 3(e)–3(h)) [10]. On the other hand, the AGG codon is absent from some metazoan mitochondria, such as fruit fly *Drosophila melanogaster* mitochondria (Table 1), and the anticodon wobble position of $tRNA^{Ser}_{GCU}$ is unmodified G [12]. In this case, the unmodified GCU anticodon of $tRNA^{Ser}_{GCU}$ seems to read the three codons AGU, AGC, and AGA as Ser. Therefore, it is summarized that G34 of Drosophila $tRNA^{Ser}_{GCU}$ base pairs with only U, C, and A in the third letter of Ser codon, and in most invertebrate mt $tRNAs^{Ser}_{GCU}$, it is prerequisite for G34 to be modified to $m^7G$ for recognizing G in the third letter of Ser codon.

The purine-purine base pairing is well discussed by Murphy and Ramakrishnan for I-A pair [43]. They reported the crystal structure of I-C and I-A base pairs in the context of the ribosomal decoding center, clearly showing that the I-A base pair is of an $I_{anti}$ -$A_{anti}$ conformation, as predicted by Crick [44] although the distance between $C_1$-$C_1$ of I and A residues is broader than the usual one. Owing to this observation, G-A and also $m^7G$-A base pairs in question would be possible to take a similar structure as that of I-A base pair (Figure 3(g)). Since $m^7G$ can form a structure in which a proton is cleaved from $HN_1$ and $O_6$ becomes $O^-$ (but G cannot form the structure), $m^7G$-G base pair can be formed in which $m^7G$ moves to the minor groove side in the context of the ribosomal decoding center (Figure 3(h)) [45]. Such base pair stacks well on the neighboring second base pair of codon-anticodon pairing, so that the whole interaction would be stabilized [46]. This speculation must be confirmed experimentally, the simplest way of which would be to use an *in vitro* translation system.

The recognition mechanism of bovine mt SerRS toward $tRNA^{Ser}$ has been elucidated well by biochemical [47, 48] as well as X-ray crystallographic studies [49]. Both results clearly demonstrated that the anticodon region of $tRNA^{Ser}$ is not involved in the identity determinant for bovine mt SerRS. Thus, it can be concluded that the presence or absence of $\tau m^5U$ in the anticodon wobble position has no influence on the recognition of SerRS toward $tRNA^{Ser}$.

*2.5. Ψ in the Anticodon Second Position of $tRNA^{Asn}_{GUU}$ Responsible for Decoding AAA Codon.* In echinoderm [32] and some platyhelminth mitochondria [50], not only the usual Asn codons AAU and AAC, but also the usual Lys codon AAA, are read as Asn by a single mt $tRNA^{Asn}$ with the anticodon GUU. Tomita et al. elucidated that starfish mt $tRNA^{Asn}$ possesses the anticodon GΨU, whose second position is modified to pseudouridine (Ψ) [42]. In contrast, mt $tRNA^{Lys}$, corresponding to another Lys codon, AAG, has the anticodon CUU. Mt tRNAs possessing anticodons closely related to that of $tRNA^{Asn}$, but responsible for decoding only two codons each ($tRNA^{His}$, $tRNA^{Asp}$, and $tRNA^{Tyr}$) (see Figure 1), were found to possess unmodified U35 in all cases, suggesting the importance of Ψ35 in $tRNA^{Asn}$ for decoding the AAU, AAC, and AAA codons. Experiments with an *E. coli in vitro* translation system confirmed that $tRNA^{Asn}_{GΨU}$ has about two-fold higher translational efficiency than $tRNA^{Asn}_{GUU}$ [42], in which $tRNA^{Asn}_{GΨU}$ was constructed by chemical synthesis and ligation, and $tRNA^{Asn}_{GUU}$ was obtained by *in vitro* run-off transcription. It is exceptional that modification at the anticodon second nucleoside is involved in the codon-anticodon interaction efficiency.

There is a report that Ψ within the base-paired region at position 35 in a model for the codon-anticodon interaction in $tRNA^{Tyr}$ increases the Tm by several degrees [51]. This is also supporting evidence for the above-mentioned phenomenon.

## 3. Relationship between Modified Nucleosides and Genetic Code Variations in the Course of Animal Evolution

In considering the evolution of the genetic code, there are so far two main hypotheses, the "codon-capture" hypothesis based on directional mutation pressure proposed by Osawa and Jukes [52], and the "ambiguous intermediate" hypothesis proposed by Schultz and Yarus [53]. Codon capture hypothesis proposes temporary disappearance of a sense codon (or stop codon) from coding frames by conversion to another synonymous codon, followed by loss of the corresponding tRNA that translates the codon. (For a stop codon, the release factor (RF) must change simultaneously so as not to recognize the stop codon.) This change may be caused by directional mutation pressure acting on genome (AT or GC pressure), changes of RF, or genome economization, which will produce an "unassigned codon." The codon reappears later by the conversion of another codon caused by directional mutation pressure and emergence of a tRNA (or RF) that translates (or recognizes) the reappeared codon with a different assignment, Thus, the codon is reassigned or captured. In the "ambiguous intermediate" hypothesis, it is presumed that reassignment of codons is facilitated by a translationally ambiguous intermediate where the transitional codon is read simultaneously as two different amino acids by two tRNAs, one cognate and the other near cognate. There has been little experimental evidence in support of the presence of these tRNAs that decode a single codon as two different amino acids simultaneously. Simulation study on codon reassignment [54]suggested that the pathway of codon reassignment in favor of either "codon capture" or "ambiguous codon" depends on the initial conditions such as genome size and number of codons in interest.

We adopt here the former hypothesis essentially to explain the genetic code variations of animal mitochondria, because the characteristics of the animal mitochondrial genome such as AT richness and genome economization would be suitable for the adoption of the codon-capture hypothesis. The hypothesis would nicely fit in explaining the UGA codon change in animal mitochondria. There has been so far no report of a release factor recognizing UGA codon [55, 56]. Such a release factor corresponding to eubacterial RF2 was probably lost in the animal mt system, so that

Table 1: Relationship between the genetic code variations and modified nucleosides in the anticodon of the corresponding tRNAs in the animal phlyla.

(a) UGA codon.

| Animal phyla | UGA specificity | Anticodon |
|---|---|---|
| Vertebrata | Trp | $\tau m^5UCA^{(a)}$ |
| Tunicata (Urochordata) | Trp | $\tau m^5UCA^{(b)}$ |
| Cephalochordata | Trp | (TCA) |
| Echinodermata | Trp | (TCA) |
| Arthropoda | Trp | $U^*CA^{(c)}$ |
| Most invertebrate phyla | Trp | $cmnm^5(s^2)UCA^{(d)}$ |
| Platyhelminthes | Trp | (TCA) |
| Cnidaria, Placozoa, Porifera | Trp | (TCA) |

(b) AUA codon.

| Animal phyla | AUA specificity | Anticodon |
|---|---|---|
| Vertebrata | Met | $f^5CAU^{(e)}$ |
| Tunicata (Urochordata) | Met | $\tau m^5UAU^{(f)}$ |
| Cephalochordata | Met | (CAT) |
| Echinodermata | Ile | (GAT) |
| Arthropoda | Met | $f^5CAU^{(g)}$ |
| Most invertebrate phyla | Met | $f^5CAU^{(h,i)}$ |
| Platyhelminthes | | |
| Most (Echinostomida, Trematoda) | Met | (CAT) |
| Rhabditiophora, Planaria | Ile | (GAT) |
| Cnidaria | Ile | —$^{(j)}$ |
| Placozoa | Ile | (GAT) |
| Porifera | Ile | $(CAT)^{(k)}$ |

(c) AAA codon.

| Animal phyla | AAA specificity | Anticodon |
|---|---|---|
| Vertebrata | Lys | (CTT) |
| Tunicata (Urochordata) | Lys | (TTT) |
| Cephalochordata | Lys | (TTT) |
| Echinodermata | Asn | $G\Psi U^{(l)}$ |
| Arthropoda | Lys | $CUU^{(m)}$/(TTT) |
| Most invertebrate phyla | Lys | (CTT/TTT) |
| Platyhelminthes | Asn | (GTT) |
| Cnidaria | Lys | —$^{(j)}$ |
| Placozoa | Lys | (TTT) |
| Porifera | Lys | (TTT) |

(d) AGA/AGG codons.

| Animal phyla | AGA/AGG specificity | Anticodon |
|---|---|---|
| Vertebrata | Term. | None |
| Tunicata (Urochordata) | Gly | $\tau m^5UCU^{(n)}$ |
| Cephalochordata | Ser | (GCT) |
| Echinodermata | Ser | $m^7GCU^{(o)}$ |
| Arthropoda | | |
| Most Arthropods | Ser | (GCT/TCT) |
| *Drosophila melanogaster* | $Ser^{(p)}$ | $GCU^{(q)}$ |
| Most invertebrate phyla | Ser | $m^7GCU^{(r)}$ |
| Platyhelminthes | Ser | (GCT/TCT) |

(d) Continued.

| Animal phyla | AGA/AGG specificity | Anticodon |
|---|---|---|
| Cnidaria | Arg | —[j] |
| Placozoa | Arg | (TCT) |
| Porifera | Arg | (TCT) |

In case that only the tRNA gene sequences are known, the anticodon sequences at DNA level are shown in parentheses. [a]*Homo sapiens* (Suzuki et al. [39]) [b]*Halocynthia roretzi* (Suzuki et al. [23]) [c]*Drosophila melanogaster* (Tomita et al. [12]). U* is an unkown modified uridine. [d]*Ascaris suum* (Sakurai et al. [15]) [e]*Bos taurus* (Moriya et al. [9]) [f]*Halocynthia roretzi* (Suzuki et al. [23]) [g]*Drosophila melanogaster* (Tomita et al. [12]) [h]*Loligo bleekeri* (Tomita et al. [30]) [i]*Ascaris suum* (Watanabe al. [31]) [j]Corresponding tRNA gene is not encoded in the mitochondrial genome. The tRNA decoding this codon is presumed to be imported from cytoplasm [40]. [k]Poriferan mt genomes encode three tRNA genes with anticodon CAT at DNA level. One of these tRNA is thought to be $tRNA^{Ile}_{LAU}$ gene (L is the modified C (lysidine) found at the anticodon first position of most bacterial $tRNA^{Ile}$ decoding AUA codon) [41]. [l]*Asterias amurensis* (Tomita et al. [42]) [m]*Drosohila melanogaster* (Tomita et al. [12]) [n]*Halocynthia roretzi* (Kondow et al. [29], Suzuki et al. [23]) [o]*Asterias amurensis* (Matsuyama et al. [10]) [p]No AGG codon appears in the *D. melanogaster* mitochondrial genome. [q]*Drosophila melanogaster* (Tomita et al. [12]) [r]*Loligo bleekeri* (Tomita et al. [11]).

the UGA codon became unassigned. When the anticodon wobble position of $tRNA^{Trp}$ changed from C to modified U (U*: $cmnm^5(s^2)U$ or $\tau m^5U$), and a part of the Trp UGG codon in reading frames was changed to UGA by AT pressure, the UGA codon was captured by $tRNA^{Trp}_{U^*CA}$ and read as Trp (Figure 4(a)). Why lower animals use $cmnm^5(s^2)U$ for U*, whereas higher animals use $\tau m^5U$ instead of $cmnm^5(s^2)U$ (Table 1(a)), and from which stage of animals conversion of $cmnm^5(s^2)U$ to $\tau m^5U$ occurs remain to be clarified.

To explain the evolutionary change of other nonuniversal codons in metazoan mitochondria, in addition to AT pressure, the genome economization effect (especially, that tRNA genes are restricted to 22~24 species, and that tRNAs are not imported from the cytoplasm in almost all metazoan mitochondria [7] except for a few cases in Cnidaria and so on) should be taken into consideration. At the same time, we should include the idea of competition between two different tRNAs toward a certain codon, to explain the role of modified nucleoside in the anticodon region of tRNA. Namely, any codon can be read by the corresponding tRNA, but if a competitor tRNA or a release factor arises that has stronger affinity toward the codon than does the original tRNA, the codon will then be read by the competitor.

The AUA codon is read as Ile in cnidarian, placozoan, poriferan, some platyhelminths, and echinoderm mitochondria, but it is read as Met in most metazoan mitochondria (Table 1(b)). $f^5C$ in most metazoan mitochondria [8, 9, 12, 31] and $\tau m^5U$ in tunicate mitochondria [23] found at the wobble position of $tRNA^{Met}$ may be important for understanding the codon reassignment from AUA-Ile to AUA-Met. Since the interaction between $tRNA^{Ile}_{GAU}$ and AUA on the ribosome might be more unstable than that between the $tRNA^{Ile}_{GAU}$ and AUY codons, when $tRNA^{Met}$ acquired the capacity to decode AUA by 5′-formylation of C or 5′-taurinometylaton of U at the wobble position, $tRNA^{Met}_{f^5CAU}$ or $tRNA^{Met}_{\tau m^5UAU}$ may have prevailed over $tRNA^{Ile}_{GAU}$ in the interaction with the AUA codon. Thus, the reassignment of Ile to Met could have easily occurred (Figure 4(b)). In echinoderm mitochondria, $f^5C$ or $\tau m^5U$ modification was lost in $tRNA^{Met}$, so that AUA is read as Ile by $tNA^{Ile}_{GAU}$ because the competitor tRNA was lost. Why most metazoan $tRNAs^{Met}$ possess $f^5C$ modification, but only tunicates $tRNA^{Met}$ possess $\tau m^5U$ modification is also to be solved.

The AAA codon is read as Lys in most metazoan mitochondria, but only in some platyhelminth and echinoderm mitochondria is read as Asn (Table 1(c)). In this case, pseudouridylation at the second position of the anticodon of $tRNA^{Asn}$ [42] was critical for the codon reassignment (Figure 4(c)). The interaction between $tRNA^{Asn}_{G\Psi U}$ and AAA may have prevailed over that between $tRNA^{Lys}_{UUU}$ or $tRNA^{Lys}_{CUU}$ (in various metazoan mitochondria such as those of echinoderms and fruit fly *Drosophila melanogaster*) and AAA. It is interesting observation that echinoderm and a part of platyhelminth mitochondria use AUA as a universal Ile codon and AAA as a nonuniversal Asn codon, while all the other animal mitochondria use AUA as a nonuniversal Met codon, and AAA as a universal Lys codon. This should be clarified in the relationship between genetic code change and animal evolution.

AGR codons are used as Ser codons in most invertebrate mitochondria, Gly codons in tunicate mitochondria and termination codons in vertebrate mitochondria (Table 1(d)). The scenario for this codon change would be as follows (Figure 4(d)). In the evolutionary process from Cnidaria, Placozoa, and Porifera to higher invertebrates, $tRNA^{Arg}_{U^*CU}$ to decode AGR codons as Arg disappeared from the mt genome, because of the reduction in genome size (in Cnidaria, most of tRNA genes are absent from the mt genome and most tRNAs are thought to be imported from the cytoplasm [40], but, in most higher animal mitochondria, import of tRNA has not been reported [7]). This has created a situation in which AGR codons cannot be translated. Since the AGR-Arg sites in the mitochondrial genomes of Protista such as *Trypanosoma* are mostly replaced with CGN-Arg codons and partially by a few other codons throughout metazoan mitochondria [57], AGR codons were converted mainly to CGN upon the deletion of $tRNA^{Arg}_{U^*CU}$, so that AGR codons became unassigned (first step). Once the anticodon wobble position (G34) of $tRNA^{Ser}_{GCU}$ was modified to $m^7G$, all four AGN codons became ready to be decoded as Ser [10, 11]. AGR codons pairing with this $tRNA^{Ser}_{m^7GCU}$ then appeared in reading frames because of the mutation of AGY-Ser codons or other codons to AGR codons, and they were captured by Ser (second step). In ancestors of tunicates and

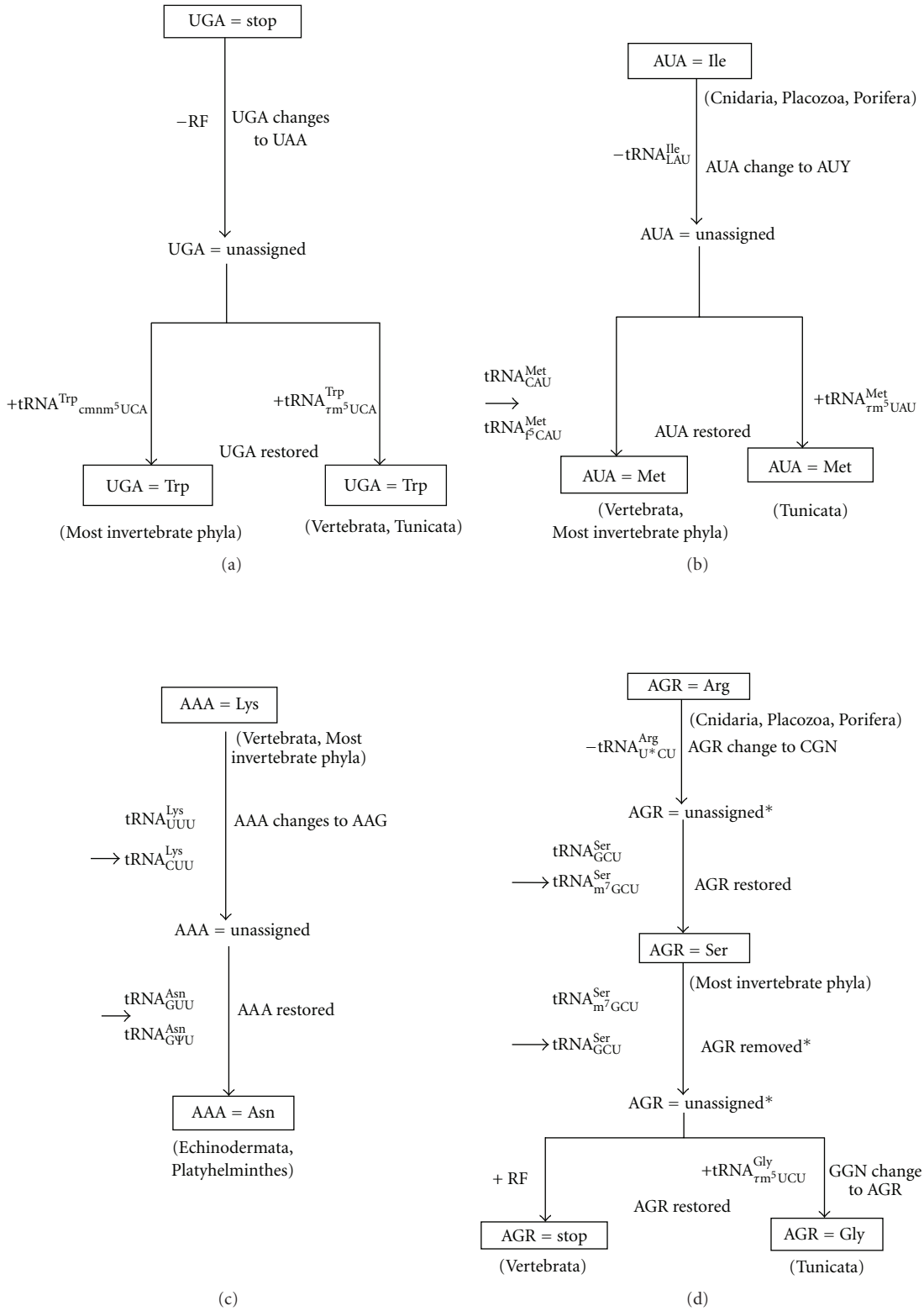


Figure 4: Schematic drawings showing the possible evolutionary changes of UGA (a), AUA (b), AAA (c), and AGR (d) codons in animal mitochondrial genomes. * In order to exclude too complicated situations, simple formulas were adopted for the process of the possible evolutionary changes of AGR codons in this figure. For details, see Text.

vertebrates, demethylation of $m^7G$ of mt $tRNA^{Ser}_{m^7GCU}$ may have occurred, so the resulting mt $tRNA^{Ser}_{GCU}$ no longer reads the AGG codon. Strong selective constraints resulting from the lost translation of AGG caused the AGG codon to change mainly to AGY or other codons, so that the AGG codon became unassigned (third step). After tunicates were separated from vertebrate ancestors, a fourth event may have occurred; the mt $tRNA^{Gly}_{UCC}$ gene was duplicated in mitochondrial DNA (mtDNA) (in fact, the ascidian mt genome has two $tRNA^{Gly}$ genes [58]), and the anticodon of one species of $tRNA^{Gly}_{UCC}$ was converted from TCC to TCT because of AT pressure, resulting in $tRNA^{Gly}_{UCU}$, which might have occurred in tunicate mt genomes. Then, the anticodon wobble position of $tRNA^{Gly}_{UCU}$ must have been modified to $\tau m^5U$ ($tRNA^{Gly}_{\tau m^5UCU}$; ([8, 23]) so as to decode AGR codons. At the same time, AT pressure caused GGN codons to change to AGR codons. Since the interaction of $tRNA^{Gly}_{\tau m^5UCU}$ with AGR is stronger than that of $tRNA^{Ser}_{GCU}$ with AGR, AGR codons are captured by $tRNA^{Gly}_{\tau m^5UCU}$, resulting in the translation of AGR codons as Gly [59]. The low GC content of the cytochrome oxydase subunit I region in ascidian (tunicate) mitochondria in comparison to that of vertebrates is consistent with the speculation that AT pressure occurred on the tunicate mt genome. In the ancestors of vertebrate mitochondria, AGR codons may have appeared in the reading frames by the deletion of U from the UAG termination codon [57], concomitantly with a functional change in the vertebrate RF so as to recognize AGR codons (a possible candidate for an mt RF capable of recognizing AGR codons was reported [56]). The RF prevails over $tRNA^{Ser}_{GCU}$ in decoding the AGA codon and, thus, changes AGR codons to termination codons (Figure 4(d)) [57]. Why $m^7G$- and $\tau m^5U$-modifications have become to be used in most metazoan and tunicate mitochondria, respectively, for decoding AGR codons are intriguing problems, which should be pursued in future.

## 4. Conclusion

The concept of the universal genetic code was forced to change since the discovery of nonuniversal codon in human mitochondria [4, 5], and it was known that not only mitochondria but also usual cellular systems (nuclear genomes) contain the nonuniversal genetic codes [60]. Nowadays, it is widely accepted that the genetic code is not universal but is changeable depending on the lineage of organisms [61]. Especially, it should be mentioned that animal mt genomes contain several variations in the genetic code, and the nonuniversal genetic codes are decoded by tRNAs which possess modified nucleosides in their anticodon first or second positions.

In animal mt genetic code, variations occur only in the 2 codon boxes where one family box is divided 2 : 2 (which may be easily understandable), and only purine-ending codon(s) are the target for codon reassignment (Figure 1). The questions may arise as to why no codon changes has occurred in two codon boxes of His(CAY)-Gln(CAR) and/or Asp(GAY)-Glu(GAR) and why pyrimidine-ending codon(s) (NNY) do not become the target for codon reassignment. For the former question, such cases may be found in future [61] and for the latter question, it may be because it is difficult to provide any modified nucleoside in the tRNA anticodon wobble position which can decode purine-ending codons (NNR) and one or both of pyrimidine-ending codons (NNY).

The way of codon-anticodon interaction in the decoding process of the nonuniversal genetic code follows the base-pairing rule based on the physiochemical property of nucleosides. It is the future problems to be answered why various modified nucleosides such as $cmnm^5(s^2)U$, $\tau m^5U$, $f^5C$, $m^7G$, and $\Psi$ have been selected as the key molecules for decoding the nonuniversal genetic code and how these modified nucleosides have been distributed among various animal mitochondria.

The genetic code consists of interaction between codon and anticodon of tRNA, and inspections of genetic code changes in animal mitochondria in which considerable number of changes occur in various animal lineages will lead to the elucidation of the basic principle of origin and evolution of the genetic code. It is expected that these studies will shed light on a way to approach the origin of life.

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