J Mol Evol (2001) 53:314–326
DOI: 10.1007/s002390010221

JOURNAL OF MOLECULAR EVOLUTION

Genetic Code Variations in Mitochondria: tRNA as a Major Determinant of Genetic Code Plasticity

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Received: 28 December 2000 / Accepted: 12 March 2001

Abstract. Characteristic features of tRNA such as the anticodon sequence and modified nucleotides in the anticodon loop are thought to be crucial effectors for promoting or restricting codon reassignment. Our recent findings on basepairing rules between anticodon and codon in various metazoan mitochondria suggest that the complete loss of a codon is not necessarily essential for codon reassignment to take place. We postulate that a possible competition between two tRNAs with cognate anticodon sequences towards the relevant codon to be varied has a potential role in codon reassignment. Our proposition can be viewed as an expanded version of the codon capture theory proposed by Osawa and Jukes (J Mol Evol 28: 271–278, 1989).

Key words: Codon capture — tRNA — Mitochondria — Wobble rule — Competition

Introduction

Since genetic code variations were first found in mammalian mitochondria (Barrell et al. 1979), a good number of variations from the "universal" code have been reported in diverse genetic systems: mitochondrial (mt), prokaryotic, and even eukaryotic nuclear-cytoplasmic (see review in Osawa et al. 1992; Ueda and Watanabe 1993; Osawa 1995; Watanabe and Osawa 1995). Although the process by which the universal genetic code system came to be established in a common ancestor of all extant organisms remains a matter of debate, it is apparent that the code is variable and is thus still evolving.

Among hypotheses advanced to explain the emergence of genetic code variations, the most widely accepted is the codon capture theory (Osawa and Jukes 1989), which can be summarized as follows. First, a certain codon disappears from the genome. Second, the tRNA corresponding to the missing codon also disappears. Third, a new tRNA that can recognize the codon that disappeared is created or recruited. If the amino acid specificity of this tRNA differs from that of the original tRNA, the "lost codon" will reappear with a different amino acid assignment. In mitochondria as well as *Mycoplasma spp.*, the driving force behind the disappearance and reappearance of a certain codon is postulated to be GC or AT directional mutation pressure. Complete loss of a codon prior to the disappearance of the cognate tRNA is regarded as necessary for the process to take place.

One problem with the codon capture theory as a hypothesis to account for codon reassignment concerns

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how scarce a codon needs to be for it to be ready for reassignment. The codon capture theory contends that in order to be reassigned, a codon should be in an unassigned state (Osawa et al. 1992; Osawa 1995). Mt-genomes, which encode around ten to several tens of protein genes, may well follow this pathway because the number of appearances of a particular codon can be few enough for it to disappear completely. However, the complete genomes of prokaryotes and eukaryotes encode several thousand to several tens of thousands of genes, most of which are protein genes (i.e., Fleischmann et al. 1995; Blattner et al. 1997). Recent extensive sequencing studies show that some codons previously thought to be unassigned based on analyses of a small number of protein genes are not in fact unassigned codons (see codon usage database; Nakamura et al. 2000). This suggests that obtaining the complete sequence data of protein genes for each genome is a prerequisite for making a final judgment on codon usage in the system. It is also important that the decoding capacity of each tRNA should be clarified, for which purpose both gene data and extensive characterization of individual tRNAs are required.

We have analyzed the characteristics of tRNA molecules related to unusual genetic codes of eukaryotic nuclei and mitochondria and ascertained that the distinctive features of tRNA, such as the anticodon sequence and modified nucleotides in the anticodon loop, play major roles in codon reassignment. In this paper, we review current knowledge on variations in the genetic code, and discuss the codon reassignment mechanism from the perspective of tRNA characteristics. Our finding may expand the codon capture theory.

Metazoan mt-Genetic Code

The metazoan mt-genetic code is a good model for studying how the genetic code has evolved. Most metazoan mtDNA is 14–18 kbp in size, circular, and generally encodes 13 protein, 2 rRNA, and 22 tRNA genes (Wolstenholme 1992; Boore 1999). The total length of 13 protein genes is less than 4,000 codons, so each codon occurs on average approximately 70 times (there are 60–62 sense codons in the code). The twenty-two tRNA genes encoded by the mt-genome should be sufficient to decoding all the codons, since the import of tRNA from cytoplasm generally appears not to occur in metazoan mitochondria (Roe et al. 1982), though an exception has been found in the case of cnidarian mitochondria (Wolstenholme 1992). It is thus concluded that each tRNA decodes two to four codons in a four-codon box. There is no metazoan mt-tRNA that decodes the same codon as another tRNA, and, as discussed above, wobble rule is much simplified. Therefore, any changes in the tRNA can directly affect the nature of genetic code through codon-anticodon interaction.

Table 1. Nucleotides found at the anticodon first position of metazoan mt-tRNAs analyzed at the RNA level

	Basepair with	Source (sequenced at RNA level)
U[a]	U, C, A, G	tRNAs with anticodon UNN for four-codon family boxes
U*[b]	U, C	tRNAs for codons NNR in two-codon families
C[c]	G	*Asterias* $tRNA^{Met}$ and $tRNA^{Lys}$, coupled with A37
C[d]	A, G	*Drosophila* $tRNA^{Lys}$ coupled with t^6A37
f^5C[e]	A, G	$tRNA^{Met}$ for *Homo, Bos, Drosophila, Loligo,* and *Ascaris*
A[f]	U, C, A, G	*Ascaris* $tRNA^{Arg}_{ACG}$
G[g]	U, C	tRNAs with anticodon GNN for NNY two-codon families
Q[d]	U, C	*Drosophila* $tRNA^{Asn}_{QUU}$
G[h]	U, C, A	*Asterias* $tRNA^{Ile}$ and $tRNA^{Asn}$, *Drosophila* $tRNA^{Ser}_{GCU}$, *Aedes* $tRNA^{Ser}_{GCU}$
m^7G[i]	U, C, A, G	$tRNA^{Ser}$ for AGN codons of *Asterias* and *Loligo*

[a] Osawa et al. (1992); Yokogawa et al. (1991). [b]Kondow et al. (1998, 1999). [c]Tomita et al. (1999b). [d]Tomita et al. (1999a). [e]Moriya et al. (1994); Watanabe et al. (1994); Tomita et al. (1997, 1999a). [f]Watanabe et al. (1997). [g]Roe et al. (1982); Ueda et al. (1985); Tomita et al. (1999a); Kondow et al. (1999). [h]Y. Ohkubo, S. Matsuyama, Y. Watanabe, S. Yokobori, T. Ueda, and K. Watanabe (unpublished result); Matsuyama et al. (1998); Tomita et al. (1999a); Dubin et al. (1984). [i]Matsuyama et al. (1998); Tomita et al. (1998)

The anticodon wobble position (position 34) of the tRNA responsible for decoding all four codons in a family box is mostly occupied by unmodified U (U34), which is seen not only in mitochondria but also in *Mycoplasma* (Andachi et al. 1989). The decoding mechanism of tRNA possessing U34 toward family-box codons has been well discussed by Yokoyama and Nishimura (1995). There are examples in which all four codons in a family box are also thought to be decoded by tRNAs with an anticodon starting with unmodified A (A34), which again is found in *Mycoplasma* (Andachi et al. 1989) as well as in nematode (*Ascaris*) mt-$tRNA^{Arg}_{ACG}$ (Watanabe et al. 1997).

The majority of tRNAs decoding NNR codons possess modified U at the 34th position (U*34). In bacterial as well as in eukaryotic cytoplasmic systems, U*34 has been well characterized in terms of its chemical structure as well as its function; it prevents basepairing with pyrimidine at the third position of the relevant codon (Yokoyama and Nishimura 1995). However, its exact structure in metazoan mt-tRNAs has not yet been elucidated. Most tRNAs decoding NNY codons possess unmodified G at the wobble position of the anticodon (G34), whereas those decoding only NNG codons have unmodified C at the position (C34) (Watanabe and Osawa 1995).

Many exceptions to the above rules have been found, which seem mostly to be related to the unusual genetic code. Table 1 includes such wobble base pairs that have been observed so far in metazoan mitochondria. In the following sections, we discuss evolutionary variations in

Table 2. Variations in metazoan mt-genetic code

	AUA	AAA	UGA	AGA	AGG
Standard	Ile	Lys	Term.	Arg	Arg
Polyfera[a] & Cidaria[b]	Ile	Lys	**Trp**	Arg	Arg
Platyhelminthes					
Rhabditophora[c]	Ile	**Asn**	**Trp**	**Ser**	**Ser**
Other platyhelmithes[d]	**Met**	Lys	**Trp**	**Ser**	**Ser**
Most invertebrates[e]	**Met**	Lys	**Trp**	**Ser**	**Ser**
Arthropoda					
Most arthropods[f]	**Met**	Lys	**Trp**	**Ser**	**Ser**
Dipteran insects[g]	**Met**	Lys	**Trp**	**Ser**	N.A.
Echinodermata[h]	Ile	**Asn**	**Trp**	**Ser**	**Ser**
Hemichordata[i]	Ile	N.A.	**Trp**	**Ser**	N.A.
Urochordata[j]	**Met**	Lys	**Trp**	**Gly**	**Gly**
Cephalochordata[k]	**Met**	Lys	**Trp**	**Ser**	N.A.
Vertebrata[l]	**Met**	Lys	**Trp**	**Term.**	**Term.**

[a] Watkins and Bechenbach (1999). [b]Pont-Kingdon et al. (1994); Beagley et al. (1998). [c]Bessho et al. (1992); Ohama et al. (1990); Garey and Wolstenholme (1989); Telford et al. (2000). [d]Telford et al. (2000). [e]Mesozoa (Watanabe et al. 1999; Telford et al. 2000), Annelida (Boore and Brown 1995), Brachiopoda (Stechmann and Schlegel 1999), Mollusca (Shimayama et al. 1990; Boore and Brown 1994), Nematoda (Okimoto et al. 1992). [f]Batuecas et al. (1988); Van Raay and Crease (1994); Flook et al. (1995); Lavrov et al. (2000). [g]de Bruijn (1983); Clary and Wolstenholme (1985); Bead et al. (1993); Mitchell et al. (1993). [h]Himeno et al. (1987); Jacobs et al. (1988). [i]Castresana et al. (1998a, b). [j]Yokobori et al. (1993, 1999). [k]Boore et al. (1999). [l]Barrell et al. (1979); Anderson et al. (1981). The bold letters show non-universal codons. N.A.: not appeared.

the metazoan mt-genetic code in relation to the characteristic features of tRNAs.

Variations in the mt-Genetic Code Dependent on Metazoan Evolution

Is it possible to trace evolutionary changes in the mt-genetic code along the course of metazoan evolution? A prerequisite to discussing the evolution of the mt-genetic code is clarifying the codon reassignment pathway in metazoan mt-evolution. Table 2 summarizes reported unusual genetic code variations found thus far in metazoan mitochondria, and Fig. 1 shows data on how the mt-genetic code has changed, incorporated in a metazoan phylogenetic tree constructed on the basis of analyses of 18S rRNA sequences (e.g., Wada and Satoh 1994; Aguinaldo et al. 1997).

Since no plant mitochondria, and not all fungus mitochondria, use the codon UGA for Trp—notwithstanding that fungi have been inferred to be closely related to metazoans (Wainright et al. 1993)—the reassignment of UGA from a stop codon to Trp would have occurred in mitochondria of a common ancestor of metazoans.

Platyhelminth mitochondria, including most basal triploblastics, use two types of mt-genetic code (Telford et al. 2000). All platyhelminth mitochondria use AGR codons for Ser, and most of them use AUA and AAA for Met and Lys, respectively, whereas, only rhabditophoran mitochondria have AUA as the Ile codon and AAA as Asn codon. From this evidence, together with the fact that mesozoan mitochondria use AUA for Met and AGR for Ser (Watanabe et al. 1999; Telford et al. 2000), rhabditophorans—including several parasitic species whose mt-genomes have been completely sequenced, as well as planaria—seem not to be the basal group of platyhelminthes (Telford et al. 2000). It has been suggested that Mesozoa belong to the triploblastics (Katayama et al. 1995). Therefore, reassignment of the AUA codon from Ile to Met, and AGR from Arg to Ser might have occurred in mitochondria of a common ancestor of triploblastics after the separation of triploblastics and diploblastics (such as polyferans and cnidarians). In this period, the "standard invertebrate mt-genetic code"—UGA, AUA, and AGR codons specifying Trp, Met, and Ser, respectively—might have been established. Other types of mt-genetic code, that have been found in rhabditophoran platyhelminthes, dipteran insects, and deuterostomes, would then have derived from this "standard" invertebrate mt-genetic code.

Evolution of UGA Codon in Metazoan Mitochondria

The UGA codon might have been captured by $tRNA^{Trp}$ in mitochondria of an ancestral metazoan, since polyferan and cnidarian mitochondria, as well as other metazoan mitochondria, use UGA for Trp (Watkins and Beckenbach 1999).

If C34 of $tRNA^{Trp}$ had changed to a U-derivative, the tRNA would have acquired the ability to decode the UGA codon, in addition to UGG, as Trp, and tryptophanyl-tRNA synthetase (TrpRS) might not have been needed to change the recognition pattern toward $tRNA^{Trp}$. One of the three termination codons, UGA, is able to disappear from the genome by changing to a UAA termination codon under the AT pressure. Concomitantly, release factor RF2 for the UAA and UGA termination codons is also able to be eliminated (Ueda and Watanabe 1993).

Planarian mitochondria have tentatively been reported to use the codon UAA for Tyr (Bessho et al. 1992), but no data for other platyhelminth mitochondria are available in the literature to confirm this (Garey and Wolstenholme 1989; Telford et al. 2000). It may be possible for $tRNA^{Tyr}$ to decode UAA if queosine (Q34) at the wobble position of $tRNA^{Tyr}$, which usually occurs in the tRNA, is eliminated—as is discussed in a later section. There is, however, no structural information on planarian mt-$tRNA^{Tyr}$, which will be required—together with more data about many protein gene sequences—to confirm the planarian mt-genetic code.

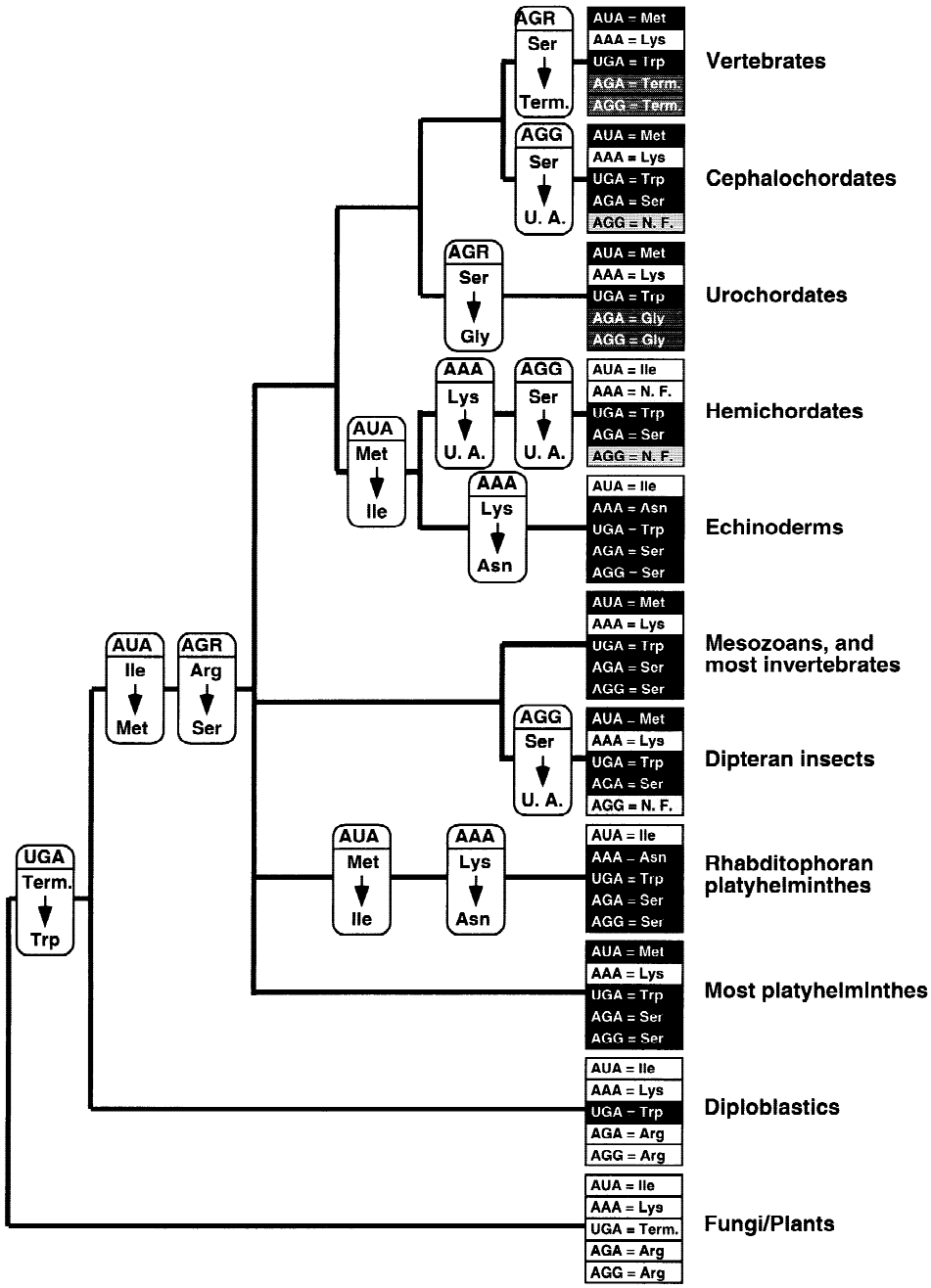

Fig. 1. Evolution of the metazoan mt-genetic code. The relationship presented is based on 18S rRNA analyses (e.g., Wada and Satoh 1994; Aguinaldo et al. 1997). Fungi and plants are treated as outgroups. Diploblastics include polyferans and cnidarians. Most invertebrates in the figure include mollusks, annelids, brachiopods, nematodes, and arthropods. Dipteran insects include *Drosophila, Anopheles,* and *Aedes.* Vertebrates, cephalochordates, and urochordates consist of chordates. Chordates, hemichordates, and echinoderms consist of deuterostomes. White letters on black backgrounds demonstrate unusual codons that deviate from the usual codons of the universal genetic code table. U.A. and N.F. mean "unassigned codon" and "not found", respectively.

Evolution of AGN Box in Metazoan Mitochondria

tRNA^{Ser}. Unmodified G at the wobble position (G34) is found in bovine and *Halocynthia* mt-tRNA$^{Ser}_{GCU}$, which is thought to correspond only to AGY codons (Arcari and Brownlee 1980; Ueda et al. 1985; Kondow et al. 1999), as suggested by the usual wobble rule (Osawa et al. 1992). However, G34 is also found in *Drosophila* mt-tRNA$^{Ser}_{GCU}$, and in the case the tRNA is thought to decode not only AGY codons but also AGA (Tomita et al. 1999a). G34 can basepair with A at the third codon position, if the A is protonated and the G takes the *syn* form (Yokoyama and Nishimura 1995).

Why do some tRNAs$^{Ser}_{GCU}$ recognize AGA while oth-

ers do not? The explanation probably lies in the presence or absence of competition for the AGA codon (Tomita et al. 1999a). If neither a tRNA nor protein factor (i.e., release factor) competes with $tRNA^{Ser}_{GCU}$ for the recognition of AGA on the ribosome, $tRNA^{Ser}_{GCU}$ will recognize the AGA codon as Ser. On the other hand, if there is any competitor stronger than $tRNA^{Ser}_{GCU}$, AGA will not be decoded as Ser. To form a basepair between G34 at the anticodon wobble position and A at the third position of codon, the location of the G34 residue needs to be changed and the A residue protonated; a G34–A* basepair is thus likely to be less stable than a U*34–A pair.

In echinoderm and molluscan mitochondria, the codon AGG is read as Ser in addition to AGY and AGA, and the $tRNAs^{Ser}$ responsible for decoding all the AGN codons have been found to possess m^7G34 (m^7G, 7-methylguanosine) at the wobble position in *Asterias* and *Loligo* mitochondria (Matsuyama et al. 1993; Tomita et al. 1998). Matsuyama et al. (1998) have proposed that the m^7G34-containing anticodon enables the AGG codon to be decoded. On the other hand, in the mitochondria of several other species, including *Apis mellifera* (Crozier and Crozier 1993) and *Ascaris* (Wolstenholme et al. 1987; Okimoto and Wolstenholme 1990; Okimoto et al. 1992), the corresponding $tRNA^{Ser}$ possesses T34 at the DNA level [unmodified U34 is found in the *Ascaris* mt-$tRNA^{Ser}_{UCU}$ (Watanabe et al. 1994)]. In these cases, $tRNA^{Ser}_{UCU}$ apparently decodes all the AGN codons according to the mt-wobble rule.

$tRNA^{Gly}_{U*CU}$ Found in Halocynthia *Mitochondria.* The $tRNA^{Gly}_{TCT}$ gene, whose product is thought to decode AGR codons, was found in the *Halocynthia* mt-genome (Yokobori et al. 1999). The $tRNA^{Gly}_{UCU}$ was determined to possess the anticodon U*CU and to accept Gly both *in vitro* and *in vivo* (Kondow et al. 1999). *Halocynthia* mitochondria therefore use AGR codons as Gly.

Reassignment of AGR Codons from Arg to Ser, and from Ser to Gly or Termination Codons. We have postulated above that the reassignment of AGR codons from Arg to Ser could have occurred in a common ancestor of all triploblastic metazoans after their separation from polyferans and cnidarians (Fig. 1). AGG might easily have been lost from the mitochondria of an ancestral triploblastic animal under AT pressure. As also noted above, if $tRNA^{Arg}_{U*CU}$ disappeared from the mt-genetic system, $tRNA^{Ser}_{GCU}$ might have decoded AGA, in addition to AGY codons, as Ser (Fig. 2A). No structural change in $tRNA^{Ser}_{GCU}$ would have been needed for the Ser codon set to be expanded from only AGY to AGY/AGA codons.

For further decoding of AGG by a single $tRNA^{Ser}$ species, modification of G34 at the anticodon wobble position to m^7G would be needed (Matsuyama et al. 1998) (Fig. 2A). In several metazoan mitochondria, such as nematode and *Apis*, the first position of the $tRNA^{Ser}$ anticodon for AGN codons is occupied by unmodified U, as discussed above (Wolstenholme et al. 1987; Okimoto and Wolstenholme 1990; Crozier and Crozier 1993; Watanabe et al. 1994), which is another device for decoding all AGN codons as Ser (Fig. 2A).

If the methylation at m^7G34 were lost, for example, by deletion of the methylation enzyme, the AGG codon would become unassigned—which is seen in dipteran (*Drosophila* and *Aedes*) mitochondria (Dubin et al. 1984; Tomita et al. 1999a) (Fig. 2A).

Hemichordate and cephalochordate mt-genomes use no AGG codon (Table 2). Although the tRNAs from mt-$tRNA^{Ser}_{GCT}$ genes have not been analyzed, their wobble position would be unmodified G. It is speculated that loss of the m^7G34 methyl group of mt-$tRNA^{Ser}_{GCU}$ occurred in a common ancestor of chordates (urochordates, cephalochordates, and vertebrates), as shown in Fig. 1. This event would have been independent of the same methyl group loss in hemichordate line. As noted earlier, if a competitor to $tRNA^{Ser}_{GCU}$ for the AGA codon appears, reassignment of AGA can easily occur (Fig. 2A).

In the cephalochordate line, no further change concerning the decoding of AGN codons would have occurred. However, in the urochordate line, a new $tRNA^{Gly}_{U*CU}$ appeared in the mt-genetic system that is capable of decoding AGG as well as AGA (Kondow et al. 1999) (Fig. 2A). We observed above that the *Halocynthia* mt-$tRNA^{Ser}_{GCU}$ is potentially able to decode AGA, but it would be defeated by the newly appeared $tRNA^{Gly}_{U*CU}$ in the competition to decode AGA on the ribosome. However, AGA would be decoded as Gly rather than Ser. In vertebrate mitochondria, an AGR codon-specific release factor (RF) is likely to have appeared in the system, competed with $tRNA^{Ser}_{GCU}$ for the decoding of AGA on the ribosome, and won the competition. As a result, AGR codons would have changed to termination codons (Fig. 2A). However, there has as yet been no report concerning an RF responsible for AGR codons; research in this area is keenly anticipated. One mt-RF from rat has so far analyzed, which seems to decode not AGR codons but UAR (Lee et al. 1987). It is known that the rat mt-genome uses no AGR codons for termination (Gadaleta et al. 1989).

Evolution of AUN Codons in Metazoan Mitochondria

$tRNA^{Met}$. $tRNA^{Met}$ in metazoan mitochondria is classified into the following four groups on the basis of the anticodon-loop sequences.

1. $tRNA^{Met}_{C*AU}$—A novel modified nucleoside f^5C (5-formylcytidine) was found at the wobble position of

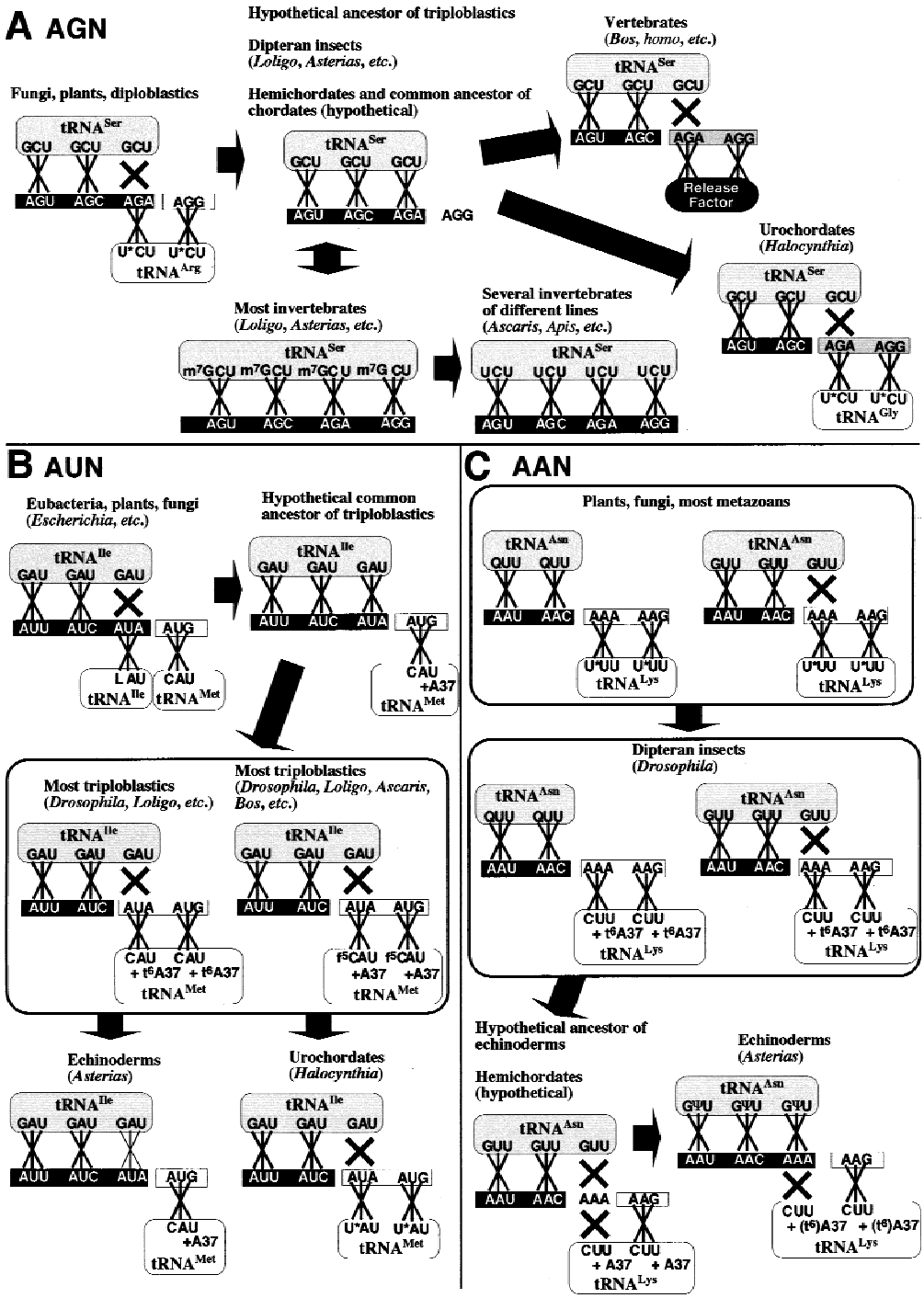

Fig. 2. Possible pathways for the reassignment of AGN (A), AUN (B), and AAN codons (C), showing the codon–anticodon interactions of each codon with the tRNA possessing the cognate or near-cognate anticodon. A large cross means that basepairing does not occur because of losing out to a competitor tRNA or release factor.

bovine mt-tRNA^{Met} (Moriya et al. 1994). *Ascaris suum, Drosophila melanogaster,* and *Loligo bleekeri* mt-tRNAs^{Met} were also demonstrated to have f^{5}C34 (Watanabe et al. 1994; Tomita et al. 1997, 1999a). All the tRNAs^{Met} possessing f^{5}C34 occur in metazoan mitochondria in which the AUA codon is read as Met. Formylation of C34 is thought to stabilize the interaction between anticodon (f^{5}CAU) and codon (AUA) (Moriya et al. 1994), though A at the codon third position must be protonated (Yokoyama and Nishimura 1995).

2. tRNA$^{Met}_{CAU}$ with t^{6}A at the 37th position—In *Drosophila* and *Loligo* mitochondria, two kinds of tRNA^{Met} have been found, one possessing f^{5}C34 and the other having unmodified C34 (Tomita et al. 1997, 1999a). An intriguing finding is that the latter species

always possesses the modified A (t^6A; N^6-threonylcarbamoyladenosine) at position 37, although the former has unmodified A37 (Tomita et al. 1997, 1999a). It has been postulated (Tomita et al. 1999) that the existence of t^6A37 stabilizes the interaction between the anticodon (CAU) and codon (AUA), since the modification is known to stabilize codon–anticodon interaction in general (Nishimura 1979).

3. $tRNA^{Met}_{U*AU}$—The $tRNA^{Met}_{TAT}$ gene is encoded on the mt-genomes of the bivalve mollusk *Mytilus edulis* and urochordate *Halocynthia roretzi* (Hoffmann et al. 1992; Yokobori et al. 1999). The gene product, $tRNA^{Met}_{U*AU}$, was demonstrated to possess U*34 (U-derivative) at the anticodon wobble position (Kondow et al. 1998), suggesting that the tRNA decodes both AUA and AUG codons, although the structure of U* is unknown.
4. $tRNA^{Met}_{CAU}$ with an unmodified nucleotide at the 37th position—The starfish *Asterias* mt-$tRNA^{Met}_{CAU}$ possesses an unmodified C34 at the anticodon first position, as do *Drosophila* and *Loligo* mt-$tRNAs^{Met}_{CAU}$ (Y. Ohkubo, S. Matsuyama, Y. Watanabe, S. Yokobori, T. Ueda and K. Watanabe, unpublished result), but the position 37 in the *Asterias* mt-$tRNA^{Met}_{CAU}$ is also not modified, whereas it is in *Drosophila* and *Loligo* mt-$tRNAs^{Met}_{CAU}$. As echinoderm mitochondria use the codon AUA for Ile, this is reasonable; because modified A at the 37th position is absent, the codon-anticodon interaction might not be stabilized, and hence AUA would not be decoded by the *Asterias* mt-$tRNA^{Met}_{CAU}$ as Met.

$tRNA^{Ile}$. All the $tRNAs^{Ile}$ reported from metazoan mitochondria (*Asterias, Homo, Loligo,* and *Drosophila*) have unmodified G34 at the anticodon wobble position (see Tomita et al. 1999a). Since the codon AUA is used for Ile in echinoderm mitochondria, the starfish *Asterias* mt-$tRNA^{Ile}_{GAU}$ should decode AUA, in addition to AUY codons, as Ile, which in principal is possible as explained above. On the other hand, the $tRNA^{Ile}_{GAU}$ in *Homo, Loligo,* and *Drosophila* mitochondria should decode only AUY codons as Ile. This can be accounted for by competition between $tRNA^{Ile}_{GAU}$ and $tRNA^{Met}$ in the decoding of AUA on the ribosome. If a $tRNA^{Met}$ capable of decoding AUA exists (it should be $tRNA^{Met}_{CAU}$ with t^6A at the 37th position, $tRNA^{Met}$ with the anticodon f^5CAU, or $tRNA^{Met}_{U*CU}$; see above), $tRNA^{Ile}_{GAU}$ would loose the competition and the AUA codon would be read as Met.

Reassignment of AUA Codon from Ile to Met, and from Met to Ile. In prokaryotic systems such as *E. coli*, there are two species of $tRNA^{Ile}$ (Osawa et al. 1989): $tRNA^{Ile}_{GAU}$ and $tRNA^{Ile}_{LAU}$ (L stands for lysidine) (Fig. 2B). $tRNA^{Ile}_{LAU}$ is also found in nonmetazoan mitochondria such as those of plants (Weber et al. 1990). Thus, it can be speculated that an ancestor of metazoan mitochondria may have possessed $tRNA^{Ile}_{LAU}$. As pointed out in the section on $tRNA^{Ser}_{GCU}$, unmodified G34 at the anticodon wobble position could basepair with A at the codon third position in the absence of competitor to decode the codon. Therefore, without changing the assignment of AUA from Ile to Met, $tRNA^{Ile}_{LAU}$ could be eliminated from the mt-genome, leaving the AUA-decoding role to $tRNA^{Ile}_{GAU}$, which otherwise decodes only AUY codons (Fig. 2B). In this pathway, the disappearance of AUA followed by its reassignment is not necessary.

The platyhelminth mt-genomes whose complete sequences are known encode both a single $tRNA^{Ile}_{GAT}$ gene and a single $tRNA^{Met}_{CAT}$ gene, as is the case in most metazoan mt-genomes (Le et al. 2000). This suggests that before platyhelminthes were separated from other "higher" triploblastics, mt-$tRNA^{Ile}_{LAU}$ had been lost. On the other hand, the cnidarian mt-genomes so far analyzed do not encode any $tRNA^{Ile}$ genes, whose product $tRNAs^{Ile}$ are capable of decoding the codon AUA, so $tRNAs^{Ile}$ are believed to be imported into mitochondria from the cytoplasm (Beagley et al. 1998; Beaton et al. 1998). In this case, $tRNA^{Ile}_{IAU}$ and $tRNA^{Ile}_{U*AU}$ (U* is unknown, but it should enable $tRNA^{Ile}_{U*AU}$ to decode only the AUA codon) are the possible candidate tRNAs for decoding the codons AUU, AUC, and AUA, because only these two species are known in the eukaryotic cytoplasmic system (see Sprinzl et al. 1998).

Interaction between $tRNA^{Ile}_{GAU}$ and AUA on the ribosome might be more unstable than that between $tRNA^{Ile}_{GAU}$ and AUY codons. Therefore, if $tRNA^{Met}$ acquired the capacity to decode AUA, reassignment of AUA from Ile to Met could easily have occurred.

Three strategies by which $tRNA^{Met}$ can acquire decoding capacity toward the AUA codon were noted above: introduction of f^5C34 or t^6A37, or replacement of C34 by U* (see Fig. 2B). The first two strategies require no change in the system of $tRNA^{Met}$ recognition by methionyl-tRNA synthetase (MetRS). Assignment of the AUA codon to either Ile or Met might be controlled by the presence or absence of a modification in $tRNA^{Met}$, so removal of f^5C34 or t^6A37 from $tRNA^{Met}$ may decrease its affinity toward AUA, and instead the codon will be decoded by $tRNA^{Ile}_{GAU}$. This is what seems to have occurred in echinoderm mitochondria, resulting in the reassignment of AUA to Ile (Fig. 2B). In the third strategy, MetRS might have changed its mechanism for recognizing the cognate tRNA, since the anticodon first position of $tRNA^{Met}$ is known to be a strong identity determinant for MetRS in prokaryotes as well as in the eukaryotic nucleous-cytoplasmic system (Ueda and Watanabe 1993).

Since all the nucleotide modification enzymes and aminoacyl-tRNA synthetases (ARSs) are imported from the cytoplasm, codon reassignment in metazoan mitochondria would have been caused by the co-evolution of tRNA derived from the mt-genome and proteins derived from nuclear genome.

Evolution of AAN Codons in Metazoan Mitochondria

tRNA^{Lys}. Since most metazoan mt-tRNA^{Lys} genes possess the anticodon TTT (e.g., Anderson et al. 1981), if the U at the first position of the anticodon is modified in tRNA^{Lys}, the tRNA might recognize both AAA and AAG codons. Indeed, the anticodon loop sequence of bovine mt-tRNA^{Lys} has been reported to be 5′-CUU*UUA*A-3′ (the anticodon is underlined) (Roe et al. 1982).

Though the codon AAA is read as Lys in arthropod mitochondria, the mt-tRNA^{Lys} genes of dipteran insects (*Drosophila*) have been found to possess the anticodon CTT (Clary and Wolstenholme 1985). Analysis of the *Drosophila* mt-tRNA^{Lys} at the RNA level showed that it possesses C34 and t^{6}A37 (Tomita et al. 1999a). As this is also the case in tRNA$^{Met}_{CAU}$ of *Loligo* and *Drosophila* mitochondria (Tomita et al. 1999a), the *Drosophila* mt-tRNA$^{Lys}_{CUU}$ should also recognize the AAA codon in addition to AAG.

tRNA^{Asn}. Q (queuosine) or its derivatives is mostly found at the anticodon first position of tRNA^{Tyr}, tRNA^{His}, tRNA^{Asn}, and tRNA^{Asp}, although the modification is incomplete (e.g. Tomita et al. 1999a). *Drosophila* mt-tRNA^{Tyr}, *Drosophila* mt-tRNA^{His}, and opossum *Didelphis virginiana* mt-tRNA^{Asp} (edited version) have been demonstrated to possess Q34 (or its derivatives) at the anticodon first position (Tomita et al. 1999b; Mörl et al. 1995). Modification of G34 to Q is known to restrict the ability of tRNA to decode NAY codons (Morris et al. 1999), which means that Q34 prevents basepairing with A at the codon third position. The minimum sequence for the Q-inserting enzyme (TGT: tRNA-guanine transglycosylase) in *E. coli* is known to be U33-G34-U35 (Nakanishi et al. 1994), so the two U's on either side of G34 should be determinants for TGT. This is known to hold for a mt case (Mörl et al. 1995).

The *Asterias* mt-tRNA^{Asn} possesses C33 instead of U33 and mostly unmodified G34 (Tomita et al. 1999b), suggesting the possibility that a tRNA not possessing Q34 is potentially capable of decoding the codon AAA. In addition, U35 at the anticodon second position is modified to Ψ (pseudouridine), which is known to strengthen codon-anticodon interaction. In fact, eukaryotic cytoplasmic tRNA^{Tyr} with the anticodon GΨA has suppressor activity toward UAR codons, although those with the anticodon QΨA or GUA do not (e.g., Johnson and Abelson 1983; Zerfass and Beier 1992). The presence of Ψ35 might help the tRNA in decoding AAA codon by intensifying codon-anticodon interaction.

Reassignment of AAA Codon from Lys to Asn. It is noteworthy that tRNA$^{Lys}_{CUU}$ in *Asterias* mitochondria possesses C34 and t^{6}A37, which could be potential effectors for the tRNA to decode AAA, although the codon is read as Asn in *Asterias* mitochondria. The Ψ35 modification could be the crucial effector that gives a decision in favor of tRNA^{Asn} over tRNA$^{Lys}_{CUU}$ in their competition to decode the AAA codon in *Asterias* mitochondria (Fig. 2C).

The AAA codon is not found in mitochondria of the hemichordate *Balanoglossus* (Castresana et al. 1998a, 1998b). As the *Balanoglossus* mt-tRNA^{Asn} gene has the sequence T33-G34-T35, recognized by TGT (Castresana et al. 1998a, 1998b), the *Balanoglossus* mt-tRNA^{Asn} is likely to possess the anticodon QUU, although the modification may not be complete. This observation strongly suggests that tRNA^{Asn} preferentially decodes AAY, but not AAA, as Asn.

In the case of rhobditophoran platyhelminth mitochondria, AAA is thought to specify Asn rather than Lys (Ohama et al. 1990; Bessho et al. 1992; Telford et al. 2000). Several complete sequences of these mt-genomes are now available (Le et al. 1999). The anticodon loop sequence of the mt-tRNAs^{Asn} of *Fasciola hepatica* (Le et al. 1999), *Echinococcus multilocularis* (genbank/EMBL/DDBJ accession number AB018440), and *Schistosoma mekongi* (Le et al. 1999) is 5′-CTGTTAA-3′, suggesting that these tRNAs can be substrates for TGT. There is likely to be some device to prevent Q modification in these tRNAs^{Asn}, because they probably decode the AAA codon as a result of winning the competition with the mt-tRNAs^{Lys}, which possess the anticodon sequence 5′-TTCTTAC-3′ in *Fasciola* and *Echinococcus* and 5′-CTCTTAA-3′ in *Schistosoma.* Since the unusual specification of AAA as Asn appeared in the rhobditophoran platyhelminth and echinoderm lines independently, it is still uncertain how rhobditphoran platyhelminth mt-tRNAs^{Asn} recognize the AAA codon.

Non-Metazoan mt-Genetic Code

Genetic code variations have been reported not only in metazoan mitochondria but also in various non-metazoan mitochondria. The most recent status of non-metazoan mt-genetic code variations is shown in Table 3. In many non-metazoan mitochondria, several tRNAs are assumed to be imported from the cytoplasm, which may make analyzing the genetic code in terms of characteristic tRNA features much more difficult.

Reassignment of Termination Codons to Sense Codons. Most codon assignment variations pertain to termination codons (UAA, UAG, and UGA) (Osawa 1995). The origin of mitochondria is thought to be an aerobic eubacterium (see Andersson et al. 1998). The present eubacteria possess two release factors, RF1 and RF2, which recognize the codons UAA/UAG and UAA/UGA, respectively (Nakamura and Ito 1998). A similar situation is likely to have existed in the mitochondria of a

Table 3. Variations in genetic codes among various non-metazoan mitochondria

Unusual genetic code reported	Organisms analyzed
UGA = Trp[a,b]	Ascomycete fungi (*Saccharomyces cervesiae, Schizosaccharomyces pombe, Neurospora crassa, Aspergllius nidulans*), amoebids (*Acanthamoeba castellanii*), kinetoplastids (*Leishmania tarentolae*), ciliates (*Paramecium aurelia, Tetrahymena pyriformis*), bicosoecids (*Cafeteria roenbergnsis*), rhodophytes (*Chondrus crispus, Phorphyra purpurea*), chlorophytes (pedinophyceans—*Pedinomonas minor*), choanoflagellates (*Monosiga brevicollis*)
UAG = Leu[c,d,e,f]	Chlorophytes (chlorophyceaens—*Scendesmus obliquus, Scendesmus quadricauda, Coelastrum microporum*), chytridiomycete fungus (*Spizellomyces punctatus*)
UAG = Ala[c]	Chlorophytes (chlorophyceaens—*Hydrodictyon reticulatum, Pediastrum boryanum, Tetraedron bitridens*)
UCA = Termination[d,e]	*Scendesmus obliquus*
CUN = Thr[a]	*Saccharomyces cervesiae*
AUA = Met[a]	*Saccharomyces cervesiae*

[a] Osawa et al. (1992). [b]Gray et al. (1998). [c]Hayashi-Ishimaru et al. (1996). [d]Kuck et al. (2000). [e]Nedelcu et al. (2000). [f]Laforest et al. (1997).

common ancestor of eukaryotes. The fact that the UAA codon can be recognized by both factors suggests that UAA is less changeable than UAG and UGA. Either of the latter codons could become unassigned if one of the factors were deleted, without changing the UAA codon. An AT-rich mt-genome prefers the UAA codon to UAG or UGA, and UAA can occur frequently even in noncoding and the spacer regions. These facts suggest that a situation in which UAG and UGA codons hardly existed could have arisen during the evolution of mt-genomes.

Since UGA is captured by $tRNA^{Trp}$ in various mitochondria from different taxa (see Table 3), the reassignment of UGA from a termination codon to one specifying Trp might have occurred independently in the different lines, as has already been discussed. In the cases of mitochondria of the chlorophyceaens *Scenedesmus obliquus, S. quadricauda,* and *Coelastrum microporum,* and that of the chytridiomycete fungus *Spizellomyces punctatus,* the UAG codon is captured by $tRNA^{Leu}$ (anticodon CUA), which might be derived from $tRNA^{Leu}_{CAA}$ (Hayashi-Ishimaru et al. 1996; Kuck et al. 2000; Nedelcu et al. 2000; Laforest et al. 1997) (Table 3). Leucyl-tRNA synthetase (LeuRS) has been shown to use no anticodon for its recognition of $tRNA^{Leu}$ (Asahara et al. 1993), which means $tRNA^{Leu}_{CUA}$ can be recognized by LeuRS. In mitochondria of three chlorophyceaens that have been analyzed (*Hydrodictyon reticulatum, Pediastrum boryanum* and *Tetraedron bitridens*), the codon UAG is used for Ala (Hayashi-Ishimaru et al. 1996). It is well known that alanyl-tRNA synthetase (AlaRS) preferentially recognizes the G3-U70 pair in the acceptor stem, ignoring the anticodon (McClain and Foss 1988; Francklyn and Schimmel 1989; Tamura et al. 1991), so a $tRNA^{Ala}$ capable of decoding the codon UAG could have been derived from the normal $tRNA^{Ala}$ (anticodon UGC).

Reassignment of CUN Codons from Leu to Thr in Saccharomyces. Two types of mt-threonyl-tRNA synthetase (mt-ThrRS) are found in *Saccharomyces cervesiae* mitochondria (Pape et al. 1985), which use CUN codons as Thr, in addition to ACN codons (Table 3). One ThrRS is for the usual $tRNA^{Thr}$ corresponding to ACN codons; the other is for an unusual $tRNA^{Thr}$ corresponding to CUN codons. LeuRS is known not to recognize the anticodon sequence of $tRNA^{Leu}$, which is classified as a class II tRNA having an extremely large variable arm in a non-metazoan mt-system (Asahara et al. 1993). The long variable arm of the non-metazoan mt-$tRNA^{Leu}$ is a major identity determinant for LeuRS (Asahara et al. 1993). Cytoplasmic LeuRS of *Saccharomyces* was recently reported to recognize the anticodon loop of the cognate tRNA (Soma et al. 1996). However, the mt-LeuRS of *Saccharomyces* is encoded by a different gene from the cytoplasmic counterpart (Tzagoloff et al. 1988), so the *Saccharomyces* mt-LeuRS might recognize the long variable arm of cognate $tRNA^{Leu}$ as in most genetic systems.

From a comparison of primary sequences, it has been inferred that the $tRNA^{Thr}$ for CUN codons could have originated from the $tRNA^{Leu}$ for CUN codons rather than from the $tRNA^{Thr}$ for ACN codons (Sibler et al. 1986). If this were the case, truncation of the variable arm of the $tRNA^{Leu}$ for CUN codons should have occurred prior to the change of identity. However, $tRNA^{Leu}$ with a truncated variable arm might not be a good substrate for LeuRS. Hence, such a tRNA might have acquired the ability to be charged with ThrRS rather than LeuRS. In this pathway, complete loss of CUN codons from the genome is necessary.

If the $tRNA^{Thr}$ for CUN codons were created from the $tRNA^{Thr}$ for ACN codons through gene duplication, no ARS might recognize the tRNA, because the $tRNA^{Thr}$ for CUN might not have retained sufficient identity determinants to be recognized by either LeuRS or ThrRS. Duplication of the mt-ThrRS gene, followed by mutations in one of the duplicates to enable the $tRNA^{Thr}$ to recognize CUN codons, would have been needed for the reassignment of CUN codons from Leu to Thr in *Saccharomyces* mitochondria, in addition to the disappearance of mt-$tRNA^{Leu}$ for CUN codons.

Reassignment of AUA Codon from Ile to Met in Ascomycetes. In the case of the reassignment of the AUA codon from Ile to Met in mitochondria of ascomycete fungi, such as *S. cerevisiae* (Table 3), a codon reassignment pathway similar to that of metazoan mitochondria can be assumed. The $tRNA^{Ile}$ specified to decode AUA is absent in these mitochondria. However, the elongator mt-$tRNA^{Met}$ has been reported to have unmodified C34 but t^6A37 (Sibler et al. 1985), which means it is possible for the AUA codon to be decoded as Met.

Reassignment of UCA from Ser to Termination in Scenedesmus. From sequence analysis evidence, in the mitochondria of the chlorophycean *Scenedesmus obliquus*, UCA is assumed to be a termination codon rather than a codon for Ser (Kuck et al. 2000) (Table 3). The *S. obliquus* mt-genome encodes no tRNA gene capable of decoding UCA and UCG. However, there has been no report of an RF capable of recognizing the UCA codon.

How Could Codon Reassignment Occur in the mt-System?

In metazoan mitochondria, genetic code variations are seen in UGA, AGN, AUN, and AAN codons. Reassignment of UGA from a termination codon to one coding Trp is well accounted for by the codon capture theory, with the main driving force being AT pressure (Osawa and Jukes 1989).

Our analysis of metazoan mt-genetic systems has revealed that several unconventional basepairings between the anticodon wobble position of tRNA (N34) and the third position of the corresponding codon (N′3) can exist on the ribosome: G34–A′3, m^7G34–A′3, m^7G34–G′3, and C34–A′3 if t^6A37 is present in the tRNA (Table 1). These basepairings are, however, easily destroyed when either a tRNA possessing U*34 or a release factor occurs in the system as a competitor. Codon reassignment pertaining to AGN, AUN, and AAN can be well explained by this new wobble rule without considering whether the codon to be reassigned was completely lost from the coding frame. In this sense, our proposition extends the codon capture theory.

UAG is read as Leu and Ala in the mitochondria of several chlorophyceaen algae species (Hayashi-Ishimaru et al. 1996). Both LeuRS and AlaRS might not recognize the anticodon sequences of cognate tRNAs. In this case, since the change of anticodon is not restricted by ARS recognition, $tRNA^{Leu}$ and $tRNA^{Ala}$ can change their codon affinity as a result of the anticodon change. If this is so, $tRNA^{His}$ and $tRNA^{Ser}$ would be possible candidates for the tRNA recognizing the UAG codon. If the cognate ARS recognizes the anticodon loop region of the tRNA, its codon specificity cannot be changed by the change in the anticodon sequence. Only the appearance of a new ARS that can recognize the mutant tRNA will enable the genetic code to change—as is seen the case of CUN codons specifying Thr in *S. cerevisiae* mitochondria (Pape et al. 1985).

In conclusion, the genetic code is changeable, but the characteristic features of tRNA such as the anticodon sequence, modified nucleotides, and identity determinants for ARS, restrict the manner in which the code can change. Because of their simplicity, metazoan mt-genetic systems will be useful for further studies aimed at elucidating the codon reassignment mechanism through the characterization of tRNA. In addition, all the genes for nucleotide-modification enzymes and synthetases for mt-tRNAs are encoded on the nuclear genome, so recent progress in whole-genome sequencing projects for metazoans—*C. elegans*, *D. melanogaster*, *H. sapiens*, and others—will likely provide us with the tools for solving the coding problems of various organisms.

Acknowledgments. We thank the late Prof. Thomas H. Jukes and Prof. Shozo Osawa for their valuable suggestions and collaboration. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and by a grant from the Human Frontier Science Program.

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