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Evolution of the Mitochondrial Genetic Code I. Origin of AGR Serine and Stop Codons in Metazoan Mitochondria

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Summary. AGA and AGG (AGR) are arginine codons in the universal genetic code. These codons are read as serine or are used as stop codons in metazoan mitochondria. The arginine residues coded by AGR in yeast or *Trypanosoma* are coded by arginine CGN throughout metazoan mitochondria. AGR serine sites in metazoan mitochondria are occupied mainly in corresponding sites in yeast or *Trypanosoma* mitochondria by UCN serine, AGY serine, or codons for amino acids other than serine or arginine. Based on these observations, we propose the following evolutionary events. AGR codons became unassigned because of deletion of tRNA Arg (UCU) and elimination of AGR codons by conversion to CGN arginine codons. Upon acquisition by serine tRNA of pairing ability with AGR codons, some codons for amino acids other than arginine mutated to AGR, and were captured by anticodon GCU in serine tRNA. During vertebrate mitochondrial evolution, AGR stop codons presumably were created from UAG stop by deletion of the first nucleotide U and by use of R as the third nucleotide that had existed next to the ancestral UAG stop.

Key words: Genetic code — Codon (capture) reassignment — Arginine codons — Serine codons — AGR stop codons — Mitochondria

Introduction

Mitochondria from simpler eukaryotes such as yeasts and protozoans use AGA and AGG as "universal" arginine codons pairing with anticodon UCU in arginine tRNA. Transfer RNA Arg (UCU)[1] is absent from metazoan mitochondria, presumably due to deletion of the gene for this tRNA during reduction of genome size. Because of this, AGA and AGG are no longer arginine codons. They are either read as serine by serine tRNA or are used as stop codons.

In mitochondria from *Ascaris* and echinoderms (nematode, starfish, and sea urchin), AGA and AGG code for serine (Himeno et al. 1987; Wolstenholme et al. 1987; Cantatore et al. 1988; Jacobs et al. 1988). In *Drosophila* mitochondria, AGA codes for serine, whereas AGG is not found (Clary and Wolstenholme 1985). In vertebrate mitochondria, AGA and AGG are not used for an amino acid and in some cases are stop codons (Anderson et al. 1981, 1982; Bibb et al. 1981; Roe et al. 1985). Presumably, AGA and AGG can be used as stop codons throughout vertebrate mitochondria.

The changes in assignments of AGR[2] codons pose two questions:

1) During early evolution of metazoan mitochondria, how did AGR become assigned to serine in place of arginine?

2) How did AGR become stop codons in vertebrate mitochondria?

AGR Codon Capture by Serine during Mitochondrial Evolution

Recently, we have proposed the codon capture hypothesis stating that the amino acid assignment for a codon may change during evolution (Osawa and

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[1] Trinucleotides in parentheses represent the anticodon for tRNA throughout this paper

[2] *Abbreviations:* R = A or G; Y = U(T) or C; N = A, G, U(T) or C

Table 1. Replacement of AGR arginine or serine sites in mitochondrial genes

	AGR Arg	CGN Arg	AGN Ser	AAA Lys	Others	NC
a) 22 arginine AGR sites of yeast						
Trypanosoma	9	1	0	1	1	10
Starfish	0	7	0	2	2	12
Drosophila	0	18	0	2	2	0
Xenopus	0	16	0	4	2	0
Mouse	0	17	0	4	1	0
Rat	0	17	0	4	1	0
Bovine	0	17	0	4	1	0
Human	0	16	0	4	2	0
b) 13 arginine AGR sites of *Trypanosoma*						
Yeast	8	0	0	1	0	4
Starfish	0	3	0	0	0	10
Drosophila	0	11	0	1	0	1
Xenopus	0	11	0	2	0	0
Mouse	0	11	0	2	0	0
Rat	0	11	0	2	0	0
Bovine	0	11	0	2	0	0
Human	0	11	0	2	0	0

	AGR Arg	CGN Arg	AGR Ser	AGY Ser	UCN Ser	ACN Thr	AAA Lys	GGN Gly	AUA Met	CCN Pro	Others	NC
c) 28 serine AGR sites of starfish												
Yeast	0	0	0	1	0	0	0	0	0	0	2	25
Trypanosoma	0	0	0	0	1	0	1	0	0	0	4	22
Drosophila	0	0	1	2	2	3	5	1	1	0	12	0
Xenopus	0	0	0	7	2	5	1	3	1	0	8	1
Mouse	0	0	0	5	3	3	1	3	3	0	9	1
Rat	0	0	0	5	3	3	1	3	3	0	9	1
Bovine	0	0	0	5	2	4	2	3	2	0	9	1
Human	0	0	0	5	1	5	2	2	1	0	11	1
d) 39 serine AGA sites of *Drosophila*												
Yeast	0	0	0	1	7	1	0	0	0	0	2	28
Trypanosoma	0	0	0	2	3	0	2	0	0	0	6	26
Starfish	0	0	1	2	9	1	1	0	0	0	5	20
Xenopus	0	1	0	7	13	3	2	2	0	3	7	1
Mouse	0	1	0	8	8	5	3	2	0	1	10	1
Rat	0	1	0	8	8	5	3	2	0	1	10	1
Bovine	0	1	0	8	9	5	1	2	1	2	9	1
Human	0	2	0	8	8	3	2	1	0	2	12	1

The DNA sequences of several mitochondrial genes (COI, COII, Cyt b, ATPase 6, ATPase 8, ND3, ND5, and ND4) from *Saccharomyces cerevisiae* (yeast), *Trypanosoma brucei* (protozoan), *Asterina pectinifera* (starfish), *Drosophila yakuba, Xenopus laevis,* mouse, rat, bovine, and human were aligned by the maximum matching method according to DNAS program. NC: Corresponding sites not available due to lack of DNA sequences or doubtful alignment by the codon positions in very poorly conserved regions. Source of DNA sequences: Yeast: Coruzzi and Tzagoloff (1979), Bonitz et al. (1980), Nobrega and Tzagoloff (1980); *Trypanosoma*: Hensgens et al. (1984); starfish: Himeno et al. (1987) and unpublished data by Asakawa et al.; *Drosophila*: Clary and Wolstenholme (1985), Roe et al. (1985); mouse: Bibb et al. (1981); rat: Grosskopf and Feldmann (1981); bovine: Anderson et al. (1982); human: Anderson et al. (1981)

Jukes 1989). A codon may become unassigned by its disappearance from reading frames and by disappearance of the corresponding tRNA, carrying the anticodon. Later, the codon reappears together with a tRNA carrying an anticodon that will translate it. The new tRNA may be for the same amino acid, but sometimes for a different amino acid. The net result is that the codon may be reassigned or captured without change in the amino acid sequence of proteins.

The essence of this scheme is in the following example taken from *Mycoplasma* (Jukes et al. 1987): AT pressure changes stop codon UGA to UAA, so that UAA and UAG become the only stop codons, but the locations of stop codons remain unchanged. Trp tRNA duplicates, and the anticodon of one duplicate, under AT pressure, becomes UCA that can pair with codons UGA and UGG by wobble pairing. Next, some tryptophan codons mutate under AT pressure to UGA that is translated by anticodon

UCA at tryptophan locations without changes in amino acid sequences. Most of these events can be explained by directional mutation pressure (AT/GC pressure). Because the genome of mitochondria is much smaller than that of complete organisms, it is possible that the mitochondria can tolerate changes in the code with accompanying changes in amino acid sequences of proteins.

There exist two possibilities in the reassignment of AGR codons from arginine to serine and stop during mitochondrial evolution, with or without accompanying changes in the protein sequences. Clary and Wolstenholme (1983, 1985) and de Bruijn (1983) pointed out that none of the *Drosophila* serine AGA codons correspond in their locations to the arginine AGR codons in the homologous mitochondrial genes of yeast, suggesting strongly that direct replacement of arginine sites by serine that leads to changes in amino acid sequence of proteins did not occur during evolution. Since then, the sequences of mitochondrial genes from additional organisms have become available, and we have reexamined the pattern of replacement for AGR codons in the genes of various mitochondria.

AGA is the most abundantly used arginine codon in mitochondria of simpler eukaryotes, presumably as a result of high AT pressure that is reflected in their high genomic AT content. For example, the amino acid sequences deduced from several genes from mitochondria of the yeast, *Saccharomyces cerevisiae,* show that 86% of arginine residues are coded by AGA, 4% by AGG, and 10% by CGN (quoted in Sibler et al. 1986). To show the pattern of replacement of these arginine sites among various mitochondria, the AGR arginine sites in several genes of mitochondria from *S. cerevisiae* and a protozoan (*Trypanosoma*) were compared with the homologous sites of the corresponding mitochondrial genes in starfish, *Drosophila,* and five vertebrate species (Table 1a and b). Most of the arginine (AGR) residues in yeast or *Trypanosoma* mitochondria are replaced by CGN arginine codons throughout metazoan mitochondria, in which AGR codons are used as either serine or stop codons, suggesting that arginine at these sites is functionally important. Only a few arginine (AGR) residues in yeast or *Trypanosoma* are not conserved in metazoan mitochondria, having been replaced by amino acids such as lysine (AAA) or others, but not by serine. Thus, no direct replacements of AGR arginine by AGR serine were revealed in the genes examined.

This conclusion is in agreement with that of Clary and Wolstenholme (1983, 1985) and de Bruijn (1983) and was strengthened by comparing AGR serine sites in starfish or *Drosophila* mitochondrial genes with the corresponding sites in those from yeast or *Trypanosoma,* revealing that AGR serine sites in metazoan mitochondria originally were occupied largely by UCN serine, AGY serine, or codons for amino acids other than arginine (Table 1c and d).

In metazoan mitochondria, tRNA Arg (UCU) for codons AGR is absent; this tRNA would have been deleted by reduction of genome size during evolution. The complete replacement of AGR by mostly CGN arginine and by a few other codons in metazoan mitochondria suggests that AGR codons were converted mainly to CGN (a silent change) upon deletion of tRNA Arg (UCU), so that AGR became unassigned.

Let us assume that the metazoan mitochondrial ancestor had a usage of AGR and CGN codons for arginine similar to that in yeast mitochondria (AGR: CGN = 9:1; Sibler et al. 1986) before AGR became unassigned, because genomes from simpler metazoan mitochondria are usually high in AT as are yeast mitochondria. The process that led AGR to become unassigned may be explained using the following equations originally proposed by Sueoka (1962, 1988) for directional mutation pressure.

$$\hat{p} = \frac{v}{u + v} \quad \text{or} \quad \frac{v}{u} = \frac{\hat{p}}{1 - \hat{p}} \tag{1}$$

where v and u were defined as the effective conversion rates of A/T to G/C and G/C to A/T, respectively and $\hat{p}$ as the G+C content at equilibrium (Sueoka 1962). For our specific example, let us define v and u as the effective conversion rates of AGR to CGN, and CGN to AGR, respectively, and $\hat{p}$ as the CGN content at equilibrium ($\hat{p} = 0.1$ in yeast mitochondria). We consider here the directional mutation pressure, D (μ_D of Sueoka 1988) and the change in translational efficiency of tRNA, T (Osawa et al. 1988) as two major factors to influence arginine codon usage. We define $v = D_v \cdot T_v$ and $u = D_u \cdot T_u$. Then Eq. (1) may be expressed as:

$$\hat{p} = \frac{D_v \cdot T_v}{D_v \cdot T_v + D_u \cdot T_u}$$

or

$$\frac{D_v \cdot T_v}{D_u \cdot T_u} = \frac{\hat{p}}{1 - \hat{p}} \tag{2}$$

D_v and D_u are constant unless the genomic GC contents change, whereas T_v and T_u are variable with the change of translation efficiency of codons by tRNA. The general formulas for T_v and T_u are not known, but when tRNA availability is at saturation, T is equal to unity, and may move to zero along with the decrease in tRNA availability. In yeast, AGR is read by major tRNA Arg (UCU), and CGN by minor tRNA Arg (ACG) (Sibler et al. 1986) (usually by UCG in other mitochondria), suggesting that T_u is a little higher than T_v. In the above argument,

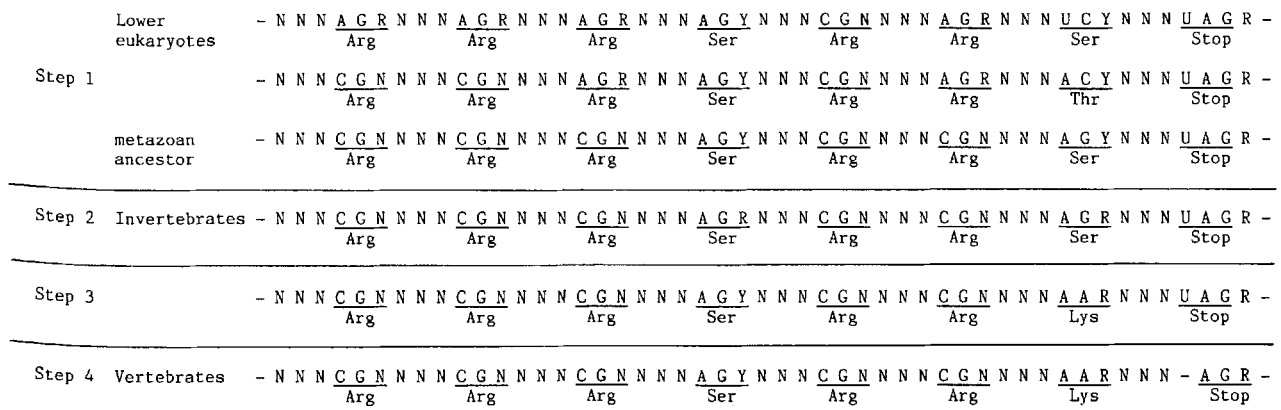

Fig. 1. Evolution of AGR codons in mitochondria. Step 1: Arginine tRNA for AGR codons gradually lost its function and finally disappeared, so that AGR became unassigned, replaced mainly by CGN arginine codons. Step 2: AGR codons were formed from AGY codons and were captured by the altered serine tRNA. Step 3: Serine tRNA gradually lost its function to translate AGR codons, so that AGR became unassigned following mutation to AGY serine or other codons. Step 4: AGR stop codon was formed from UAG stop by deletion of U. The sequences in all the steps are only for illustration.

selective constraints on phenotype have not been treated as a major factor and are considered as neutral.

In the metazoan mitochondrial ancestor, AGR at the important arginine sites mutated even in the presence of AT pressure ($D_u > D_v$) to CGN arginine codons by completely selective constraints ($T_u = 0$), because conversion of AGR to CGN was the only way to conserve these arginine residues. However, the removal of tRNA Arg (UCU) would not have occurred by one-step deletion. If it had occurred, predominant AGR codons would become untranslatable, which certainly would be deleterious. What probably happened would be that tRNA Arg (UCU) gradually lost pairing ability with AGR codons ($T_u \rightarrow 0$), so that u decreased. Finally, the gene for this tRNA disappeared ($T_u = 0$), so that no more conversion of CGN to AGR could occur ($u = 0$). Gradual disappearance of a tRNA has been reported in one of the trytophan tRNAs in *Mycoplasma* (Yamao et al. 1988) and one of the proline tRNAs in liverwort chloroplast (Umesono and Ozeki 1987). By this time, all AGR codons disappeared from reading frames, by conversion mainly to CGN ($\hat{p}$ becomes 1), and the tRNA for CGN codons presumably would have increased in amount adaptively. In this way, AGR became, without disruption, an unassigned codon pair available for subsequent capture by an amino acid (step 1 in Fig. 1).

Following this, the structure of tRNA Ser (GCU), pairing primarily with AGY codons, was modified, so that the altered serine tRNA translated AGR codons (see below). AGR (mainly AGA) codons, pairing with this serine tRNA, then appeared in the reading frames by mutations of AGY serine codons and other codons. AGR codons were thus captured by serine (step 2 in Fig. 1).

The AGR codons that have been captured by serine are translated either by tRNA Ser (UCU) (in *Ascaris*) or by tRNA Ser (GCU) (in other invertebrates). These tRNAs have undergone structural changes, bringing about the observed G:R pairing between anticodon GCU and codons AGR (Watanabe 1986; Himeno et al. 1987). The anticodon UCU in *Ascaris* mitochondria would have reappeared with a new assignment by mutation of anticodon GCU under AT pressure (Osawa and Jukes 1989) and seems to translate all AGN codons as serine by four-way wobbling.

Removal of AGR Serine Codons from Reading Frames

The two "new" serine codons AGG and AGA should have been removed from reading frames in messenger RNA, before these codons became stop codons, because appearance of stop codons at serine sites in reading frames results in cessation of translation, which is deleterious. As noted above, AGG codons are not found in *Drosophila* mitochondria, but we do not know whether AGG can be used as a stop codon in mitochondria of other insects.

Shortly before, or in the early phase of vertebrate mitochondrial evolution, tRNA Ser (GCU) underwent further changes leading to abolishment of pairing ability with codons AGR (Himeno et al. 1987). Table 1 (c and d) shows that all AGR (AGA) codons in *Drosophila* were replaced in vertebrates mainly by serine AGY, serine UCN, lysine AAA, threonine ACN, and others. Presumably, AGR codons were first removed by mutation mainly to AGY or to ACA by strong selective constraints resulting from the loss of AGR translation by anticodon GCU, so that AGR became unassigned codons (step 3 in Fig. 1). AGY and ACA would further mutate to AGG, ACU, and ACC, and thence to UCN. The replacement of AGR by other codons is not unidirectional,

i.e., A/T to G/C or G/C to A/T. It could result from gradually increased GC pressure during vertebrate mitochondrial evolution (Jukes and Bhushan 1986). The unassigned AGA and AGG codons appeared as stop codons in vertebrate mitochondrial genomes.

Origin of AGR Stop Codons in Vertebrate Mitochondria

The simplest way to use unassigned AGR as stop codons would be that AGR appears at the translation-termination site by one-step mutation from the preexisting stop codon. However, the standard mitochondrial stop codons UAA and UAG cannot change to AGR by single nucleotide replacement. Two-step mutations, e.g., UAR → UGR → AGR cause a disappearance of the stop codon at the first mutation step, because UGR is not a stop codon and is read-through as tryptophan until the next stop codon appears, as a result of which a longer peptide is produced. This possibility is not very likely in such an economized system as vertebrate mitochondria.

Four AGR stop codons are known in vertebrate mitochondrial genes; AGA in human COI, AGG in human ND6 (Anderson et al. 1981), AGA in bovine cytochrome b (Anderson et al. 1982), and AGA in *Xenopus* ND6 (Roe et al. 1985). The appearance of these AGR stops is explained in the following way: The ancestor for the gene had used UAG as a stop codon with the sequence for termination region 5′–NNNUAGR–3′. Deletion of U from the UAG stop by one-step mutation resulted in the present sequence 5′–NNNAGR–3′ without disruption of amino acid sequence or of position of the stop codon (step 4 in Fig. 1). This scheme can be applied to all of the known cases as illustrated in Fig. 2. In the human mitochondria COI gene, the third nucleotide A of the AGA stop codon is complementary to the 3′-terminal U of the antisense sequence for serine tRNA gene. Deletion of U from the ancestral UAG stop did not result either in change of amino acid sequence of COI or of the sequence of serine tRNA. In the case of *Xenopus,* another putative protein gene ND5 is encoded within the opposite strand of ND6 and their terminal regions are overlapped. The terminal sequence of ND6 is 5′–UGCGUUAGAA–3′, and the antisense sequence of ND6 is 5′–UUCUAACGCA–3′ where UAA is used as stop codon for ND5. If we assume that the ancestral terminal sequence of ND6 is 5′–UGCGUUUAGAA–3′, from which the present sequence 5′–UGCGUUAGAA–3′ resulted by deletion of U from UAG stop, the ancestral sequence of the antisense strand should be 5′–UUCUAAACGCA–3′. The present antisense sequence 5′–UUCUAACGCA–3′ was derived by deletion of A at the middle position of the ancestral UAA stop. This deletion produced a new stop codon UAA, because the nucleotide next to the ancestral UAA stop is A. Thus, the amino acid sequences as well as the positions of stop codons for both genes were not affected.

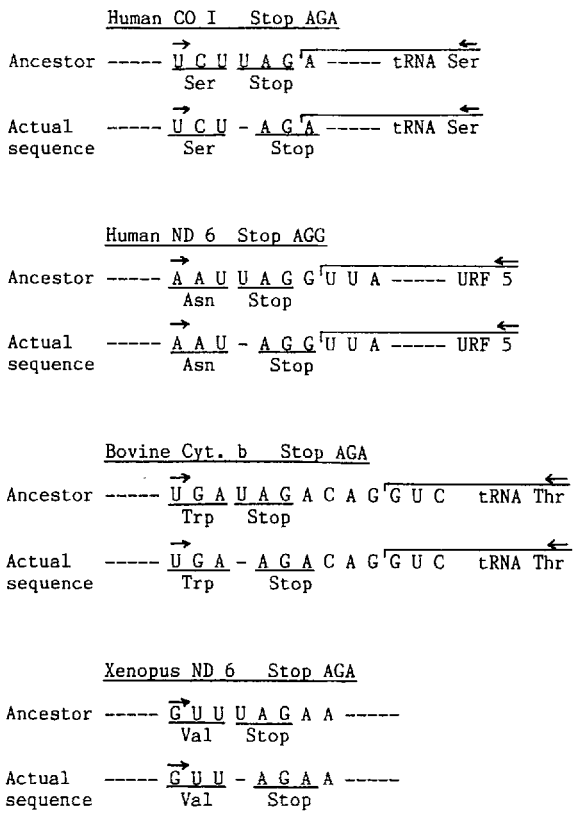

Fig. 2. Possible mechanism for conversion of UAG to AGR stop codons in vertebrate mitochondria. — = Deletion.

Another, but not mutually exclusive, possibility would be as follows. When the ancestral terminal sequence for a given gene was 5′–NNNUGRUAR–3′, or 5′–NNNGGRUAR–3′, where tryptophan (UGR) or glycine (GGR) was functionally dispensable, UGR or GGR could mutate to AGR stop by single mutation. This produced the sequence having double stops as AGRUAR. UAR could then change its sequence, because the second stop UAR was no longer needed and was therefore free to mutate. Conversion of UGR, especially UGA, to AGR could occur, as the tryptophan codon UGA at the terminal region could be a vestigial remnant of the ancestral bacterial double stop UGAUAR and is functionally unimportant.

Conclusion

The change in assignments of AGR codons from arginine to serine or to stop in metazoan mitochon-

dria is neutral in the sense that the change itself did not cause alterations in amino acid sequences of mitochondrial proteins.

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