The presence of pseudouridine in the anticodon alters the genetic code: a possible mechanism for assignment of the AAA lysine codon as asparagine in echinoderm mitochondria

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ABSTRACT

It has been inferred from DNA sequence analyses that in echinoderm mitochondria not only the usual asparagine codons AAU and AAC, but also the usual lysine codon AAA, are translated as asparagine by a single mitochondrial (mt) tRNAAsn with the anticodon GUU. Nucleotide sequencing of starfish mt tRNAAsn revealed that the anticodon is GΨU, U35 at the anticodon second position being modified to pseudouridine (Ψ). In contrast, mt tRNALys, corresponding to another lysine codon, AAG, has the anticodon CUU. mt tRNAs possessing anticodons closely related to that of tRNAAsn, but responsible for decoding only two codons each—tRNAHis, tRNAThr and tRNAArg—were found to possess unmodified U35 in all cases, suggesting the importance of Ψ35 for decoding the three codons. Therefore, the decoding capabilities of two synthetic Escherichia coli tRNAAla variants with the anticodon GΨU or GUU were examined using an E.coli in vitro translation system. Both tRNAs could translate not only AAC and AAU with similar efficiency, but also AAA with an efficiency that was 2-fold higher in the case of tRNAAla GΨU than tRNAAla GUU. These findings imply that Ψ35 of echinoderm mt tRNAAsn actually serves to decode the unusual asparagine codon AAA, resulting in the alteration of the genetic code in echinoderm mitochondria.

INTRODUCTION

Diversification in the genetic code is one of the characteristics of the mitochondrial (mt) gene expression system. Some codons in the organelles are hot spots for genetic code variations (1,2). For example, AUA codes for methionine in most animal mitochondria, but for isoleucine in echinoderm or platyhelminth mitochondria; AGA and AGG are codons for termination, serine and glycine in mitochondria of vertebrates, most invertebrates and tunicates, respectively, instead of the usual arginine; and AAA codes for asparagine in echinoderm and platyhelminth mitochondria, but for lysine in the other animal mitochondria reported so far (reviewed in 1,2).

Mitochondria are generally considered to use a unique codon–anticodon pairing rule that allows unmodified uridine at the anticodon first position to base-pair with all four nucleotides at the third codon position in four-codon boxes (3); in a two-codon set, G forms a base-pair with pyrimidine, and modified uridines such as 5-carboxymethylaminomethyl-uridine can discriminate purines from pyrimidines. Although this rule is applicable to most codon–anticodon pairings of animal mitochondria, the following three exceptional cases have been noted, all of which, interestingly, are related to the above-mentioned hot spots.

The first case is the codon AUA. Adenosine at the third letter of this codon is presumed to be translated by 5-formylcytidine (f5C) at the wobble position of tRNA Met in bovine, nematode and squid mitochondria (4–6). The second case is AGR (R: A or G). In invertebrate mitochondria, serine tRNA with the anticodon GCU (tRNA Ser GCU) is considered to be responsible for decoding AGR in addition to AGY (Y: U or C) as serine. We recently found that 7-methylguanosine (m7G) is located at the wobble position in tRNA Ser m7GCU isolated from starfish (7) and squid mitochondria (8) and proposed that it is responsible for enabling tRNA Ser m7GCU to decode AGR as well as AGY codons. The third case is AAA in echinoderm (9–14) and platyhelminth (15,16) mitochondria, which is regarded as an asparagine codon instead of the usual lysine. Sequence determination of some echinoderm mt genomes followed by comparison of the deduced protein gene sequences with those of other species already elucidated has revealed that AAA is translated as asparagine instead of the usual lysine of the universal genetic code. The gene sequences of the tRNAs responsible for decoding AAN (N: A, G, U, C) codons show that lysine and asparagine tRNAs have the anticodon sequences CUU and GUU, respectively (9–14). Although it is reasonable to assume that lysine tRNA with the anticodon CUU translates only AAG, the problem remains as to how tRNAAsn with the

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antidromon GUU translates AAA in addition to the usual asparagine codons AAC and AAU.

Here, we present a possible explanation for the third of the exceptional cases, outlined above, with respect to echinoderm mitochondria, focusing mainly on the intriguing antidromon sequence of tRNA\textsuperscript{Asn}. Based on the RNA sequences of tRNA\textsuperscript{Asn} and related tRNAs as well as on in vitro translation assays, we propose that the post-transcriptional modification at the antidromon second position of tRNA\textsuperscript{Asn} (Ψ(35)) is involved in decoding the unusual AAA codon as asparagine.

**MATERIALS AND METHODS**

**Chemicals and enzymes**

\[
[\gamma-\text{3}^2\text{P}]\text{ATP} \quad (111 \text{ TBq/mmol}), \ [5',3'-\text{P}]\text{cytidine-3',5'-bisphosphate} \quad (111 \text{ TBq/mmol}) \quad \text{and} \quad [2,3-\text{H}]\text{alanine} \quad (1.85 \text{ TBq/mmol})
\]

were purchased from Amersham. RNase T1 and RNase U2 were from Sigma, RNase PhyM was from Pharmacia, RNase CL3 from Boehringer and Nucleose P1 from Yamasa-Shoyou, Japan. T4 polynucleotide kinase and Escherichia coli A19 alkaline phosphatase were obtained from Toyobo and Takara, Japan, respectively. T4 RNA ligase was from Takara, and streptavidin-agarose from Gibco BRL. Other enzymes were from either Takara or Toyobo. Fully-protected ribonucleoside β-cyanoethyl-phosphoramidites and CPG-packed columns were purchased from Perspective Biosystems. Pseudouridine phosphoramidite was a generous gift from Dr. H. Takaku of the Chiba Institute of Technology.

**Preparation of total tRNAs from starfish ovaries**

Preparation of total tRNAs from starfish (Asterias amurensis) ovaries was carried out as described (17) with a slight modification. For column chromatography, Q-Sepharose (strain A19) ovaries was carried out as described (21) with an additional U at the 3′-end was purchased from Genset, Japan. These 5′- and 3′-half fragments were ligated according to the methods already reported (20) and the resulting full-length tRNA\textsuperscript{Ala} was finally purified by 10% PAGE under denaturing conditions. The counterpart, a 3′-half fragment (C38–A76) of tRNA\textsuperscript{Ala} with an additional U at the 3′-end was purchased from Amersham. RNase T1 and RNase U2 were from Sigma, RNase PhyM was from Pharmacia, RNase CL3 from Boehringer and Nucleose P1 from Yamasa-Shoyou, Japan. T4 polynucleotide kinase and Escherichia coli A19 alkaline phosphatase were obtained from Toyobo and Takara, Japan, respectively. T4 RNA ligase was from Takara, and streptavidin-agarose from Gibco BRL. Other enzymes were from either Takara or Toyobo. Fully-protected ribonucleoside β-cyanoethyl-phosphoramidites and CPG-packed columns were purchased from Perspective Biosystems. Pseudouridine phosphoramidite was a generous gift from Dr. H. Takaku of the Chiba Institute of Technology.

**Purification of tRNAs by the hybridization method**

3′-biotinylated oligonucleotide probes with the following DNA sequences were purchased from Sci-Media, Japan and used for the purification of mt tRNAs: 5′-CTGAGCTGGCAAGTATGTTA TTAGTTTAACTAGTTTCTT-3′ for tRNA\textsuperscript{Asn}, 5′-TTAATGCTTGGCATTA TAAGCTTATCAAG-3′ for tRNA\textsuperscript{Ala}G, 5′-TGGCAAGAAA GGATTAACACTTTTATTTTA-3′ for tRNA\textsuperscript{Ala} A, 5′-GACTATAG ATCAGATTITAAACTGATATAATT-3′ for tRNA\textsuperscript{Ala}S, and 5′-ATCC AGTGTAAATTGTAATTTTACTGTTTCT-3′ for tRNA\textsuperscript{Ala}MP. These probes are complementary to the 30 nt regions at the 3′ or 5′ ends of mt tRNA genes. Individual mt tRNAs were isolated from the tRNA fraction by the solid-phase hybridization method (18–20). The RNA purified by this method was further purified by 12% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions.

**Nucleotide sequence determination of mitochondrial tRNAs**

Purified mt tRNAs were first analyzed by Donis-Keller’s method (21). The sequences of the mt tRNAs isolated as described above were revealed to be identical to the corresponding mt tRNA gene sequences with the exception of certain modifications. The purified mt tRNAs were further analyzed by the method of Kuchino et al. (22) to determine the nucleotide sequences, including those of the modified nucleotides.

**Preparation of synthetic E.coli tRNA\textsuperscript{Ala} variants with antidromon GUU and GPyU**

A synthetic E.coli tRNA\textsuperscript{Ala} (23) variant with the anticodon GPyU (tRNA\textsuperscript{Ala}GPyU) was prepared using microsurgery techniques as follows. A synthetic GPyU-containing fragment (G1–A37) of tRNA\textsuperscript{Ala} with anticodon GPyU was synthesized by an Applied Biosystems 381A DNA/RNA synthesizer and deprotected as described (24). The fragment was purified first by YM-10 A4-P HPLC (25) followed by 10% PAGE under denaturing conditions. The counterpart, a 3′-half fragment (C38–A76) of tRNA\textsuperscript{Ala}GPyU with an additional U at the 3′-end was purchased from Amersham. RNase T1 and RNase U2 were from Sigma, RNase PhyM was from Pharmacia, RNase CL3 from Boehringer and Nucleose P1 from Yamasa-Shoyou, Japan. T4 polynucleotide kinase and Escherichia coli A19 alkaline phosphatase were obtained from Toyobo and Takara, Japan, respectively. T4 RNA ligase was from Takara, and streptavidin-agarose from Gibco BRL. Other enzymes were from either Takara or Toyobo. Fully-protected ribonucleoside β-cyanoethyl-phosphoramidites and CPG-packed columns were purchased from Perspective Biosystems. Pseudouridine phosphoramidite was a generous gift from Dr. H. Takaku of the Chiba Institute of Technology.

**In vitro translation assay using synthetic mRNAs**

Escherichia coli (strain A19) ribosomes (70S) and the S100 fraction were prepared as described (27,28). Escherichia coli alanyl-tRNA synthetase (AlaRS) was partially purified from the S100 fraction by DEAE-Sepharose column chromatography. Each tRNA\textsuperscript{Ala} variant prepared as described above was aminoacylated with [3H]alanine by repeated ethanol precipitation. The mRNAs used in this study (Fig. 3a) were prepared by in vitro transcription. Synthetic DNAs containing a Shine–Dalgarno sequence, an AUG initiation codon, eight repeats of phenylalanine codons (UUU)\textsubscript{8} and three repeats of AAN test codons (AAN)\textsubscript{3} were constructed in a frame downstream of the T7 promoter, which were double-stranded by PCR and then ligated into pUC18. These plasmids were digested with HindIII and subjected to in vitro run-off transcription using T7 RNA polymerase as described above. The mRNAs thus obtained were purified by QIAquick column (Qiagen) according to the manufacturer’s protocol. In vitro cell-free translation was carried out in 55 μl of a solution containing 50 mM HEPES, pH 7.8, 8 mM MgCl\textsubscript{2}, 60 mM NH\textsubscript{4}Cl, 1 mM ATP, 200 μM GTP, 20 μM methionine, 40 μM phenylalanine, 35 μg/ml folic acid, 20 A\textsubscript{260} U/ml 70S ribosomes, 10 A\textsubscript{260} U/ml E.coli tRNA\textsuperscript{Phe} and initiator tRNA\textsuperscript{Met}, 5 mM phosphoenolpyruvate, 30 μg/ml pyruvate kinase, 1/10 vol of 1000 fold, 400 μg/ml mRNA and 40 000 c.p.m. [3H]alanine-tRNA\textsuperscript{Ala} variant. The reaction mixture was incubated at 37°C and a 16 μl aliquot was withdrawn at an appropriate time, followed by the addition of 10 μl 1 N NaOH and incubation at 37°C for 30 min. After TCA precipitation, TCA-insoluble material was collected by filtration on a GF/C filter (Whatman), which was washed twice with cold TCA and once with ethanol, and then dried. Radioactivity on the filter was measured by a scintillation counter.
decoding of tRNA Asn toward the codon AAA arises from the modification (Fig. 1c). Thus, it was speculated that the unusual tRNA Lys from A. amurensis had the CUU anticodon, all the remaining parts of the tRNAs being identical.

Because the Q base has a positive charge, the two mt tRNA anticodons were closely related to that of tRNA Asn (anticodon GUG), tRNA Asp (GUC) and tRNA Tyr (GUU), and their efficiencies toward AAC and AAU were easily separated by PAGE under denaturing conditions. In the case of mt tRNA Tyr, only a single tRNA species with the anticodon GUA was identified.

The nucleotide sequences of these three tRNAs clearly show that U35 is never modified to Ψ, although G34 is partially modified to Q in tRNA His (Fig. 2a) and tRNA Asp (Fig. 2b). Hence, it is most probable that the reason why starfish mt tRNA Asn has a modified U (Ψ) at the anticodon second position is because tRNA Asn is required to translate not only the usual asparagine codons AAU and AAC, but also the lysine codon AAA as asparagine in the starfish mt translation system. By contrast, the other three tRNAs—tRNA His, tRNA Asp and tRNA Tyr—are responsible for translating only two codons, so they do not need the modified U.

In vitro translation assays
To examine the effect of Ψ35 in starfish mt tRNA Asn on AAN codon recognition, we prepared two synthetic E. coli tRNA Ala variants with the anticodons G4 and GUU, respectively, and analyzed their decoding capabilities toward synthetic mRNAs containing AAN test codons in the reading frame using an E. coli in vitro translation system. The results are shown in Figure 3. tRNA Ala G4 and tRNA Ala GUU could efficiently translate both AAC (d) and AAU (e) in the reading frame of the mRNAs (a) in this translation system, although the translational efficiencies seemed to depend to a greater or lesser degree on the codon species; the efficiency of tRNA Ala G4 toward codon AAU was almost 1.5 times higher than that of the others.

RESULTS
Nucleotide sequences of mt tRNA Asn and tRNA Lys
The complete nucleotide sequence of mt tRNA Asn of A. amurensis was determined by a combination of the methods of Donis-Keller (21) and Kuchino et al. (22) and is shown in Figure 1. The anticodon sequence is GGUU, in which U at the second position is post-transcriptionally modified to pseudouridine (Ψ35), while G at the wobble position (G34) remains unmodified (Fig. 1a and b). In the alkaline hydrolysis employed in Donis-Keller’s method, the second nucleotide of the anticodon gave a rather faint band (data not shown), suggesting that U35 is fully modified to Ψ. Unlike the cases of tRNA His and tRNA Asp (see below), neither queosine (Q) nor any of its derivatives were detected at position 34. The corresponding tRNA Asn of A. pectinifera was found to have the same anticodon (G4U3P) as that of A. amurensis mt tRNA Asn (data not shown). The nucleotide sequence of mt tRNA Lys from A. amurensis had the CUU anticodon with no modification (Fig. 1c). Thus, it was speculated that the unusual decoding of tRNA Asn toward the codon AAA arises from the pseudouridylate at the anticodon second position. This possibility was examined in further experiments.

Nucleotide sequences of mt tRNA Tyr, tRNA His and tRNA Asp
mt tRNAs with anticodons closely related to that of tRNA Asn—tRNA His (anticodon GUG), tRNA Asp (GUC) and tRNA Tyr (GUU)—were analyzed (Fig. 2a–c). Unlike tRNA Asn, these three tRNAs are considered to translate only two codons (N′AU and N′AC; N′ = C, G, U). After purification by the hybridization method (18), two electrophoretically distinct tRNA species were identified for both mt tRNA His and tRNA Asp. Sequence analyses of all these species indicated that their heterogeneity arose from the partial modification of G to Q at the wobble position of the anticodon, all the remaining parts of the tRNAs being identical. Because the Q base has a positive charge, the two mt tRNA species for tRNA Asp and those for tRNA His were easily separated by PAGE under denaturing conditions. In the case of mt tRNA Tyr, only a single tRNA species with the anticodon GUA was identified.

Figure 1. (a) Nucleotide sequence of A. amurensis mt tRNA Asn in clover-leaf form. The numbering of each residue conforms to the proposal of Sprinzl et al. (44). (b) Analysis of nucleotides around the anticodon region [from positions 31 to 39 in (a)] of A. amurensis mt tRNA Asn by two-dimensional thin-layer chromatography (22). The solvents used were isobutyric acid/concentrated ammonia/water (66:1:33 v/v/v) for the first dimension, and 2-propanol/HCl/water (70:15:15 v/v/v) for the second dimension. (c) Nucleotide sequence of A. amurensis mt tRNA Lys in clover-leaf form. The A at position 37 is partially modified to ΨA.
**Figure 2.** Nucleotide sequences of *A. amurensis* (a) mt tRNA^{His}GUG, (b) mt tRNA^{Asp}GUC and (c) mt tRNA^{Tyr}GUA in clover leaf form.

**Figure 3.** The mRNA sequence used for translation assays and predicted peptide sequence is shown in (a). *In vitro* translation of AAN test codons by tRNA^{Ala}GUU (■) and tRNA^{Ala}ΨU (●) using AAA (b), AAG (c), AAC (d) and AAU (e) as test codons.

addition, the AAA-dependent incorporation of [3H]alanine by tRNA^{Ala}ΨU and tRNA^{Ala}GUU was inhibited by adding an excess amount of *E. coli* lysyl-tRNA^{Ala}U*UU (U*; 5-methylaminomethyl-2-thiourdidine) (data not shown). In contrast, neither tRNA^{Ala}ΨU nor tRNA^{Ala}GUU could decode the AAG codon (c).

A quantitative comparison of the translational efficiencies of the variants is shown in Figure 4. Taking the translational efficiencies of tRNA^{Ala}ΨU and tRNA^{Ala}GUU toward codon AAC (Fig. 3d) as 1.0 in each case, tRNA^{Ala}ΨU translated codons AAA and AAU about twice as efficiently as tRNA^{Ala}GUU did (compare Fig. 4a and b). These results imply that Ψ35 serves to strengthen the non-Watson–Crick base-pairing between bases at the anticodon first position and at the codon third position (G34:U3 and G34:A3).

**DISCUSSION**

Understanding how the usual lysine codon AAA is decoded as asparagine in starfish mitochondria is a long-standing problem (1,2,11). It has been speculated that the wobble position of tRNA^{Asn} responsible for this unusual decoding might have been modified to inosine or its equivalent (G*) so as to form a base-pair with A in addition to U and C at the third position of the codon (1,2,14). However, our findings clearly refute this speculation. We have shown that it is not the first (wobble) position but the second position of the anticodon of tRNA^{Asn} that is modified (Fig. 1a and b). The tRNA^{Lys} corresponding to another lysine codon, AAG, has the anticodon CUU (Fig. 1c), which is consistent with the wobble rule as it has been established so far:
that unmodified C at the wobble position can recognize only G at the third position of the codon.

From the above, it appeared likely that the modification from U to Ψ at the second position of the anticodon of tRNA^Asn was the key event that enabled tRNA^Asn to be involved in the unusual decoding of the AAU codon as asparagine, in addition to the usual decoding of the AAU and AAC codons. In fact, other relevant tRNAs, whose anticodons are closely related to that of tRNA^Asn but decode only two codons —tRNA^His, tRNA^Asp and tRNA^Tyr—were found to have no Ψ at the anticodon second position (Fig. 2).

To obtain experimental evidence for this hypothesis, we devised a method of utilizing E.coli tRNA^Ala with the anticodon GΨU, and comparing it with tRNA^AlaGUU, in an E.coli in vitro translation system. We chose this option rather than using mt tRNA^Asp in a mt in vitro translation system for the following reasons. (i) We have not yet succeeded in constructing an efficient mt in vitro translation system in which the decoding of any codon can be tested. (ii) It is not so easy to isolate mt tRNA^Asn from starfish in an amount sufficient for translation assays. (iii) Even if synthetic mt tRNA^Asp with the anticodon GΨU or GUU can be prepared using microsurgery techniques, it is very difficult to charge the tRNA with mt asparaginyl-tRNA synthetase (AsnRS) because we have not yet procured active mt AsnRS and it cannot be substituted by the E.coli enzyme. (iv) It is relatively easy to prepare anticodon variants of E.coli tRNA^Ala in which the original anticodon is replaced by either GΨU (for mt tRNA^Asn) or GUU (as a control) by a combination of in vitro transcription using T7 RNA polymerase and microsurgery techniques. Because these tRNAs have the identity determinant (G3–U70) for E.coli AlaRS (29,30), they are easily chargeable with the E.coli enzyme. In fact, both synthetic tRNA^AlaGUU and tRNA^AlaGUU were alanylated as efficiently as synthetic wild-type tRNA^Ala to the almost same extents by partially purified E.coli AlaRS (~800–900 pmol/μg protein).

The experimental results obtained using the E.coli system show that tRNA^AlaGUU decodes the AAA codon more efficiently than tRNA^AlaGUU does (Fig. 3b), thus confirming that Ψ at the second position of the anticodon of tRNA^AlaGUU actually serves to bring about this unusual decoding, although the translational efficiencies are one-half and one-third of those toward AAU and AAC, respectively. Even tRNA^AlaGUU with unmodified Ψ35 appears to have the potential to decode the AAA codon (Fig. 3b).

Since mitochondria do not possess a tRNA^Asp with the anticodon U^ΨUU (U^Ψ; U derivatives) competing with tRNA^AspGΨU in decoding AAA, tRNA^AspGUU should originally have been able to decode the AAA codon by itself. However, its decoding ability may not be sufficient to work in the mt translation system (this remains to be clarified), so that the non-Watson–Crick codon–anticodon interactions would have been strengthened by the modification of Ψ35 to Ψ35 (Fig. 4).

Involvement of Ψ35 in the unusual decoding of certain codons has already been reported: a eukaryotic cytoplasmic tRNA^ Tyr with the anticodon GΨA (tRNA^ TyrGΨA) has suppressor activity toward the stop codons UAA and UAG, in addition to decoding the usual tyrosine codons UAY (33–37). The finding that tRNA^ Tyr having either Ψ34 or Ψ35 in the anticodon (33–37) or GUU (in an in vitro transcript) (37) as an anticodon has no such suppressor activity demonstrates the prerequisites of the unmodified G34 and modified Ψ35 to the G34:A33 (A3 means A at the third position of the codon) or the G34:G35 wobble pairing. This is consistent with our finding that tRNA^AlaGUU decodes the AAA codon more efficiently than tRNA^AlaGUU does (Fig. 3b), and that Q34 is never found in tRNA^AspGΨU responsible for decoding three codons, including the unusual AAA (Fig. 1a), but it is observed to exist partially in tRNA^His and tRNA^Asp, which are known to decode only two codons (Fig. 2a and b).

The role of Ψ35 in such unusual decoding may lie in the stabilization of U35:A33 pairing. However, involvement of Ψ35 in the stabilization of U35:A33 pairing by modifying U35 with Ψ has been reported as the thermostability of a polyA-polyΨ duplex over a polyA-polyU duplex (38), and of tRNA^His from an E.coli hisT mutant (39), although there is a report that the thermostability of the U1/mRNA 5′ splice site duplex does not differ between duplexes containing Ψ and U (40). An alternative possibility is that the intramolecular base-pairing between the N1 imino-hydrogen of Ψ and the phosphate oxygen of the backbone—the 3′ phosphate oxygen of G34 located 5′-adjacent to Ψ35—contributes to the stabilization of the codon–anticodon interaction by stacking or other forces (40,41). Further structural studies on codon–anticodon interactions are necessary to clarify these possibilities.

The presence of unmodified G34 of tRNA^Asn may be related to C33 replacing the usual U33 (14) (Fig. 1a), which seems to serve as an antideterminant for the Ψ-inserting enzymes. The finding that both tRNA^His and tRNA^Asp with the usual U33 possess Q34, although only partially modified, indicates that Q-inserting enzymes, in which tRNA-guanine transglycosidase (TGT) should be the enzyme that catalyzes the first step in the Q-modification pathway, are probably involved in the Q-insertion of mt tRNAs. The recognition site of tRNA by E.coli TGT is known to be the U33–G34–U35 sequence in the anticodon loop (42,43). Assuming that mt TGT is endowed with the same recognition mechanism as that of the E.coli enzyme, C33 of tRNA^Asn may play a role as a negative determinant toward TGT, although at present we have no rational explanation as to why Q is lacking in starfish mt tRNA^ Tyr having the U33–G34–U35 sequence (Fig. 2c).
The presence of C33 has so far been observed only in mt tRNA\textsuperscript{Asn} genes of echinoderms, in which AAA is assigned as an asparagine codon, while U is present in the case of other animal mt tRNA species which utilize AAA as a usual lysine codon (\textit{Drosophila yakuba}) (51), sea urchin (\textit{Strongylocentrotus purpuratus}) (10) and \textit{Pisaster ochraceus} (9) and starfish \textit{(Pisaster ochraceus)} (11), A.pectinifera (14) and \textit{A.amurensis)} (13). All the echinoderm mt tRNA\textsuperscript{Asn} genes (gray background) have C33, S'-adjacent to the anticodons (black background).

![Figure 5.](https://example.com/figure5.png)

Figure 5. Comparison of nucleotide sequences of mt tRNA\textsuperscript{Asn} genes from various organisms: mouse (47), chicken (48), codfish (\textit{Gadus morhua}) (49), fruit fly (\textit{Drosophila yakuba}) (50), blue mussel (\textit{Mytilus edulis}) (51), sea urchin (\textit{Strongylocentrotus purpuratus}) (10) and \textit{Pisaster ochraceus} (9) and starfish \textit{(Pisaster ochraceus)} (11), A.pectinifera (14) and \textit{A.amurensis)} (13). All the echinoderm mt tRNA\textsuperscript{Asn} genes (gray background) have C33, S'-adjacent to the anticodons (black background).

The change of AAA from a lysine to an asparagine codon in echinoderm mitochondria can be explained by the codon capture theory\textsuperscript{(45)}, according to which the following events can be posited to have occurred in mt tRNA\textsuperscript{Asn} during the disappearance of codon AAA in the progenitor of echinoderm mitochondria (Fig. 6). First, T33 mutated to C33 in the mt tRNA\textsuperscript{Asn} gene, as a result of which G34 of mt tRNA\textsuperscript{Asn} was no longer modified to Q,\textsuperscript{(2)} which in turn, enabled the formation of the G34:A3 wobble pairing together with the modification of U35 to W\textsuperscript{35}. On the basis of these speculations, it is possible to hypothesize a possible evolutionary process of the genetic code change in the AAN codon box mediated by the post-transcriptional modification, as illustrated in Figure 6.

![Figure 6.](https://example.com/figure6.png)

Figure 6. A possible scheme for reassigment of the AAA codon from lysine to asparagine in echinoderm mitochondria, as compared with that in other animal mitochondria. Mutation of T33 to C33 in the echinoderm mt tRNA\textsuperscript{Asn} gene 1) and the appearance of W\textsuperscript{35} synthase in echinoderm mitochondria 2) result in the modification of U35 to W\textsuperscript{35}.

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