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) tRNA[Asn] with the anticodon GUU. Nucleotide sequencing of starfish mt tRNA[Asn] revealed that the anticodon is GΨU, U35 at the anticodon second position being modified to pseudouridine (Ψ). In contrast, mt tRNA[Lys], corresponding to another lysine 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]—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* tRNA[Ala] 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 tRNA[Ala]GΨU than tRNA[Ala]GUU. These findings imply that Ψ35 of echinoderm mt tRNA[Asn] 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 (f^{5}C) 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 (m^{7}G) is located at the wobble position in tRNA[Ser]GCU isolated from starfish (7) and squid mitochondria (8) and proposed that it is responsible for enabling tRNA[Ser]GCU 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 tRNA[Asn] with the

anticodon 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 anticodon sequence of tRNA^{Asn}. Based on the RNA sequences of tRNA^{Asn} and related tRNAs as well as on *in vitro* translation assays, we propose that the post-transcriptional modification at the anticodon second position of tRNA^{Asn} ($\Psi35$) is involved in decoding the unusual AAA codon as asparagine.

MATERIALS AND METHODS

Chemicals and enzymes

$[\gamma\text{-}^{32}P]ATP$ (111 TBq/mmol), $[5'\text{-}^{32}P]$cytidine-$3',5'$-bisphosphate (111 TBq/mmol) and $[2,3\text{-}^3H]$alanine (1.85 TBq/mmol) were purchased from Amersham. RNase T1 and RNase U2 were from Sigma, RNase PhyM was from Pharmacia, RNase CL3 from Boehringer and Nuclease P1 from Yamasa-Shoyu, 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* and *Asterina pectinifera*) ovaries was carried out as described (17) with a slight modification. For column chromatography, Q-Sepharose (Pharmacia) was employed instead of DEAE–cellulose and total tRNAs were eluted from the column with a buffer containing 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 0.6 M NaCl.

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′-CTGAGCTGGCAAGTATTG-ATCTTGCTATC-3′ for tRNA^{Asn}, 5′-TTAATGCTTGCCATTA-TAAGCTTATCAAAG-3′ for tRNA^{Lys}, 5′-TGGCAAGAAAA-GGAATTAAACCTTTATTTA-3′ for tRNA^{Tyr}, 5′-GACTATAG-ATCAGATTTTAACTGATAATTT-3′ for tRNA^{His} and 5′-ATCC-AGTGTTATTAGTTTAACTAGTTTCTT-3′ for tRNA^{Asp}. 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^{Ala} variants with anticodons GΨU and GUU

A synthetic *E.coli* tRNA^{Ala} (23) variant with the anticodon GΨU (tRNA^{Ala}GΨU) was prepared using microsurgery techniques as follows. A synthetic 5′-half fragment (G1–A37) of tRNA^{Ala} with anticodon GΨU was synthesized by an Applied Biosystems 381A DNA/RNA synthesizer and deprotected as described (24). The fragment was purified first by YMC-pack C4-Ap HPLC (25) followed by 10% PAGE under denaturing conditions. The counter-part, a synthetic 3′-half fragment (C38–A76) of tRNA^{Ala}GΨU 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^{Ala}GΨU was finally purified by 10% PAGE under denaturing conditions. The sequence of tRNA^{Ala}GΨU thus obtained was confirmed by Donis-Keller's method. A synthetic *E.coli* tRNA^{Ala} variant with the anticodon GUU (tRNA^{Ala}GUU) was prepared by *in vitro* run-off transcription (26) of a chemically synthesized template DNA containing the tRNA^{Ala}GUU gene downstream of the T7 promoter. The tRNA^{Ala}GUU was finally purified by PAGE under denaturing conditions.

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^{Ala} variant prepared as described above was aminoacylated with [^3H]alanine by AlaRS. The alanyl-tRNA^{Ala} variants were separated from free [^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 $(UUU)_8$ and three repeats of AAN test codons $(AAN)_3$ 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 *Hin*dIII and subjected to *in vitro* run-off transcription using T7 RNA polymerase as described above. The mRNAs thus obtained were purified by QIAquick column Tip-500 (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 $MgCl_2$, 60 mM NH_4Cl, 1 mM ATP, 200 μM GTP, 20 μM methionine, 40 μM phenylalanine, 35 μg/ml folic acid, 20 A_{260} U/ml 70S ribosomes, 10 A_{260} U/ml *E.coli* tRNA^{Phe} and initiator tRNA^{Met}, 5 mM phosphoenolpyruvate, 30 μg/ml pyruvate kinase, 1/10 vol of S100 fraction, 400 μg/ml mRNA and 40 000 c.p.m. [^3H]alanyl-tRNA^{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.
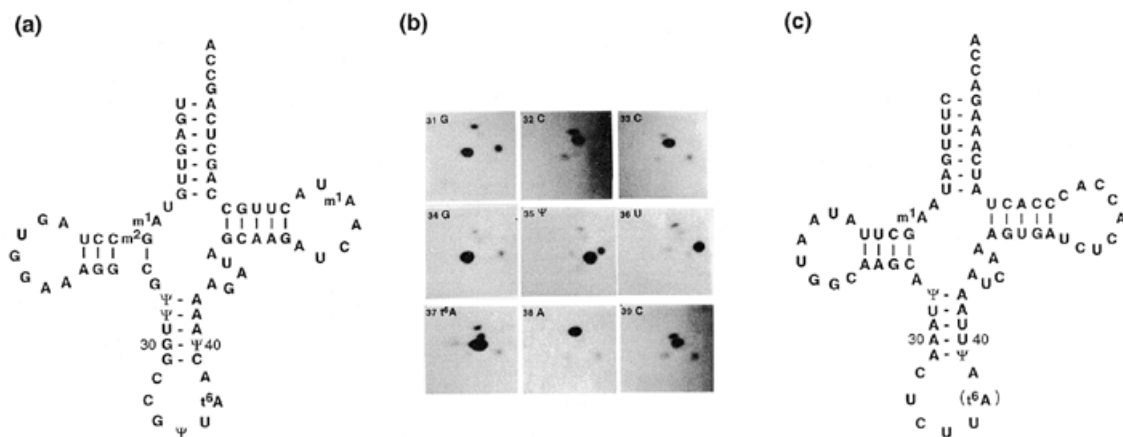
Figure 1. (**a**) Nucleotide sequence of *A.amurensis* mt tRNA^AsnGΨU in clover-leaf form. The numbering of each residue conforms to the proposal of Sprinzl *et al.* (44). (**b**) Analysis of nucleotides around the anticodon region [from positions 31 to 39 in (a)] of *A.amurensis* mt tRNA^AsnGΨU by two-dimensional 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^LysCUU in clover-leaf form. The A at position 37 is partially modified to t⁶A.

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 GΨU, 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 (GΨU) 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 pseudouridylation 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 (GUA)—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.

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 (Ψ35) 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 GΨU (tRNA^AlaGΨU) and GUU (tRNA^AlaGUU), 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^AlaGΨU and tRNA^AlaGUU 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^AlaGΨU toward codon AAU was almost 1.5 times higher than that of tRNA^AlaGUU (e), while their efficiencies toward AAC were reversed (d). It was found that the two tRNAs could translate the AAA codon to an appreciable extent, as shown in (b), although the translational efficiency was apparently lower than those toward AAC and AAU. It should be noted that tRNA^AlaGΨU could translate AAA ~2-fold more efficiently than tRNA^AlaGUU. In
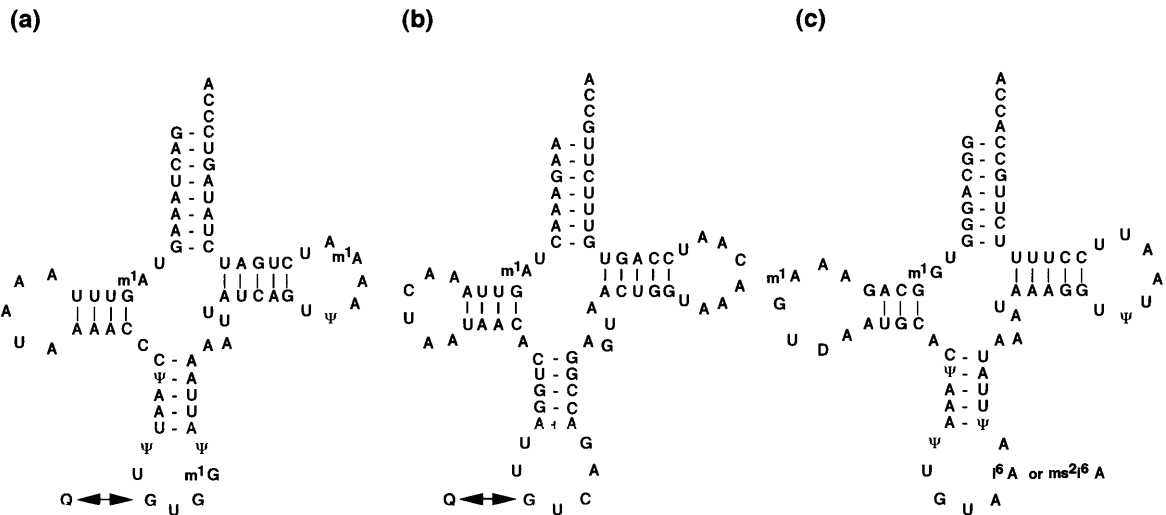
Figure 2. Nucleotide sequences of *A.amurensis* (**a**) mt tRNA[His]G/QUG, (**b**) mt tRNA[Asp]G/QUC and (**c**) mt tRNA[Tyr]GUA in clover leaf form.
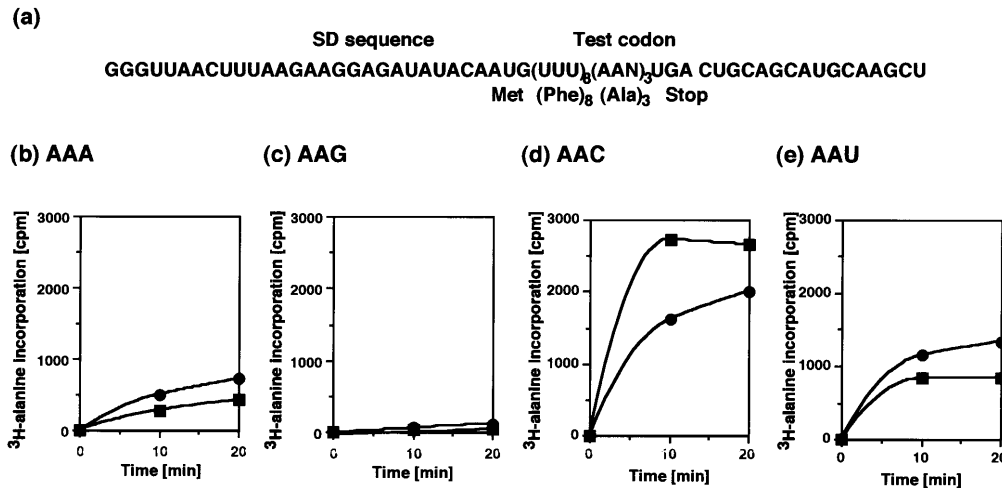

Figure 3. The mRNA sequence used for translation assays and predicted peptide sequence is shown in (**a**). *In vitro* translation of AAN test codons by tRNA[Ala]GUU (■) and tRNA[Ala]GΨU (●) using AAA (**b**), AAG (**c**), AAC (**d**) and AAU (**e**) as test codons.

addition, the AAA-dependent incorporation of [³H]alanine by tRNA[Ala]GΨU and tRNA[Ala]GUU was inhibited by adding an excess amount of *E.coli* lysyl-tRNA[Lys]U*UU (U*; 5-methylamino-methyl-2-thiouridine) (data not shown). In contrast, neither tRNA-[Ala]GΨU 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]GΨU and tRNA[Ala]GUU toward codon AAC (Fig. 3d) as 1.0 in each case, tRNA[Ala]GΨU 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:

(a) tRNA^{Ala}GUU
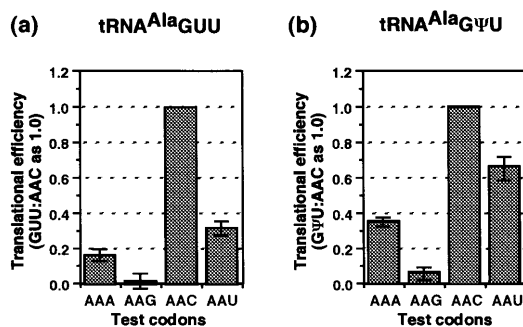
(b) tRNA^{Ala}GΨU

Figure 4. Summary of translational efficiencies using tRNA^{Ala}GUU (**a**) and tRNA^{Ala}GΨU (**b**). The efficiency using tRNA^{Ala}GUU and tRNA^{Ala}GΨU toward codon AAC was taken as 1.0. Each set of data represents the average of three independent experiments, with bars showing the SD.

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 AAA 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^{Ala}GUU, in an *E.coli in vitro* translation system. We chose this option rather than using mt tRNA^{Asn} 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^{Asn} 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^{Ala}GΨU and tRNA^{Ala}GUU were alanylated as efficiently as synthetic wild-type tRNA^{Ala} to the almost same extents by partially purified *E.coli* AlaRS (~800–900 pmol/A$_{260}$ unit). (v) We have recently confirmed in an *in vitro* translation assay of mt tRNA^{Met} toward the synthetic mRNAs (AUG)$_6$ and (AUA)$_6$ (Takemoto *et al.*, in preparation) that ribosomes are exchangeable between *E.coli* and mitochondria. Since it is already known that *E.coli* tRNA can work in mt ribosomes (31,32), it is reasonable to assume that codon–anticodon interactions in mitochondria can be examined using *E.coli* ribosomes and tRNAs whose anticodon is properly modified.

The experimental results obtained using the *E.coli* system show that tRNA^{Ala}GΨU decodes the AAA codon more efficiently than tRNA^{Ala}GUU does (Fig. 3b), thus confirming that Ψ at the second position of the anticodon of tRNA^{Ala}GΨU 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^{Ala}GUU with unmodified U35 appears to have the potential to decode the AAA codon (Fig. 3b). Since mitochondria do not possess a tRNA^{Lys} with the anticodon U*UU (U*; U derivatives) competing with tRNA^{Asn}GΨU in decoding AAA, tRNA^{Asn}GUU 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 U35 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^{Tyr}GΨA) 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 QΨA (in wheat germ) (36,37) or GUA (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:A3 (A3 means A at the third position of the codon) or the G34:G3 wobble pairing. This is consistent with our finding that tRNA^{Ala}GΨU decodes the AAA codon more efficiently than tRNA^{Ala}GUU does (Fig. 3b), and that Q34 is never found in tRNA^{Asn}GΨU 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:A2 pairing by modifying U35 with Ψ (37), as reported for the thermostability of a polyA:polyΨ duplex over a polyA:polyU duplex (38), and of tRNA^{Phe} from an *E.coli his*T mutant (39), although there is a report that the thermostability of a U1/mRNA 5′ splice site duplex does not differ between duplexes containing Ψ and U (40). An alternative possibility is that the intramolecular water bridge 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 interaction 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 Q-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).

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                       anticodon
                  stem  loop  stem
                  #####
                              #####
        Mouse      TTTAG CTGTTAA CTAAA
        Chicken    TTTAG CTGTTAA CTAAA
        Gadus morhua    CTTAG CTGTTAA CTAAG
        Drosophila yakuba   TATCA CTGTTAA TGATA
        Mytilus edulis   TTGAG CTGTTAA TTCAA
        Strongylocen. purp.  CTTGG CCGTTAA CCAAG
        Paracentrotus liv.   CTTGG CCGTTAA CCAAG
        Pisaster ochraceus   TTTGG CCGTTAA CTAAA
        Asterina pectini.    ATTGG CCGTTAA CCAGG
        Asterina amurensis   TTTGG CCGTTAA CTAAA
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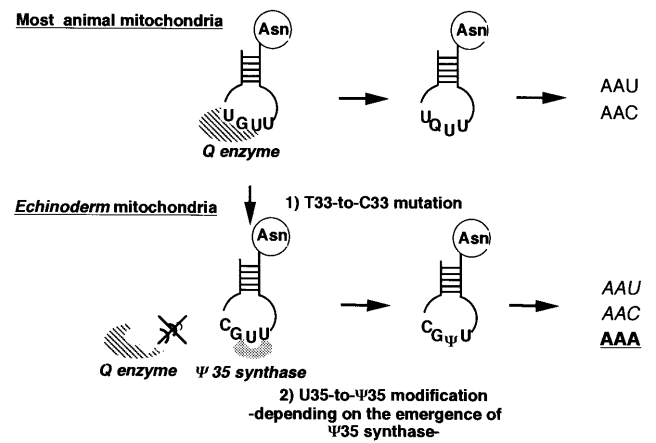
Figure 5. Comparison of nucleotide sequences of mt tRNA^Asn genes from various organisms: mouse (47), chicken (48), codfish (*Gadus morhus*) (49), fruit fly (*Drosophila yakuba*) (50), blue mussel (*Mytilus edulis*) (51), sea urchin [*Strongylocentrotus purpuratus* (10) and *Paracentrotus lividus* (9)] and starfish [*Pisaster ochraceus* (11), *A.pectinifera* (14) and *A.amurensis* (13)]. All the echinoderm mt tRNA^Asn genes (gray background) have C33, 5′-adjacent to the anticodons (black background).

Figure 6. A possible scheme for reassignment 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^Asn gene 1) and the appearance of Ψ35 synthase in echinoderm mitochondria 2) result in the modification of U35 to Ψ35.

The presence of C33 has so far been observed only in mt tRNA^Asn 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 (Fig. 5) (44). Thus, the replacement of U33 by C33 may have been indispensable to prevent the modification of G34 to Q, which may, in turn, have enabled the formation of the G34:A3 wobble pairing together with the modification of U35 to Ψ35. 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.

The change of AAA from a lysine to an asparagine codon in echinoderm mitochondria can be explained by the codon capture theory (45), according to which the following events can be posited to have occurred in mt tRNA^Asn during the disappearance of codon AAA in the progenitor of echinoderm mitochondria (Fig. 6). First, T33 mutated to C33 in the mt tRNA^Asn gene, as a result of which G34 of mt tRNA^Asn was no longer modified to Q by the Q-inserting enzyme. During this event, tRNA^Lys responsible for decoding AAA lost this function, causing AAA to become an unassigned codon. The second event was the post-transcriptional modification of U35 to Ψ35 in mt tRNA^Asn, caused by the emergence of a unique Ψ35 synthase in echinoderm mitochondria, which enabled the G34:A3 wobble pairing to occur. Finally, the AAA codon reappeared in the genome to be captured by an altered tRNA^Asn and assigned as asparagine. In this manner, AAA could have changed from a codon that decodes lysine to one that decodes asparagine.

Recently, Castresane *et al.* found that no AAA codon appeared in the whole mt genome of the hemichordate, *Balanoglossus carnosus* (46), suggesting that AAA is an unassigned codon. Phylogenetically, hemichordates (AAA = unassigned) and echinoderms (AAA = asparagine) derive from a common progenitor α, which in turn shares a common progenitor β with vertebrates (AAA = lysine). This progenitor β is thought to use AAA as a lysine codon. It is thus reasonable, according to the codon capture theory (45), to postulate that the codon became unassigned in progenitor α and that this usage was probably taken over by hemichordates.

REFERENCES

1 Osawa,S., Jukes,T.H., Watanabe,K. and Muto,A. (1992) *Microbiol. Rev.*, **56**, 229–264.
2 Watanabe,K. and Osawa,S. (1995) In Söll,D. and RajBhandary,U. (eds), *tRNA: Structure, Biosynthesis and Function*. American Society for Microbiology, Washington, pp. 225–250.
3 Heckmann,R.J., Sarnoff,J., Alzner-DeWeerd,Y.S. and RajBhandary,U.L. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 3159–3163.
4 Moriya,J., Yokogawa,T., Wakita,K., Ueda,T., Nishikawa,K., Crain,P.F., Hashizume,T., Pomerantz,S.C., McCloskey,J.A., Kawai,G., Hayashi,N., Yokoyama,S. and Watanabe,K. (1994) *Biochemistry*, **33**, 2234–2239.
5 Watanabe,Y., Tsurui,H., Ueda,T., Furushima,R., Takamiya,S., Kita,K., Nishikawa,K. and Watanabe,K. (1994) *J. Biol. Chem.*, **269**, 22902–22906.
6 Tomita,K., Ueda,T. and Watanabe,K. (1997) *Nucleic Acids Res. Symp. Ser.*, **37**, 197–198.
7 Matsuyama,S., Ueda,T., Crain,P.F., McCloskey,J. and Watanabe,K. (1998) *J. Biol. Chem.*, **273**, 3363–3368.
8 Tomita,K., Ueda,T. and Watanabe,K. (1998) *Biochim. Biophys. Acta*, **1399**, 78–82.
9 Cantatore,P., Roberti,M., Rainaldi,G., Gadaleta,M.N. and Saccone,C. (1989) *J. Biol. Chem.*, **264**, 10965–10975.
10 Jacobs,H.T., Elliott,D.J., Math,V.B. and Farquharson,A. (1988) *J. Mol. Biol.*, **202**, 185–217.
11 Smith,M.J., Banfield,D.K., Doteval,K., Gorski,S. and Kowbel,D.J. (1990) *J. Mol. Evol.*, **31**, 195–204.
12 Himeno,H., Masaki,H., Ohta,T., Kumagai,I., Miura,K. and Watanabe,K. (1987) *Gene*, **56**, 219–230.
13 Araki,T., Asakawa,S., Kumazawa,Y., Miura,K. and Watanabe,K. (1988) *Nucleic Acids Res. Symp. Ser.*, **20**, 93–94.
14 Asakawa,S., Himeno,H., Miura,K. and Watanabe,K. (1995) *Genetics*, **140**, 1047–1060.
15 Ohama,T., Watanabe,K., Osawa,S. and Juckes,T.H. (1992) *J. Mol. Evol.*, **30**, 329–332.
16 Bessho,Y., Ohama,T. and Osawa,S. (1992) *J. Mol. Evol.*, **34**, 331–335.
17 Ueda,T., Ohta,T. and Watanabe,K. (1985) *J. Biochem.*, **98**, 1275–1284.

18 Tsurui,H., Kumazawa,Y., Sanokawa,R., Watanabe,Y., Kuroda,T. and Wada,A. (1994) *Anal. Biochem.*, **269**, 22902–22906.
19 Wakita,K., Watanabe,Y., Yokogawa,T., Kumazawa,Y., Nakamura,S., Ueda,T., Watanabe,K. and Nishikawa,K. (1994) *Nucleic Acids Res.*, **22**, 345–353.
20 Ohtsuki,T., Kawai,G., Watanabe,Y., Kita,K., Nishikawa,K. and Watanabe,K. (1996) *Nucleic Acids Res.*, **24**, 662–667.
21 Donis-Keller,H. (1980) *Nucleic Acids Res.*, **8**, 3133–3142.
22 Kuchino,Y., Hanyu,N. and Nishimura,S. (1987) *Methods Enzymol.*, **155**, 379–396.
23 Lund,E. and Dahlberg,J.E. (1977) *Cell*, **11**, 247–262.
24 Scaringe,S.A., Francklyn,C. and Usman,N. (1990) *Nucleic Acids Res.*, **18**, 5433–5441.
25 Kevin,R.W., Yousif,S., William,K. and Eleanor,K.S. (1991) *Biotechniques*, **11**, 658–661.
26 Sampson,J.R. and Uhlenbeck,O.C. (1988) *Proc. Natl Acad. Sci. USA*, **84**, 2185–2188.
27 Speeding,G. (ed.) (1990) *Ribosomes and Protein Synthesis*. Oxford University Press, New York, pp. 1–29.
28 Watanabe,K., Ohshima,T., Iijima,K., Yamaizumi,Z. and Nishimura,S. (1980) *J. Biochem.*, **87**, 1–13.
29 Hou,Y.M. and Schimmel,P. (1988) *Nature*, **333**, 140–145.
30 McClain,W.H. and Foss,K. (1988) *Science*, **240**, 793–796.
31 Kumazawa,Y., Schwattzbach,C.J., Liao,H.-X., Mizumoto,K., Kaziro,Y., Miura,K., Watanabe,K. and Spremulli,L.L. (1991) *Biochim. Biophys. Acta*, **1090**, 167–172.
32 Takemoto,C., Koike,T., Yokogawa,T., Benkowski,L., Spremulli,L.L., Ueda,T., Nishikawa,K. and Watanabe,K. (1995) *Biochimie*, **77**, 104–108.
33 Bienz,M. and Kubli,E. (1981) *Nature*, **294**, 188–190.
34 Johnson,P.F. and Abelson,J. (1983) *Nature*, **302**, 681–678.
35 Beier,H., Barciszewska,M., Krupp,G., Mitnacht,R. and Gross,H.J. (1984) *EMBO J.*, **3**, 351–356.
36 Beier,H., Barciszewska,M. and Sickinger,H.-D. (1984) *EMBO J.*, **3**, 1091–1096.
37 Zerfass,K. and Beier,H. (1992) *Nucleic Acids Res.*, **20**, 5911–5918.
38 Pochon,F., Michelson,A.M., Grunberg-Manago,M., Cohn,W.E. and London,L. (1964) *Biochim. Biophys. Acta*, **80**, 441–447.
39 Davis,D.R. and Poulter,C.D. (1991) *Biochemistry*, **30**, 4223–4231.
40 Hall,K.B. and McLaughlin,L.W. (1991) *Biochemistry*, **30**, 1795–1801.
41 Davis,D.R. (1995) *Nucleic Acids Res.*, **23**, 5020–5026.
42 Nakanishi,S., Ueda,T., Hori,H., Yamazaki,N., Okada,N. and Watanabe,K. (1994) *J. Biol. Chem.*, **269**, 32221–32225.
43 Curnow,A.W. and Garcia,G.A. (1995) *J. Biol. Chem.*, **270**, 17246–17247.
44 Sprinzl,M., Steegborn,C., Hubel,F. and Steinberg,S. (1996) *Nucleic Acids Res.*, **24**, 68–72.
45 Osawa,S. and Juckes,T.H. (1989) *J. Mol. Evol.*, **28**, 271–278.
46 Castresana,J., Feldmaier-Fuchs,G. and Pääbo,S. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 3703–3707.
47 Bibb,M.J., VanEtten,R.A., Wright,C.T., Walberg,M.W. and Clayton,D.A. (1981) *Cell*, **26**, 167–180.
48 Desjardins,P. and Morias,R. (1990) *J. Mol. Biol.*, **212**, 599–634.
49 Johansen,S., Guddal,P.H. and Johansen,T. (1990) *Nucleic Acids Res.*, **18**, 411–419.
50 Clary,D.O. and Wolstenholme,D.R. (1985) *J. Mol. Evol.*, **22**, 252–271.
51 Hoffmann,R.J., Boore,J.L. and Brown,W.M. (1992) *Genetics*, **131**, 397–412.