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An extra tRNA^{Gly}(U*CU) found in ascidian mitochondria responsible for decoding non-universal codons AGA/AGG as glycine

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ABSTRACT

Amino acid assignments of metazoan mitochondrial codons AGA/AGG are known to vary among animal species; arginine in Cnidaria, serine in invertebrates and stop in vertebrates. We recently found that in the mitochondria of the ascidian *Halocynthia roretzi* these codons are exceptionally used for glycine, and postulated that they are probably decoded by a tRNA(UCU). In order to verify this notion unambiguously, we determined the complete RNA sequence of the mitochondrial tRNA(UCU) presumed to decode codons AGA/AGG in the ascidian mitochondria, and found it to have an unidentified U derivative at the anticodon first position. We then identified the amino acids attached to the tRNA(U*CU), as well as to the conventional tRNA^{Gly}(UCC) with an unmodified U34, *in vivo*. The results clearly demonstrated that glycine was attached to both tRNAs. Since no other tRNA capable of decoding codons AGA/AGG has been found in the mitochondrial genome, it is most probable that this tRNA(U*CU) does actually translate codons AGA/AGG as glycine *in vivo*. Sequencing of tRNA^{Ser}(GCU), which is thought to recognize only codons AGU/AGC, revealed that it has an unmodified guanosine at position 34, as is the case with vertebrate mitochondrial tRNA^{Ser}(GCU) for codons AGA/AGG. It was thus concluded that in the ascidian, codons AGU/AGC are read as serine by tRNA^{Ser}(GCU), whereas AGA/AGG are read as glycine by an extra tRNA^{Gly}(U*CU). The possible origin of this unorthodox genetic code is discussed.

INTRODUCTION

Various non-universal codons are known to be used in the mitochondrial (mt) genetic code (1). Sequence determination of metazoan mt protein genes followed by comparison of the deduced amino acid sequences with those of other species already determined has demonstrated that the amino acid assignment of codons AGA/AGG differs in various metazoans (1–3). Instead of arginine as in the standard genetic code, AGA/AGG specify serine in most invertebrates, including platyhelminths (4,5), nematodes (6), molluscs (7,8), annelids (9), arthropods (10,11) and echinoderms (12); exceptionally, the protein genes of dipteran insects such as *Drosophila melanogaster*, *Drosophila yakuba*, *Anopheles gambiae* and *Anopheles quadrimaculatus* (13) contain no codon AGG. In vertebrates, one or both of the codons is absent or they are used as rare stop codons (1). In 1993, Yokobori *et al*. first reported that in the ascidian *Halocynthia roretzi* codons AGA/AGG correspond to glycine (14), and this was later found also to be the case in another ascidian, *Pyura stolonifera* (15). While codon AGA (but not AGG, which is unassigned; 1) in the mt DNA of the amphioxus *Branchiostoma lanceolatum* is also read as glycine (16), it has recently been reported that in another amphioxus, *Branchiostoma floridae*, AGA (AGG is again unassigned) specifies serine (17).

However, except for *H.roretzi* and *B.lanceolatum*, known metazoan mt DNAs do not code an extra tRNA gene that can recognize only codons AGA/AGG (13). There has been no report of any of nuclear-coded tRNA species in the cytoplasm that might be able to overcome this deficiency being imported into the organelle (18). Therefore, while tRNA^{Ser}(GCU) usually corresponds only to codons AGU/AGC, it is thought to become recognizable also to AGA/AGG in most invertebrate mitochondria (1,2), which may be enabled by the presence of 7-methylguanosine (m^7G) at the anticodon first position (position 34) of the tRNA^{Ser}(GCU) (19,20).

The mt DNA of the ascidian *H.roretzi* contains an extra mt tRNA gene with the anticodon UCU (S.-i.Yokobori, T.Ueda, G.Feldmaier-Fuchs, S.Pääbo, R.Ueshima, A.Kondow, K.Nishikawa and K.Watanabe, submitted), which is capable of decoding codons AGA/AGG. We previously purified and partially determined the nucleotide sequence of this extra tRNA(U*CU), including the anticodon sequence, and reported that the modification was 5-carboxymethylaminomethyluridine ($cmnm^5U$) on the basis of two-dimensional thin layer chromatography (TLC) analysis using two different solvent systems (21). In the present study, we attempted to examine

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this modification further by nucleoside analysis of the tRNA(U*CU) using high-performance liquid chromatography (HPLC). However, despite the TLC finding, no cmnm^{5}U was detected by HPLC. These TLC and HPLC results suggest that the modified nucleotide at the anticodon first position might be an unknown species of modified U with the same RF values as those of cmnm^{5}U on the TLC plates, which can apparently discriminate purine from pyrimidine in a similar manner to cmnm^{5}U (21). Although these findings led us to assume that the tRNA (U*CU) is responsible for decoding codons AGA/AGG as glycine, we did not know from our previous work whether the tRNA(U*CU) is actually charged with glycine. The purpose of the work reported here was to ascertain this by analyzing *in vivo* the amino acid attachment to the tRNA(U*CU) as well as to the conventional tRNA^{Gly}(UCC). We also determined the complete sequence of the ascidian mt tRNA(GCU) specific for serine to confirm its capability of discriminating codons AGU/AGC from AGA/AGG.

MATERIALS AND METHODS

Chemicals and enzymes

[1-^{14}C]Acetic anhydride (2.04 GBq/mmol) was purchased from American Radiolabeled Chemicals. Uniformly labeled [^{14}C]glycine (3.81 GBq/mmol), [^{14}C]serine (5.59 GBq/mmol), [^{14}C]arginine (11.84 GBq/mmol) and [γ-^{32}P]ATP (111 TBq/mmol) were from Amersham and [5'-^{32}P]pCp (111 TBq/mmol) from NEN. 3'-Biotinylated DNA probes, Gly (I): 5'-TGCATCAAAGGGACCAACCCTAACTTATAC-3' for purification of tRNA^{Gly}(UCU), Gly (II): 5'-TACTACGTCCCTAAAGGATCCCATGATTGG-3' for tRNA^{Gly}(UCC), and Ser: 5'-ATCAAAAAAATAGCAACTTTCTAACATTCCTAATCACCCTT-3' for tRNA^{Ser}(GCU), were synthesized by Sci. Media, Japan. A chimeric oligonucleotide for an RNase H cleavage splint, SerG2: 5'-rArUrCrArArArArArArArUrArGrCdAdAdCdUdUdUdC-3' (rN and dN denote ribonucleotide and deoxyribonucleotide, respectively), was from Genset. T4 polynucleotide kinase was obtained from Toyobo. T4 RNA ligase, *Escherichia coli* A19 alkaline phosphatase and RNase H were from Takara Shuzo. RNase T1 was from Sankyo, RNase U2 from Sigma, RNase PhyM from Pharmacia, RNase CL3 from Boehringer Mannheim and Nuclease P1 from Yamasa. DEAE Sepharose Fast Flow resin was from Pharmacia. Streptavidin agarose was from Gibco BRL. Glycogen was purchased from Boehringer Mannheim. The ascidian, *H.roretzi*, was kindly provided by Drs M. Hoshi and M. Matsumoto of Tokyo Institute of Technology or purchased from Tsukiji market in Tokyo. Other chemicals used were of analytical or biochemical grade. Analysis of the amino acids attached to tRNA in the cell was carried out at the Radioisotope Center belonging to The University of Tokyo.

Preparation of tRNA and aminoacyl-tRNA from ascidian muscle

Total RNA was prepared as described previously (22) from ascidian muscle and purified sequence-specifically using solid-phase-attached DNA probes (23). NTE buffer [20 × NTE buffer consisting of 4 M NaCl, 100 mM Tris–HCl (pH 7.5) and 50 mM EDTA] was used instead of SSC. The total RNA was dissolved in 50 mM EDTA at a tRNA concentration of 50–300 A_{260}/ml and incubated at 65°C for 10 min prior to purification using the solid-phase-attached DNA probes described above. The probes annealed with total RNA were washed with 0.1× NTE at 40°C in order to remove non-specifically attached tRNAs. The tRNAs were further purified by 10% PAGE in the presence of 7 M urea. Aminoacyl-tRNA was prepared according to the literature (24) with the following modifications. In the phenol extraction step, 100 mM NaoAc (pH 4.5), 10 mM EDTA, 100 mM LiCl and 1% SDS was used as the extraction buffer (25). After ethanol precipitation, the precipitate was resolved in ice-cold 50 mM NaOAc (pH 5.0) and centrifuged at 100 000 *g* for 15 h to remove a white precipitate, presumably containing polysaccharide. The supernatant was applied onto a DEAE Sepharose Fast Flow column which was then washed with 50 mM NaOAc, 0.2 M NaCl and 8 mM $MgCl_2$. Total aminoacyl-tRNA was eluted with 50 mM NaOAc, 0.5 M NaCl and 16 mM $MgCl_2$. The aminoacyl-tRNA thus prepared was analyzed by 10% 7 M urea, 100 mM NaOAc (pH 5.0) PAGE followed by northern hybridization analysis (26). For latter analysis, a 5'-^{32}P-labeled DNA probe was used with the same sequence as the solid-phase-attached DNA probes used for purification.

Sequencing of ascidian mt tRNAs

Purified tRNAs were sequenced by Donis-Keller's method (27) and the post-labeling method of Kuchino *et al*. (28). To determine the nucleotide at the first letter of the anticodon (position 34), tRNA^{Ser}(GCU) was cleaved sequence-specifically at the 5' of the position using RNase H according to the method of Inoue *et al*. (29,30), the 2'-*O*-methyl RNA in the chimeric oligonucleotide splint being replaced by the unmodified RNA. Purified tRNA^{Ser}(GCU) was annealed with the chimeric oligonucleotide splint (SerG2) in 40 mM Tris–HCl (pH 7.7), 1 mM DTT, 0.003% BSA and 4% glycerol. Two units of RNase H and 4 mM $MgCl_2$ (final concentration) were added to the reaction mixture and it was incubated at 37°C for 2 h. The cleaved tRNA was purified by 10% PAGE with 7 M urea. The purified tRNA was labeled with [γ-^{32}P]ATP at the 5' end as described previously (28) and further purified by 12% PAGE with 7 M urea. The 5'-labeled tRNA^{Ser}(GCU) was digested with Nuclease P1 and the derived nucleotides were analyzed by two-dimensional TLC (28).

In vitro aminoacylation

Aminoacylation was performed at 30°C in a reaction mixture containing 100 mM Tris–HCl (pH 8.0), 15 mM $MgCl_2$, 2 mM ATP, 20 mM KCl, 0.2 mM spermine, 0.36 µM purified tRNA, 24 µM U-[^{14}C]glycine, and partially purified bovine mitochondrial glycyl-tRNA synthetase (GlyRS) (equivalent to 3.2 mg/ml protein), which was prepared from bovine liver and purified by a DEAE Sepharose Fast Flow and a Mono S column (a kind gift of Dr N. Takeuchi of our laboratory).

Analysis of amino acid(s) attached to mt tRNA(U*CU) and tRNA(UCC) *in vivo*

All the procedures were carried out according to Suzuki *et al*. (24) with slight modifications. To 50 µl of the total aminoacyl-tRNA (200–600 A_{260}/ml) was added 12.3 MBq of [1-^{14}C]acetic anhydride (2.04 GBq/mmol) diluted in 10 µl of diethylformamide. Since ascidian mt tRNA(U*CU) could not be purified by the conventional method, the total acetylaminoacyl-tRNA was

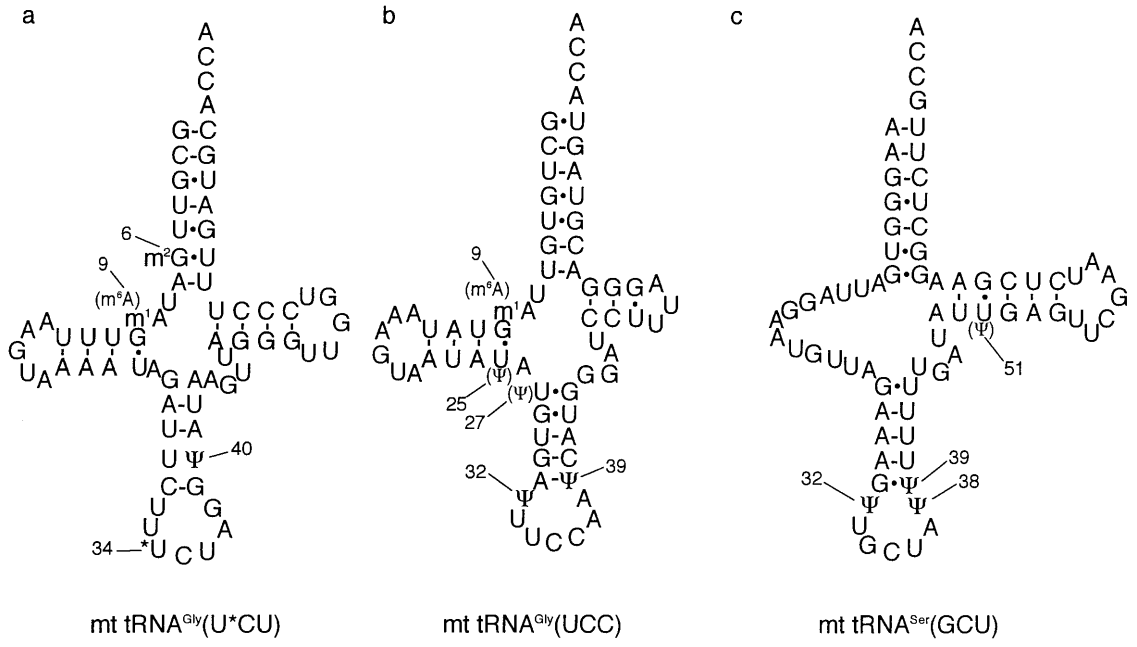

Figure 1. Nucleotide sequences of tRNA^{Gly}(U*CU) (**a**), tRNA^{Gly}(UCC) (**b**) and tRNA^{Ser}(GCU) (**c**) shown in the cloverleaf form (33). In tRNA^{Gly}(UCC), we tentatively propose a 3-nt loop in the T arm because such a tRNA with a hairpin loop of 3 nt has been reported (44). Letters in parentheses show residues with partial modification (in the case of Ψ) or putative base conversion during sequencing analysis [in the case of m^{6}A in tRNA^{Gly}(UCU) and tRNA^{Gly}(UCC)]. – indicates a Watson–Crick base pair and • indicates a non-Watson–Crick base pair. The numbering of each residue conforms to the rule proposed by Sprinzl *et al*. (33).

dissolved in 50 mM EDTA and incubated for 20 min at 65°C in order to denature its structure before purification using the solid-phase-attached DNA probes. The acetylaminoacyl-tRNA purified in this manner was subjected to 10% PAGE with 7 M urea and 100 mM NaOAc (pH 5.0) and eluted with 400 µl of 50 mM NaOAc (pH 5.0) for 3 h at 4°C. The eluted acetyl amino-acyl-tRNA was deacylated in 0.1 N NH_4OH, 37°C for 3 h and the released amino acid(s) was analyzed by TLC (24).

RESULTS

Nucleotide sequences of ascidian mt tRNAs

To verify our earlier assumption that ascidian mt tRNA(U*CU) corresponds to the non-universal codons AGA/AGG, which was based on the partial sequencing of ascidian mt tRNA(U*CU) and tRNA(UCC) possessing the anticodons U*CU and UCC, respectively (21), we determined the entire sequences of these tRNAs by the Donis-Keller and post-labeling methods (27,28). The nucleotide sequence of ascidian mt tRNA with the anticodon GCU was newly determined in this report in order to clarify whether it could discriminate between codons AGU/AGC and AGA/AGG. The first nucleotide of the anticodon was confirmed by sequence-specific cleavage with RNase H using the method of Inoue *et al*. (29,30) followed by with 5'-end nucleotide analysis.

Figure 1 shows the complete nucleotide sequences of ascidian mt tRNA(U*CU), tRNA(UCC) and tRNA(GCU) in the clover leaf form. In the case of tRNA(U*CU), 2-methyl guanosine (m^{2}G) at position 6 and 1-methyladenosine (m^{1}A) at position 9 were newly detected; in addition pseudouridine (Ψ) was found at position 40 as previously reported. We also corrected the sequence with respect to two positions. First, no 1-methyl-guanosine (m^{1}G) was found at position 45 as was reported previously (21). Second, we previously reported the presence of cmnm^{5}U at position 34 (21) based on the fact that a radio-active spot derived from the nucleotide at position 34 was observed at almost the same position as that reported for cmnm^{5}U on the standard TLC plate (31). However, we recently found that an additional radioactive spot appearing at a different position always accompanies the spot identified as that of cmnm^{5}U. We attempted to determine this modified nucleoside by HPLC analysis of the tRNA(U*CU), but we were unable to detect cmnm^{5}U (data not shown). These TLC and HPLC results raise the possibility that the modified U is in fact a novel modification that differs from cmnm^{5}U. We observed a similar result in the case of the first anticodon nucleo-side of tRNA^{Met}(UAU), which probably possesses U* instead of cmnm^{5}U as reported previously (32). It is possible, therefore, that U* may also exist in other tRNA species in ascidian mito-chondria.

Two modified nucleotides were newly determined in the tRNA(UCC): m^{1}A9 and Ψ32. In addition, the existence of Ψ39 was confirmed and the nucleotides at positions 25 and 27, which had been reported as Ψ (21), were found to be a mixture of Ψ and U. The tRNA(GCU) sequence was found to possess only Ψ at positions 32, 38, 39 and 51 (partially modified). Also, position 37, 3'-adjacent to the anticodon, is adenosine and not modified to *N*^{6}-threonylcarbamoyladenosine (t^{6}A) or its derivatives, which has been reported to be conserved among metazoan mt tRNAs(GCU) (19,20,33,34), while the first nucleotide of the anticodon is unmodified guanosine.

Identification of amino acids attached to ascidian mt tRNAs

We determined the complete sequences of ascidian mt tRNA(U*CU) and tRNA(UCC), and found that position 34 of the tRNA(U*CU) was occupied by U* (Fig. 1). According to the proposed codon–anticodon pairing rule for metazoan mitochondria, U* at the anticodon first position should recognize both A and G at the codon third position (3). In order to ascertain that the extra tRNA(U*CU) corresponding to codons AGA/AGG is actually responsible for decoding these codons as glycine, an *in vitro* aminoacylation assay was attempted. Since we have never succeeded in detecting GlyRS activity in an ascidian mitochondrial extract in spite of every endeavor to do so, the corresponding enzyme from bovine mitochondria was used for the assay, although only $tRNA^{Gly}$(UCC) is encoded in the bovine mt genome (35). Ascidian mt tRNA(U*CU) and tRNA(UCC) were incubated with partially purified bovine mt GlyRS in the presence of [^{14}C]-labeled glycine. tRNA(UCC) accepted 230 pmol of [^{14}C]-labeled glycine per $1A_{260}$ unit of tRNA after 10 min of incubation, while tRNA(U*CU) accepted only 6.3 pmol. The possibility that tRNA(U*CU) lacks one or more nucleotide at the 3'-end was excluded by the finding that tRNA(U*CU) labeled with ^{32}P at the 3'-end showed the mobility of the full-length tRNA on the sequencing gel (data not shown). Therefore, ascidian mt tRNA(U*CU) may be aminoacylated only by ascidian mt GlyRS, but not by bovine mt GlyRS.

Since *in vitro* aminoacylation activity could not be examined because of the difficulty in preparing GlyRS from ascidian mitochondria as mentioned above, the amino acid attached to tRNA(U*CU) *in vivo* was analyzed by the method of Suzuki *et al*. (24) with slight modifications. First, total aminoacyl-tRNA was subjected to acid PAGE followed by northern hybridization analysis, from which it was found that approximately half of the tRNA(U*CU) was aminoacylated (Fig. 2a). [^{14}C]-labeled acetylaminoacyl-tRNAs were purified sequence-specifically using solid-phase-attached DNA probes (Fig. 2b). The tRNAs were further purified by acid PAGE and 0.5–5 ng of both tRNAs [tRNA(U*CU) and tRNA(UCC)] were obtained from 100 g of ascidian muscle. After purification, each species of acetylaminoacyl-tRNA was deacylated, followed by analysis of its acetyl amino acids by TLC (Fig. 3). Radioactivities were detected with an imaging plate by exposing the TLC plates for 26 days.

A spot derived from acetylaminoacyl-$tRNA^{Gly}$(U*CU) showed the same mobility as that of the acetylglycine marker (Fig. 3a). We purified 0.2 pmol of tRNA(U*CU) by acid PAGE, and this was analyzed by TLC after the acetyl amino acid was released from tRNA(U*CU); 0.05 pmol of acetylglycine was detected. This low level of acetyl amino acid recovery may have been due to a low tRNA aminoacylation level at the beginning of the preparation (50%), loss of some of the tRNA sample during the recovery from gel, and/or deacylation during the purification procedure. The radioactivity remaining at the origin on the TLC plate for tRNA(U*CU) was ~8-fold that detected for acetylglycine, which may be indicative of direct acetylation of nucleobases or riboses in the tRNAs (24). This is substantiated by the fact that other ^{14}C-labeled RNAs longer than tRNAs, such as 5S rRNA, were also detected in the PAGE (Fig. 2b).

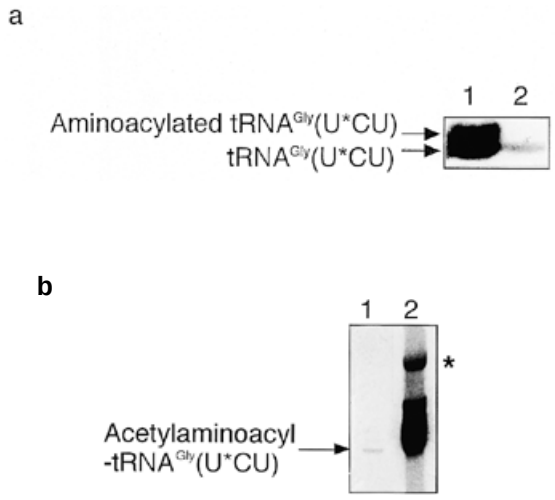

Figure 2. (**a**) Northern hybridization analysis of ascidian total RNA fraction against a DNA probe complementary to the 30 3'-terminal nucleotides of $tRNA^{Gly}$(U*CU). Lane 1, total aminoacyl-RNA prepared under acidic conditions below 4°C as described in Materials and Methods. Lane 2, total RNA prepared at neutral pH and room temperature. The low level of aminoacyl-tRNA may be due to deacylation during aminoacyl-tRNA preparation, or to lack of freshness of the ascidians since ~20 h passed after their capture until they were frozen in liquid nitrogen to prevent aminoacyl-tRNA deacylation. (**b**) PAGE of [^{14}C]acetylaminoacyl-$tRNA^{Gly}$(U*CU) of ascidian *H.roretzi* mitochondria before (lane 2) and after (lane 1) purification with a solid-phase DNA probe. The composition of the gel was 10% polyacrylamide with 7 M urea. After electrophoresis, the gel was dried. Radioactivities were detected with an imaging plate and visualized by an imaging analyzer (BAS-1000; Fuji Film). Both lanes derived from the same gel. An asterisk indicates the position of 5S rRNA.

A spot derived from acetylaminoacyl-$tRNA^{Gly}$(UCC) corresponding to the universal glycine codons GGN (N: U, C, A, G) also showed the same mobility as that of the acetylglycine (Fig. 3b). For tRNA(GCU), radioactivity was detected at the position with the same mobility as acetylserine (data not shown). These results indicate that in addition to $tRNA^{Gly}$(UCC) corresponding to the universal codons GGN, there is an extra $tRNA^{Gly}$(U*CU) in ascidian mitochondria possessing modified U34, and hence the non-universal codons AGA/AGG are translated as glycine.

DISCUSSION

The mt genome sequence of the ascidian *H.roretzi* indicates that the amino acid assignment of codons AGA/AGG has changed from the usual codons—serine in most invertebrates or stop in vertebrates (1–3)—to glycine in ascidian (14). Another feature of the ascidian mt genome is that an additional tRNA gene with the anticodon U*CU, thought to decode codons AGA/AGG, is encoded and transcribed *in vivo* (S.-i.Yokobori, T.Ueda, G.Feldmaier-Fuchs, S.Pääbo, R.Ueshima, A.Kondow, K.Nishikawa and K.Watanabe, submitted; 21) (Fig. 1). These results led us to infer that this tRNA(U*CU) is most likely

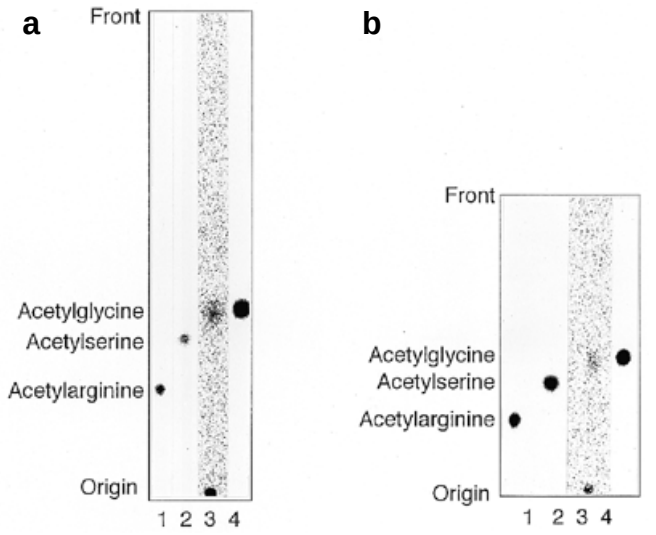

Figure 3. Analysis of acetyl amino acids attached to tRNA^{Gly}(U*CU) (**a**) and tRNA^{Gly}(UCC) (**b**). Acetyl amino acids released from the tRNAs were spotted on silica gel plates and developed by n-butanol/acetic acid/dH_2O (4:0.9:1, v/v/v) (lane 3), together with [^{14}C]-labeled acetylarginine (lane 1), acetylserine (lane 2), and acetylglycine (lane 4) markers, which were prepared by acetylating [^{14}C]-labeled aminoacyl-tRNA with cold acetic anhydride followed by deacylation. Each lane in (a) or (b), respectively, derived from the same TLC plate although the exposure time periods toward the imaging plate were different.

responsible for the change of the amino acid assignment by being charged with glycine and that it decodes codons AGA/AGG. The findings of the present work have verified that the mt tRNA(U*CU) is indeed charged with glycine *in vivo*. The supposition that mt tRNA(U*CU) decodes codons AGA/AGG was also confirmed by the fact that ascidian mt tRNA^{Ser}(GCU) was found to have unmodified G at position 34. The presence of tRNA(GCU) with unmodified G34 at the anticodon first position is consistent with previous knowledge concerning the mt codon–anticodon pairing rule (1). Thus, our results support the assumption that the additional tRNA^{Gly}(U*CU) in ascidian mitochondria is responsible for using AGA/AGG as non-universal glycine codons. To verify directly that tRNA^{Gly}(U*CU) recognizes codons AGA/AGG, *in vitro* translation analysis would be useful; however, this is not yet fully possible with metazoan mitochondrial systems except for that of bovine mitochondria (C.Takemoto, T.Ueda, T.Yokogawa, L.A.Benkowski, L.L.Spremulli and K.Watanabe, submitted; 36)

tRNA(U*CU) has another characteristic feature. Almost every tRNA reading codon ANN (N: U, C, A, G) possesses a modified adenosine derivative at position 37 (37). Modifications of the nucleotide 3'-adjacent to the anticodon (position 37) are known to affect codon–anticodon interaction (38). Strikingly, ascidian tRNA(U*CU) decoding codons AGA/AGG has an unmodified A37, whereas tRNA^{Arg} of yeast (*Saccharomyces cerevisiae*) mitochondria decoding the same codons have has t^{6}A37 (33). However, there are also several other exceptional tRNAs. tRNA^{Ser}(GCU) of ascidian and yeast mitochondria harbors unmodified A37, unlike metazoan mitochondrial tRNA^{Ser}(GCU) or tRNA^{Ser}(UCU) which have t^{6}A or its derivatives at position 37 (33) (Fig. 1). Other exceptions are known among the initiator tRNA^{Met} with the anticodon CAU of prokaryotes, chloroplasts and mitochondria (most of which harbor f^{5}CAU in metazoan mitochondria, f^{5}C being 5-formyl cytidine), *Mycoplasma capricolumn* elongator tRNA^{Met}, and mt tRNA^{Ile} (1,33).

Ascidian mt tRNA(U*CU) was shown to be aminoacylated with glycine *in vivo*, whereas, it was not aminoacylated *in vitro* by the bovine mt GlyRS fraction. This suggests that the recognition mechanism of ascidian mt GlyRS differs from that of its bovine counterpart. One possible explanation is that the nucleotide at position 36 (the anticodon third letter) is one of the major determinants of bovine mt GlyRS, as in the cases of the bacterial and yeast systems (39,40) and, therefore, tRNA^{Gly}(U*CU) was not aminoacylated with glycine. In both *Thermus thermophilus* and yeast, mutation at this position leads to a 5000-fold loss of efficiency *in vitro* (40). Although C36 has an influence on glycylation, other major determinants have been reported for GlyRS: the discriminator base at position 73 and C35 of the anticodon. A at position 73, known to be conserved in eukaryotic cytoplasmic tRNA^{Gly}, is also conserved in most of metazoan mt tRNAs^{Gly}, including both of the ascidian mt tRNAs^{Gly} (33). Two or three of the first three G–C or C–G base pairs of the acceptor stem have also been reported to play an important role in *E.coli*, *T.thermophilus*, yeast and human GlyRS recognition (39,40), although these base pairs are not well conserved among metazoan mt tRNAs^{Gly}. Contrary to most other metazoans, both of the ascidian mt tRNAs^{Gly} possess a C2–G71 base pair (33). These facts lead us to presume that A73 and C2–G71 might contribute to the recognition of ascidian mt GlyRS.

The results of *in vitro* aminoacylation of the ascidian tRNAs^{Gly} imply the possibility that along with the emergence of tRNA(U*CU), ascidian mt GlyRS has changed its tRNA recognition ability. Alternatively, another mt GlyRS specific for tRNA(U*CU) may have appeared in the ascidians—similar to the case of the non-universal codons CUN (N:U, C, A, G) in mitochondria of *S.cerevisiae*, belonging to Hemiascomycetes, which have been shown to possess an extra threonyl-tRNA responsible for reading the leucine codons CUN as threonine (41,42) and in which, it has been suggested, two threonyl-tRNA synthetases corresponding to each of the tRNAs^{Thr} exist (43).

The reassignment of codons AGA/AGG from serine in most invertebrate mitochondria to glycine in ascidian mitochondria could be explained by the codon capture hypothesis (1). First, a tRNA^{Ser}(GCU) that can read both codons AGC/AGU and AGA/AGG changes to one able to read only AGC/AGU, probably through some structural rearrangement, and concomitantly the codons AGA/AGG become unassigned. Then, a tRNA^{Gly} with the anticodon U*CU corresponding to codons AGA/AGG appears in the mt genome and these codons come to be assigned as glycine. This process would require either a recognition change of GlyRS so as to charge the newly appearing tRNA^{Gly}(U*CU), in addition to the usual tRNA^{Gly}(UCC), with glycine, or the emergence of a new GlyRS specific for tRNA^{Gly}(U*CU). To examine this speculation further, it is a prerequisite to purify active ascidian mt GlyRS(s) and characterize it (them).

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