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Codon reading patterns in *Drosophila melanogaster* mitochondria based on their tRNA sequences: a unique wobble rule in animal mitochondria

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ABSTRACT

Mitochondrial (mt) tRNA^{Trp}, tRNA^{Ile}, tRNA^{Met}, tRNA^{Ser}GCU, tRNA^{Asn} and tRNA^{Lys} were purified from *Drosophila melanogaster* (fruit fly) and their nucleotide sequences were determined. tRNA^{Lys} corresponding to both AAA and AAG lysine codons was found to contain the anticodon CUU, C34 at the wobble position being unmodified. tRNA^{Met} corresponding to both AUA and AUG methionine codons was found to contain 5-formylcytidine (f^{5}C) at the wobble position, although the extent of modification is partial. These results suggest that both C and f^{5}C as the wobble bases at the anticodon first position (position 34) can recognize A at the codon third position (position 3) in the fruit fly mt translation system. tRNA^{Ser}GCU corresponding to AGU, AGC and AGA serine codons was found to contain unmodified G at the anticodon wobble position, suggesting the utilization of an unconventional G34-A3 base pair during translation. When these tRNA anticodon sequences are compared with those of other animal counterparts, it is concluded that either unmodified C or G at the wobble position can recognize A at the codon third position and that modification from A to t^{6}A at position 37, 3′-adjacent to the anticodon, seems to be important for tRNA possessing C34 to recognize A3 in the mRNA in the fruit fly mt translation system.

INTRODUCTION

All mitochondria, except for those of plants, are known to use a unique wobble rule (1): in family boxes unmodified U at the anticodon wobble position (U34) recognizes all four nucleotides at the third position of the cognate codon (N3; where N is A, G, C or U), in two-codon sets unmodified G34 recognizes only pyrimidines (Y is U or C), and modified U34 discriminates purines (R is A or G) from pyrimidines. Even in this wobble rule unmodified C34 has been believed to recognize only G3 of the cognate codon (2).

Sequence analyses of non-plant mitochondrial (mt) genomes from various organisms have revealed that there are some exceptions to the above-mentioned mitochondria-specific wobble rules (3,4 and references therein). Most of these exceptions are often observed in the decoding of the non-universal genetic codes, which is one of the unique features of the non-plant mt translation systems. The first case is the Ile codon AUA, in which A3 is presumed to be recognized by 5-formylcytidine (f^{5}C34) of tRNA^{Met} in bovine, nematode (*Ascaris suum*) and squid (*Loligo bleekeri*) mitochondria (5–7), and thus the AUA codon is translated as Met. The second exception is the Arg codons AGR (R is A or G). In most invertebrate mitochondria, serine tRNA with the anticodon GCU (tRNA^{Ser}GCU) is considered to be involved in decoding both AGR and AGY codons as Ser, because no tRNA^{Arg} gene exists in the mt genome that is capable of decoding the AGR codons as Arg. There is also no evidence showing that the relevant cytoplasmic tRNA is imported into animal mitochondria (8). We recently found that 7-methylguanosine (m^{7}G) is located at the anticodon wobble position of tRNA^{Ser}GCU from mitochondria of starfish (*Asterias amurensis*) and squid (*L.bleekeri*) (9,10), and proposed that the modification from G to m^{7}G permits a single tRNA to decode all four AGN codons. The third exception is the Lys codon AAA. In echinoderm and platyhelminth mitochondria, tRNA^{Asn}GUU is considered to translate the AAA codon, in addition to the usual AAU and AAC Asn codons, as Asn. We recently found that starfish (*A.amurensis* and *Asterina pectinifera*) mt tRNA^{Asn} has the GΨU anticodon sequence, and pseudouridylation at the anticodon second position enhances recognition of the AAA codon (11).

Sequence determination of the *Drosophila melanogaster* mt genome and comparison of the protein genes with those of other animal counterparts elucidated so far, led to the notion that codons AUA, AGA and UGA are read by the corresponding

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specific tRNAs as Met, Ser and Trp, respectively (12,13). To clarify these assumptions in greater detail, we attempted to elucidate the decoding properties of several fruit fly mt tRNAs that are thought to be involved in these unusual codon recognition events (tRNA^{Trp} for the UGR codon set, tRNA^{Ile} and tRNA^{Met} for the AUN family box, tRNA^{Ser}GCU for the AGN family box, and tRNA^{Asn} and tRNA^{Lys} for the AAN family box) by determining their nucleotide sequences including the modified nucleotides. By comparing their anticodon sequences with those of other animal counterparts, the unique codon reading patterns in most animal mt translation systems are deduced. A rationale for these codon reading patterns is presented.

MATERIALS AND METHODS

Chemicals and enzymes

[γ-^{32}P]ATP (111 TBq/mmol) and [5′-^{32}P]cytidine-3′,5′-bisphosphate (111 TBq/mmol) were purchased from Amersham. RNase T1 and RNase U2 were purchased from Sigma (Japan). RNase PhyM and T4 RNA ligase were purchased from Pharmacia (Japan). RNase CL3 was purchased from Boehringer Mannheim (Japan), and nuclease P1 was purchased from Yamasa-shoyu (Japan). T4 polynucleotide kinase and *Escherichia coli* A19 alkaline phosphatase were from Toyobo and Takara (Japan), respectively. RNase H was from Takara (Japan). Streptavidin-agarose was purchased from Gibco BRL (Japan).

Purification of mt tRNAs from *D.melanogaster* by the hybridization method

Preparation of total tRNA was carried out as described previously (14) from the whole body of adult fruit flies. The tRNA fractions were purified by DEAE-cellulose column chromatography using a buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 0.8 M NaCl.

Individual mt tRNAs were isolated from total tRNA fractions by the solid-phase hybridization method (15,16). 3′-Biotinylated oligonucleotide probes, whose DNA sequences are complementary to the 3′-terminal 30 nt of the respective mt tRNA genes (12,13), were purchased from Sci-Media (Japan) and used for the purification of mt tRNAs. The DNA sequences of the 3′-biotinylated probes are as follows: 5′-TAAGGCTTAA-AGAAATTTCTTTATTTATAG-3′ for tRNA^{Trp}, 5′-CAAT-GAATGCAGAAAACTGCATGATTTACC-3′ for tRNA^{Ile}, 5′-TAAAAAGAAAAGGATTATAACCTTTATAAA-3′ for tRNA^{Met}, 5′-AGAAATATAAATGGAATTTAACCATTA-AAA-3′ for tRNA^{Ser}GCU, 5′-CTTAATTGGAATTTAAAAT-TCAATTAT-3′ for tRNA^{Asn} and 5′-TCATTTGAAGTAAGT-GCTAATTTACTATTA-3′ for tRNA^{Lys}. The isolated tRNAs were further purified by 15% polyacrylamide gel electrophoresis (PAGE) under denaturing conditions.

Sequence determination of mt tRNAs

The purified mt tRNAs thus obtained were first analyzed by Donis-Keller's method (17). Their sequences were identical to the corresponding mt tRNA gene sequences except for modified nucleotides, which were further analyzed by the method of Kuchino *et al.* (18).

To confirm that tRNA^{Lys} possesses unmodified C at the anticodon wobble position, tRNA^{Lys} was digested with RNase H using an RNA–DNA chimera splint oligonucleotide (5′-rArA-rArGrGrUrUrUrArArGdAdGdAdCdC-3′; purchased from Genset) as described previously (19,20), which was designed to cleave the phosphodiester bond between U33 and C34. Twenty-five picomoles of the RNA–DNA chimera splint was annealed to 8.5 pmol (~200 ng) of purified mt tRNA^{Lys}, in 45 µl of a reaction buffer containing 40 mM Tris–HCl, pH 7.5, 0.5 mM NaCl, 0.1 mM DTT, 3 µg/ml BSA and 0.4% (v/v) glycerol, by heating at 65°C for 10 min, then placed at room temperature for 30 min. Next, 4 µl of 50 mM $MgCl_2$ and 1 µl of RNase H (6 U) were added and incubated at 30°C for 2 h. The resultant 3′-fragment (from position 34 to 76) of mt tRNA^{Lys} was purified by PAGE under denaturing conditions. The 5′-end was dephosphorylated, labeled with [γ-^{32}P]ATP by polynucleotide kinase and the labeled fragment was analyzed by Donis-Keller's method (17). The labeled 5′-end produced by nuclease P1 digestion of the fragment was also determined by two-dimensional thin-layer chromatography (2D-TLC).

Directly combined high-performance liquid chromatography/electrospray ionization mass spectrometry (LC/MS) of mt tRNA^{Lys}

The nucleotide composition of the purified mt tRNA^{Lys} was analyzed by directly combined high-performance LC/MS using a Hewlett-Packard 1090 DAD liquid chromatograph interfaced to a Fisons Instruments Quattro II triple quadrupole mass spectrometer (Micromass Inc., Beverly, MA), as described (9). Four micrograms of tRNA^{Lys} was digested sequentially with nuclease P1 and snake venom phosphodiesterase I, and dephosphorylated with bacterial alkaline phosphatase (21). The nucleosides in the hydrolysates were separated by reversed-phase chromatography and their identities determined from retention times by measurement of UV absorbance, and from their electrospray ionization mass spectra.

RESULTS

Purification of individual mt tRNAs by the solid-phase hybridization method

Mitochondrial tRNA^{Trp}, tRNA^{Ile}, tRNA^{Met}, tRNA^{Ser}GCU, tRNA^{Asn} and tRNA^{Lys} were isolated from *D.melanogaster* by the solid-phase hybridization method (15,16), followed by purification with PAGE. Isolation of mt tRNA^{Trp} is shown as an example in Figure 1. The solid-phase hybridization method is quite useful for isolating single species of tRNA present in very low amounts, such as mt tRNA, although the gene sequences of the target tRNAs must be known for preparing the DNA probes. Moreover, unless RNA editing has occurred, all possible tRNA species derived from a particular gene can, in principle, be isolated so that heterogeneity of the tRNA caused by undermodification and/or lack of an intact CCA terminus can be detected. In fact, the electrophoretic analyses of tRNA^{Asn} indicated that subspecies were present, which resulted from partial modification of the nucleotide at the anticodon first position, as described below.

Nucleotide sequences of six species of fruit fly mt tRNAs

The complete nucleotide sequences of six species of mt tRNAs from *D.melanogaster* were determined by a combination of the methods of Donis-Keller (17) and Kuchino *et al.* (18). The

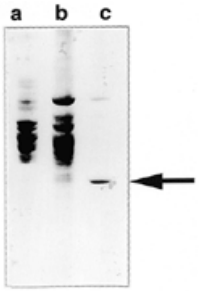

Figure 1. PAGE of *D.melanogaster* mt tRNA^{Trp} isolated by solid-phase hybridization. (a) Unfractionated *E.coli* total tRNAs, (b) fruit fly total tRNAs and (c) mt tRNA^{Trp} isolated from unfractionated fruit fly total tRNAs were electrophoresed in a 15% polyacrylamide gel containing 7 M urea and stained with toluidine blue. The arrow indicates the band of mt tRNA^{Trp}.

sequences are shown in Figure 2, and identities of the corresponding position-34 nucleotides are shown in Figure 3. Mitochondrial tRNA^{Lys} was further analyzed by RNase H digestion using a DNA–RNA chimera, as well as by LC/MS of a total nuclease digest.

The anticodon sequence of mt tRNA^{Trp} was U*CA (Fig. 2a), where U34 is modified to an unknown derivative of uridine that is the same as one found at the anticodon first position of the ascidian *Holocynthia rorentzi* mt tRNA^{Gly}U*CU (22), judging from its mobility on the TLC plates (Fig. 3a). Since in ascidian mitochondria codons AGA and AGG are thought to be translated as glycine instead of arginine in the universal genetic code, by the tRNA^{Gly}U*CU (22), this unknown derivative of uridine U* should recognize purines (A and G) at the codon third position, according to the proposed mt wobble rule described above (1). Therefore, the fruit fly mt tRNA^{Trp} with the same modified uridine at the anticodon first position should translate both UGA and UGG codons as Trp.

The anticodon sequence of mt tRNA^{Ile} was GAU (Fig. 2b), and we conclude that both Ile codons AUU and AUC are translated by this tRNA^{Ile} having GAU as the anticodon sequence.

tRNA^{Met} was found to contain CAU and f^{5}CAU (f^{5}C; 5-formylcytidine) anticodon sequences, as described below (Fig. 2c). The sequence analysis of mt tRNA^{Met} by the method of Kuchino *et al.* (18) strongly suggested that tRNA^{Met} consisted of two subspecies; one having N^6-threonylcarbamoyladenosine (t^{6}A37) at position 37 and the anticodon CAU

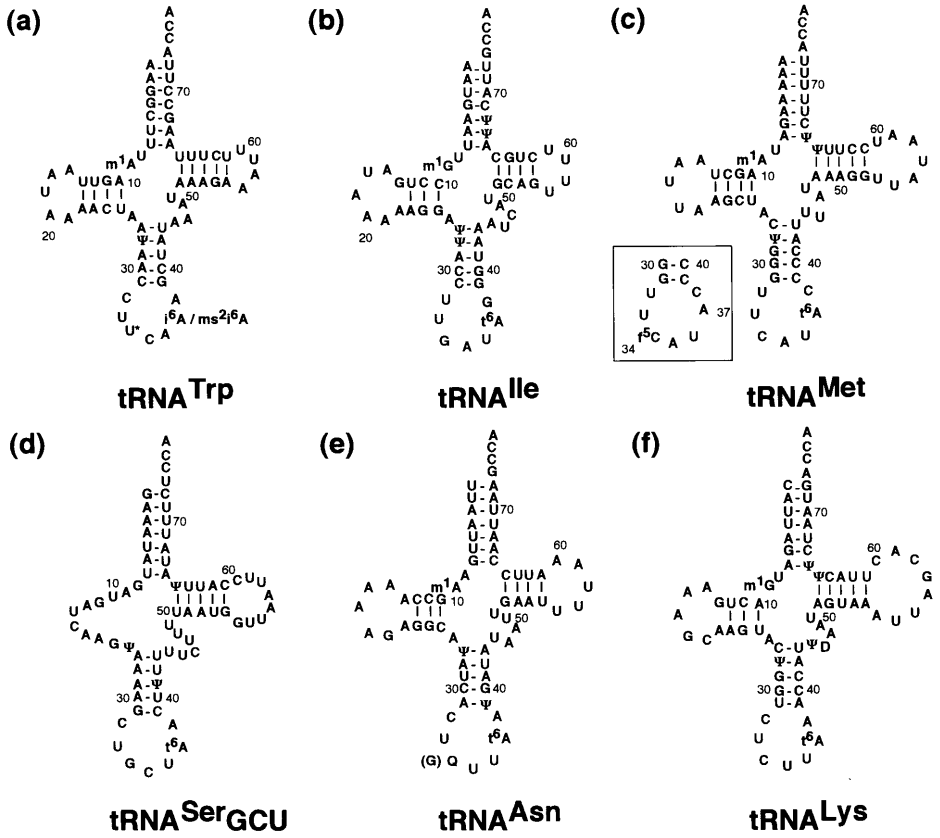

Figure 2. Nucleotide sequences of *D.melanogaster* mt tRNAs in clover-leaf forms. The numbering of each residue conforms to the proposal of Sprinzl *et al.* (37). (**a**) tRNA^{Trp}, (**b**) tRNA^{Ile}, (**c**) tRNA^{Met}, (**d**) tRNA^{Ser}GCU, (**e**) tRNA^{Asn} and (**f**) tRNA^{Lys}. tRNA^{Met} comprises two species [tRNA^{Met}1 (boxed) and tRNA^{Met}2]. U at the anticodon wobble position of tRNA^{Trp} is modified to an unknown derivative of uridine (U*) and G at the anticodon wobble position of tRNA^{Asn} was partially modified to Q.

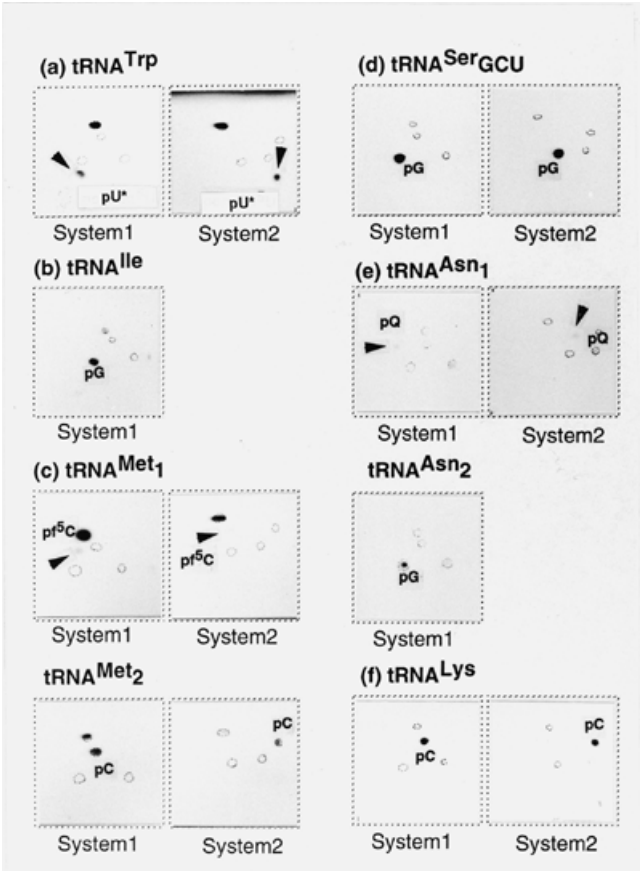

Figure 3. 2D-TLC analyses of the nucleotides at the anticodon wobble positions of *D.melanogaster* mt tRNAs. (**a**) $tRNA^{Trp}$, (**b**) $tRNA^{Ile}$, (**c**) $tRNA^{Met}1$ and 2 (Fig. 2c), (**d**) $tRNA^{Ser}GCU$, (**e**) $tRNA^{Asn}1$ and 2 (Fig. 2e) and (**f**) $tRNA^{Lys}$. The samples for $tRNA^{Met}1$ and 2 were respectively derived from the adjacent two bands in the ladder produced by the method of Kuchino *et al.* (18) (see Results). The solvents used were isobutyric acid/concentrated ammonia/water (66:1:33 v/v/v) for the first dimension in both systems, 2-propanol/HCl/water (70:15:15 v/v/v) for the second dimension in system 1, and ammonium sulfate/0.1 M sodium phosphate, pH 6.8/1-propanol (60 g:100 ml:2 ml) for the second dimension in system 2.

[$tRNA^{Met}$(C34/t^6A37)] (Fig. 2c), while the other had A37 and the anticodon f^5CAU [$tRNA^{Met}$(f^5C34/A37)] (Fig. 2c, boxed).

We first identified the spot of t^6A on the TLC plate, which is usually located at position 37 in $tRNA^{Met}$. From the position of t^6A, the sequence in the anticodon region was read as 3′-t^6A37U36A35C34-5′, the nucleotide at the anticodon first position being unmodified C34 (Fig. 3c, lower panel). However, we found a faint f^5C spot on the TLC plate, which was derived from the neighboring band with slower mobility, together with a strong spot of A (Fig. 3c, upper panel). Since f^5C has been found at the first position of the anticodon of bovine, squid and nematode mt $tRNAs^{Met}$ (5–7), it is most appropriate to judge that f^5C is located at position 34 also in this case and the concomitantly appearing A should be located at position 35. This assumption is reasonable because t^6A has a positive charge, so both of the 3′ fragments [^{32}P]f^5C34A35U36A37- CCA76 and [^{32}P]A35U36t^6A37- CCA76 should have the same mobility on the polyacrylamide gel under the condition used, so that these two fragments should be included within the same electrophoretic band in the ladder.

Therefore, C at position 34 and A at position 37 are partially modified to f^5C and t^6A, respectively, and there must exist two mt $tRNAs^{Met}$ [$tRNA^{Met}$(C34/t^6A37) and $tRNA^{Met}$(f^5C34/A37)] in *Drosophila* mitochondria. These two $tRNAs^{Met}$ could not be separated from each other by PAGE. The same result was also obtained in the course of sequence determination of squid mt $tRNA^{Met}$ (K.Tomita, T.Ueda and K.Watanabe, unpublished results). From these results, we conclude that both codons AUG and AUA are probably read by $tRNAs^{Met}$ with the anticodon loop sequences f^5CAUA and/or CAUt^6A (see Discussion).

The anticodon sequence of mt $tRNA^{Ser}$GCU was GCU (Fig. 2d), where G34 was not modified (Fig. 3d), so that the anticodon GCU should recognize the unusual serine codon AGA in addition to the usual serine codons AGU and AGC. No AGG codon exists in the mt genome of the fruit fly, so that AGG is an unassigned codon (13).

The $tRNA^{Asn}$ sample purified by the hybridization method gave two distinct bands on the electrophoresis gel (data not shown). The sequence analyses of these two species indicated that the heterogeneity arises from partial modification of G to queuosine (Q) at the anticodon wobble position (Figs 2e and 3e), otherwise the two tRNAs were identical. Because the Q base has a positive charge, the two species of $tRNA^{Asn}$ were easily separated by PAGE under denaturing conditions. Thus, the anticodon sequence of $tRNA^{Asn}$ was determined as GUU and QUU, which should recognize both Asn codons AAU and AAC.

In the case of $tRNA^{Lys}$, sequence analysis by the methods of Donis-Keller (17) and Kuchino *et al.* (18) indicated that the anticodon is CUU (Figs 2f and 3f). There was a possibility that the nucleotide at the anticodon wobble position might have been edited from C34 to U34, so that the edited anticodon UUU can recognize both of the lysine codons AAA and AAG. To exclude this possibility, we first amplified a cDNA fragment of the anticodon region of the purified $tRNA^{Lys}$ by RT–PCR and the amplified cDNA fragment was digested by *Dra*I, which recognizes the TTTAAA sequence. If C34 were edited to U34 in $tRNA^{Lys}$, the T34T35T36A37A38A39 sequence would appear in the amplified cDNA fragment (Fig. 2f), which would be digested with *Dra*I. However, no cut was detected following *Dra*I digestion of the cDNA fragment, as followed by Southern hybridization (data not shown).

Another possibility is that there is a C derivative at the first anticodon position that recognizes both AAA and AAG codons, because the gene sequence of $tRNA^{Lys}$ indicates C at this position. Thus, the nucleotide composition of $tRNA^{Lys}$ was analyzed by LC/MS. Figure 4 shows the LC analysis data, and indicated that the $tRNA^{Lys}$ contained only four modified nucleosides—dihydrouridine (D), pseudouridine (Ψ), 1-methylguanosine (m^1G) and N^6-threonylcarbamoyladenosine (t^6A)—which were identified unequivocally by the MS analysis. No modified C was detected (Fig. 4).

Finally, to confirm that an unmodified C exists actually at the anticodon wobble position, $tRNA^{Lys}$ was specifically cleaved at position between 33 and 34 by RNase H using an RNA–DNA chimera splint. The resultant 3′ fragment was labeled with ^{32}P at the 5′-end and analyzed by Donis-Keller's method (17) as shown in Figure 5a. After hydrolysis with

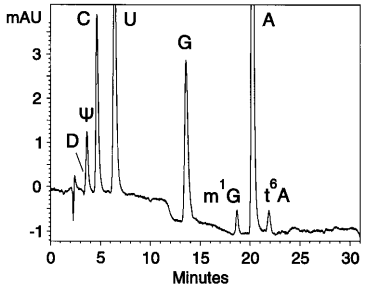

Figure 4. Reversed-phase chromatographic separation of nucleosides derived from *D.melanogaster* mt $tRNA^{Lys}$ (UV detection at 254 nm, mAU is 0.001 A254 U/ml). Mitochondrial $tRNA^{Lys}$ contained four minor modified nucleoside, pseudouridine (Ψ), dihydrouridine (D), 1-methylguanosine (m^1G) and N^6-threonylcarbamoyladenosine (t^6A), in addition to the major nucleosides A, G, C and U. Identities of each nucleoside were established from their electrospray mass spectra (21).

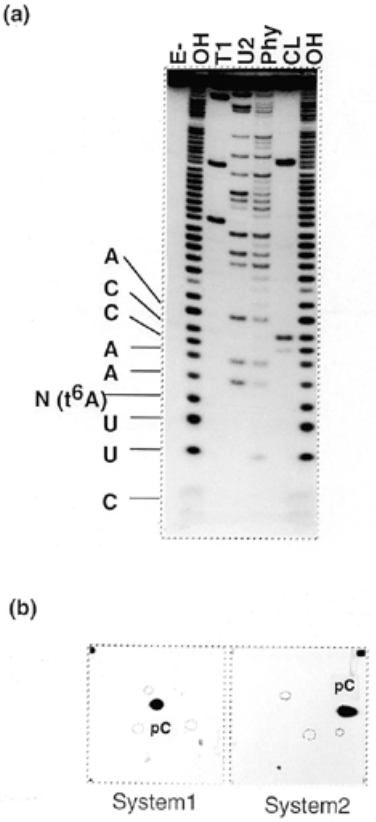

Figure 5. (**a**) Sequence analysis of a 3′-fragment (from 34 to 76) of *D.melanogaster* mt $tRNA^{Lys}$ by Donis-Keller's method (17). Purified mt $tRNA^{Lys}$ was cleaved between 33 and 34 by RNase H using a DNA–RNA chimera oligonucleotide (Materials and Methods). (**b**) 2D-TLC analyses of the 5′ end of the resultant 3′ fragment (nucleotide at position 34 in Fig. 2f). The solvent systems used were the same as those in Figure 3.

nuclease P1, the ^{32}P-labeled nucleotide at the 5′-end, which corresponds to the wobble nucleotide, was analyzed by 2D-TLC and identified as unmodified C (Fig. 5b). It should be noted that t^6A exists at position 37 in this $tRNA^{Lys}$. From these

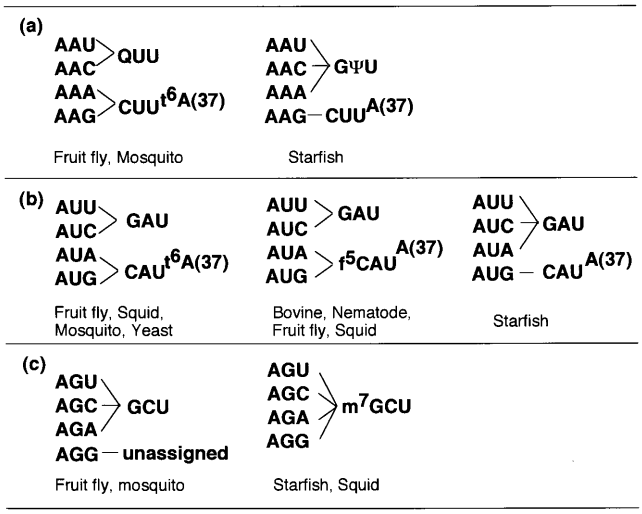

Figure 6. Inferred codon–anticodon relations in decoding of (**a**) AAN, (**b**) AUN and (**c**) AGN codons by the corresponding tRNA anticodons in various mt translation systems, which are based on the RNA sequence analysis; cow (5), nematode (6), squid (7,10; K.Tomita, T.Ueda and K.Watanabe, unpublished results), mosquito (31,32,38), yeast (30), starfish (9,11; T.Ohkubo, S.Matsuyama, Y.Watanabe, S.Yokobori, K.Tomita, T.Ueda and K.Watanabe, unpublished results) and fruit fly (this study). AGG is an unassigned codon in fruit fly and mosquito mitochondria (12,13).

results, it is most probable that $tRNA^{Lys}$ possessing the anticodon CUU together with t^6A37 recognizes both the codon AAA and AAG as lysine.

DISCUSSION

In the present study, we found possible new codon–anticodon base pairing patterns in fruit fly mitochondria, which are summarized in Figure 6, together with already known ones found in other non-plant mitochondria (1–4).

We have already proposed that both the modified C (f^5C) and G (m^7G) at the anticodon wobble position are capable of base pairing with both A and G at the codon third position (5–7,9,10). f^5C has been found so far at the anticodon wobble position of bovine (5), nematode (6) and squid (7) mt $tRNAs^{Met}$. Recently, it was demonstrated in our laboratory that bovine mt $tRNA^{Met}$ with anticodon f^5CAU can recognize both codons AUG and AUA by using a bovine mt *in vitro* translation system (23, C.Takemoto, T.Ueda, T.Yokogawa, L.A.Benkowski, L.L.Spremulli and K.Watanabe, manuscript in preparation). Thus, it is most likely that in the fruit fly mitochondria the Ile codon AUA is read as Met by $tRNA^{Met}$ with the anticodon f^5CAU (Figs 2c and 6). This modification is also expected to play an important role in the decoding of the AUA codon as Met in most other mt translation systems, where AUA is identified as a Met codon and the corresponding $tRNA^{Met}$ gene possesses the anticodon CAT at the DNA level.

On the other hand, how the Arg codons AGA/AGG are read by $tRNA^{Ser}GCU$ as Ser in most invertebrate mitochondria has been a long-standing question (3,4). We recently found that m^7G occurs at the anticodon first position of $tRNA^{Ser}GCU$

from starfish and squid mitochondria and proposed that this modification might be responsible for decoding all four nucleotides at the codon third position (9,10).

In this study we found new cases for the fruit fly mitochondria, in which unmodified C and G occur at the anticodon wobble position of tRNAs and they might be involved in decoding the codons AAR, AUR and AGA. Since these codons are thought to correspond to Lys, Met and Ser, respectively, it is reasonable to assume that C34 and G34 are capable of recognizing A at the codon third position, depending on the modification at position 37 of the tRNA (in the case of C34) or absence of the competitor tRNA against the relevant tRNAs (in the case of G34) (9–11), as discussed below.

There have been several experimental results indicating that unmodified C34 of *E.coli* tRNAs may be able to recognize A at the codon third position. (i) *Escherichia coli* elongator $tRNA^{Met}$ in which the anticodon ac^4CAU (ac^4C; 4-acetylcytidine) was altered to CAU by treatment with sodium bisulfite, can translate both codons AUG and AUA (24); (ii) *E.coli* $tRNA^{Gln}$ with anticodon CUG recognizes both CAG and CAA codons (25); (iii) *E.coli* $tRNA^{Trp}$ with anticodon CCA can read both UGG and UGA codons (26,27); and (iv) *E.coli* mutant $tRNA^{Tyr}$ with anticodon CUA can suppress UAG and UAA codons (28,29).

Similar base-pairing rule seems to hold for some mt translation systems: (i) *Saccharomyces cerevisiae* mt elongator $tRNA^{Met}$ (30) and mosquito and squid mt $tRNAs^{Met}$ (31, K.Tomita, T.Ueda and K.Watanabe, unpublished results) responsible for decoding AUA and AUG codons have anticodon CAU; and (ii) mosquito mt $tRNA^{Lys}$ for codons AAA and AAG has CUU anticodon (32). It should be noted that all these mt tRNAs possess the modified adenosine t^6A at position 37. It is implied that the modification status of the nucleotide at position 37 influences the suppressor efficiency as well as stability of the codon–anticodon interaction (33–35). Therefore, C-A base-pairing, although unconventional, might proceed efficiently and stably for tRNAs with modification at position 37 as described previously (33,35). On the basis of these results, it is most probable that mt $tRNA^{Lys}$ possessing both the anticodon CUU and t^6A37 can read AAG and AAA codons, and that $tRNA^{Met}$ possessing the anticodon CAU and t^6A37 can read both AUG and AUA codons in fruit fly mitochondria. If position 37 is occupied by unmodified A in tRNAs with unmodified C34, such tRNAs may only decode codons ending with G, because starfish mt $tRNA^{Lys}$ (11) and $tRNA^{Met}$ (T.Ohkubo, S.Matsuyama, Y.Watanabe, S.Yokobori, K.Tomita, T.Ueda and K.Watanabe, unpublished results) possessing both unmodified C34 and A37 are thought to read only AAG and AUG codons, but not AAA and AUA codons, respectively (Fig. 6).

We have already proposed that in mt translation systems unmodified G at the anticodon first position is potentially capable of decoding not only codons ending with C and U but also those codons ending with A (9–11). Thus, it is reasonable to consider that when codons in a family box are translated by two tRNAs with anticodons GNN and CNN, codons ending with A could be recognized by both tRNAs. In such cases, which of the tRNAs (with anticodon CNN or GNN) will read a codon ending with A *in vivo*? It may be most plausible to assume that it depends on whether or not competition exists between these tRNAs for the codon. If there exists no competitor tRNA which recognizes a codon ending with A, anticodon GNN would recognize all three codons NNC, NNU and NNA. However, when there exists any competitor tRNA in the system, such a tRNA would read codon NNA, so that the anticodon GNN will read only codons NNC and NNU.

At first, as already described, it is most likely that tRNAs possessing the anticodon CNN together with t^6A37 are more effective competitors for NNA codons than tRNAs possessing the anticodon GNN. $tRNA^{Met}CAU$ and $tRNA^{Lys}CUU$ possessing t^6A37 (or $tRNA^{Met}$ with anticodon f^5CAU and A37) win the competition with $tRNA^{Ile}GAU$ and $tRNA^{Asn}GUU$, respectively, toward the codon ending with A in fruit fly mitochondria. It is already known that the anticodon QNN reads only NNU and NNC codons (38), which is the case for mt $tRNA^{Asn}$ of fruit fly (Fig. 6a). If position 37 is not modified, such tRNAs possessing unmodified C34 would fail the competition with tRNAs possessing G34 toward the recognition of the codons ending with A (compare fruit fly tRNAs with starfish counterparts in Fig. 6).

Next, let us consider other cases either with different kinds of competitor or without competitor. In vertebrate mitochondria, AGA/AGG codons are considered to be termination codons, which are preferentially recognized by a release factor (a strong competitor), so that $tRNA^{Ser}GCU$ recognizes only codons AGU/AGC. In the ascidian (*H.rorentzi*) mitochondria, AGA/AGG codons are considered to be read as glycine (36) by $tRNA^{Gly}$ with anticodon U*CU (22) (also a strong competitor). Therefore, $tRNA^{Ser}GCU$ recognizes only the usual serine codons AGU/AGC. On the other hand, since there exists no tRNA to decode AGA/AGG codons in fruit fly mitochondria, and moreover, because AGG is an unassigned codon (12,13), the AGA codon will be read by $tRNA^{Ser}GCU$ with the anticodon GCU.

Elucidation of the molecular mechanisms of these decoding patterns requires further detailed study.

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