Wobble modification defect in tRNA disturbs codon–anticodon interaction in a mitochondrial disease

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We previously showed that in mitochondrial tRNA^{Lys} with an A8344G mutation responsible for myoclonus epilepsy associated with ragged-red fibers (MERRF), a subgroup of mitochondrial encephalomyopathic diseases, the normally modified wobble base (a 2-thiouridine derivative) remains unmodified. Since wobble base modifications are essential for translational efficiency and accuracy, we used mitochondrial components to estimate the translational activity *in vitro* of purified tRNA^{Lys} carrying the mutation and found no mistranslation of non-cognate codons by the mutant tRNA, but almost complete loss of translational activity for cognate codons. This defective translation was not explained by a decline in aminoacylation or lowered affinity toward elongation factor Tu. However, when direct interaction of the codon with the mutant tRNA^{Lys} defective anticodon was examined by ribosomal binding analysis, the wild-type but not the mutant tRNA^{Lys} bound to an mRNA–ribosome complex. We therefore concluded that the anticodon base modification defect, which is forced by the pathogenic point mutation, disturbs codon–anticodon pairing in the mutant tRNA^{Lys}, leading to a severe reduction in mitochondrial translation that eventually could result in the onset of MERRF.

Keywords: anticodon/mitochondrial disease/
mitochondrial translation/mitochondrial tRNA/
post-transcriptional modification

Introduction

Mutations in tRNA genes encoded on mitochondrial DNA (mtDNA) are associated with a wide spectrum of human pathologies caused by mitochondrial disorders (Schon *et al.*, 1997). A point mutation in the tRNA^{Lys} gene at nucleotide position 8344, which is responsible for myoclonus epilepsy associated with ragged-red fibers (MERRF) (Fukuhara *et al.*, 1980)—a major subgroup of the mitochondrial encephalomyopathies—was the first

reported disease-related tRNA gene mutation (Shoffner *et al.*, 1990). On the other hand, point mutations at nucleotide positions 3243 and 3271 in the tRNA^{Leu(UUR)} (R = A and G) gene were found in patients with another major subgroup—mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (Goto *et al.*, 1990, 1991; Kobayashi *et al.*, 1990).

By establishing cybrid cell clones in which mutant mtDNA derived from patients was transferred intercellularly into human mtDNA-lacking (ρ^0) cells (King and Attardi, 1989; Hayashi *et al.*, 1991), the above three mutations (i.e. those at nucleotide positions 8344, 3243 and 3271) were revealed to be directly involved in mitochondrial dysfunction. In particular, the A to G transition at nucleotide position 8344 in the tRNA^{Lys} gene was demonstrated to cause severe impairment of mitochondrial protein synthesis and respiratory deficiency in cultured myoblasts from MERRF patients (Boulet *et al.*, 1992) and in ρ^0 transformants (Chomyn *et al.*, 1991; Yoneda *et al.*, 1994). Peptide analysis carried out to try and explain the molecular mechanism suggested the occurrence of premature translation termination resulting from a decrease in aminoacyl-tRNA^{Lys} (Enriquez *et al.*, 1995). On the other hand, in tissue biopsies from MERRF patients harboring the 8344 mutation, neither the relative abundance nor the aminoacylation of the mutated tRNA^{Lys} was found to be affected (Börner *et al.*, 2000), which raises the question of whether the amount of aminoacyl-tRNA^{Lys} significantly affects the molecular pathogenesis of MERRF.

Recently, we found that a modification normally present at the anticodon wobble nucleotide is absent in mitochondrial tRNA^{Lys} with the MERRF mutation [tRNA^{Lys}(A8344G)] (Yasukawa *et al.*, 2000a), while the other modified nucleotides remain normal in the mutant tRNA^{Lys} (Figure 1). In addition, modification at the wobble position is also defective in tRNA^{Leu(UUR)} with either the MELAS 3243 [tRNA^{Leu(UUR)}(A3243G)] or 3271 [tRNA^{Leu(UUR)}(U3271C)] mutation (Yasukawa *et al.*, 2000b). The wild-type tRNAs have novel uridine derivatives at the wobble position, whose chemical structures have been identified recently (T.Suzuki, T.Suzuki, T.Wada, K.Saigo and K.Watanabe, in preparation). Both tRNAs have a taurinomethyl group at position 5 of the uridine, while tRNA^{Lys} additionally has a thio-group at position 2 [tRNA^{Lys}_{sU*UU} and tRNA^{Leu(UUR)}_{U*AA}, respectively]. Uridine modification at the wobble position has been studied in terms of its apparent importance in codon–anticodon interaction (Yokoyama and Nishimura, 1995; Takai *et al.*, 1996; Hagervall *et al.*, 1998; Krüger *et al.*, 1998). Ashraf *et al.* (1999) have also shown that modification of the wobble uridine, particularly thiolation at position 2, is essential for tRNA^{Lys}_{sU*UU} species having 2-thiouridine derivatives at the wobble position

Fig. 1. Cloverleaf structures of human mitochondrial tRNA^{Lys} from wild-type cells (left) and from MERRF patient-derived cells with the A8344G mutation (right). sU* indicates the modified uridine, and U on a round black background, unmodified uridine. G on a square black background represents the point mutation. The other modified nucleosides were determined previously: 1-methyladenosine (m¹A), 2-methylguanosine (m²G), pseudouridine (Ψ) and *N*⁶-threonylcarboxyladenosine (t⁶A) (Helm *et al.*, 1998; Yasukawa *et al.*, 2000a).

(5-methylaminomethyl-2-thiouridine (mm⁵s²U) in *Escherichia coli* tRNA^{Lys} and 5-methoxycarbonylmethyl-2-thiouridine in human cytoplasmic tRNA^{Lys}³, respectively) to interact with codons on ribosomes.

These findings prompted us to speculate that the MERRF-mutant tRNA^{Lys} intrinsically loses its translational activity due to an impairment of codon–anticodon interaction, eventually leading to mitochondrial dysfunction. To verify our speculation, we estimated the translational ability of tRNA *in vitro* using mitochondrial translation components and were able to obtain clear experimental evidence that the mutant tRNA^{Lys} with the unmodified anticodon UUU (tRNA^{Lys}_{UUU}) is actually unable to translate its cognate codons AAR. Furthermore, taking advantage of ribosomal binding analysis, we show that the mutant tRNA^{Lys} does not bind to AAA-programmed ribosomes. Our findings lead us to conclude that the wobble modification defect is primarily responsible for dispossessing the mutant tRNA^{Lys} of its cognate codon binding affinity, forcing the mutant tRNA^{Lys}_{UUU} to become translationally inactive, which subsequently results in mitochondrial dysfunction. This is the first evidence that a post-transcriptional modification deficiency causes a human disease.

Results

Mitochondrial translation and oxygen consumption in MERRF-mutant cybrid cells

The cybrid cell lines used were constructed previously by the intercellular transfer of mtDNA from a MERRF patient, or from fetal human fibroblasts as a control, to ρ⁰ HeLa cells (King and Attardi, 1989; Hayashi *et al.*, 1991). A mutant cybrid clone (ME1-4) exclusively harboring mtDNA with the A8344G MERRF mutation consumed oxygen at a significantly lower rate (1.7 ± 0.2 fmol/min/cell) than the control cybrid (Ft2-11) with the wild-type mtDNA (5.3 ± 0.8 fmol/min/cell) when the oxygen

Fig. 2. (A) [³⁵S]Methionine incorporation into the Ft2-11 wild-type cybrid (Wt) versus the ME1-4 MERRF-mutant cybrid (MERRF). CO, cytochrome *c* oxidase subunit; ND, NADH-ubiquinone oxidoreductase subunit; A, ATP synthase subunit. The arrow indicates the reported premature translation termination product of COI (Enriquez *et al.*, 1995). (B) Western blots of cytochrome *c* oxidase subunits (COI, COII and COIV), dihydrolipoamide succinyltransferase (DLST) and translocase of the outer membrane 40 (TOM 40) in Ft2-11 and ME1-4 cybrids.

consumption was measured as described by King and Attardi (1989). This finding is consistent with previous observations (Chomyn *et al.*, 1991; Yoneda *et al.*, 1994). Figure 2A shows the mitochondrial translation products labeled with [³⁵S]methionine for 50 min in the presence of a cytoplasmic translation inhibitor, emetine, in which the polypeptides were identified as reported previously (Hayashi *et al.*, 1993). The labeling efficiency of all the polypeptides was markedly reduced, indicating that the overall rate of protein synthesis in ME1-4 cells was very slow as compared with that in Ft2-11 cells. A faint but reproducible additional band was detected (indicated by an arrow in Figure 2A), which probably corresponds to the premature translation termination product of cytochrome *c* oxidase subunit I (COI) as has been reported (Chomyn *et al.*, 1991; Boulet *et al.*, 1992; Yoneda *et al.*, 1994; Enriquez *et al.*, 1995). The intensity of the abnormal band may depend on the nuclear background of the cybrid cells. Pulse labeling for 30 min and 2 h gave similar results (not shown). Western blot analysis (Figure 2B) revealed severely decreased steady-state levels of not only mitochondrially encoded cytochrome *c* oxidase subunits I and II (COI and COII) but also of nuclearly encoded subunit IV (COIV) in the mutant cells. The steady-state levels of internal control mitochondrial proteins, dihydrolipoamide succinyltransferase (DLST) (Nakano *et al.*, 1993) and a translocase of the outer mitochondrial membrane (TOM 40) (Pfanner and Geissler, 2001), were comparable in the mutant and wild-type cybrids.

Steady-state amounts and extent of aminoacylation of mutant and wild-type tRNA^{Lys} in cybrid cells

Northern blot analysis showed no reduction in the steady-state amount of the mutant tRNA^{Lys}(A8344G) in the ME1-4 cybrid compared with that of the wild-type tRNA^{Lys} in the Ft2-11 cybrid (Figure 3, lanes 1 and 4, and Figure 4 at time 0). The lysylation of the wild-type and mutant

Fig. 3. Quantitative analysis of aminoacyl-tRNA^{Lys} in cells. The upper and lower bands correspond to aminoacyl and uncharged tRNA^{Lys}, respectively. Lanes 2 and 3 depict RNA samples prepared under acidic conditions at 4°C from Ft2-11 and ME1-4 cybrids, respectively; lanes 1 and 4 show the Ft2-11 RNA and ME1-4 RNA, respectively, both treated with alkali.

tRNAs^{Lys} was also examined by separating aminoacylated and uncharged tRNAs using acid urea polyacrylamide gel electrophoresis followed by northern hybridization (Varshney *et al.*, 1991). Although the amount of uncharged tRNA^{Lys} in the mutant cybrid was more than that in the control, the extent of lysylation in the mutant tRNA^{Lys} appeared not to be markedly reduced (Figure 3), being 80% in the mutant cybrid and 93% in the control as determined by careful and repeated quantification.

Stability of mutant tRNA^{Lys} and its wild-type counterpart in cells

To verify the finding of comparable steady-state amounts of tRNA^{Lys} in mutant and wild-type cybrid cells, we further examined the degradation rates of the tRNAs^{Lys} and, as a control, of tRNA^{Ile} in the presence of a potential mitochondrial transcription inhibitor, ethidium bromide (EtBr) (Hayashi *et al.*, 1990). Both the wild-type tRNA^{Lys} and tRNA^{Ile} in the Ft2-11 cells appeared to degrade slightly faster than their respective counterparts in the ME1-4 cells (Figure 4A), which might result from a difference in sensitivity to EtBr between the two cell lines. However, the amounts of remaining tRNA^{Lys} normalized at each time point by that of the remaining tRNA^{Ile} in the respective cybrid cells were comparable (Figure 4), showing that the point mutation occurring in the TΨC loop of tRNA^{Lys} affects neither the stability of the tRNA nor the steady-state level *in vivo*.

Aminoacylation efficiency and affinity for elongation factor Tu of mutant tRNA^{Lys} *in vitro*

Aminoacylation and elongation factor Tu (EF-Tu)-dependent hydrolysis protection assays were performed *in vitro* to observe the aminoacylation efficiency and affinity of tRNAs^{Lys} toward EF-Tu, respectively. For the following *in vitro* experiments, both the wild-type and mutant tRNAs^{Lys} were purified homogeneously by a solid-phase probing technique (Wakita *et al.*, 1994) from respective mass cultures of wild-type and mutant cells. The initial rates of tRNA^{Lys} lysylation were determined using mitochondrial lysyl-tRNA synthetase (KRS_{mt}) purified from bovine liver by a conventional purification method (see Materials and methods). As shown in Table I, the kinetic parameters for the lysylation of the wild-type and mutant tRNAs^{Lys} did not differ significantly.

For the hydrolysis protection assay, recombinant bovine mitochondrial EF-Tu (EF-Tu_{mt}) was used and preparative

Fig. 4. Time-dependent degradation of tRNA in the respective cybrid clones in the presence of a potential inhibitor of mitochondrial transcription. (A) Examples of northern hybridization for tRNA^{Lys} and tRNA^{Ile} in the wild-type cybrid (left panels) and in the MERRF cybrid (right panels). (B) Time courses of quantitative analysis for the wild-type tRNA^{Lys} (filled circles) and the mutant tRNA^{Lys} (filled squares), normalized with tRNA^{Ile} in the respective cybrid clones. The average values at the starting time were defined arbitrarily as 100. Each set of data represents the average of at least three independent northern blotting experiments (SD bars are within the circles and squares).

Table I. Kinetic parameters in aminoacylation of wild-type and MERRF-mutant tRNA^{Lys}

Substrate	K_m (μ M) ^a	V_{max} (μ M/s)	V_{max}/K_m (relative)
Wild-type tRNA ^{Lys}	0.71	2.9×10^{-3}	1
MERRF tRNA ^{Lys} (A8344G)	0.18	2.4×10^{-4}	0.32

^aThe apparent K_m values are given since the KRS_{mt} used was a partially purified fraction.

aminoacylation of native tRNA^{Lys} with or without the A8344G mutation was carried out with sufficient amounts of KRS_{mt} and [³H]lysine. The stability of the ternary complex (Lys-tRNA^{Lys}·EF-Tu_{mt}·GTP) against non-enzymatic deacylation under an alkaline pH condition was estimated by measuring the remaining radioactivity of [³H]Lys-tRNA^{Lys} in the complex (Pingoud *et al.*, 1977). Although the half-life of the wild-type tRNA^{Lys} was about double that of the mutant tRNA^{Lys} in the presence of each amount of EF-Tu_{mt} used (Figure 5C), the plots in Figure 5A and B show that both tRNAs^{Lys} were recognized efficiently by EF-Tu_{mt}.

Translational activities of tRNAs estimated *in vitro* using bovine mitochondrial translation components

We examined whether the MERRF mutant tRNA^{Lys}-(A8344G), as well as the MELAS mutants tRNA^{Leu}(UUR)₋(A3243G) and tRNA^{Leu}(UUR)(U3271C), all lacking the modification only at the wobble position, could function in the translation elongation process. The *in vitro* assay was performed basically according to the literature (Eberly *et al.*, 1985; Takemoto *et al.*, 1995) using bovine

Fig. 5. Stability of the ternary complex $[^3\text{H}]\text{Lys-tRNA}^{\text{Lys}}\cdot\text{EF-Tu}_{\text{mt}}\cdot\text{GTP}$ against non-enzymatic deacylation. Time courses of remaining radioactivity of (A) wild-type and (B) mutant $[^3\text{H}]\text{Lys-tRNA}^{\text{Lys}}$ determined at each indicated time point in the presence of the following amounts of EF-Tu_{mt}: 0.5 pmol (open triangles), 5 pmol (open circles), 25 pmol (filled squares), 37.5 pmol (filled triangles) and 50 pmol (filled circles), and in the absence of EF-Tu_{mt} (open squares). Values at the starting time (0 min) were defined as 100. (C) Half-life values ($T_{1/2}$) of the wild-type $[^3\text{H}]\text{Lys-tRNA}^{\text{Lys}}$ (open circles) and mutant $[^3\text{H}]\text{Lys-tRNA}^{\text{Lys}}$ (open squares) plotted against the ratio of EF-Tu_{mt}/ $[^3\text{H}]\text{Lys-tRNA}^{\text{Lys}}$ initially added to the reaction mixture.

mitochondrial components. To compare the translational efficiencies, all the tRNAs used were purified from the respective cell lines and equal amounts of the ^3H -labeled aminoacyl-tRNAs prepared were put into the reaction mixtures as described in Materials and methods. Since we wished to emphasize the codon reading efficiency of the wild-type and mutant tRNAs, instead of natural mRNAs we used synthetic RNAs containing 30 repeats of cognate (AAA and AAG) and non-cognate (AAC and AAU) codons for tRNA^{Lys}, and of cognate (UUA and UUG) codons for tRNA^{Leu(UUR)}, respectively. Figure 6A and B shows that the mutant tRNA^{Lys}_{UUU}(A8344G) could not translate AAA and AAG codons, whereas the translation reaction proceeded quite efficiently with the wild-type tRNA^{Lys}. Neither the mutant nor the wild-type tRNA^{Lys} showed translational activity for non-cognate codons under the conditions used (Figure 6C and D). In addition, the translational activities of the two MELAS-mutant tRNAs^{Leu(UUR)} for the cognate codons also decreased to a greater or lesser extent (Figure 6E and F). This result clearly supports our hypothesis that the MERRF-mutant tRNA^{Lys} loses its translational activity, at least *in vitro*.

Binding affinity of mutant tRNA^{Lys}_{UUU} for AAA-programmed ribosomal small subunits

Although the above experiment showed that the mutant tRNA^{Lys}_{UUU} almost completely lost its translational

Fig. 6. Summary of *in vitro* translational elongation activities. (A–D) Translational efficiencies of the wild-type tRNA^{Lys}_{UUU} (Wt) and mutant tRNA^{Lys}_{UUU} with the A8344G mutation (MERRF) toward codons AAA, AAG, AAC and AAU. (E and F) Translational efficiencies of the wild-type tRNA^{Leu(UUR)}_{UAA} (Wt) and two mutant tRNAs^{Leu(UUR)}_{UAA} with the A3243G (MELAS 3243) or U3271C (MELAS 3271) mutation toward codons UUA and UUG. The radioactivity of the $[^3\text{H}]\text{aminoacyl-tRNA}$ initially added to the reaction mixture was defined arbitrarily as 100. Each set of data represents the average of three independent experiments, with bars showing the SD.

activity *in vitro*, it was essential to prove that this translational deficiency arose from the wobble modification defect itself but was not a direct result of the A to G replacement in the T Ψ C loop (Figure 1). For this purpose, we considered it appropriate to apply the binding assay of AAA-programmed *E. coli* ribosomal small subunits. Since a crystallographic analysis has shown that only the tRNA anticodon stem-loop (ASL) region, but not the T Ψ C loop, is located on the small subunits when tRNA binds to the ribosomes (Ogle *et al.*, 2001; Yusupov *et al.*, 2001), the possibility of the mutant nucleotide directly affecting

Fig. 7. Binding of the wild-type tRNA^{Lys}_{sU*UU} (filled circles) and the MERRF-mutant tRNA^{Lys}_{UUU} (filled squares) to AAA-programmed ribosomal small subunits. Three independent experiments were performed and the average values are plotted on the graph, with the bars indicating the SD.

codon–anticodon pairing in this binding assay can be excluded. As is clearly shown in Figure 7, the wild-type tRNA^{Lys}_{sU*UU} bound efficiently to AAA-programmed small subunits, but the mutant tRNA^{Lys}_{UUU} did not. This finding suggests that the defective translational activity of the MERRF-mutant tRNA^{Lys} is caused by an inability to form codon (AAA)–anticodon (unmodified UUU) base pairs on the ribosomes.

Discussion

As has been reported previously (Chomyn *et al.*, 1991; Yoneda *et al.*, 1994; Enriquez *et al.*, 1995), the overall rate of mitochondrial protein synthesis was shown to be severely impaired in MERRF-mutant cybrid cells (Figure 2A). As expected, the steady-state amounts of mitochondrially encoded COI and COII proteins were very low (Figure 2B). The level of nuclear-encoded COIV was also found to decrease markedly in the mutant cybrid, which presumably can be accounted for by enhanced intramitochondrial degradation previously observed in ρ^0 cells in which mitochondrial subunits were absent, and as a consequence cytochrome *c* oxidase could not be built up (Nijtmans *et al.*, 1995). This might also be the case with NADH-ubiquinone oxidoreductase, because seven of the subunits are produced from mtDNA. It is possible to speculate that if a very small number of mitochondria exclusively had the wild-type tRNA^{Lys}, while the vast majority had only the mutant counterpart, the faint radiolabeled protein bands in Figure 2A would be derived from normal mitochondrial translation provided by a small number of mitochondria.

Enriquez *et al.* (1995) found decreased availability of aminoacyl-tRNA^{Lys} with the 8344 mutation, which they postulated most probably brought about the translational defect in their mutant osteosarcoma-derived cybrid clones. In contrast, we found that the stability and steady-state amount of the mutant tRNA^{Lys}(A8344G) in the ME1-4 cybrid remained unchanged when compared with its wild-type counterpart in the Ft2-11 cybrid (Figure 4).

Furthermore, the extent of aminoacylation of the mutant tRNA^{Lys} was comparable to that of the wild-type in the respective cybrids (Figure 3). Consistent with our findings, Börner *et al.* (2000) reported that neither the relative abundance nor the aminoacylation of mutated tRNA^{Lys} was reduced in tissue biopsies from MERRF patients. The reason for the discrepancy with the result reported by Enriquez *et al.* (1995) is unknown, but it may be related to the cell types used. Nevertheless, our findings and those of Börner *et al.* (2000) both indicate that even without a reduction of aminoacylation, respiration declines in MERRF patients' cells.

While the above analysis is salient, it was still considered important to elucidate the reason for the slightly reduced aminoacylation level in the mutant cybrid (Figure 3). The apparent kinetic parameters determined by a standard *in vitro* aminoacylation assay using bovine KRS_{mt} showed that the lysine accepting activities (V_{max}/K_m values) of the wild-type and mutant tRNAs^{Lys} were similar, suggesting that the reduced aminoacylation level has no pathogenic significance (Table I). Because bovine KRS_{mt} had the activity to aminoacylate human tRNA^{Lys} as well as its bovine counterpart and the kinetic parameters in these aminoacylations were comparable (data to be published elsewhere), the region around the mutation point may not include an identity element(s) of the human, or bovine, tRNA^{Lys} toward the corresponding KRS_{mt}. For these reasons, the parameters obtained using bovine KRS_{mt} would appear to be reasonable.

Since bovine EF-Tu_{mt} bound efficiently to human aminoacyl-tRNA^{Lys}, as would be expected from the fact that bovine and human EF-Tu_{mt} are 95% identical in their sequences (Worix *et al.*, 1995), we used bovine EF-Tu_{mt} instead of its human counterpart for an EF-Tu-dependent hydrolysis protection analysis. Figure 5C shows that the half-life of Lys-tRNA^{Lys} was shortened in the case of the mutant tRNA^{Lys}, which implies that the binding affinity of EF-Tu_{mt} toward the mutant tRNA decreased to some extent. This finding is intriguing in seeking an explanation for the slight decrease in the ratio of aminoacyl-tRNA^{Lys} to total tRNA^{Lys} in mutant cybrid cells (Figure 3). The reduced affinity of the aminoacylated mutant tRNA^{Lys} toward EF-Tu_{mt} presumably could be a candidate for explaining the decreased aminoacylation in the osteosarcoma background cybrid reported by Enriquez *et al.* (1995). However, in this study, the difference in affinity toward EF-Tu_{mt} between the wild-type and mutant tRNAs^{Lys} would not affect the translational efficiency *in vivo* since the ratio of aminoacylated tRNA^{Lys} to total tRNA^{Lys} was not greatly reduced in the mutant compared with that in the control cells (Figure 3). Additionally, in the *in vitro* translation reaction mixture without mRNA, the rates of deacylation in the wild-type and mutant tRNA^{Lys} were observed to be almost identical (not shown). Since, for technical reasons, we used bovine enzymes in the two *in vitro* experiments above, the possibility that the results were due to heterogeneous enzymes cannot be excluded completely. Nevertheless, taking the *in vivo* and *in vitro* analyses together, it is reasonable to consider that the amount of aminoacylated tRNA is not the main factor underlying mitochondrial dysfunction in MERRF-mutant cells.

We have already noted that the rate of mitochondrial protein synthesis was severely impaired in MERRF-mutant cybrid cells. At the same time, it has been proven that modification of a wobble uridine is indispensable for the tRNA^{Lys}_{sU*UU} species to bind poly(A) on ribosomal small subunits (Ashraf *et al.*, 1999), and we recently found that the wobble modification is lacking in the MERRF-mutant tRNA^{Lys} (Yasukawa *et al.*, 2000a). Modifications of wobble bases are, in general, known to be very important for regulating the translational efficiency and accuracy of codon–anticodon base pairing. In addition, as shown in Figure 3, the absence of the wobble modification in the mutant tRNA^{Lys} did not affect the aminoacylation, which is similar to the case in an *in vivo* study in which aminoacylation of *E. coli* tRNA^{Lys} was shown to be unaffected by a specific lack of the wobble uridine modification (mnm⁵s²U) (Hagervall *et al.*, 1998). Therefore, we examined whether the mutant tRNA^{Lys} is deprived of translational activity. For this purpose, we estimated the mutant tRNA^{Lys}_{UUU} activity in the translation elongation process *in vitro* using mitochondrial components and demonstrated that the mutant tRNA^{Lys}_{UUU} did indeed lose a significant amount of its essential function of translating lysine codons (Figure 6A and B). The magnitude of the decline in the translational activity of the mutant tRNA may have been overestimated to a certain extent, because unlike the actual circumstances in the mitochondrion, the mRNAs used contained repeats of the test codon. Furthermore, the *in vitro* conditions were heterogeneous. Nevertheless, we consider the *in vitro* assay appropriate to emphasize the defect of the mutant tRNA. Mistranslation of non-cognate codons was not observed in this system (Figure 6C and D). This finding is, unexpectedly, different from the cases of many mitochondrial and *Mycoplasma* tRNAs containing unmodified uridine at the wobble position (U34) that are specific for amino acids corresponding to four codon boxes (Samuelsson *et al.*, 1983; Inagaki *et al.*, 1995; Watanabe and Osawa, 1995).

To elucidate the molecular basis of the tRNA^{Lys} dysfunction, we next examined *in vitro* the binding affinity of the tRNAs for mRNA-programmed ribosomal small subunits, which is a widely accepted method for examining codon–anticodon interactions (von Ahsen *et al.*, 1997; Ashraf *et al.*, 1999; Yarian *et al.*, 2000). We excluded the possibility that the pathogenic point mutation (G at nucleotide position 8344) in the TΨC loop may affect the codon–anticodon pairing by using ribosomal small subunits. It is known that only the ASL region of tRNA interacts with mRNA on the small subunits, and the TΨC loop is not involved in the interaction (Ogle *et al.*, 2001; Yusupov *et al.*, 2001). Furthermore, ASL minihelices with appropriate modifications showed binding equivalent to intact tRNAs toward messenger-programmed small subunits (Ashraf *et al.*, 1999; Yarian *et al.*, 2000). We used *E. coli* small ribosomal subunits instead of the human mitochondrial counterparts since it has been shown that mitochondrial tRNA enables translation to proceed on *E. coli* ribosomes and, conversely, that *E. coli* tRNA does likewise on mitochondrial ribosomes (Schwartzbach and Spremulli, 1989; Kumazawa *et al.*, 1991). Similar to the result shown in Figure 6, in an *in vitro* assay using *E. coli* ribosomes instead of their mitochondrial counterparts, the

wild-type mitochondrial tRNA^{Lys} allowed the translation reaction to proceed efficiently, whereas the MERRF-mutant tRNA^{Lys} did not (data not shown). Therefore, this binding assay system permitted us to compare the physicochemical interactions of the wild-type and mutant tRNAs with AAA-programmed small subunits. In fact, the experiment clearly showed that while the wild-type tRNA^{Lys} with the modified uridine bound efficiently to the cognate codon, the mutant tRNA^{Lys}_{UUU} did not under the same conditions (Figure 7). From this result, we infer that the defective translational activity of the mutant tRNA^{Lys}_{UUU} is derived from its inability to form codon–anticodon base pairs, at least *in vitro*, most probably due to the lack of the wobble modification.

On the other hand, there is a possibility that the base substitution in the TΨC loop could itself directly affect the translational activity of the tRNA^{Lys}, because the mutation would have some influence on the interaction of the TΨC loop with intact ribosomes, thereby altering the translational activity of the mutant tRNA. Such an effect, if it exists, may not, however, be critical for the defective translation of the mutant tRNA observed in this study (Figure 6A and B), since the TΨC loop sequence is not conserved among mammalian mitochondrial tRNA^{Lys} (Sprinzl *et al.*, 1998). Moreover, as shown in Figure 7, the translational defect can be explained by the apparent inability for an interaction to occur between codon and the mutant tRNA^{Lys} on the ribosomal small subunit.

Another possibility to be considered is that the observed translational deficiency may arise indirectly from a structural alteration, especially in the anticodon region, induced by the mutation. If a putative modification enzyme(s) of the wobble base is able to recognize the structure of the anticodon region for modifying the base, this possibility cannot be ruled out. However, structural alteration seems unlikely because the tRNA maturation, including processing and base modifications, was normal except for the specific lack of the wobble modification in the mutant tRNA^{Lys} (Yasukawa *et al.*, 2000a) and the aminoacylation and affinity toward EF-Tu_{mt} were not significantly affected (Table I; Figures 3 and 5). Moreover, the lifetime of the mutant tRNA was not changed (Figure 4), suggesting that the structural stability of the mutant tRNA was not influenced by the point mutation. Therefore, we conclude that the mutant tRNA could have the point mutation without incurring structural alteration and, even if such alteration does occur, the translational activity is likely to be affected only to a small extent.

The ability of an unmodified ASL minihelix of human cytoplasmic tRNA^{Lys3}_{UUU} to bind to AAA-programmed *E. coli* small ribosomal subunits was reported to be restored by the introduction of a single modified nucleotide, N⁶-threonicarboxyladenosine (t⁶A), to position 37, 3'-adjacent to the anticodon third letter (Yarian *et al.*, 2000). This does not agree with the case of the mutant mitochondrial tRNA^{Lys}_{UUU}, probably because the anticodon stem sequence is not at all conserved between the cytoplasmic tRNA^{Lys3} and the mitochondrial tRNA^{Lys} (Figure 1).

The translational activity appeared to decrease more severely in the cases of AAG and UUG than AAA or UUA (Figure 6), indicating that G at the third position of the codon was recognized less efficiently than A by the

unmodified U34 (Takai *et al.*, 1996). The data in Figure 6 suggest that the modification at position 5 (taurinomethyl group) of U34 in both tRNA^{Lys}_{sU*UU} and tRNA^{Leu(UUR)}_{U*AA} is very important for codon–anticodon interaction and stabilization, particularly of the wobble pair, presumably because it is required for the anticodon to be arranged into an appropriate structure. The tendency observed in this study is in accordance with a report that lack of the modification at position 5 and thiolation at position 2 of the uridine base at the wobble position in *E. coli* tRNA^{Glu} reduces the translation rate of codons ending with G (Krüger *et al.*, 1998). Figure 6 also indicates that the MERRF mutation had the most drastic effect on translational ability, which may be related to the fact that in addition to the modification at position 5 of U34, thiolation at position 2 is necessary for tRNA^{Lys}, but not for tRNA^{Leu(UUR)}, to be functional. This postulation is supported by a report that thiolation at position 2 of U34 plays a crucial role in the ribosomal binding of tRNA^{Lys} species (Ashraf *et al.*, 1999) as well as by the finding that this modification is particularly important for the recognition of A at the third letter of the codon in the *in vivo* translation system of *E. coli* (Krüger *et al.*, 1998). In contrast to the case of MERRF, the stability and aminoacylation of both mutant tRNAs^{Leu(UUR)} were found to decrease (Yasukawa *et al.*, 2000b), suggesting that the molecular pathogenesis of MELAS could be a combination of a lowered availability of aminoacyl-tRNA^{Leu(UUR)} and defective translation.

The defective translational activity of the MERRF-mutant tRNA^{Lys}_{UUU} can easily explain the exponential decrease in the rate of mitochondrial protein synthesis with increasing lysine content and premature termination of translation, which were elegantly described by Attardi and colleagues (Enriquez *et al.*, 1995). Lysine codons in 12 mRNAs encoded on mtDNA seem to be distributed almost uniformly in each mRNA, and larger polypeptides tend to have more lysines (Anderson *et al.*, 1981). Impairment of mitochondrial translation may result from stalling of ribosomes with an empty A-site at lysine codons due to a deficiency in the interaction of the mutant tRNA^{Lys}_{UUU} with lysine codons, leading to premature termination. Another possibility is that frameshifting may occur at lysine codons and abortive proteins might then be produced. Prematurely terminated proteins and polypeptides with abnormal sequences are probably not assembled in the complexes for the electron transport chain but instead are degraded rapidly by ATP-dependent mitochondrial proteases (Käser and Langer, 2000). Even if some peptides are produced in their full length (e.g. ND4L, which does not contain lysine), the nascent polypeptides are likely to be in danger of degradation—as happened with COIV (Figure 2B)—by proteases, since the loss of other peptides may disturb the normal assembly of intact subunits in the complex.

In conclusion, our study has unraveled the essential molecular mechanism causing mitochondrial dysfunction in MERRF pathology. A point mutation at nucleotide position 8344 in the mtDNA results in a modification defect at the anticodon first letter of the mutant tRNA^{Lys} molecule, which subsequently disturbs codon–anticodon interaction, severely impairing mitochondrial translation.

This is the first proof that a post-transcriptional modification deficiency causes a human disease.

Materials and methods

Cybrid cell lines

The mutant cybrid cell lines, which were constructed by the intercellular transfer of MERRF or MELAS patient mtDNA to p⁰ HeLa cells (EB8) (King and Attardi, 1989; Hayashi *et al.*, 1991), were described previously (Yasukawa *et al.*, 2000a,b). The ME1-4, ML2-2-2 and ML5-1-13 cell lines exclusively contain mtDNA with the A8344G, A3243G and T3271C point mutations, respectively. The Ft2-11 cell line, used as a wild-type control, harbors mitochondria containing wild-type mtDNA derived from fetal human fibroblasts (Hayashi *et al.*, 1991). The cells were cultured in normal medium [Dulbecco's modified Eagle's medium/F-12 (1:1) (Gibco-BRL), 10% fetal calf serum (FCS)].

Oxygen consumption

The rate of oxygen consumption by $0.4\text{--}1 \times 10^7$ exponentially growing cells in 2 ml of the normal medium was measured at 37°C using a Clark-type electrode as described (King and Attardi, 1989).

Mitochondrial protein synthesis *in vivo*

[³⁵S]methionine incorporation into mitochondrially encoded proteins was analyzed essentially as described (Chomyn *et al.*, 1991; Hayashi *et al.*, 1993). Semiconfluent cells were labeled with [³⁵S]methionine (43.5 TBq/mmol) (American Radiolabeled Chemicals) containing 0.2% FCS in the presence of emetine (0.2 mg/ml) for 50 min. The mitochondrial fraction was separated by SDS–PAGE. The gel was dried and exposed to an imaging plate, and the labeled polypeptides were located with a bioimaging analyzer (BAS 1000, Fuji Photo Film).

Western blotting analysis

Western blotting was performed essentially as described (Towbin *et al.*, 1979). Total mitochondrial protein was separated on an SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore). Subsequently, the proteins were visualized specifically using antibodies against COI, COII (Molecular Probes), COIV (Clontech Laboratories), DLST and TOM 40.

Evaluation of tRNA aminoacylation levels in cybrids

Total RNA from semiconfluent cultured cybrid cells was prepared under acidic conditions at 4°C in order to prevent deacylation. A portion of the RNA sample was incubated at 37°C for 3 h in a buffer containing 50 mM Tris–HCl pH 9.5 for deacylation of tRNA. The same amount of total RNA containing aminoacyl-tRNAs or forcibly deacylated tRNAs was electrophoresed at 4°C through an acid urea polyacrylamide gel to separate aminoacyl-tRNA and uncharged tRNA (Varshney *et al.*, 1991) and the RNA was blotted onto a nylon membrane (Hybond N, Amersham Pharmacia Biotech). Northern hybridization for tRNA^{Lys} was performed using a ³²P-5'-end labeled probe specific for mitochondrial tRNA^{Lys}: 5'-TCACTGTAAAGAGGTGTTGG-3' (not including the mutation position). Aminoacyl- and non-acylated tRNAs^{Lys} were quantified by exposing the membrane to an imaging plate, followed by analysis with a Fuji BAS1000 bioimaging analyzer.

Analysis of tRNA stability in cybrids

The procedure was essentially identical to that described previously (Yasukawa *et al.*, 2000b). Equal amounts of semiconfluent cells were put into four dishes and cultured in a medium containing EtBr (250 ng/ml) to block specifically mitochondrial transcription (Hayashi *et al.*, 1990). At the indicated times, total RNA was isolated from one of the dishes by Isogen (Nippon Gene, Toyama, Japan). A sample (5 µg) of total RNA was electrophoresed on a denaturing polyacrylamide gel. Northern hybridization and quantification of RNAs were performed as described above. A ³²P-5'-end labeled probe specific for mitochondrial tRNA^{Leu} (5'-TAG-AAATAAGGGGGTTTAAGCTCCTATTAT-3') was used to detect tRNA^{Leu}.

Purification of tRNAs from cybrid cells by the solid-phase probe method

About 1000–2000 A₂₆₀ units (the actual amount varied depending on the cell line) of total RNA were obtained from a large-scale culture of the semiconfluent cell. A portion of the preparation for wild-type tRNAs was isolated from HeLa cells. The tRNA^{Lys} and tRNA^{Leu(UUR)} with or without

the mutations were purified homogeneously from the respective total RNA as described (Yasukawa *et al.*, 2000a,b).

Partial purification of bovine mitochondrial Lysyl-tRNA synthetase and in vitro aminoacylation of tRNA^{Lys}

Mitochondria were prepared from fresh bovine liver (Matthews *et al.*, 1982; Schwartzbach *et al.*, 1996), and fractionated on a DEAE–Sepharose Fast Flow column (Amersham Pharmacia Biotech) with a linear gradient of KCl between 20 and 300 mM in a buffer containing 20 mM Tris–HCl pH 7.6, 1 mM MgCl₂, 0.1 mM EDTA, 6 mM β-mercaptoethanol and 10% glycerol. The fractions containing KRS_{mt} activity were enriched at a protein concentration of 6 mg/ml with Centriprep (Millipore). All the procedures were performed at 4°C.

Aminoacylation reactions to determine the kinetic parameters were carried out at 37°C in a buffer consisting of 10 mM Tris–HCl pH 8.1, 15 mM MgCl₂, 2 mM ATP, 12 mM KCl and 15.4 μM [¹⁴C]-lysine (12 GBq/mmol) (Amersham Pharmacia Biotech). The initial rates of aminoacylation were determined by using five different concentrations of both tRNA^{Lys} (0.1, 0.3, 0.5, 0.7 and 0.9 μM) at a fixed concentration of partially purified KRS_{mt}, which gave reasonable kinetic plots for determining the apparent kinetic parameters.

Preparation of [³H]aminoacyl-tRNAs

Preparative aminoacylation of the wild-type or mutant tRNA^{Lys} was performed with [³H]-lysine (1.85 Mbq/mmol) (American Radiolabeled Chemicals) and a sufficient amount of KRS_{mt} under the reaction conditions as described above. The charged tRNA was extracted by phenol and passed through an NAP-5 column (Amersham Pharmacia Biotech) to remove proteins, ATP and uncharged lysine. The purified [³H]Lys-tRNA^{Lys} was stored in an acidic buffer at –80°C to prevent deacylation. The wild-type or mutant [³H]Leu-tRNA^{Leu(UUR)} was prepared by the same procedure except that [³H]-leucine (1.85 Mbq/mmol) (American Radiolabeled Chemicals) and recombinant human mitochondrial leucyl-tRNA synthetase (LRS_{mt}) were used. KIAA0028 (Nomura *et al.*, 1994) containing the full-length human LRS_{mt} gene was provided by Kazusa DNA Research Institute, Japan; the gene was cloned into pET 15b, expressed in *E.coli* and highly purified using a nickel affinity resin.

EF-Tu-dependent hydrolysis protection assay

The protection experiment was carried out basically as reported (Pingoud *et al.*, 1977). A plasmid containing full-length bovine EF-Tu_{mt} cloned into pET-24c(+) was kindly provided by Dr L.L.Spremulli (University of North Carolina) (Woriar *et al.*, 1995). The EF-Tu_{mt} was expressed in *E.coli* BL21(DE3) and highly purified using a nickel affinity resin. The reaction mixture (40 μl) contained 75 mM Tris–HCl pH 8.1, 75 mM NH₄Cl, 15 mM MgCl₂, 7.5 mM dithiothreitol (DTT), 0.1 mM GTP, 60 μg/ml bovine serum albumin (BSA), 2.25 mM phosphoenolpyruvate (PEP), 2.3 U/ml pyruvate kinase (PK) (Sigma), 0.5 pmol [³H]Lys-tRNA^{Lys} and various amounts of EF-Tu_{mt} (0, 0.5, 5, 25, 37.5 and 50 pmol). The mixture was kept on ice for 6 min to allow formation of a ternary complex, and incubation was then started at 30°C.

In vitro translation using bovine mitochondrial translation components

Using fresh bovine liver, mitochondrial ribosomes were prepared according to the method of Matthews *et al.* (1982) with the modifications described by Eberly *et al.* (1985), and a partially purified mitochondrial EF-G (EF-G_{mt}) was obtained (Chung and Spremulli, 1990). Poly(AAN)₃₀ and poly(UUR)₃₀ (N = A, G, C or U) were synthesized *in vitro* using T7 RNA polymerase. The reaction mixture (20 μl) contained 50 mM Tris–HCl pH 8.6, 7 mM MgCl₂, 5 mM KCl, 1 mM DTT, 2 mM spermine (SP), 2.5 mM PEP, 2.5 U/ml PK, 0.5 mM GTP, 15 pmol EF-Tu_{mt}, 0.25 μg/μl EF-G_{mt}, 2 pmol mitochondrial ribosome, 4 μg of poly(AAN)₃₀ or poly(UUR)₃₀ and 0.1 pmol [³H]Lys-tRNA^{Lys} or [³H]Leu-tRNA^{Leu(UUR)}, respectively. The amounts of aminoacyl-tRNAs were adjusted by measuring the radioactivity of the amino acid attached to tRNA. The mixture was incubated at 37°C for 20 min and the radioactivity of polymerized amino acids was measured as described (Gradner *et al.*, 1962; Ravel and Shorey, 1971).

Binding of tRNA^{Lys} to small ribosomal subunits

A portion of wild-type or mutant tRNA^{Lys} was labeled at the 5' end with ³²P and mixed with the non-labeled respective counterpart. *Escherichia coli* small ribosomal subunits were prepared and activated according to Ericson *et al.* (1995). The non-enzymatic binding reaction was performed at 0°C for 1 h in 20 μl of a mixture consisting of 50 mM Tris–HCl pH 7.5,

30 mM MgCl₂, 60 mM KCl, 1 mM DTT, 2 mM SP, 4 pmol 30S subunits, 0.1 μg/μl poly(A) (Sigma) and four different amounts (0.5, 1, 1.5 or 2 pmol) of either the wild-type or mutant tRNA^{Lys}. The amount of bound tRNA was determined according to the method of Ashraf *et al.* (1999) with a slight modification.

Acknowledgements

We would like to express our thanks to Dr L.L.Spremulli (University of North Carolina) for kindly providing an EF-Tu_{mt} expression vector, to Dr J.-I.Hayashi (Tsukuba University) for giving us an original mutant cybrid cell line of the ME1-4 clone, and to Dr K.Mihara (Kyushu University) for kindly allowing us to use anti-TOM 40 antibody. We are also grateful to Dr P.F.Agris and Ms C.Yarian (North Carolina State University) for valuable discussions, to Dr T.Kanamori (Nippon Medical School) for providing anti-DLST antibody, and to Mr T.Hanada and Mr T.Suzuki (Tokyo University) for their technical assistance and helpful advice. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (Japan).

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Received February 20, 2001; revised July 5, 2001;
accepted July 9, 2001