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Wobble modification deficiency in mutant tRNAs in patients with mitochondrial diseases

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Abstract **Point mutations in mitochondrial (mt) tRNA genes are associated with a variety of human mitochondrial diseases. We have shown previously that mt $tRNA^{Leu(UUR)}$ with a MELAS A3243G mutation and mt $tRNA^{Lys}$ with a MERRF A8344G mutation derived from HeLa background cybrid cells are deficient in normal taurine-containing modifications [$\tau m^5(s^2)U$; 5-taurinomethyl-(2-thio)uridine] at the anticodon wobble position in both cases. The wobble modification deficiency results in defective translation. We report here wobble modification deficiencies of mutant mt tRNAs from cybrid cells with different nuclear backgrounds, as well as from patient tissues. These findings demonstrate the generality of the wobble modification deficiency in mutant tRNAs in MELAS and MERRF.**
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Keywords: Mitochondrial disease; Mitochondrial tRNA; Patient tissue; Post-transcriptional modification; Taurine

1. Introduction

Mitochondrial DNA (mtDNA) mutations are responsible for a wide spectrum of human diseases caused by mitochondrial dysfunction [1]. An A to G mutation either at nucleotide position (np) 3243 in the $tRNA^{Leu(UUR)}$ gene or at np 8344 in the $tRNA^{Lys}$ gene has been shown to be responsible for mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonus epilepsy associated with ragged-red fibers (MERRF), respectively. These mutations, which are responsible for two major subgroups of the mitochondrial encephalomyopathies [2–4], were demonstrated to be the direct cause of reductions in oxygen consumption and mitochondrial protein synthesis [1] using a cybrid cell system in which mutant mtDNAs derived from patients were intercellularly transferred into recipient cells lacking mtDNA (ρ^0 cells) [5]. Several cybrid cell lines have been constructed with different nuclear backgrounds, including 143B osteosarcoma, HeLa and A549 lung carcinoma cells [5–7].

We have previously shown that several disease-associated mitochondrial tRNA gene mutations are associated with a lack of taurine wobble base-modification in the mutant tRNAs. In HeLa cybrid cells homoplasmic for pathogenic mtDNA mutations, the taurine-containing modified uridine (τm^5U; 5-taurinomethyluridine) [8], which normally occurs at the anticodon wobble position of mt $tRNA^{Leu(UUR)}$, remains unmodified in mt $tRNA^{Leu(UUR)}$ bearing either the A3243G or T3271C MELAS mutation (Fig. 1) [9]. Similarly, the mt $tRNA^{Lys}$ with the A8344G MERRF mutation also lacks normal taurine-modification (τm^5s^2U; 5-taurinomethyl-2-thiouridine) at the wobble position (Fig. 1) [8,10]. It is known that uridine modification at the wobble position is responsible for precise and efficient codon recognition [11–13]. In the case of MERRF, we previously showed that the mutant $tRNA^{Lys}$ lacking the wobble modification loses translational activity for both AAA and AAG codons [14]. In the case of MELAS, we recently reported that the mt $tRNA^{Leu(UUR)}$ lacking the taurine-modification showed severely reduced UUG translation but no decrease in UUA translation [15,16]. We thus concluded that the UUG codon–specific translational defect of the mutant mt $tRNA^{Leu(UUR)}$ is the primary cause of MELAS at the molecular level. This result could explain the defective translation of UUG-rich genes, such as ND6, leading to complex I deficiency as observed clinically in MELAS. These findings strongly suggest that lack of the wobble modification is the primary molecular pathogenesis causing these mitochondrial diseases. However, the possibility remains that the

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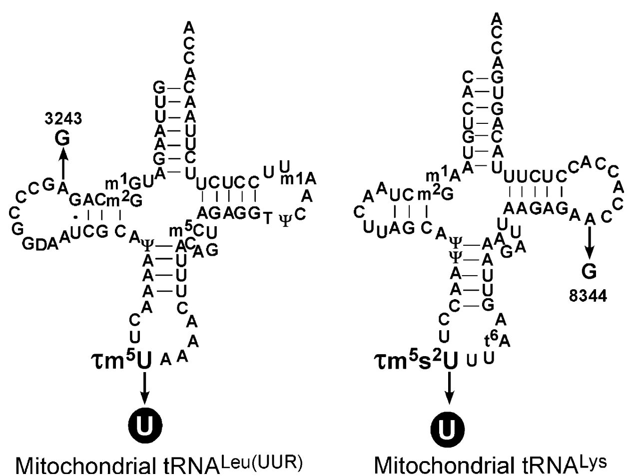

Fig. 1. Cloverleaf structure of human mitochondrial $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$. The wobble modified uridines in the wild-type $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ are 5-taurinomethyluridine (τm^5U) and 5-taurinomethyl-2-thiouridine (τm^5s^2U), respectively [8]. The MELAS and MERRF point mutations, A3243G in $tRNA^{Leu(UUR)}$ and A8344G in $tRNA^{Lys}$, respectively, are shown. The U on a round black background indicates the unmodified uridine present in the mutant tRNAs. The other modified nucleosides were determined previously: 1-methyladenosine (m^1A), N^6-threoninocarbonyladenosine (t^6A), 1-methylguanosine (m^1G), 2-methylguanosine (m^2G), pseudouridine (Ψ), ribothymidine (T) dihydrouridine (D), and 5-methylcytidine (m^5C) [9,10,35].

wobble modification deficiency depends on the nuclear background of cybrid cell lines and does not reflect the situation in vivo, because behavior of mtDNA often depends upon the nuclear background in cybrids [17,18].

To assess the generality of the modification deficiency in mutant tRNAs, we analyzed the mutant tRNAs in actual tissues of MELAS and MERRF patients. Here, we describe the confirmation of the wobble modification deficiency in mutant tRNAs from cybrid cells with different nuclear backgrounds, as well as from patient tissues.

2. Materials and methods

2.1. Cell lines and cell culture

An A549 lung carcinoma cybrid containing 99% mtDNA with the A3243G mutation in the $tRNA^{Leu(UUR)}$ gene [18] was cultured in Dulbecco-modified Eagle's medium (DMEM) with 10% fetal bovine serum and uridine at 50 μg/mL. The 143B osteosarcoma cybrid lines used were: R1–C3, carrying predominantly the A8344G mutation; R2-1, the wild-type counterpart of R1–C3; 43B, carrying predominantly the A3243G mutation; 94I, the wild-type counterpart of 43B [19,20]. The 143B cybrids were kindly provided by Dr. Attardi and Dr. Chomyn (California Institute of Technology, Pasadena, CA) and were cultured in DMEM with 10% fetal bovine serum.

2.2. Patient tissues

A patient died at the age of 22 in 1971 at Niigata University Hospital, Niigata, Japan and was reported as the first case of MERRF in 1980 [21]. The specimen from the autopsied liver (15 mg) of the MERRF patient contained 76% mutant and 24% wild-type mtDNA. Another patient was diagnosed with MELAS at Saigata National Hospital, Saigata, Joetsu, Niigata, Japan and died at the age of 17. The autopsied sample (34 mg) from the liver was maintained at −80 °C and was also heteroplasmic, containing both the wild-type and 66% np 3243 mutated mtDNA.

2.3. 3′-End splint labeling of tRNA

Total RNA from frozen tissue sections or semiconfluent cybrid cells was extracted using Isogen (Nippon Gene, Toyama, Japan), followed by deacylation of aminoacyl-tRNAs [9]. Total RNA was first incubated at 37 °C for 30 min in a reaction mixture containing 50 mM HEPES–KOH (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, and T4 polynucleotide kinase for dephosphorylation of the 3′-end of the tRNAs. Subsequently, 3′-end CCA repair of tRNAs was performed at 37 °C for 1 h with recombinant human mitochondrial CCA-adding enzyme [22] in the presence of 1 mM ATP, 1 mM CTP, and 100 mM KCl. Then, the desired tRNAs were specifically labeled at their 3′ termini by the splint labeling technique [23]. Briefly, total RNA mixed with 20 pmol oligonucleotide probe was incubated at 75 °C for 2 min and then cooled to 37 °C for annealing in 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM DTT and 0.5 mM EDTA. The probes used were 5′-GTGGTCACTGTAAAGAGGTGTTGG-3′ for $tRNA^{Lys}$, 5′-GTGGTGTTAAGAAGAGGAATTGAACC-3′ for $tRNA^{Leu(UUR)}$, 5′-GTGGTATTCTCGCACGGACTACAA CC-3′ for $tRNA^{Glu}$, and 5′-GTGGCTAGGACTATGAGAATCGAACC-3′ for $tRNA^{Gln}$. After annealing, 10 mM $MgCl_2$, 1.11 MBq [α-^{32}P]dCTP and 10 U of Sequenase (Amersham Pharmacia Biotech, Piscataway, NJ) were added to the mixture and the reaction was incubated at 37 °C for 30 min. The ^{32}P-labeled tRNA was separated by electrophoresis in a 7 M urea-polyacrylamide gel and then eluted from the gel.

2.4. Sequencing of tRNA

The labeled tRNA was purified by gel electrophoresis and sequenced as described previously [9] according to the methods of Donis-Keller [24]. The gel was exposed on an imaging plate and the radio active bands were visualized using a BAS5000 bioimaging analyzer (Fuji Film, Tokyo, Japan).

2.5. APM gel electrophoresis and Northern blotting for the detection of the thiouridine modification

Total RNA containing 3′-end splint labeled $tRNA^{Lys}$, $tRNA^{Glu}$, or $tRNA^{Gln}$ was electrophoresed in a 10% polyacrylamide gel containing 0.016 mg/mL [(N-acryloylamino)phenyl]mercuric chloride (APM) as described previously [25–27]. This method was originally developed by Igloi [28]. Radioactive bands were detected with a BAS5000 bioimaging analyzer (Fuji Film).

3. Results

3.1. Wobble modification deficiency in mutant mt tRNAs from cybrid cells with different nuclear backgrounds

The mutant mt $tRNA^{Leu(UUR)}$ was isolated by the solid phase DNA probe method [26,29] from a large scale culture of A549 lung carcinoma cybrid cells bearing 99% mtDNA with the A3243G mutation. The purified tRNA was 5′-end labeled and subjected to enzymatic sequencing [24]. As expected, the mutant mt $tRNA^{Leu(UUR)}$ contained a G at position 3243 (Fig. 2, upper panel). In the anticodon region of the wild-type mt $tRNA^{Leu(UUR)}$ (Fig. 2, lower panel), the band at the wobble position in the alkaline ladder was slightly up-shifted due to the taurine-modification. In addition, no band was observed in the RNasePhyM (A and U-specific) lane since τm^5U34 is resistant to RNase digestion [9]. However, no shift of the band corresponding to the wobble position and a clear band of RNasePhyM digestion could be observed in the mutant mt $tRNA^{Leu(UUR)}$. This result demonstrates that the wobble base is an unmodified uridine. We also analyzed 143B osteosarcoma cybrid lines with mutant mt $tRNA^{Leu(UUR)}$ bearing the A3243G mutation and found no modification of the wobble base as observed in the mutant tRNA from the A549 lung carcinoma cybrid cells (see Supplementary Information). Furthermore, we analyzed the mutant mt $tRNA^{Lys}$ bearing the A8344G mutation from 143B cybrid lines and found that the

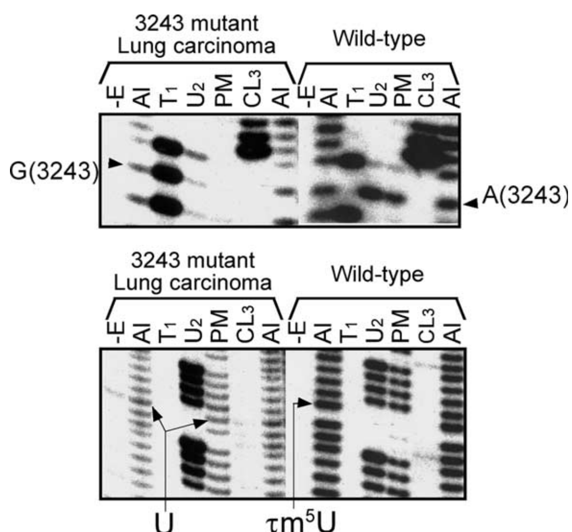

Fig. 2. Sequence ladders obtained by the Donis-Keller method [24] for 5′-end labeled wild-type and A3243G mutant tRNA$^{Leu(UUR)}$ from A549 lung carcinoma cybrid cells. Ladders from the regions around the point mutation (upper panel) and anticodon loop (lower panel) are shown. E, Al, T_1, U_2, PM, and CL_3 indicate no treatment, and treatments by alkali, RNase T_1 (specific for G), RNase U_2 (for A > G), RNase PhyM (for A and U), or RNase CL_3 (for C), respectively. Arrowheads show the position of the MELAS 3243 mutation in the upper panel. In the lower panel, arrows indicate the bands at the wobble position. The wild-type tRNA shows an up-shifted band in the alkaline ladder and no band in RNase PhyM lane due to the taurine modification (τm^5U), whereas the mutant tRNA shows a normal ladder in the alkali lane and clear band of RNasePhyM digestion corresponding to unmodified uridine (U).

wobble base remains unmodified (see Supplementary Information). Together with our previous results using HeLa cybrid cells [9,10], these data indicate that the wobble modification deficiency of the mutant tRNAs from MELAS 3243 and MERRF 8344 mutations is a universal phenomenon and does not depend on the nuclear background of the cybrid cell lines.

3.2. *Wobble modification deficiency of mutant mt tRNA$^{Leu(UUR)}$ in MELAS patient tissue*

Since a limited amount of patient tissues were available, direct isolation of the mutant mt tRNA for nucleotide modification analysis was impractical. Specific labeling of the mutant tRNA was achieved instead using the 3′-end splint labeling technique [23]. This approach to labeling the tRNAs allowed us to work directly with unfractionated total RNA from a limited amount of patient tissue.

A liver autopsy specimen of a MELAS patient was heteroplasmic and contained both the wild-type mtDNA and 66% np 3243 mutated mtDNA. Ninety micrograms total RNA was isolated from 34 mg tissue. The mutant tRNA$^{Leu(UUR)}$ was 3′-end labeled by the splint labeling technique (see Section 2). The labeled tRNA was subjected to enzymatic RNA sequencing [24] and produced bands not only in the presence of RNase T_1 (specific for G), but also when treated with RNase U_2 (for A > G) and RNase PhyM (Fig. 3, upper panel), indicating the coexistence of the mutant and the wild-type tRNA$^{Leu(UUR)}$.

In the anticodon region of the alkaline-treated ladders (Fig. 3), a band at the wobble position (position 34, according to the tRNA numbering system [30]) in the wild-type tRNA was up-shifted due to the taurine-modification as shown in Fig. 2. In contrast, the wobble position from the MELAS patient's tissue reproducibly showed an irregular band pattern between positions 31 and 33 (Fig. 3, lower panel) and was clearly different from the ladder pattern produced by the wild-type tRNA. The fact that tRNA$^{Leu(UUR)}$ from the patient's tissue is a mixture of the wild-type and the mutant tRNA$^{Leu(UUR)}$ (A3243G), and that the latter has been shown to lack the wobble modification in three different nuclear background cybrids (this study and [9]), suggests that this irregular band pattern in the mutant is caused by the coexistence of RNA fragment containing unmodified U from the tRNA$^{Leu(UUR)}$(A3243G) and fragment containing τm^5U from the wild-type tRNA$^{Leu(UUR)}$. This band pattern in the alkaline ladder was also observed in mutant tRNA$^{Leu(UUR)}$ obtained from a heteroplasmic HeLa cybrid cell bearing 50% of the A3243G mutation (data not shown). We could not observe a clear band after RNase PhyM digestion of the mutant mt tRNA$^{Leu(UUR)}$ due to the heteroplasmic A3243G mutation (66%) and low labeling efficiency of the mutant tRNA from the MELAS patient (Fig. 3).

3.3. *Wobble modification deficiency in mutant mt tRNA^{Lys} from MERRF patient tissue*

We obtained 50 μg total RNA from 15 mg of liver autopsy tissue from a MERRF patient, whose mutation frequency in mt DNA was determined to be 76% for mt tRNA^{Lys}. Total RNA was used for splint labeling of the 3′-end of the mt tRNA^{Lys}. Sequence analysis revealed that the mutant tRNA^{Lys}

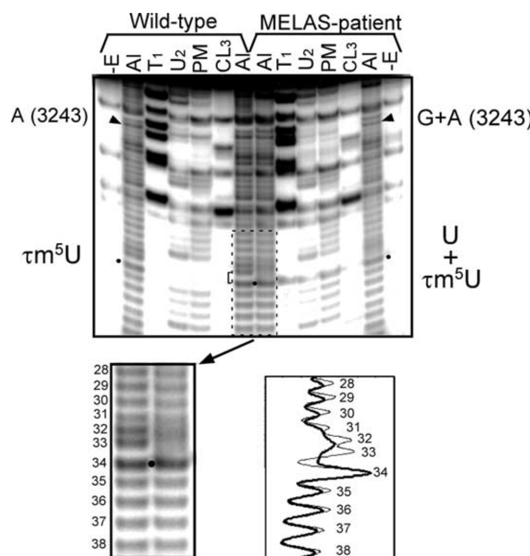

Fig. 3. MELAS patient-derived tRNA$^{Leu(UUR)}$ sequencing was compared to the wild-type tRNA$^{Leu(UUR)}$. Not only RNases T_1 but also U_2 and PhyM gave bands at the 3243 mutation point in the patient tRNA (arrowhead), confirming the coexistence of the mutant and wild-type tRNA$^{Leu(UUR)}$. The wobble position is indicated by dots. The alkali ladders around the anticodon loop are highlighted in the lower panel (left) and a densitometric analysis is shown (right). tRNA numbering was according to the usual method described in the literature [30]. Unusual up-shifting of alkali bands was observed at position 33 of the wild-type tRNA$^{Leu(UUR)}$ because of the wobble modification. Additionally, the AI ladders of the wild-type tRNA$^{Leu(UUR)}$ gives clear bands, whereas those of patient-derived tRNA is smeared at positions 31–33, suggesting the coexistence of unmodified and modified uridines at position 34 (wobble position).

from the MERRF patient's tissue had an A to G mutation at np 8344, thereby directly demonstrating the existence of the mutant tRNA in the mitochondria of the patient's tissue (Fig. 4A). In the anticodon region, the sequence ladders showed that the wobble position of the patient-derived $tRNA^{Lys}$ was sensitive to digestion by RNasePhyM, demonstrating that the wobble base is an unmodified uridine (Fig. 4A). In contrast, the same position of the wild-type $tRNA^{Lys}$ is resistant to this RNase due to the τm^5s^2U-modification (Fig. 4A). This result is consistent with our previous observations in cybrid cells [10].

To confirm the absence of the wobble modification in the mutant $tRNA^{Lys}$ from the MERRF patient's tissue, we employed APM ([(*N*-acryloylamino)phenyl] mercuric chloride) gel electrophoresis to separate the mutant tRNA from the wild-type tRNA containing the 2-thio modification of τm^5U [25–28]. In this analysis, the electrophoretic mobility of the major portion of tRNAs containing the thiolated nucleotide is significantly retarded in the presence of APM, due to a specific interaction between the thiocarbonyl group and the mercuric compound in the gel. As shown in Fig. 4B, the majority of the wild-type mt $tRNA^{Lys}$ (C) in the gel is retarded due to the 2-thio group of the τm^5s^2U at the wobble position, whereas the mutant mt $tRNA^{Lys}$ (M) obtained from MERRF cybrid cells containing 96% A8344G mutations in mt DNA showed no retardation in the APM gel since the tRNA lacks the 2-thio modification. In mutant mt $tRNA^{Lys}$ (P) obtained from MERRF patient's tissue with 76% A8344G mutations in the mt DNA, a small portion of the tRNA was retarded in the gel, but most of the tRNA showed normal mobility.

These results provide evidence that the mutant $tRNA^{Lys}$ in the patient's tissue specifically lacks the 2-thio modification of τm^5s^2U at the wobble position. To rule out the possibility that the wobble modification deficiency of the patient-derived mutant $tRNA^{Lys}$ was caused by decreased activity of the RNA-modifying enzyme responsible for 2-thio modification of τm^5s^2U, we verified 2-thio modification of other mt tRNAs that normally have a τm^5s^2U [8] using APM gel analysis. As shown in Fig. 4C, mt tRNAs for Glu and Gln in the total RNA from the MERRF patient tissue showed significant retardation, similar to that of mt tRNAs from wild-type cells, suggesting that the absence of the wobble modification in mt $tRNA^{Lys}$ was the direct result of a pathogenic point mutation and not a change in RNA-modifying enzyme activity. These results demonstrate that mt $tRNA^{Lys}$ carrying the A8344G mutation lacks the τm^5s^2U modification at the wobble position in patient tissues.

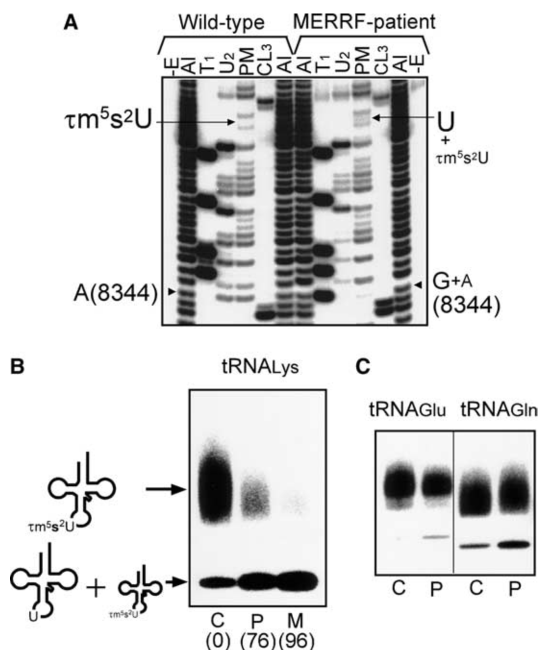

Fig. 4. (A) RNA sequence ladders from 3′-end labeled wild-type and MERRF patient-derived $tRNA^{Lys}$. The regions around the point mutation and anticodon loop are shown. Arrowheads show the position of the MERRF 8344 mutation. Bands corresponding to unmodified uridine (U) are present at the wobble position in the patient-derived $tRNA^{Lys}$, whereas resistance against RNase PhyM digestion was observed at the same position in the wild-type $tRNA^{Lys}$ because of the τm^5s^2U modification (arrows). (B) Detection of thiolated nucleotides in tRNA by APM gel electrophoresis. Total RNA containing 3′-end labeled $tRNA^{Lys}$ derived from the wild-type cybrid cell (C), the MERRF patient tissue (P), and the 8344 mutant cybrid cell (M), were separated by APM gel electrophoresis (origin at top). The retarded, smeared band contains $tRNA^{Lys}$ with the wobble modified τm^5s^2U, whereas the lower band contains tRNA without the thiolated uridine and residual amounts of tRNA with the thiolated uridine. The numbers in parentheses indicate the mutation ratio of mtDNA in each sample. (C) 3′-End labeled $tRNA^{Glu}$ and $tRNA^{Gln}$ derived from wild-type cybrid (C) or MERRF patient tissue (P) were separated by APM gel electrophoresis. Both $tRNA^{Glu}$ and $tRNA^{Gln}$ showed equivalent extents of band retardation between the wild-type cybrid and the MERRF patient sample, indicating the specificity of the wobble modification deficiency in the mutant $tRNA^{Lys}$ from the patient sample.

4. Discussion

Since the behavior of mtDNA often depends upon the nuclear background in cybrids [17,18], it is substantial to confirm that the modification deficiency in mutant mt tRNAs is due to the mutations even under the different nuclear backgrounds and that the deficiency is not specific in cybrids. We here describe a wobble modification deficiency in the mutant mt $tRNA^{Leu(UUR)}$ (A3243G) from three different nuclear background cybrids, and in the mutant mt $tRNA^{Lys}$ (A8344G) from two different nuclear background cybrids (this study and [9,10]). The wobble modification deficiency in the mutant tRNAs from MELAS and MERRF cybrid cells has been firmly established.

The A3243G and A8344G mutations work as a negative determinant for the biosynthesis of τm^5U in $tRNA^{Leu(UUR)}$ and τm^5s^2U in $tRNA^{Lys}$, respectively. This indicates that the RNA-modifying enzyme responsible for the 5-taurinomethyl group, which has not been identified, is a class of enzyme that recognizes the whole tertiary structure of tRNA, because both the np 3243 and 8344 are far from the wobble position (Fig. 1).

Studies using cultured cells have consistently demonstrated the wobble modification deficiency, but confirmation that the same phenomenon occurs in patient tissues has not been reported previously. By overcoming the difficulty in sequencing tRNAs from small amounts of tissue, we were able to show that patient tissues lacked the wobble modification in the mutant $tRNA^{Leu(UUR)}$ (A3243G) and $tRNA^{Lys}$ (A8344G). This finding provides the first link between our biochemical observations and clinical analysis, which is a necessary first step in

the development of diagnostic procedures and possible therapeutic measures.

Mitochondrial dysfunctions such as MELAS or MERRF could have multiple causes, including impaired termination of transcription, decreased RNase P processing, decreased tRNA stability, aminoacylation, or abnormal conformations [31,32], which would lead to decreased steady-state levels of the normal aminoacylated tRNAs and result in reduced mitochondrial protein synthesis. However, particularly in the case of MELAS, the reduced mitochondrial protein synthesis in cells with these pathogenic mutations cannot explain the decreased respiratory activity or oxygen consumption, since the extent of the reduction in protein synthesis does not parallel the defective enzymatic activity [7,20,33,34]. Thus, reduced protein synthesis caused by quantitative defects of the mutant tRNAs does not appear to be the direct cause of the clinical symptoms presented by MELAS. Qualitative defects are likely to arise from the wobble modification deficiency, since the mutant tRNAs lacking wobble modifications show codon-specific decoding disorders [15]. This suggests that the lack of wobble modification is a major causative factor of these mitochondrial diseases.

In conclusion, the present study excluded the possibility that the modification deficiency of the wobble position is limited to HeLa cybrids and confirmed that the wobble modification deficiency occurs in patient tissues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.04.038.

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