Mutations in mtDNA are responsible for a variety of mitochondrial diseases, where the mitochondrial tRNALeu(UUR) gene has especially hot spots for pathogenic mutations. Clinical features often depend on the tRNA species and/or positions of the mutations; however, molecular pathogenesis elucidating the relation between the location of the mutations and their leading phenotype are not fully understood. We report here that mitochondrial tRNALeu(UUR) harboring one of five mutations found in tissues from patients with symptoms of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (A3243G, G3244A, T3258C, T3271C, and T3291C) lacked the normal taurine-containing modification (5-taurinomethyluridine) at the anticodon wobble position. In contrast, mitochondrial tRNALeu(UUR) with different mutations found in patients that have mitochondrial diseases but do not show the MELAS symptoms (G3242A, T3250C, C3254T, and A3280G) had the normal 5-taurinomethyluridine modifications. These observations were made by using a modified primer extension technique that can detect the modification deficiency in the extremely limited quantities of mutant tRNAs obtainable from patient tissues. These results strongly suggest deficient taurine modification could be a key molecular factor responsible for the phenotypic features of MELAS, which can explain why the different MELAS-associated mutations result in indistinguishable clinical features.

Specific correlation between the wobble modification deficiency in mutant tRNAs and the clinical features of a human mitochondrial disease

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Mutations in mtDNA are responsible for a variety of mitochondrial diseases, where the mitochondrial tRNALeu(UUR) gene has especially hot spots for pathogenic mutations. Clinical features often depend on the tRNA species and/or positions of the mutations; however, molecular pathogenesis elucidating the relation between the location of the mutations and their leading phenotype are not fully understood. We report here that mitochondrial tRNALeu(UUR) harboring one of five mutations found in tissues from patients with symptoms of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (A3243G, G3244A, T3258C, T3271C, and T3291C) lacked the normal taurine-containing modification (5-taurinomethyluridine) at the anticodon wobble position. In contrast, mitochondrial tRNALeu(UUR) with different mutations found in patients that have mitochondrial diseases but do not show the MELAS symptoms (G3242A, T3250C, C3254T, and A3280G) had the normal 5-taurinomethyluridine modifications. These observations were made by using a modified primer extension technique that can detect the modification deficiency in the extremely limited quantities of mutant tRNAs obtainable from patient tissues. These results strongly suggest deficient taurine modification could be a key molecular factor responsible for the phenotypic features of MELAS, which can explain why the different MELAS-associated mutations result in indistinguishable clinical features.

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; m5U, 5-taurinomethyluridine.
common the lack of taurine modification of their respective mutant tRNAs. Thus, these point mutations may hinder the biosynthesis of the wobble taurine modification of mitochondrial tRNAs. It is known that uridine modifications at the wobble position are required for precise and efficient codon recognition (22–24). Recently, we reported that mitochondrial tRNAs\textsubscript{Leu(UUR)} lacking the taurine modification showed severely reduced UUG translation but no decrease in UUA translation (25). We thus concluded that the UUG codon–specific translational defect of the mutant mitochondrial tRNAs\textsubscript{Leu(UUR)} could play an important role in the molecular pathogenesis of MELAS. This finding could explain the translational defect of UUG-rich genes such as ND6 that leads to the complex I deficiency observed clinically in MELAS patients.

To investigate the relationship between the clinical features of MELAS and taurine modification deficiency, we wanted to analyze the modification deficiency in actual tissues from patients with various mutations in the mitochondrial tRNAs\textsubscript{Leu(UUR)} gene. However, because of the extremely limited amounts of mitochondrial tRNAs available from patient tissues, this objective required the development of a technique that would detect with high sensitivity the modification deficiency. We thus devised a technique based on primer extension and here describe that the mutant mitochondrial tRNAs\textsubscript{Leu(UUR)} obtained from MELAS patient tissues bear the wobble modification deficiency, unlike the mutant mitochondrial tRNAs\textsubscript{Leu(UUR)} obtained from patients with other diseases. This finding clearly reveals the specific correlation between the wobble modification deficiency and the clinical features of MELAS. This finding will aid the development of appropriate therapeutic measures and diagnostic applications.

**Methods**

**Patient Tissues and Cell Lines.** Approximately 0.5 g of liver and cardiac muscle was obtained from the A3243G MELAS patient (3). The other tissues used were frozen sections of patient skeletal muscles (1–10 mg) (5, 7, 8, 26). The mutant cybrid cell lines that had been constructed by the intercellular transfer of MELAS patient mtDNA into \(\rho^0\) HeLa cells have been described (19). The ML2-2-2 and ML5-1-13 cell lines exclusively contain mtDNA with the A3243G and T3271C point mutations, respectively. The cells were cultured in normal medium [DMEM/F-12 (1:1) (GIBCO/BRL), 10% FCS].

**Primer Extension Method to Detect the Taurine Modification of Wobble Uridines.** Crude RNAs from frozen tissue sections or cybrid cells were obtained by using Isogen (Nippon Gene, Tokyo) according to the manufacturer's instructions. The 5'-33P-labeled primer (0.1 pmol) was incubated with 0.1–0.5 \(\mu\)g of the total RNAs in a 10-\(\mu\)l solution containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA at 80°C for 2 min and then allowed to stand at room temperature for 1 h. Subsequently, 1.5 \(\mu\)l of ddH\(_2\)O, 4 \(\mu\)l of 5X reaction buffer for reverse transcription (Toyobo, Osaka), 0.5 \(\mu\)l of d/ddNTP mix containing 1.5 mM

analyses of RNA samples from cybrid cells with the A3243G mutation (>99%) and WT cybrid cells, respectively. (c) The primer extension reaction with total RNA composed of various ratios of A3243G mutant mitochondrial tRNAs\textsubscript{Leu(UUR)} relative to WT tRNAs\textsubscript{Leu(UUR)}. Modification deficiency values were calculated on the basis of the intensities of the upper and lower bands. The ratio of the upper band intensity for WT tRNA was subtracted from the band intensity the basis of the intensities of the upper and lower bands. The ratio of the upper band intensity for WT tRNA was subtracted from the band intensity

\[ \text{modification deficiency} = \frac{\text{intensity}_{WT} - \text{intensity}_{mutant}}{\text{intensity}_{WT}} \times 100 \]

Fig. 1. The primer extension method used to detect the taurine modification deficiency of wobble uridines in A3243G mutant mitochondrial tRNAs\textsubscript{Leu(UUR)}. (a) A highly sensitive primer extension method was used to detect the taurine modification (\(\text{m}^5\text{U}\)) deficiency of A3243G mutant mitochondrial tRNAs\textsubscript{Leu(UUR)}. The labels 3243 cybrid and WT cybrid indicate the analyses of RNA samples from cybrid cells with the A3243G mutation (>99%) and WT cybrid cells, respectively. The reverse transcription from the primer terminates at position 32 because of the incorporation of ddGTP when the template tRNA lacks \(\text{m}^5\text{U}\) at position 34 (lane 3243 cybrid). In contrast, the extension is hindered when the template tRNA contains \(\text{m}^5\text{U}\). This yields a position 33-specific band (lane WT cybrid). The primer is shown as a solid line next to the anticodon region of the tRNAs, and the nascent cDNAs synthesized from the primer are indicated as gray lines. Lanes G, A, C, T, and Ladder represent reactions carried out in the presence of ddGTP, ddATP, ddCTP, ddTTP, and ddNTP mix, respectively. (b) The primer extension method using unlabeled primer and [\(\alpha\)-32P]dATP. The labels 3243 cybrid and WT cybrid indicate the

analyses of RNA samples from cybrid cells with the A3243G mutation (>99%) and WT cybrid cells, respectively. (c) The primer extension reaction with total RNA composed of various ratios of A3243G mutant mitochondrial tRNAs\textsubscript{Leu(UUR)} relative to WT tRNAs\textsubscript{Leu(UUR)}. Modification deficiency values were calculated on the basis of the intensities of the upper and lower bands. The ratio of the upper band intensity for WT tRNA was subtracted from the band intensity ratio for each mutant tRNA as a background. The calculated data were normalized on the basis of the A3243G cybrid data, which were set at 100. Each data set represents the average of three independent experiments, with bars showing the SD. The percentage of input A3243G mutant tRNAs\textsubscript{Leu(UUR)} relative to total tRNAs\textsubscript{Leu(UUR)} in each sample was determined by a conventional primer extension method (see Fig. 5).
each of dATP, dTTP, and ddGTP (ultrapure products, Amer-
sham Pharmacia), 3 μl of 25 mM MgCl₂, and 1 μl of Moloney
murine leukemia virus reverse transcriptase (RNase H⁻) (40
units/μl, Toyobo) were added, and the mixture was incubated at
42°C for 1 h. The reaction mixture was subjected to 15% PAGE
containing 7 M urea (40 cm). The radiolabeled bands were
visualized by a BAS5000 bioimaging analyzer (Fuji). The
synthetic DNA primers used were as follows: 5'-ACCTCTGACCT-
GTAAGG-3' for 3271 mutant tRNALeu(UUR), 5'-ACCTC-
CGACTGTAAG-3' for 3280 mutant tRNALeu(UUR), and 5'-
ACCTCTGACTGTAAG-3' for other mutant tRNAs.

For the primer extension method with unlabeled primer and
[α-32P]dATP (Fig. 1b), 0.1 pmol of unlabeled primer, 0.25 μM
[α-32P]dATP, 0.15 mM dATP, and 1.5 mM each of dTTP and
ddGTP were used.

Results

It was previously not possible to analyze the mitochondrial tRNA
modifications in patients by conventional techniques as that
would require the isolation of sufficient amounts of mutant
mitochondrial tRNAs directly from the patient tissues, which
is not practicable given the limited amounts of patient tissues or
biopsy specimens that are available. Furthermore, as the patient
tissues often bear heteroplasmic point mutations in their
mtDNA, the amounts of the mutant tRNA that can be isolated
depends on the frequency with which the mutation occurs and
the stability of the mutant tRNA in the mitochondria. To
overcome these difficulties, we have established a sensitive
method for detecting the wobble modification deficiency in
mutant mitochondrial tRNALeu(UUR) molecules. This method
was developed by optimizing a primer extension technique (Fig.
1a) with the 5'-32P phosphorylated DNA primer that is com-
plementary to positions 39–54 of mitochondrial tRNALeu(UUR)
and that specifically hybridizes to mitochondrial tRNALeu(UUR)
in the total RNAs obtained from cybrid cells or patient tissues.

After hybridization, reverse transcription of the tRNA from
the primer is then carried out in the presence of deoxy GTP. When total RNA from WT cells was used as the template in
this experiment, the m5U34 modification in mitochondrial
tRNALeu(UUR) hinders the extension by acting as a roadblock.
As a result, a specific band ending at U33 is observed, as shown by
the lower band in Fig. 1a. However, when total RNA from
A3243G cybrid cells was used, the extension continued through
the unmodified U34 residue and stopped at C32 because of the
insertion of deoxy guanosine (Fig. 1a, upper band).

We confirmed that the reverse transcription on mitochondrial
tRNALeu(UUR) was correctly performed by deoxy sequencing
reactions using in vitro transcribed mitochondrial tRNALeu(UUR)
as a template (Fig. 1a). In addition, the same experiment
was performed with unlabeled primer and [α-32P]dATP to confirm
that the extension terminated at U33 when the template tRNA
contains m5U (Fig. 1b). By adjusting the total RNAs from
patient and WT tissues that contain the same amount of mitochondrial tRNALeu(UUR), the radioactivity of the lower band
ending at U33 of WT tRNA was almost the same as that of the
upper band ending at C32 of A3243G mutant tRNA (Fig. 1b),
which suggests that m5U hindered the reverse transcription by
incorporating two dAMPs at m5U34 and U33. Furthermore, we
performed this experiment with total RNAs composed of vari-
ous amounts of the mutant mitochondrial tRNALeu(UUR) with
A3243G mutation relative to the WT tRNA (Fig. 1c).

The upper and lower band intensities were quantified, and the ratios
were calculated to yield values of modification deficiency that were
almost proportional to the ratio of input A3243G mutant
mitochondrial tRNALeu(UUR) (Fig. 1c). Thus, this method allows
us to detect the modification deficiency by measuring the ratio
of the intensities of the upper and lower bands. Most impor-
tantly, we can analyze the wobble modification deficiency of the

![Fig. 2.](attachment:image_url) The primer extension method using a discriminating primer detects the taurine modification deficiency in T3271C mutant mitochondrial tRNALeu(UUR) with particularly high sensitivity. The wobble taurine modification deficiency in T3271C mutant mitochondrial tRNALeu(UUR) was detected by the primer extension method by using a discriminating primer that contains guanine in its 3' region for specifically analyzing T3271C mitochondrial mutant tRNALeu(UUR). The C on a round black background in the anticodon stem indicates the T-to-C point mutation at np 3271. The primer is shown as a solid line next to the anticodon region of the tRNAs, and the nascent cDNAs synthesized from the primer are indicated as gray lines. The percentage of mutant tRNA in each sample was determined by the primer extension method (Fig. 5).

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**Fig. 5.** Major method for detecting the wobble modification deficiency in mitochondrial tRNALeu(UUR) with high sensitivity. The primers are shown as solid lines next to the anticodon region of the tRNAs, and the nascent cDNAs synthesized from the primer are indicated as gray lines. The percentage of mutant tRNA in each sample was determined by the primer extension method (Fig. 5).
A3243G or T3271C mutation with our primer extension technique revealed the marked presence of the upper band that indicates the lack of modification (Fig. 3a). Modification deficiency values are shown in Fig. 3c. These results are consistent with our previous observations found in the mutant tRNAs from the cybrid cells (19). Lower modification deficiency values were observed for the A3243G (liver), A3243G (cardiac muscle), and T3271C (skeletal muscle) tissues. This finding can be explained by the low mutation frequency of the heteroplasmic mtDNAs in these tissues, which generated the mutant tRNAs at a ratio of only 57%, 70%, and 85%, respectively (Fig. 3 and Table 1). In contrast, when the \( m^\text{U}34 \) modification is absent, the transcription stops at C32. The molecular mechanism by which the \( m^\text{U}34 \) modification hinders reverse transcription differs from that used by the general method described above, because the modification at the C5 position of uridine does not block the Watson–Crick base pairing in the nascent cDNA resulting from the reverse transcription.

By using this detection system, we succeeded in identifying the wobble modification deficiency of mutant mitochondrial tRNAs\( \text{Leu(UUR)} \) in extremely limited amounts of the total RNA (0.5 \( \mu \)g) obtained from patient tissues (\( \sim \)0.1–1 mg). This experiment revealed that the mitochondrial tRNAs\( \text{Leu(UUR)} \) with five different point mutations (A3243G, G3244A, T3258C, T3271C, and T3291C) derived from patients showing the clinical features of MELAS lack the taurine modification (although G3244A and T3291C mutants may only partially lack the modification) (Table 1 and Fig. 4 Right). Thus, each of these point mutations acts as a negative determinant for \( m^\text{U}34 \) biosynthesis. In contrast, the mitochondrial tRNAs\( \text{Leu(UUR)} \) with four different mutations (G3242A, T3250C, T3254T, and A3280G) from patients showing non-MELAS symptoms retained normal taurine modifications (Table 1 and Fig. 4 Left). These results clearly indicate the strong association between deficient taurine modification and the clinical features of MELAS.

Many studies have shown defective aspects of MELAS mutant mitochondrial tRNAs\( \text{Leu(UUR)} \) other than the wobble modification, including decreased aminoacylation and stability, impaired pre-tRNA processing, and abnormal conformation (29, 30). These dysfunctions commonly lead to the quantitative decrease of the aminoacylated mitochondrial tRNAs\( \text{Leu(UUR)} \), which in turn leads to the decreased translation of both UUA and UUG codons. Meanwhile, the most remarkable observation in this study is that all five different point mutations associated with MELAS showed the lack of the wobble modification in the mitochondrial tRNAs\( \text{Leu(UUR)} \) harvested from the tissues of patients with these mutations. This finding strongly suggests deficient wobble modification could be a key molecular factor responsible for the phenotypic features of MELAS, although the mitochondrial dysfunction in MELAS could arise from multiple causes. We have already demonstrated the characteristic translational disorder caused by wobble modification deficiency of MELAS mutant mitochondrial tRNAs\( \text{Leu(UUR)} \) (25). Mitochondrial tRNAs\( \text{Leu(UUR)} \) that does not bear a pathogenic mutation but lacks the taurine modification, which was constructed from

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Source</th>
<th>Tissue</th>
<th>Proportion of mutant tRNA, %</th>
<th>Taurine modification</th>
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<tr>
<td>G3242A</td>
<td>MM</td>
<td>Skeletal muscle</td>
<td>&gt;99</td>
<td>+</td>
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<tr>
<td>A3243G (L)</td>
<td>MELAS</td>
<td>Liver</td>
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<td>−</td>
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<td>87</td>
<td>−</td>
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<tr>
<td>T3250C</td>
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<td>&gt;99</td>
<td>+</td>
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<tr>
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<td>+</td>
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<tr>
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<td>&gt;99</td>
<td>+</td>
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<tr>
<td>T3291C</td>
<td>MELAS</td>
<td>Skeletal muscle</td>
<td>62</td>
<td>−</td>
</tr>
</tbody>
</table>

†The precise clinical report on the patients with C3254T mutation is unpublished (Y.G.).

Table 1. Tissues of patients with mitochondrial diseases analyzed in this study
native WT human mitochondrial tRNALeu(UUR) by a molecular surgery technique, showed a UUG codon-specific decoding disorder. Thus, the taurine modification at the C5 position of wobble uridine in mitochondrial tRNALeu(UUR) appears to play a crucial role in the decoding of the UUG codon by stabilizing the U:G wobble base pairing. This result could nicely explain the translational defect of the respiratory complex I component ND6, whose gene is rich in UUG, that was observed in the MELAS cybrid cells (12, 17). Because complex I deficiency is a typical phenotype of MELAS symptom (31, 32), it appears the wobble modification deficiency of the mutant mitochondrial tRNALeu(UUR) molecule is a major molecular contributor to MELAS pathogenesis. In addition, we estimated the negative effect on decoding the cognate UUA codon the pathogenic point mutation has by itself, independent of the wobble modification deficiency (25). The MELAS mitochondrial tRNALeu(UUR) with the A3243G mutation showed a more severe reduction in UUA decoding than the mitochondrial tRNALeu(UUR) with the T3271C mutation. This result is consistent with the translational activities of MELAS cybrid cells with these point mutations (11, 12). The negative effect of these pathogenic point mutations may arise from the fragile relaxed structure caused by the mutations, because A3243G could disrupt the potential tertiary interaction in the tRNA (33–35), whereas the T3271C mutation destabilizes the anticodon stem (36). Therefore, it is assumed that each of the three other MELAS mutations (G3244A, T3258C, and T3291C) results in a similar decoding disorder, albeit perhaps to different extents. The level of the translational defect imposed by each mutation may be determined by a combination of the pathogenic nature of each MELAS mutation and the wobble modification deficiency. In the case of the four other mutations (G3242A, T3250C, C3254T, and A3280G) found in patients with mitochondrial diseases showing non-MELAS symptoms, because the mitochondrial tRNALeu(UUR) with each of these mutations had the normal m5U modification, it appears that these mutations are directly responsible for tRNA disorders that lead to mitochondrial dysfunction, although it remains a possibility that these mutations may induce deficiencies in other tRNA modifications.

**Fig. 3.** Detection of the wobble taurine modification deficiency in mitochondrial tRNALeu(UUR) from patient tissues and cybrid cells that bear pathogenic point mutations by using the primer extension technique. (a) The primer extension method was used to detect the taurine modification (m5U) deficiency in actual patient tissues. Cybrid and patient indicate the analyses of RNA samples from cybrid cells and patient tissues, respectively. Normal indicates the analysis of total RNA from the oral mucosal cells of a healthy person in the laboratory. 3243 patient (L) and 3243 patient (C), respectively, indicate the analyses of RNA samples from the liver and cardiac muscle of the A3243G MELAS patient shown in Table 1. (b) The primer extension analyses to detect the wobble modification deficiency in mutant tRNALeu(UUR) obtained from various patient tissues bearing pathogenic point mutations. (c) Modification deficiency values of mutant tRNALeu(UUR). Each data set represents the average of three independent experiments, with bars showing the SD. The percentage of each mutant mitochondrial tRNALeu(UUR) relative to total mitochondrial tRNALeu(UUR) in each sample as determined by the primer extension method (Fig. 5) is shown in parentheses. 3243(L) and 3243(C), respectively, indicate the liver and cardiac muscle of the A3243G MELAS patient (see Table 1).

**Fig. 4.** Pathogenic point mutations in the cloverleaf structure of human mitochondrial tRNALeu(UUR) that result in deficient wobble taurine modification or have no effect. (Left) Mutations not causing the wobble modification deficiency are shown. (Right) MELAS mutations that cause the wobble modification deficiency are shown. m1A, 1-methyladenosine; t6A, Nε-threoninocarbonyladenosine; m1G, 1-methylguanosine; m2G, 2-methylguanosine; ψ, pseudouridine; T, ribothymidine; D, dihydrouridine; m3C, 5-methylcytidine (19).
To date, we have found five MELAS and one myoclonus epilepsy associated with ragged-red fibers mutation to be associated with the wobble modification deficiency. This finding suggests that the deficiencies in RNA modifications may be frequently caused by the pathogenic point mutations in mitochondrial tRNAs. The direct destruction of tRNA function by a point mutation would be best obtained by introducing a mutation into any of the anticodon bases or discriminator bases that are critical for codon recognition and aminoacylation. However, of the 90 pathogenic point mutations in mitochondrial tRNA genes that have been reported to be associated with mitochondrial diseases (MITOMAP, www.mitomap.org) (2), only a few occur that have been reported to be associated with mitochondrial development and only manifest themselves later as mitochondrial diseases. Supporting this idea is that it is known that most RNA modifications are nonessential and work to modulate or fine-tune RNA functions.

Our observations have provided an understanding of the molecular pathogenesis behind the human mitochondrial diseases arising from RNA modification disorders, although how impaired mitochondrial translation leads to the development of pathogenic cells and tissues to cause the observed biochemical defect remains to be elucidated. In retrospect, it is not surprising that a qualitative disorder of RNA molecules can cause disease because noncoding RNAs are functional molecules that must mature by undergoing posttranscriptional modifications. The primer extension method for detecting the modification deficiency can be used to detect other modifications in the limited quantities of RNAs obtainable from patient tissues.

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