ARTICLE

Decreased aminoacylation of mutant tRNAs in MELAS but not in MERRF patients

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Mutations in human mitochondrial tRNA genes are associated with a number of multisystemic disorders. Using an assay that combines tRNA oxidation and circularization we have determined the relative amounts and states of aminoacylation of mutant and wild-type tRNAs in tissue samples from patients with MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes) and MERRF syndrome (myoclonus epilepsy with ragged red fibers), respectively. In most, but not all, biopsies from MELAS patients carrying the A3243G substitution in the mitochondrial tRNALeu(UUR) gene, the mutant tRNA is under-represented among processed and/or aminoacylated tRNAs. In contrast, in biopsies from MERRF patients harboring the A8344G substitution in the tRNALys gene neither the relative abundance nor the aminoacylation of the mutated tRNA is affected. Thus, whereas the A3243G mutation may contribute to the pathogenesis of MELAS by reducing the amount of aminoacylated tRNALeu, the A8344G mutation does not affect tRNALys function in the same way.

INTRODUCTION

Human cells contain hundreds of mitochondrial DNA (mtDNA) molecules, each of which encodes 13 polypeptides essential for cell respiration, as well as two ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs), which constitute a complete set of RNAs necessary for mitochondrial translation (for a review see ref. 1). To date, >30 mutations in mitochondrial tRNA genes have been reported to be associated with chronic degenerative diseases affecting brain, heart, muscle, kidney and endocrine glands (2). The most studied of these mutations are an A→G transition at position 8344 in the tRNALys gene, causing the MERRF syndrome (myoclonus epilepsy with ragged red fibers) (3), and an A→G transition at position 3243 in the tRNALeu(UUR) gene, which is frequently associated with the MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes) (4,5), but also with other clinical manifestations such as chronic progressive external ophthalmoplegia and certain forms of diabetes mellitus (4–7).

Although characteristic defects in mitochondrial protein synthesis have been observed in cell lines carrying the MELAS or the MERRF mutations (8–14), it is still poorly understood how these mutations interfere with tRNA function. We have taken advantage of the fact that most disease-associated mutations in mitochondrial tRNA genes are heteroplasmic in patient tissues, i.e. they occur as a mixture with wild-type mtDNA (15). Any effect of a mutation on tRNA processing, tRNA stability or aminoacylation should therefore be observable as a shift in the relative abundance of the mutant when mtDNA, total tRNAs and aminoacylated tRNAs are compared. Using an assay that combines tRNA oxidation and circularization we show that the tRNALeu(UUR) carrying the A3243G mutation is under-represented relative to the tRNA with the wild-type sequence among aminoacylated tRNAs in patient tissue, indicating a specific defect of the mutant molecule in stability and/or aminoacylation. In contrast, no such decrease is observed for the tRNALys carrying the A8344G mutation. Our results suggest that reduced levels of

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RESULTS

The OXOCIRC assay

To determine the relative abundance of the mutant sequence in mtDNA, total cellular tRNAs and aminoacylated tRNAs in heteroplasmic patient tissue, we applied the OXOCIRC assay (16) (Fig. 1). In this assay, total cellular RNAs are isolated under conditions that minimize deacylation (i.e. pH 5 and low temperature) and incubated with NaIO$_4$ which specifically oxidizes the cis-diol present in the 3$'$ terminal ribose of non-aminoacylated RNAs while leaving aminoacylated tRNAs unaffected. The tRNAs are subsequently deacylated and incubated with RNA ligase, allowing the formerly aminoacylated molecules to undergo intramolecular ligation to the 5$'$-phosphate, thereby generating circular tRNA molecules (16,17). In contrast, formerly non-aminoacylated tRNAs with oxidized 3$'$-ends remain in their linear form (16). tRNAs are subsequently reverse transcribed and amplified by PCR using primers located such that only circularized (formerly aminoacylated) tRNAs yield amplification products. To generate an RT–PCR product representing all tRNAs irrespective of their aminoacylation state, an aliquot of the RNA preparation is deacylated and circularized without oxidation. The tRNAs are then reverse transcribed and amplified by PCR using primers located such that only circularized (formerly aminoacylated) tRNAs yield amplification products. To generate an RT–PCR product representing all tRNAs irrespective of their aminoacylation state, an aliquot of the RNA preparation is deacylated and circularized without oxidation. Finally, (RT–)PCR products generated from mtDNA, total circularized tRNAs and oxidized circularized tRNAs are analyzed for the relative abundance of mutant and wild-type sequence by a primer extension assay in the presence of an informative ddNTP (Figs 2b and 3b).

Figure 1. Schematic representation of the OXOCIRC assay. Filled circles, aminoacylated 3$'$ ends; double bar, oxidized 3$'$ ends; dashed arrows, circularization; black arrowheads, primers for reverse transcription and PCR; shaded arrowheads, primers for PCR. Letters in parentheses refer to the control experiment in Figure 2d where a mixture of aminoacylated tRNA$^{Amp}$ with anticodon GGC and non-aminoacylated tRNA$^{Amp}$ with anticodon GLC were analyzed with the OXOCIRC assay.

Figure 2. OXOCIRC assay on tRNA$\text{Leu}$(UUR) from MELAS patients. (a) Detection of circular tRNA$^{Amp}$ by RT–PCR. The black arrowhead indicates the primer (#1620) used for reverse transcription whereas shaded arrowheads indicate the primers (#1904 and #1905) used for PCR together with primer #1620. (b) Detection of the A3243G mutation in (RT–)PCR products by primer extension with primer #1973 in the presence of ddTTP and the three other dNTPs. (c) Relative abundance of mutant (MT) and wild-type (WT) in patient tissues carrying the A3243G mutation analyzed by the OXOCIRC assay followed by primer extension. Extension products were analyzed by electrophoresis in a 20% polyacrylamide gel. D, mtDNA; T, total small RNAs circularized without oxidation; A, total small RNAs circularized with previous oxidation. The quantitation is described in Materials and Methods. (d) Control of the OXOCIRC assay. Primer extension on RT–PCR products derived from a mixture of non-aminoacylated tRNA$^{Amp}$ and glycylated tRNA$^{Amp}$ that was circularized without (lane 2) and with (lane 3) previous oxidation. See Figure 1 for a diagram. The upper band is derived from tRNA$^{Amp}$ and glycylated tRNA$^{Amp}$ and tRNA$^{Amp}$ in lanes 1 and 4, respectively. The relative abundance of tRNA$^{Amp}$ is 71% before and 24% after oxidation in the experiment shown. The band immediately above the primer results from non-specific incorporation of nucleotides by thermosequenase. Apart from this band, no extension products are observed in the absence of the specific template sequence, indicating that the intensity of the two informative bands is not affected by non-specific incorporation (data not shown).

functional tRNA$\text{Leu}$(UUR) contribute to the pathogenesis of the MELAS syndrome.
Effects of the A3243G mutation in MELAS patients

The OXOCIRC assay was performed on seven muscle biopsies and one lymphoblastoid cell line from patients who carry the A3243G mutation in the tRNALeu(UUR) gene and display MELAS or related symptoms (Fig. 2a and b) (see Materials and Methods for case histories). Representative results are shown in Figure 2c and the results from all patients are summarized in Table 1. A decrease of the relative amounts of the mutant in the processed tRNA pool (Fig. 2c, lanes T) compared with its abundance in the mtDNA fraction (lanes D) is observed in four samples (patients 1, 10, 12 and 13) whereas four other samples exhibit no or only minor deviations from the ratios in mtDNA (patients 9, 11, 14 and 17). Oxidation of tRNAs prior to circularization (Fig. 2c, lanes A) results in a lower relative abundance of the mutant sequence in six samples (patients 1, 9, 10, 11, 13 and 17), whereas no effect is apparent in the tRNAs from the lymphoblastoid cell line and one muscle biopsy (patient 14). Thus, in seven of eight patient samples the tRNA Leu carrying the A3243G mutation is under-represented in the (RT–)PCR products derived from aminoacylated tRNA compared with its abundance among those derived from mtDNA.

Control experiments for MELAS samples

Several controls were performed to ensure that the variable effect of the A3243G mutation on aminoacylation in different patient samples is not due to variations in the assay system. To monitor the efficiency of oxidation of non-aminoacylated tRNAs, in vitro aminoacylated glycy1-tRNAAsp(C) from opossum, which carries the anticodon GCC, and a non-aminoacylated tRNAAsp(U), which carries the anticodon GUC but otherwise the same sequence as the glycyl-tRNAAsp(C) were mixed and divided into two aliquots (16,18) (Fig. 1). While one aliquot was first deacylated, then circularized with RNA ligase, and amplified by RT–PCR, the other aliquot was oxidized with NaIO₄ prior to deacylation, circularization and RT–PCR. Both RT–PCR products were analyzed by primer extension, and the relative abundance of non-aminoacylated tRNAAsp(U) was found to be 71% in the non-oxidized and 24% in the oxidized aliquot, confirming the heteroplasmic status of the latter (data not shown). D, mtDNA; T, total small RNAs circularized without oxidation; A, total small RNAs circularized with previous oxidation. (d) pCP 3’ end-labeling (16,27) of total small RNAs from MERRF patient sample 2B before (lane 1) and after (lane 2) oxidation. The arrow indicates the position of 5S tRNA.

Figure 3. OXOCIRC assay on tRNAlys isolated from MERRF patients. (a) Detection of circular tRNAlys by RT–PCR. The black arrowhead indicates the primer (#1593) used for reverse transcription whereas shaded arrowheads indicate the primers (#2050 and #2049) used for RT–PCR. Note that primer #1747 was used only for secondary amplification of products generated with primers #2050 and #2049, ensuring the selective amplification of cDNAs derived from circular tRNAs. (b) Detection of the A8344G mutation in (RT–)PCR products by primer extension with primer #1974. (c) Relative abundance of mutant (MT) and wild-type (WT) tRNAlys in MERRF patient tissues analyzed as described in (a) and (b). Extension products were analyzed on a 20% polyacrylamide gel and quantitated as described in Materials and Methods. When a PCR product generated from a bacterial plasmid carrying the mutant tRNAlys was analyzed in an identical fashion, the resulting ratio of lower to upper band was ∼400:1, much higher than in the case of the patients with the highest ratio of mutant (∼20:1 or 95%, respectively), confirming the heteroplasmic status of the latter (data not shown). D, mtDNA; T, total small RNAs circularized without oxidation; A, total small RNAs circularized with previous oxidation. (d) pCP 3’ end-labeling (16,27) of total small RNAs from MERRF patient sample 2B before (lane 1) and after (lane 2) oxidation. The arrow indicates the position of 5S tRNA.
an at least 6.1 (±1.4)-fold relative decrease of the non-
aminoacylated tRNA species in the three samples. [The tRNA\textsuperscript{Amp}G/tRNA\textsuperscript{Amp}C ratios were changed from 2.4, 1.3 and 1.6 without oxidation to 0.32, 0.28 and 0.27 with oxidation.] It should also be noted that the observed variation between the control experiments is too small to explain the differences in effects observed among the patient samples. For example, in patient 11, the mutant tRNA\textsuperscript{Leu} was shifted from 78 to 49% (patient 11) indicating a 3.5-fold decrease in its relative abundance (the mutant/wild-type ratio was ∼3.5 without oxidation and ∼1.0 with oxidation) whereas in patient 14 the mutant sequence was decreased (∼3.4-fold decrease) and from 58 to 29% in patient 11 (∼3.4-fold decrease; data not shown). In the same samples, no change in the relative abundance of mutant tRNA\textsuperscript{Leu} was observed in patient 14 whereas in patient 11 the mutant sequence was decreased ∼3.5-fold (Table 1 and Fig. 2c). Thus, in a sample where oxidation did not result in a decrease of the mutant tRNA\textsuperscript{Leu}, oxidation had been as efficient as in a sample where oxidation resulted in a clear decrease of the mutant tRNA. These results lead us to conclude that the tRNA\textsuperscript{Leu} carrying the A3243G mutation is under-represented among aminoacylated tRNAs in some patients whereas no such effect is detected in others. In fact, among the eight samples analyzed, all possible combinations of effects are seen. In patients 1, 10, 11 and 13, the mutant is decreased in the total tRNA fraction compared with the DNA fraction, and again in the aminoacylated tRNA fraction. In patients 9 and 17, the relative abundance of mutant molecules in the DNA fraction, and again in the aminoacylated tRNA fraction. In

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<td>pT3/LB64</td>
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\textsuperscript{a}Means ± standard deviation based on primer extension analysis of two or more PCR products derived from the same extract.

\textsuperscript{b}Means ± standard deviation based on primer extension analysis of two or more RT–PCR products derived from the same circularization (9, 12, 2A, 22, 23, pT3/pT1, pT3/LB64) or from two different circularizations (1, 11, 14, 2B, 5, 24) of the same extract. Generation of RT–PCR products succeeded only once for samples 13 and 17.

\textsuperscript{c}Means ± standard deviation based on primer extension analysis of two or more RT–PCR products derived from the same oxidation/circularization (9, 12, 22, 23, pT3/pT1, pT3/LB64) or from two independent oxidations/circularizations (1, 11, 14, 2A, 5, 24) of the same extract. Generation of RT–PCR products succeeded only once for samples 13, 17, 2B and 25.

\textsuperscript{d}Samples previously used for thin sectioning.
No effects of the A8344G mutation in MERRF patients

In further experiments, five muscle biopsies and two fibroblast cell lines from MERRF patients containing the A8344G mutation in the tRNA^Lys^ gene were analyzed in a way analogous to that of the MELAS samples (see Fig. 3a and b for details). Figure 3c shows the extension products generated from (RT–)PCR products derived from mtDNA, total and aminoacylated tRNAs from three representative samples. Table 1 summarizes the findings. In all cases, there are only minor deviations in the abundance of the mutant sequence from that in mtDNA. Although the mutant is slightly decreased in some cases from mtDNA to total RNAs (e.g. samples 5, 22, 23 and 25), oxidation results in a slight increase of the mutant sequence. Thus, in the samples from six MERRF patients, the mutated tRNA^Lys^ is present in total RNAs and in the aminoacylated tRNA fraction at approximately the same abundance as would be anticipated from the mtDNA. The mutation per se seems to have no or only minor effects on either tRNA steady state levels or in vivo aminoacylation.

Control experiments for MERRF samples

As previously described for tRNA^Lys^, the efficiency of oxidation was monitored by adding a mixture of aminoacylated opossum glycyl-tRNA^Glu^ and non-aminoacylated tRNA^Ap^ to the tRNA preparations from patients 2B and 5. When the mixture of these tRNAs was co-oxidized with tRNAs isolated from patient sample 2B, the relative abundance of RT–PCR products derived from non-aminoacylated tRNA^Ap^ changed from 47 to 9% whereas the abundance of the mutant tRNA^Lys^ remained virtually unaffected (91 and 93% before and after oxidation, respectively). Similarly, in patient 5, the non-aminoacylated tRNA^Ap^ was decreased from 47 to 17% whereas the relative abundance of mutant tRNA^Lys^ was 87 and 90% before and after oxidation, respectively (data not shown). Furthermore, 3’-end-labeling with [32P]PipCp of total small RNAs from patient 2B showed a 26-fold decrease of 3’ ends in the 5S tRNA after oxidation relative to labeled tRNAs indicating both efficient oxidation and protection of tRNAs by aminoacylation (Fig. 3d).

Effect of the A8344G mutation in cybrid cell lines

The absence of a detectable effect of the A8344G MERRF mutation on abundance or aminoacylation of the tRNA^Lys^ was surprising since previous studies comparing cybrid cell lines homoplasmic for the A8344G transition or the wild-type sequence had reported a reduction of tRNA^Lys^ in processed (11,12) and in aminoacylated (12) tRNAs for the mutant cell lines. (Cybrids have the same nuclear background but carry different mitochondria.) To determine whether the apparently contradictory results were due to differences in cell material or in experimental procedures, we performed the OXOCIRC assay on total small RNAs isolated from the pT1 and the LB64 cybrid cell lines carrying the A8344G mutation. Initial analysis of (RT–)PCR products derived from mtDNA, circularized or circularized/oxidized tRNAs by primer extension showed that no wild-type sequence was detectable in these cell lines, indicating that they contain ≥98% of mtDNAs carrying the A8344G transition (Fig. 4 and data not shown). To compare the effects of the MERRF mutation in cybrids and patient tissue, total small RNAs from a cell line homoplasmic for the wild-type sequence at mitochondrial position 8344 (pT3) were mixed with extracts from mutant cell lines (pT1 or LB64) at ratios similar to those found in patient tissues. The mixtures were subsequently analyzed by the OXOCIRC assay.

When DNA/RNA extracts from wild-type (pT3) and mutant (pT1) cybrids are mixed such that ~95% of the mtDNAs carry the MERRF mutation, the abundance of the mutation is decreased to ~69% in the fraction of tRNAs that can be circularized (Fig. 4). Oxidation prior to circularization does not change the abundance of the mutant suggesting that mutant and wild-type tRNA^Lys^ are aminoacylated to a similar extent in the two cell lines (Fig. 4, lanes 1–3). To test whether the OXOCIRC assay would have detected decreased aminoacylation of the mutant tRNA^Lys^, isolates from the pT3 and pT1 cell lines were mixed at a similar ratio as before (~84% MT) but prior to mixture the aliquot from the pT1 (mutant) cell line had been deacylated by incubation at 37°C in 20 mM Tris (pH 8.3) for 30 min. When the abundance of the mutant was assayed in this aliquot, it was decreased to ~62% prior to oxidation to ~43% (Fig. 4, lanes 3–6). This shows that a decrease in aminoacylation of the mutant tRNA would have been detected by the assay. Furthermore, it confirms that a substantial fraction of the tRNA^Lys^ was aminoacylated in the extracts from the mutant cybrids. Our results suggest that, whereas the steady-state level of tRNA^Lys^ is decreased in the mutant cybrid cell line pT1 compared with the respective wild-type cell line, the mutation does not affect the level of aminoacylation.

The same set of experiments was performed with RNAs isolated from another 143B-derived cybrid cell line carrying the A8344G mutation in homoplasmic form (LB64). In a mixture containing ~90% of mutation at the mtDNA level (wild-type pT3), the mutation was present at ~83% in the (RT–)PCR product derived from processed tRNA^Lys^ and at ~78% in the RT–PCR product derived from oxidized/circularized tRNA^Lys^ (Fig. 4, lanes 7–9). Deacylation of the LB64 RNAs prior to mixture with those from pT3 and subsequent oxidation resulted in a shift from ~84 to ~64% confirming that a substantial proportion of the mutant tRNA^Lys^ is aminoacylated in this cell line (Fig. 4, lanes 10–12). These results suggest that cybrid cell lines carrying the mutant tRNA^Lys^ gene may indeed contain a lower level of aminoacylated tRNA^Lys^ compared with a cell line homoplasmic for the wild-type sequence (11,12). This decrease is due mainly to a lower amount of processed tRNAs. No similar effect is detectable in the seven tissue samples from MERRF patients analyzed in this study.
aminoacylated tRNALeu(UUR) is the primary defect in MELAS is leucine codons, mistranslation of UUR codons or translational anticodon of the other mitochondrial tRNA Leu(CUN) gene in a supported by the recent isolation of a suppressor mutation in the patients the relative abundance of tRNALeu carrying the A3243G suggests important differences in the molecular pathogenesis of samples fall close to the line of expected fit (Fig. 5b). This detectable in biopsies from MERRF patients where all patient aminoacylated tRNAs (Fig. 5a). In contrast, no similar effect is is decreased on average 4-fold in the aminoacylated tRNA fraction compared with its abundance in the mtDNA fraction due to the combined decrease of the mutant tRNA in total and/or aminoacylated tRNAs (Fig. 5a). In contrast, no similar effect is detectable in biopsies from MERRF patients where all patient samples fall close to the line of expected fit (Fig. 5b). This suggests important differences in the molecular pathogenesis of the MELAS and MERRF syndromes.

In the case of MELAS, half of the tissue samples examined show a decrease of tRNAs carrying the A3243G mutation. In six patients the relative abundance of tRNA\textsuperscript{Leu(UUR)} carrying the A3243G mutation is (further) decreased in aminoacylated tRNAs compared with total tRNAs. Assuming that the level of gene expression of mtDNA molecules carrying the wild-type sequence is unaffected by the presence of the A3243G mutation in heteroplasmic tissue, it seems reasonable to extrapolate these relative decreases in the mutant tRNA\textsuperscript{Leu(UUR)} among aminoacylated tRNAs to the absolute amount of available aminoacylated tRNA\textsuperscript{Leu(UUR)} (MT/WT). Under this assumption, the average mitochondrial in biopsies from all but one patient should contain <70% of aminoacylated tRNA\textsuperscript{Leu(UUR)} (mutant plus wild-type) compared with a situation where all mtDNA molecules are wild-type. In a case like patient 11, the level of aminoacylated tRNA\textsuperscript{Leu} may be as low as 30% of wild-type. In muscle fibers containing an accumulation of mutant mtDNA (19), a decreased level of aminoacylated tRNA\textsuperscript{Leu(UUR)} may result in stalling of ribosomes at leucine codons, mistranslation of UUR codons or translational frame shifting (20). The possibility that decreased levels of aminoacylated tRNA\textsuperscript{Leu(UUR)} is the primary defect in MELAS is supported by the recent isolation of a suppressor mutation in the anticodon of the other mitochondrial tRNA\textsuperscript{Leu(CUN)} gene in a A3243G lung carcinoma cell line (21). Although the suppressor tRNA is efficiently aminoacylated in the lung carcinoma cell line, aminoacylation of the tRNA\textsuperscript{Leu(UUR)} is severely decreased (22).

The decreased availability of aminoacylated tRNA\textsuperscript{Leu(UUR)} may be enough to explain the pathogenicity of the A3243G mutation. At the same time, our results suggest that this mutation has a variable effect on the expression and aminoacylation levels of tRNA\textsuperscript{Leu(UUR)} in the patient population indicating that additional factors may contribute to the molecular pathogenesis of MELAS. It is possible that the extent of effects induced by the A3243G mutation vary between different tissues or parts of tissues in a given patient, or in a given tissue over time. In addition, nuclear and mitochondrial background might modulate the effects of the mutation on tRNA function. More extensive work, including patients with milder as well as more restricted symptoms, will be required before insights about the exact correlation between clinical symptoms and the amount and state of aminoacylation of tRNA\textsuperscript{Leu(UUR)} can be achieved.

In the case of MERRF, our results, in contrast to previous studies of homoplasmic cybrid cell lines, indicate that the A8344G mutation exerts its effects (11–14) in the tissues of affected individuals by mechanisms other than reduced accumulation of tRNA\textsuperscript{Lys} and/or reduced availability of aminoacylated tRNA\textsuperscript{Lys}. Such effects could include, for example, partial misacylation and/or incorrect interactions between the mutant tRNA\textsuperscript{Lys} and translation factors.

DISCUSSION

The effects of the MELAS and the MERRF mutations on the relative abundance of the respective mutations in aminoacylated tRNAs are summarized in Figure 5. In the case of tissue from MELAS patients, the tRNA\textsuperscript{Leu(UUR)} carrying the A3243G mutation is decreased on average 4-fold in the aminoacylated tRNA fraction compared with its abundance in the mtDNA fraction due to the combined decrease of the mutant tRNA in total and/or aminoacylated tRNAs (Fig. 5a). In contrast, no similar effect is detectable in biopsies from MERRF patients where all patient samples fall close to the line of expected fit (Fig. 5b). This suggests important differences in the molecular pathogenesis of the MELAS and MERRF syndromes.

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MATERIALS AND METHODS

Case histories

Patients with the A3243G mutation. Patient 1: male, 35 years, juvenile onset, seizures, bilateral hearing loss, proximal myopathy, mental deterioration, polyneuropathy; patient 9: female, 45 years, typical MELAS syndrome, sister of patient 13; patient 10: male, juvenile onset, progressing dementia, insulin-dependent diabetes, episodes of hemianopsia, hemiparesis, motor aphasia, ataxia, frontal headaches, generalized tonic-clonic seizures (grand-mal), hypertrophic cardiomyopathy, death at 42 years from cardiac failure; patient 11: female, complete MELAS syndrome, onset at age 19; patient 12: male, severe MELAS syndrome from age 1 year, death at age 3 years; patient 13: male, 39 years, typical MELAS syndrome, juvenile onset, brother of patient 9; patient 14: female, 18 years, mental retardation, short stature, adult onset of seizures, muscles with ragged red fibers; patient 17: female, 28 years, epileptic seizures, muscle atrophy of the legs, moderate dementia, muscles with ragged red fibers.

Patients with the A8344G mutation. All but one sample (patient 22) are from patients exhibiting typical MERRF syndrome. Sample 22 is from an asymptomatic relative with ragged red fibers, whose mother and maternal uncle had adult-onset muscle weakness. Samples 2A and 2B are two different biopsies from the same patient.

Cell lines and media

The pT1 and pT3 human cell lines, which are homoplasmic for the mutant G and the wild-type A, respectively, in the mitochondrial tRNA\textsuperscript{Lys} gene at position 8344, were derived from a 143B206(p\textsuperscript{+}) lung carcinoma cell line, LB64 human cell line, which is efficiently aminoacylated in the lung carcinoma cell line, aminoacylation of the tRNA\textsuperscript{Lys(UUR)} is severely decreased (22). The increased aminocacylation of tRNA\textsuperscript{Lys(UUR)} may be enough to explain the pathogenicity of the A3243G mutation. At the same time, our results suggest that this mutation has a variable effect on the expression and aminoacylation levels of tRNA\textsuperscript{Lys(UUR)} in the patient population indicating that additional factors may contribute to the molecular pathogenesis of MELAS. It is possible that the extent of effects induced by the A3243G mutation vary between different tissues or parts of tissues in a given patient, or in a given tissue over time. In addition, nuclear and mitochondrial background might modulate the effects of the mutation on tRNA function. More extensive work, including patients with milder as well as more restricted symptoms, will be required before insights about the exact correlation between clinical symptoms and the amount and state of aminoacylation of tRNA\textsuperscript{Lys(UUR)} can be achieved.

In the case of MERRF, our results, in contrast to previous studies of homoplasmic cybrid cell lines, indicate that the A8344G mutation exerts its effects (11–14) in the tissues of affected individuals by mechanisms other than reduced accumulation of tRNA\textsuperscript{Lys} and/or reduced availability of aminoacylated tRNA\textsuperscript{Lys}. Such effects could include, for example, partial misacylation and/or incorrect interactions between the mutant tRNA\textsuperscript{Lys} and translation factors.
line carrying in homoplasmic form the mutant G at position 8344 was also derived from 143B206 and was generated as described for other A8344G cell lines by Masucci et al. (11) (a gift from E. Schon, Columbia University). Cell lines were cultured in Dulbecco's modified Eagle's medium containing 4.5 mg glucose/ml and 110 µg pyruvate/ml supplemented with 10% fetal bovine serum.

Oligonucleotides

Nucleotide numbers of human primers are according to Anderson et al. (23) and of opossum primers (#537, 740, 2169) according to Janke et al. (24). L denotes primers with L-strand sequences, H those with H-strand sequences. Primer 1747 spans the ligation site between the 5′ and 3′ ends of the RNA153. Post-transcriptionally added 3′ CCA sequences are underlined.


Isolation of small RNAs

All biopsies had been frozen immediately after removal from patients and stored at −80°C. Samples 14, 17, 22 and 23 had been previously surrounded by a plastic matrix to affix them during thin sectioning in a cryostat. This matrix was removed prior to homogenization. Thin sectioning requires no chemical treatment of the samples, so these samples were processed in the same way as other samples. Frozen muscle samples (20–40 mg) which had been frozen as pellets had been frozen immediately after removal from patients and stored at −80°C.

Oxidation, deacylation and circularization of tRNAs from patient samples were essentially as described (16). Eluates from the DEAE matrix were divided into two aliquots and precipitated with 3 vol of ethanol. One pellet was resuspended in 32 µl of ddH2O containing 10 µl of 4× circularization buffer (200 mM Tris−HCl pH 7.7, 40 mM MgCl2, 0.4 µg/ml bovine serum albumin), deacylated by heating for 5 min to 70°C and cooling for 30 min at 5°C and subsequently circularized for 2 h at 37°C by adding 0.6 µl of 10 mM ATP, 6 µl of DMSO and 40 U of T4 RNA ligase (New England Biolabs, Beverly, MA). The other pellet was resuspended in 20 µl of oxidation buffer (10 mM sodium acetate, 10 mM MgCl2, 1 mM EDTA, 15 mM β-mercaptoethanol) (25), and 20 µl of 65 mM NaIO4 in oxidation buffer was added and incubated for 20 min at room temperature in the dark. Since <5 µg of small cellular RNAs could be isolated from muscle biopsies, this equals a ≥1000-fold excess of NaIO4 over 3′ ends. Higher NaIO4 concentrations do not increase the oxidation efficiency yet interfere with subsequent enzymatic manipulations (data not shown). Oxidation was stopped by the addition of glucose in equimolar amounts to NaIO4 followed by two subsequent ethanol precipitations in the presence of 20 µg of glycogen. The pellet of oxidized small RNAs was resuspended in 32 µl of ddH2O containing 10 µl of 4× circularization buffer, deacylated and circularized as described above. To estimate the efficiency of circularization, total cellular RNAs from HeLa cells were circularized, separated by electrophoresis on a 10% polyacrylamide gel and analyzed by RT−minisequencing (16) of RNAs eluted from gel slices. Approximately 25% of the human tRNA153(UUR) and tRNA153, respectively, were detected in a circular fraction migrating above the respective linear tRNAs (data not shown). When a 5′-32P-labeled in vitro transcript of a mitochondrial tRNA153(16) was included in the circularization of 5 µg of total tRNAs from HeLa cells and analyzed by gel electrophoresis, 90% of the in vitro transcript was shifted to a single additional band above the linear RNA, indicating that circularization is favored over RNA multimerization under these conditions (data not shown).

cDNA synthesis

One microliter from the circularization reactions with oxidized/non-oxidized tRNAs was transferred into a 20 µl cDNA synthesis assay containing 0.5 µM of the respective gel-purified primer, 50 µM dNTPs and the appropriate buffer. After annealing (92°C, 2 min; 24°C, 10 min; 0°C, 10 min) AMV reverse transcriptase (Pharmacia, Uppsala, Sweden) was added and the reaction was incubated at 42°C for 30 min. cDNA synthesis on circular human tRNA153(UUR), circular human tRNA153, and circular marsupial tRNA153 was performed with primers #1620, #1593 and #537, respectively.

PCR of cDNAs

One microliter from the cDNA synthesis was transferred into a 30 µl PCR reaction. PCR reactions contained 67 mM Tris−HCl pH 8.9, 4 mM MgCl2, 16 mM (NH4)2SO4, 10 mM β-mercaptoethanol, 0.5 µg/ml bovine serum albumin (26) and 0.3 µM of the respective primers. cDNAs derived from circular human tRNA153 were amplified as follow. cDNAs from patients 1 and 11 were amplified with primers #1620 and #1905 (92°C, 2 min; and 40 cycles of 94°C,
30°C; 48°C, 30 s; 72°C, 30 s). The remaining MELAS samples were amplified for 20 cycles with primers #1620 and #1904 (92°C, 2 min; 94°C, 30 s; 48°C, 30 s; 72°C, 30 s), and 1 μl of the reaction was transferred into a new PCR reaction and cycled for another 20 cycles with the nested primer pair #1620 and #1905 (same conditions as before). cDNAs derived from circular human tRNA^Lys were amplified as follows. cDNAs from patient 2A and from cybrid cell lines (pT1, pT3, LB64) were amplified by PCR with primers #2049 and #2050 (92°C, 2 min; and 40 cycles of 94°C, 30 s; 48°C, 30 s; 72°C, 30 s). cDNAs from other patients were amplified by running 20 cycles with primers #2049 and #2050 (92°C, 2 min; 94°C, 30 s; 48°C, 30 s; 72°C, 30 s). After transferring 1 μl into a new PCR reaction, another 20 cycles with primers #1747 and #2049 followed. cDNAs generated from opossum tRNA^Lys were amplified for 40 cycles with primers #537 and #740 (92°C, 2 min; 94°C, 30 s; 42°C, 30 s; 72°C, 30 s). In all cases, the appropriate controls (circularization reaction to which no tRNAs had been added followed by cDNA synthesis) were included. When circularization was omitted or the circularization reaction was treated with RNase A prior to cDNA synthesis, no PCR product of the expected length was generated (data not shown).

PCR of mtDNA and cloned tRNA genes

PCR products representing the tRNA^Leu(UUR)^{16} gene were generated with primers #914 and #915, recognizing sequences in the 16S rRNA gene and in the ND1 gene, respectively (92°C, 2 min; and 35 cycles of 94°C, 30 s; 54°C, 30 s; 72°C, 30 s). PCR products representing the tRNA^Lys gene were generated with primers SIDS and #2049, recognizing sequences in the ATPase 6 gene and the tRNA^Lys gene, respectively (92°C, 2 min; and 35 cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 30 s). PCR products representing tRNA^Lys with the A8344G mutation cloned into pUC18 were generated with primers #553 and #554 which recognize sequences adjacent to the multiple cloning site (92°C, 2 min; and 35 cycles of 94°C, 30 s; 54°C, 30 s; 72°C, 30 s).

Primer extension

Non-incorporated primers and nucleotides were removed from (RT-)PCR products by three washes with 200 μl of ddH₂O during ultracentrifugation (Ultrafree 30; Millipore, Bedford, MA). Subsequently, the PCR products were resuspended in 20 μl of ddH₂O, 5 μl of which were included in the detection reaction. The extension was done in 13 μl containing 4 mM MgCl₂, 50 mM Tris–HCl pH 8.6, 5 U of Thermosequenase (Amersham, Little Chalfont, UK) and 0.1 pmol of the appropriate gel-purified 5'-32P-labeled primer. The concentration of the informative ddNTP was 175 μM and those of the other three ddNTPs were 70 μM. PCR products derived from tRNA^Leu(UUR)^{16} were analyzed with primer #1973 with ddITTP and the three other ddNTPs, and those derived from tRNA^Lys were analyzed with primer #1974 plus ddCTP and the three other ddNTPs. Primer #2169 plus ddCTP and the three other ddNTPs were used to analyze PCR products derived from the tRNA^Lys from opossum. After three to five cycles between 92 and 56°C, 1 min each, reactions were stopped with loading buffer, analyzed on a 20% polyacrylamide gel and quantitated with a Fuji-Phosphorimager. Primer extension analysis of RT–PCR products derived from pure wild-type tRNA^Lys and pure mutant tRNA^Leu(UUR)^{16} reveals a weak ladder of bands that decrease in intensity with increasing length leading up to the major extension product (data not shown and Figs 2c and 3c). To account for these unspecified extension products (presumably generated by premature termination), in each lane the extension signals immediately below the wild-type and mutant bands were subtracted as background from the major extension signals, respectively. Only tissue samples where both mutant and wild-type sequences could be detected in the mtDNA fraction were further analyzed.

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