Pathophysiology of the MELAS 3243 Transition Mutation*

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Single base substitutions of the mitochondrial genome are associated with a variety of metabolic disorders. The myopathy, encephalopathy, lactic acidosis, stroke-like episodes syndrome, most frequently associated with an A to G transition mutation at position 3243 of the mitochondrial tRNALeu(UUR) gene, is characterized by biochemical and structural alterations of mitochondria. To investigate the pathophysiology of the mutation, we established distinct Epstein-Barr virus-transformed B-cell lines for analyses that harbored 30–70% of the mutated genome. Interestingly, neither an alteration of the processing of primary transcripts nor a general impairment of individual mitochondrial protein subunit synthesis rates could be observed. Nevertheless, a marked decrease of cytochrome-c oxidase activity and reduced content of mitochondrial encoded subunits in the assembled respiratory complex IV was recorded on the cell line harboring 70% mutated mtDNA. Quantitative analysis of incorporation rates of the amino acid leucine into newly synthesized mitochondrial proteins, representing the functionality of the tRNA\textsuperscript{Leu(UUR)} in protein biosynthesis, revealed a specific decrease of this amino acid in distinct mitochondrial translation products. This observation was supported by a variation in the proteolytic fingerprint pattern. Our results suggest that the malfunctioning mitochondrial tRNA\textsuperscript{Leu(UUR)} leads to an alteration of amino acid incorporation into the mitochondrialy synthesized subunits of the oxidative phosphorylation system, thus altering its structure and function.

Mitochondrial encephalomyopathies are often associated with a variety of alterations of the mitochondrial DNA (1). The investigation of potential pathogenic mutations in the mitochondrial genome has revealed a complex relationship between patient’s genotype and the clinically defined multisystemic disorders (2). One of the best characterized diseases is the MELAS\textsuperscript{1} syndrome (mitochondrially encoded, lactic acidosis, stroke-like episodes) (3). Characterized by biochemical and morphological abnormalities of muscle mitochondria, the MELAS syndrome has been linked to multiple mitochondrial DNA alterations (4–6). Although mutations in protein genes have been reported to be associated with the disease, single base replacements in the tRNA\textsuperscript{Leu(UUR)} gene have been identified to play the key role in developing this devastating disorder. Among the tRNA\textsuperscript{Leu(UUR)} mutations, the pathogenic mechanism of the A to G transition mutation at position 3243 is discussed controversially in the literature. Embedded in the middle of a tridecamer sequence necessary for the formation of the 3’ ends of the 16 S ribosomal RNA, a severe impairment of 16 S transcription termination has been demonstrated \textit{in vitro} (7). The molecular defect in patients presenting the mutation could be the inability to produce the correct type and quantity of rRNA relative to other mitochondrial transcripts. Cytoplasmic transfer experiments, performed with mitochondria harboring the 3243 mutation and fused to a cell line devoid of endogenous mtDNA, showed a decreased synthesis rate of mitochondrial translational products in steady-state levels and the appearance of a novel RNA species (termed RNA 19), derived from transcription of the 16 S rRNA/tRNA\textsuperscript{Leu(UUR)}/ND1 gene (8). In a similar approach performed on a mutation at position 3302 in the tRNA\textsuperscript{Leu(UUR)} gene, mitochondrial RNA processing appears to be tissue-specific; while an unprocessed RNA 19 was detectable in muscle cells, this transcript was not detected in fibroblasts, implying that a tissue-specific mitochondrial RNA processing contributes to the generation of RNA 19 (9). In contrast, RNA transfer hybridization experiments on transformed cells harboring the 3243 mutation revealed no significant change in the steady-state level of the two rRNA species encoded upstream of the termination motif and of the mRNA species encoded downstream to the mutation (10). Thus, the suggested pathogenic mechanisms seems to be inconclusive.

To investigate the pathophysiology of the 3243 mutation, we established distinct EBV-transformed B-cell lines that harbored 30–70% of the mutated genome. Interestingly, neither an alteration of quality nor quantity of the primary transcripts could be observed. In addition, our experiments carried out on mitochondrial translation revealed no impairment of overall mitochondrial protein synthesis rate, although specific OXPHOS complexes exhibited a marked decrease of enzyme activity and assembly of mitochondrial subunits of the cell line harboring 70% mutated mtDNA. Quantitative analysis of incorporation of the amino acid leucine, monitoring the functionality of the tRNA\textsuperscript{Leu(UUR)} in protein biosynthesis, revealed a specific decrease of this amino acid in mitochondrially encoded and synthesized protein subunits. This observation was supported by heteroplasmic variations in the proteolytic fingerprint pattern of these subunits in the assembled OXPHOS-complexes. Our results are leading us to the hypothesis that the crucial step in molecular pathogenicity of MELAS is an alteration of the translation process at the mitochondrial ribosomes, induced by the malfunctioning mitochondrial...
subunits of the oxidative phosphorylation system, thus altering acid leucine incorporation into the mitochondrially synthesized chondria (14). Briefly pyruvate.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from Life Technologies, Inc. Additional materials used in molecular biological studies were obtained from Perkin-Elmer (PCR), U.S. Biochemical Corp. (DNA sequencing), Boehringer Mannheim (Northern hybridization, RFLP analyses, reverse transcription), or Amersham Buchler (radiochemicals).

Cell and Tissue Culture—Human B-lymphoblastoid cell lines were established by isolation of lymphocytes from blood of patients presenting with MELAS syndrome and controls. The EBV-transformed lymphocytes (11) were grown in glucose-rich media (RPMI 1640), supplemented with 10% fetal calf serum, 50 μg/ml uridine, and 100 μg/ml pyruvate.

Enzyme Kinetics—Standard enzymatic assays for OXPHOS enzymes were performed as described previously (12, 13). Cells were also used for polarographic assays to determine the respiratory capacity of mitochondria (14). Briefly 10^6 cells were either resuspended in cell culture medium or prepared by digitonin treatment according to Granger and Lehninger (15). 5 × 10^6 cells were resuspended in the appropriate assay buffer, and mitochondrial respiration was determined using a small scale Clarke-type oxygen electrode (Biolytic M81; RE K1–1N) (16, 17).

As reference enzyme for oxidative phosphorylation, cytochrome-c oxidase activity was additionally assayed by using the turnover number using reversed Edie-Hofstei plot (18).

DNA and RNA Preparation, Enzymatic Amplification, and Sequencing—Total DNA was extracted from 1 × 10^6 cultured cells using a standard SDS/proteinase K protocol (19). Total RNA was isolated from 1 × 10^7 cells by guanidinium thiocyanate-phenol:chloroform extraction (20). PCR amplification of the mitochondrial DNA fragments encompassing the 3243 point mutation was performed using the oligonucleotides displayed in Table I. tRNALeu−1, 1 and 2; pre-tRNALeu (displaying tRNA, oligo 9; detection of 12 S rRNA/tRNA Val primary transcript, oligo 9; detection of 12 S rRNA/tRNAβ primary transcript (26), oligo 10; NADH dehydrogenase subunit 1, oligo 11.

Analysis of Mitochondrial Translation Products—5 × 10^6 cells were harvested and incubated for 30 min in starvation medium. The starvation medium was specifically formulated using the RPMI 1640 medium (deficient in arginine, cysteine, glutamine, leucine, and methionine) (Life Technologies, Inc.) according to the conditions described by the supplier, thereby depleting the amino acids that will be subsequently used to label the proteins. To shut down cytoplasmic protein synthesis, 130 μg/ml cycloheximide were added, and incubation was continued for an additional 10 min. The labeling reaction was initiated by adding the following amino acids: for 35S labeling a mixture of 70% L-[35S]methionine, 30% L-[35S]cysteine; >100 Ci/mmol (Pro-MixTM, Amersham Corp.) was used. 3H labeling was performed by adding 700 kBq/ml either L-[3H]leucine (120–190 Ci/mmol) or l-[4,5-3H]lysine or l-[3,4,5,6]-H-phenylalanine. The labeling reactions were performed for 30 min (only using the 35S-amino-acids to measure the rate of protein synthesis) or for 3 h (using also 3H-labeled amino acids to evaluate their rates of incorporation) at 37 °C, followed by a 20-min chase with standard medium, containing 130 μg/ml cycloheximide and the 4-fold concentrations of the depleted amino acids.

Protein Extraction and Gel Electrophoresis—After washing the cells twice in ice-cold phosphate-buffered saline, the labeled cells were processed for subsequent analysis. For denaturing SDS-PAGE (21), 1 × 10^6 cells were resuspended in a 10-fold volume of 10 mM bis-tris/HCl, pH 7, 5.0 mM MgCl2, 1.0 mM phenylmethylsulfonyl fluoride, and 1.0 μg/ml aprotinin. After addition of 50 units/ml DNase I, the suspension was incubated at 25 °C for 1 h, dissolved in detergent buffer (750 mM aminocaproic acid, 1 mM bis-tris/HCl, pH 7.0, and 1% laurylmaltoside), and centrifuged for 30 min at 100,000 × g. Protein concentration was determined according to a Lowry assay kit following the manufacturer’s protocol (Pierce). 100–150 μg of protein (approximately 4 × 10^4 dpm) were mixed with loading buffer (22), incubated at 40 °C for 10 min, and used for 12.5% 7% SDS-PAGE on a 1.5 × 120 × 230-mm vertical SGMA electrophoresis unit. For native PAGE separation of mitochondrial translation products, 10% or 15% Bis-Tris/Tricine gels were replaced in isolation buffer (28) and homogenized with a tight fitting glass-Teflon potter. After two centrifugation steps at 2,500 and 15,000 × g the mitochondrial pellet was resuspended in 750 mM aminocaproic acid, 50 mM bis-tris/HCl, pH 7.0, and 1% laurylmaltoside and centrifuged as described above. The supernatant was supplemented with Coomassie Blue G-250 and loaded on a 7–16% linear gradient gel system (29). The separated mitochondrial proteins were electroblotted on 0.1-μm nitrocellulose membranes by semi-dry blotting (Schleicher & Schull) in a discontinuous buffer system (30).

Quantitation of Labeling Efficiency—After denaturing SDS-gel electrophoresis and subsequent electroblotting as described above, the individual protein subunits were localized by autoradiographic detection (24 h exposure to Kodak Biomax MR film) and isolated by excision. For scintillation counting, the nitrocellulose membranes were dissolved in 2 ml of acetonitrile and resuspended in 8 ml of scintillation solution (Insta-Fluor; Canberra Packard). The fractions were analyzed for their 3H and 35S content by spectral scintillation counting. The relative proportions of incorporated amino acids were calculated by using the following calibration: reference spectra of each single radioisotope were recorded under identical experimental conditions specific channels for 35S and 3H were defined. An experimental correction factor was applied on 3H radiation recorded within the 3H window.

Protein Fingerprinting—The proteolytic digestion pattern of the mitochondrial subunits was performed by a modification of the protocol of Cleveland and colleagues (31). After separation of whole cell lysates in the first dimension, mitochondrial subunits were excised and incubated...
for 10 min in 200 mM Tris-Cl, pH 8.3, 0.1% SDS, 10% glycerol. The SDS gel for separation in the second dimension was prepared as described but using preparative combs and 0.05% SDS in the stacking gel and cathode buffer. 25 μg of endoproteinase Glu-C (Boehringer Mannheim) was dissolved in 200 mM ammonium carbonate, 0.025% SDS, 10% glycerol, pH 8.9, 0.0001% bromphenol blue. After loading, electrophoresis was performed until the endoproteinase had entered the gel. The excised gel fragments were positioned in the sample slots between the gel plates and overlaid by another portion of the endoproteinase solution containing 35 μg of endoproteinase Glu-C. Electrophoresis was resumed until the marker reached the end of the stacking gel. After a pause of 30 min, electrophoresis was again resumed and continued to its end. The cleavage pattern was visualized by autoradiography of the blotted gel as described above.

RESULTS

To elucidate the pathogenic mechanism underlying the A to G transition mutation in the mitochondrial tRNA^{Leu(UUR)} gene at position 3243, we established EBV-transformed lymphoblastoid cell lines from patients carrying heteroplasmic populations of the mutated genome. After initial screening, two stable cell lines were obtained, harboring 30 and 70% mutated DNA. These were subjected to biochemical and genetic analyses. While enzymatic activities and polarographic studies of cells presenting with 30% mutant genome showed no significant differences in activity (data not shown), cells harboring 70% mutated DNA exhibited significant reduction in cytchrome-c oxidase, with other OXPHOS enzymes activity lying at the lower limit of standard deviation (see Table II). These data were supported by polarographic studies carried out on permeabilized cells; whereas respiration capacity from cells with 30% mutation rate were almost indistinguishable from control cells, a 20% reduction in oxygen consumption was recorded for permeabilized cells harboring 70% mutated genome (see Fig. 1).

To prove whether malfunctioning enzymes are responsible for the observed abnormalities, we decided to measure the turnover number of cytchrome-c oxidase as a marker for enzyme integrity. The analysis of cytchrome-c oxidase activity under different concentrations of cytchrome-c revealed a significantly decreased turnover number of the enzyme at low substrate concentrations in cells presenting with 70% mutated genotype (see Fig. 2).

To exclude that an additional mitochondrial DNA variation could contribute to this effect, the mitochondrial encoded COX genes were sequenced (see "Experimental Procedures"). By comparing the obtained sequence to the published sequence, no variation was identified that could account for the enzymatic alterations. Hence, the altered enzyme integrity must be related to the mutation in the tRNA^{Leu(UUR)} gene itself.

To determine whether the mutation in the tRNA^{Leu(UUR)} gene influences the transcript level of mutated and wild-type tRNA in heteroplasmic cells, we analyzed the degree of heteroplasm on DNA, tRNA, and primary RNA transcript level by RFLP analysis (see "Experimental Procedures"). The quantitative determination of the proportion of mutated versus wild-type nucleic acids in patient’s cells revealed corresponding levels so that for each cell line the amount of mutated DNA (genotype) matched its transcript level (see Fig. 3a). This was observed for the primary transcripts as well as for their processed counterparts. Hence, neither a malfunctioning processing of primary transcripts nor a difference in stability of the mutated versus the wild-type tRNA could be observed.

To verify that the ratio of mitochondrial to nuclear transcripts was unaltered, mitochondrial and nuclear expression levels were characterized by Northern analyses. A difference in mitochondrial RNA contents was not detectable for primary transcripts or rRNAs or mRNAs, or processed ribosomal RNA, or mature tRNAs (Fig. 3b). There was no detectable accumulation of a precursor-like abnormal primary transcript (termed RNA 19) as has been described for ρ- cells with a high percentage of altered mtDNA (32).

Although levels of mutant and wild-type transcripts showed no alteration, further analyses were extended onto the mitochondrial translation process. The reduced enzyme activities could be caused by two effects: a reduction in the abundance of enzymes or by malfunctioning components of the complexes. Thus, mitochondrial protein labeling experiments were carried out in the presence of [35S][methionine] and [35S][cysteine] as described under "Experimental Procedures." Evaluation of the protein synthesis rates of cells affected by 70% mutation expression showed no differences in the amounts of mitochondrial subunits (see Fig. 4 for laser densitometric scanning of the protein banding pattern). This result was underlined by experiments utilizing a 30-, 60-, and 180-min pulse. Neither condition applied resulted in an alteration of our primary result. Hence, neither the rate of synthesis (represented by a 30-min pulse) nor the accumulation of peptides in stable complexes (as monitored by the 180-min pulse) revealed significant differences. Hence, at least up to levels of 70% mutated genotype, the overall protein synthesis rate of individual subunits seems not
To prove that the defect was tRNA(Leu(UUR)) specific and to evaluate the possibility of conservative substitution of the UUR-encoded leucine by phenylalanine, encoded by the related UUN codon, we carried out identical experiments using 3H-labeled phenylalanine in conjunction with similar amounts of [35S]methionine/[35S]cysteine in the more severely affected MELAS cell line. Experiments were also performed by using the tritiated amino acids phenylalanine, lysine, and proline, encoded by the codons UUN, AAR, and CCW. Analysis of these co-labeling experiments was performed with the abundant mitochondrial subunits COX I and COX II, also presenting suitable MELAS cell line and wild-type cells could be observed. After separation of the assembled mitochondrial subunits by second dimension SDS-PAGE, no differences in the relative amount of nearly all mitochondrial encoded subunits were detectable. However, an altered pattern of mitochondrial encoded subunits of respiratory complex IV could be observed in the mutated cells (see Fig. 6). Analysis of mitochondrial encoded and co-labeled smaller subunits isolated from this gel system also showed a significant [3H]leucine reduction in the 70% cell line relative to controls, monitoring assembly of altered subunits in the OXPHOS complexes.

To analyze if the altered peptides participate in the assembly of the OXPHOS complexes, we utilized blue native gel electrophoresis to separate assembled OXPHOS complexes prior to SDS-PAGE analysis. These qualitative and quantitative analyses of assembled OXPHOS complexes were carried out by using mitochondrial fractions derived from digitonin-permeabilized cells. No differences in the relative amount of the individual OXPHOS enzyme complexes between the more severely affected MELAS

![Image](https://example.com/image.png)
The treatment resulted in a specific cleavage pattern of the labeled subunits within the second dimension of a two-dimensional gel system. Subtractive images of the endoproteinase Glu-C fingerprints were created by superimposition of the autoradiograms from the 70% affected cell line and the control cell line (see Fig. 8). As expected from the labeling experiment, fingerprints revealed different cleavage patterns that derived from the accessible subunits of ND5, COX I, and ND4, showing an alteration of the primary amino acid sequence of mitochondrially synthesized proteins.

**DISCUSSION**

The MELAS syndrome is one of the best examined and most explored phenotypes among mitochondrial encephalopathies that are associated with tRNA point mutations of the mitochondrial genome. A number of tissue and cell culture experiments have been performed to elucidate the mechanism evoking the serious clinical manifestations and to find a relationship between the disease and the observed point mutations of the mitochondrial DNA. An impairment of 16 S transcription termination, resulting in an altered structure and quantity of heavy strand transcripts, could not be confirmed by our data obtained on cell lines carrying 30 or 70% mutated genome. Additionally, the novel RNA species, termed RNA 19 (8), was not detectable in our system. However, this was not completely unexpected, since an altered processing status of mitochondrial RNA in muscle tissue compared with fibroblasts has been reported (9). Hence, processing of mitochondrial transcripts appears to be tissue- and cell type-specific, with fibroblasts and lymphoblastoid cells exhibiting a marked difference when compared with muscle tissue.

Quantitative analyses of the transcription levels of mutated and wild-type tRNA was not detectable by our data obtained on cell lines carrying 30 or 70% mutated genome. Additionally, the novel RNA species, termed RNA 19 (8), was not detectable in our system. However, this was not completely unexpected, since an altered processing status of mitochondrial RNA in muscle tissue compared with fibroblasts has been reported (9). Hence, processing of mitochondrial transcripts appears to be tissue- and cell type-specific, with fibroblasts and lymphoblastoid cells exhibiting a marked difference when compared with muscle tissue.

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products. The qualitative composition of mitochondrial protein syntheses period) and puzzled by the decreased enzyme activity that was quantitative difference in synthesis rates of individual mitochondrial protein subunits (from pulse labeling up to a 3-h labeling duration) revealed no quantitative differences in the mitochondrial protein biosynthesis products.

The analysis of the OXPHOS complexes in the inner mitochondrial membrane revealed no quantitative differences in separated and stained enzyme complexes between the 70% MELAS cell line and wild-type cells, monitoring functional import and assembly behavior of the nuclear-encoded OXPHOS subunits (33). After denaturing gel electrophoresis of the complexes in the second dimension, altered electrophoretic mobility of labeled mitochondrial subunits of respiratory complex IV could be observed in the 70% MELAS cell line (see Fig. 6). These results were pointing toward altered assembly of mitochondrial encoded subunits and impaired functionality of affected OXPHOS complexes (34).

While measuring protein synthesis rates of individual mitochondrial encoded subunits in the presence of [3H]leucine and [35S]methionine/cysteine, we observed a decreased incorporation rate of [3H] in the patient cell lines when compared with controls. Hence, also presenting apparently with the same molecular weight, mitochondrial translation products of cells harboring the tRNA\text{Leu(UUR)} mutation at position 3243 were deficient in leucine content. If this observation was correct, this could account not only for the so far unrecognized qualitative defects in mitochondrial translation but could also explain the enzymatic defects and the alteration of OXPHOS complex integrity (e.g. alteration in the turnover number of COX), caused by a blueprint modification of the primary amino acid sequence on the translational level. This is also supported by the remarkable correspondence of the decreased leucine content with the genotype of the cell. Cells with 70% mutant genotype exhibited a higher deficiency in leucine content as cells with 30% mutant genotype. Although incorporation of leucine into mitochondrial proteins is mediated by two distinct tRNA\text{Leu(UUR)} that recognize different codons (UUR or CUN), a decrease in leucine content of the translational products should be proportional to the number of UUR codons of the mRNAs. In fact, assuming a hindering of tRNA\text{Leu(UUR)}-mediated amino acid incorporation, a strikingly similar distribution can be recognized when comparing the experimental data with our theoretical estimations (see Table III). The most extensive alteration in \( ^{3}\text{H}/^{35}\text{S} \) ratio for example has been recorded for the subunits ND3 and ND6. This is consistent with the theoretical data, since these subunits contain 14 UUR/3 CUN or 10 UUR/18 CUN codons, respectively, and the \( ^{3}\text{H}/^{35}\text{S} \) ratio is mainly determined by the influence of the defective tRNA\text{Leu(UUR)}. Since the ND4L transcript carries only one UUR, but 22 CUN codons, no significant alteration of the \( ^{3}\text{H}/^{35}\text{S} \) ratio should be expected. Analyses of the co-labeled translation product of ND4L were corresponding to this theoretical consideration (see Fig. 5).

To further support our findings, we carried out a proteolytic fingerprint on mitochondrially synthesized subunits. As expected, the fingerprint pattern of the cell line exhibiting 70% mutated DNA revealed a heteroplasmic appearance. This reflects the heteroplasmic genotype of the cells, resulting in different proportions of mitochondrial protein biosynthesis products synthesized by the wild-type and mutated tRNAs.

Mitochondrial translation takes advantage of only a limited set of 22 tRNAs that can serve all codons by keeping the third position of the codon variable (“wobble hypothesis”). Assuming that the “wobbling mechanism” could lead to a misrecognition of the leucine UUR codons by a tRNA exhibiting a related anticodon, as for instance tRNA\text{Phe(UUN)}, an alteration (increase) of the \( ^{3}\text{H}/^{35}\text{S} \) ratio using labeled phenylalanine could be expected. In fact, our analyses revealed no evidence that tRNA\text{Phe(UUN)} contributes as a substitution for the defective tRNA\text{Leu(UUR)}. Since the \( ^{3}\text{H}/^{35}\text{S} \) ratio from 30 or 70% mutated cells was not significantly different when compared with controls. A substitution of tRNA\text{Leu(UUR)} by more distant related tRNAs, as shown by incorporation of amino acid lysine or proline, revealed no further evidence for defects of the mitochondrial translation system. Limited activity, abundance, and low incorporation rates of other radiolabeled amino acids used for co-labeling did not allow further evaluation of these results. Sequence analysis from labeled mitochondrial subunits as well as from proteolytic fragments also requires new experimental methods. This is due to the limited amount of sample material as well as to the heterogeneous and heteroplasmic appearance of the mitochondrially synthesized protein subunits in cell preparations, reducing the applicability of experimental procedures for their isolation and analysis.

Nevertheless the results from this study together with the reports found in literature lead us to extend the pathomechanistic models for MELAS assuming direct interference of altered tRNA\text{Leu(UUR)} with the mitochondrial translation processes.

The tRNA\text{Leu(UUR)} Substitution Model—Assuming that the...
mutated mitochondrial tRNA<sub>Leu(UUR)</sub> does not take part in the process of mitochondrial translation and assuming that another mitochondrial tRNA harboring a UUR-related or UUR-like anticodon could take over tRNA<sub>Leu(UUR)</sub>'s function, mitochondrial translation products could be synthesized exhibiting another amino acid at UUR recognition motifs.

**The Aminocacyl-tRNA Synthetase Model**—Assuming that the mutated tRNA<sub>Leu(UUR)</sub> actively takes part in the process of mitochondrial translation, this process could occur on a mischarged or an uncharged tRNA level. In both cases, a malfunctioning charging of the tRNA induced by the mutation must be assumed. This would result in translation products with altered or disturbed amino acid sequence, presenting proteins with same apparent molecular weight but of different amino acid composition, depending on the leucine (UUR) content of the translation product.

**The Ribosomal Mistranslation Model**—Assuming that the mutated mitochondrial tRNA<sub>Leu(UUR)</sub> does not take part in the process of mitochondrial translation and assuming that no other mitochondrial tRNA recognizes the UUR codon and takes over tRNA<sub>Leu(UUR)</sub>'s function, a reduced leucine content of the mitochondrial translation products could occur on ribosomal mistranslation at UUR codons on mRNAs. Mechanistically, this could be induced by a translational frameshift, so that proteins synthesized would be deficient in leucine at UUR codons, depending on the degree of heteroplasmy within the environment (mitochondria). Although this model would lead to ultimate changes of the primary amino acid sequence, the apparent molecular weight of these proteins would be slightly shorter, depending on the leucine (UUR) content of the translation product.

All three models discussed here are able to explain the decreased leucine content of the mitochondrial translation products in cell lines harboring the 3243 mutation. In our study, conservative amino acid substitution was neither observed with the functionally related tRNA<sub>Phle(UUN)</sub> nor with other hydrophobic amino acids closely related to leucine (proline and lysine). These results did not support a decisive role of this mechanism in MELAS.

Although ribosomal frameshifting (35) has been known for procaroytic (36) and eucaroytic systems (37), a report from Fox et al. (38) suggested a similar mechanism occurring also in yeast mitochondria. Appealing to the hypothesis that frameshifting could occur on ribosomes in vertebrate mitochondria is the fact that translational accuracy of the procaroytic ribosomes can be influenced by energy and/or amino acid (39, 40) or aminoaoyl-tRNA content (41, 42). It is known that ribosomal frameshifting can be induced by starving a system for functional tRNAs (43). The starvation is postulated to interfere with ribosomal composition (44), translational accuracy (45), as well as processivity (46). An elevated proportion of mutated and thus nonfunctional tRNA transcripts could consequently initiate alterations of the ribosomal translation process. Further on, additional effects on ribosomal accuracy due to subsequent energy deprivation of the mitochondrial compartment, also interfering with the tRNA charging processes, could be induced. The observations described in literature together with our results could present, in the case of tRNA point-mutations, a sort of ribosomal bottleneck. In cells harboring an elevated level of mutated mitochondrial tRNA<sub>Leu(UUR)</sub> alterations at the translating ribosome would be induced, leading to modified translation products. Assembled to multi-enzyme complexes, these functionally altered peptides would lead to subsequent energy deprivation of the affected mitochondria, resulting in further impairment of mitochondrial protein biosynthesis. Reaching a threshold level, these cumulative effect could finally lead to the characteristic phenotype in the affected cells and tissues.

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