

Two Novel Pathogenic Mitochondrial DNA Mutations Affecting Organelle Number and Protein Synthesis

Is the tRNA^{Leu(UUR)} Gene an Etiologic Hot Spot?

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Abstract

We identified two patients with pathogenic single nucleotide changes in two different mitochondrial tRNA genes: the first mutation in the tRNA^{Asn} gene, and the ninth known mutation in the tRNA^{Leu(UUR)} gene. The mutation in tRNA^{Asn} was associated with isolated ophthalmoplegia, whereas the mutation in tRNA^{Leu(UUR)} caused a neurological syndrome resembling MERRF (myoclonus epilepsy and ragged-red fibers) plus optic neuropathy, retinopathy, and diabetes. Both mutations were heteroplasmic, with higher percentages of mutant mtDNA in affected tissues, and undetectable levels in maternal relatives. Analysis of single muscle fibers indicated that morphological and biochemical alterations appeared only when the proportions of mutant mtDNA exceeded 90% of the total cellular mtDNA pool. The high incidence of mutations in the tRNA^{Leu(UUR)} gene suggests that this region is an "etiologic hot spot" in mitochondrial disease. (*J. Clin. Invest.* 1993; 92:2906–2915.) Key words: mitochondrial DNA • mitochondrial disorders • tRNA identity • diabetes • ophthalmoparesis

Introduction

Several mitochondrial DNA (mtDNA) alterations have been linked to human diseases. Most alterations are heteroplasmic, i.e., both mutant and wild type mtDNAs coexist within cells. Large-scale mtDNA deletions, which are usually nonfamilial, are commonly associated with progressive external ophthalmoplegia (PEO).¹ In addition to PEO, patients may have the multisystem Kearns-Sayre syndrome (with pigmentary retinopathy, ataxia, heart block, and high CSF protein content) (1, 2). Deletions of mtDNA have also been observed in infants with the Pearson syndrome, affecting bone marrow and pan-

creas (3). The pathogenesis of syndromes with mtDNA deletions seems related to severe impairment in mitochondrial protein synthesis (4–5).

Maternal inheritance is commonly observed in diseases associated with mtDNA point mutations. Several pathogenic mutations in the mitochondrial tRNA^{Leu(UUR)} gene have been described (6–12). One of these, an A → G transition at nt-3243, is seen most frequently in patients with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes). Two point mutations at nt-8344 and nt-8356, within the tRNA^{Lys} gene, have been associated with myoclonus epilepsy with ragged-red fibers (MERRF syndrome) (13–16). The mitochondrial dysfunction in MELAS and MERRF has also been attributed to impaired mitochondrial protein synthesis (17–20).

We noted that impairment of mitochondrial protein synthesis in skeletal muscle was invariably accompanied by mitochondrial proliferation, which is easily detectable by cytochemical staining. Muscle fibers with massive mitochondrial proliferation are called ragged-red fibers (RRF). RRF are observed in patients with mtDNA deletions (deletions including tRNA or rRNA genes (1–2), mtDNA point mutations in tRNA genes (8, 13, 21, 22), and mtDNA depletion (23–24). On the other hand, with one possible exception (25), mutations in protein-coding genes, such as those observed in Leber hereditary optic neuropathy (LHON) (26), or in a condition characterized by neuropathy, ataxia, and retinitis pigmentosa (NARP), are typically not associated with RRF (27).

Any pathogenic mtDNA mutation is expected to affect oxidative phosphorylation. Therefore, the signal for mitochondrial proliferation may not be the impairment of oxidative phosphorylation per se, but rather a direct response to defective mitochondrial protein synthesis (21, 22). To test this hypothesis, we have analyzed protein synthesis-related mtDNA sequences (e.g., rRNAs and tRNAs) from 10 patients with mitochondrial proliferation in muscle (RRF) who did not have any of the known pathogenic mtDNA mutations. We found two patients with novel pathogenic alterations in two different tRNA genes.

Methods

Patients. 10 patients with mitochondrial myopathy or encephalomyopathy were selected primarily for the presence of RRF in their muscle biopsies. Although none of them had definite evidence for maternal inheritance, they all had symptoms and signs that suggested a mitochondrial disorder.

Patient 1 was a 27-yr-old African-American woman with severe ocular myopathy. Bilateral ptosis was noted at age 9 mo and diminished eye movements by the age of 2 yr. She had about 10 syncopal events between 8 and 15 yr of age. At age 27 weakness was restricted to

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1. Abbreviations used in this paper: COX, cytochrome c oxidase; CSF, cerebrospinal fluid; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibers; PEO, progressive external ophthalmoplegia; SDH, succinate dehydrogenase.

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ocular muscles, but she complained of easy fatigability for one year. Family history was negative for neuromuscular disorders. She was very thin (160-cm tall, 40.5 kg) and electromyography tests were compatible with a myopathy. Deep tendon reflexes were present and the muscle biopsy showed many RRF.

Patient 9 was a 45-yr-old Caucasian man with a progressive multi-system disorder. His birth and early life were unremarkable until age 28 yr when he had generalized tonic-clonic seizures that responded to carbamazepine therapy. In his thirties, he developed ptosis, ophthalmoparesis, hearing loss, and diabetes mellitus. At age 40, a physician diagnosed Hashimoto thyroiditis with hypothyroidism and placed the patient on T4 replacement therapy. At age 42, he lost central vision bilaterally over 2 wk. Examination revealed temporal pallor of the optic disks, and retinal pigment degeneration in a "bull's-eye" pattern surrounding the relatively unaffected macula. Limb myoclonus, mild weakness of the neck muscles and deltoids, and mild ataxia (heel-to-shin and tandem gait testing) were also noted. Reflexes and sensations were normal. Laboratory studies revealed: an elevated resting venous lactate = 3.9 mM/l (normal 0.5–2.2); normal CSF protein and glucose, but elevated CSF pyruvate = 0.19 mM/l (normal 0.04–0.13) and CSF lactate = 3.04 mM/l (normal 0.90–2.80); brain MRI with mild diffuse atrophy with possible optic nerve atrophy; and nerve conduction and electromyography studies consistent with a myogenic process. He subsequently developed vertigo with nausea. There was no family history of neurological disorders. Patient 9 was described as patient 2 in reference 41 as a possible case of MERRF with PEO.

Materials. Restriction enzymes were from Boehringer Mannheim Biochemicals (Indianapolis, IN) and New England Biolabs (Beverly, MA). Klenow fragment of *Escherichia coli* DNA polymerase I was from Boehringer Mannheim. RNase A and chemicals were from Sigma Chemical Co., St. Louis, MO). [α - 32 P]- and [γ - 32 P]-dATP (800 Ci/mmol) were from New England Nuclear (Boston, MA).

Single-stranded oligonucleotide primers were from Genosys (Houston, TX).

Direct sequencing of PCR products. Mitochondrial DNA sequences were amplified by PCR from genomic DNA preparations, purified by Gene Clean (Bio-101, Inc., Vista CA), and sequenced directly using 32 P-labeled primers with a cycle-sequencing kit (BRL, Bethesda, MD). Amplified fragments ranged from 0.2 kb to 1.8 kb. Sequencing primers were spaced by approximately 250 nt. Sequencing reactions were electrophoresed through a 6% denaturing polyacrylamide gel, dried under vacuum, and exposed to x-ray films for 12–24 h.

Determination of the proportion of normal and mutant mtDNAs. Approximately 0.5 μ g of genomic DNA were submitted to PCR amplification (1 min 94°C, 1 min 55°C, 0.75 min 72°C, 30 cycles), using appropriate primers (see below). One extra cycle was then performed (2 min 94°C, 1 min 55°C, 12 min 72°C) after the addition of 10 μ Ci of [α - 32 P]dATP, 50 pmol of each primer, and 2.0 U of Taq polymerase (21). The digestion products were electrophoresed through a 12% non-denaturing polyacrylamide gel, and the radioactive fragments quantitated by scanning the gel in a Betascope 603 Blot Analyzer (Betagen, Inc., Framingham, MA).

RFLP analysis of mtDNA mutations from purified DNA and single-muscle fibers. The following sets of primers and restriction enzymes were used. For the detection of the A \rightarrow G 3243 mutation: light-strand positions 3116–3134, and heavy-strand positions 3353–3333 (numbers according to reference 28), digestion with HaeIII (21). For the detection of the A \rightarrow G 8344 mutation: a mismatched primer was used in order to create a BanII site in combination with the A \rightarrow G transition at position 8344 ("mismatch PCR"; reference 15).

For the detection of the G \rightarrow A mutation at position 5703: primers corresponding to light-strand positions 5472–5492 and heavy-strand positions 5798–5776 were used to amplify a 326-bp fragment that was subsequently digested with DdeI (the 5703 mutation disrupts a DdeI

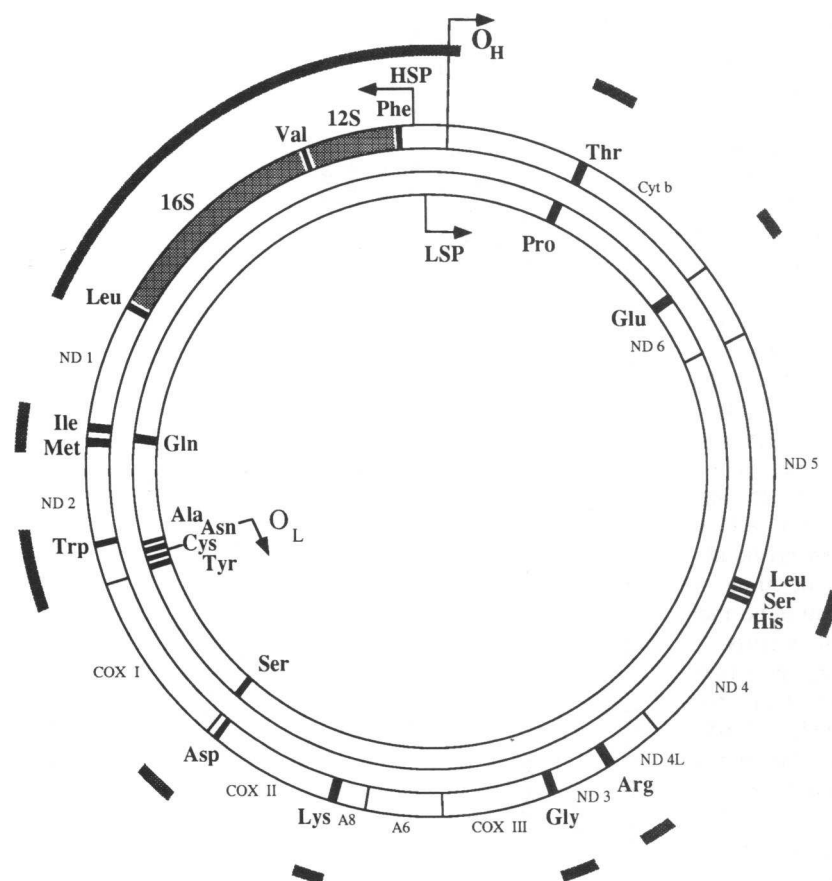


Figure 1. Sequence analysis of mtDNA regions associated with mitochondrial protein synthesis. Map of the 16.5-kb circular human mitochondrial genome showing the structural genes for the mitochondrial-encoded subunits of NADH-coenzyme Q oxidoreductase (ND), cytochrome *c* oxidase (COX), cytochrome *b* (Cyt *b*), and ATP synthase (A), the 12S and 16S ribosomal RNAs, and the 22 tRNAs (3 letter code). The outside arcs indicate regions that were PCR-amplified and sequenced from DNA extracted from muscle of the 10 patients. All tRNAs, rRNAs, promoters of transcription (HSP and LSP), origins of replication (O_H and O_L) and other regulatory *cis*-acting regions, thought to be involved in mitochondrial protein synthesis, were analyzed.

restriction site). Fragments amplified from mutated sequences digested with DdeI yielded 5 fragments of 110, 81, 62, 41, and 32-bp, while fragments amplified from wild-type sequences digested with DdeI yielded 6 fragments (the 110-bp fragment was digested into a 98 and a 12-bp fragment; on our electrophoresis conditions the smaller fragments ran off the polyacrylamide gel).

For the detection of the T → C mutation at nt-3256, a modified light-strand primer, corresponding to mtDNA positions 3230–3255 (5' GTTAAGATGGCAGCGCCCGTAAAGCG 3'), had the underlined modifications from the normal sequence in order to create two HinPI restriction sites (at positions 3243 and 3255) when the wild-type sequence was amplified. The modified primer was used in combination with a heavy-strand primer corresponding to positions 3353–3332. The amplified wild-type 123-bp fragment was digested by HinPI into 99, 13, and 11-bp fragments. Fragments originated from mutant sequences had only one HinPI site (at position 3243), yielding a 110- and a 13-bp fragment.

Single muscle fibers were isolated and subjected to PCR amplification as described (21). "Last-cycle hot PCR" and restriction fragment length polymorphism (RFLP) analysis were performed as described above.

Enzyme activities in muscle homogenates. Respiratory chain enzymes and citrate synthase activities were measured spectrophotometrically in 10% muscle homogenates as described (29).

Cytochemistry and immunocytochemistry. 8-μm-thick muscle biopsy sections were used for cytochemistry, and 4-μm-thick sections for immunocytochemistry. Staining for cytochrome c oxidase (COX), succinate dehydrogenase (SDH), and indirect immunofluorescence against NADH-dehydrogenase subunit I (ND1), COX subunit II (COX II), and COX subunit IV (COX IV) were performed as described (24).

Results

Sequence analysis of protein synthesis-related mtDNA regions. Preliminary mtDNA analysis showed that all 10 patients with RRF were negative for mtDNA point mutations at nt-3243, 8344, and 8993 or for large-scale mtDNA deletions that have been associated with mitochondrial disorders (30). To search for novel mtDNA alterations, we sequenced PCR products from all mtDNA regions directly related to mitochondrial protein synthesis (Fig. 1), including: the two origins of mtDNA replication, conserved sequence boxes possibly associated with the replication process, promoters of transcription, binding sites for mitochondrial transcription factor mtTF-1, rRNA genes, and tRNA genes (28, 31, 32). Partial sequences of protein-coding genes contiguous to these regions were also analyzed. Table I summarizes the differences observed between the patients' mtDNA sequences and the reference "Cambridge" mtDNA sequence (28). All 9 patients had 2 polymorphisms that are probably more common in the human population than are the corresponding "Cambridge" assignments: the first was an A → G at position 750, and the second an A → G at position 1438 (both alterations have been noted previously (33, 34). We also detected small insertions and deletions in noncoding regions, most of which were also present in normal individuals and were therefore considered neutral variants.

We excluded nonpathogenic single base change polymorphisms by establishing the following minimal criteria for pathogenicity: (a) the base change must be absent in unaffected individuals, and (b) the base change must be in an evolutionarily conserved region. These criteria sufficed to eliminate all but two point mutations, which were observed in patients 1 and 9.

Potentially pathogenic mtDNA mutations. Patient 1 had a large number of polymorphisms, due probably to her African

Table I. mtDNA Polymorphisms in Patients with Mitochondrial Disorders

Patients	1	2	3	4	5	6	7	8	9	10
Polymorphisms	G → A 185 ⁿ A → G 200 ⁿ G → A 247 ⁿ C ins. 311 ⁿ G → A 709 ^r G → A 769 ^r C → T 1048 ^r A → G 2245 ^r A → G 2758 ^r A → G 2951 ^r C → T 5603 ^r G → A 7521 ^r C → T 8468 ^{ps}	A → G 189 ⁿ T → C 236 ⁿ A → G 263 ⁿ CA del. 520 ⁿ A → G 663 ^r A → G 750 ^r A → G 1018 ^r A → G 1438 ^r A → G 2706 ^r T → C 2885 ^r C → T 4312 ^r G → A 5703 ^r C → T 8428 ^{ps} A → G 10398 ^r G → A 12007 ^{ps}	A → G 263 ⁿ C ins. 311 ⁿ C ins. 353 ⁿ A → G 750 ^r A → G 1438 ^r A → G 1438 ^r A → G 2706 ^r A → G 5656 ⁿ A → G 12308 ^r	A → G 263 ⁿ A → G 750 ^r A → G 1438 ^r 9bp ins. 8280 ⁿ T → C 14766 ^p	A → G 263 ⁿ C ins. 311 ⁿ A → G 750 ^r A → G 1438 ^r T → C 14766 ^p	A → G 263 ⁿ C ins. 311 ⁿ A → G 750 ^r A → G 1438 ^r C → T 2283 ^r G → A 7521 ^r T → C 14766 ^p	AA del. 290 ⁿ C ins. 311 ⁿ T → C 489 ⁿ A → G 493 ⁿ CA del. 520 ⁿ A → G 750 ^r A → G 1438 ^r A → G 2706 ^r C → T 4242 ^{ps} A → G 10398 ^p C → T 10400 ^{ps}	A → G 263 ⁿ C ins. 311 ⁿ T → C 471 ⁿ CA ins. 520 ⁿ A → G 750 ^r A → G 1438 ^r G → A 3010 ^r T → C 14766 ^p	A → G 263 ⁿ C ins. 303 ⁿ C ins. 311 ⁿ G → A 709 ^r A → G 750 ^r A → G 1438 ^r C → T 3256 ^r T → C 14766 ^p	A → G 263 ⁿ CC ins. 303 ⁿ C ins. 311 ⁿ A → G 750 ^r A → G 1438 ^r A → G 2706 ^r T → C 14766 ^p A → G 15954 ⁿ

The table lists the differences in nucleotide sequences between selected mtDNA regions from 10 patients with muscle mitochondrial proliferation and the reference "Cambridge" mtDNA sequence.²⁸ The nucleotide position (according to reference 28) is followed by a letter code indicating the functional location: n, non-coding region; r, rRNA gene; t, tRNA gene; p, protein coding gene; ps, protein coding gene (synonymous mutation); ins, insertion; del., deletion. Potentially pathogenic alterations are italicized.

origin (the reference mtDNA sequence is Caucasian [28]). Most of these changes were located in evolutionarily nonconserved regions and were considered neutral polymorphisms, including 3 mutations in tRNA genes (Table I). However, a G → A transition at mtDNA position 5703, located in the anticodon stem of the tRNA^{Asn} gene (Fig. 2), fulfilled the initial requirements for a pathogenic mutation. The nt-5703 mutation disrupts the first base pair of the anticodon stem, a secondary structure highly conserved throughout evolution (Fig. 3). This transition also disrupts a DdeI restriction endonuclease site, allowing for easy detection of the mutation by RFLP analysis of PCR fragments. RFLP analysis of DdeI recognition sites has been used extensively in establishing the origins of human populations. From a total of 813 individuals examined, 64 of which were of African ancestry, there were no reports of a DdeI polymorphic site at position 5703 (35–40). We have analyzed 57 additional patients with mitochondrial disease and none possessed the nt-5703 alteration seen in the muscle of patient 1.

Patient 9 had a C → T transition at position 3256 within the mitochondrial tRNA^{Leu(UUR)} gene (Fig. 2). This alteration was not observed in 44 other patients nor in 20 controls. The C

at position 3256 pairs with a G in the D-stem; both nucleotides are highly conserved throughout evolution (Fig. 3).

None of the two tRNA gene alterations described above were described in previous reports, which included 19 additional individuals (33, 34). Once the initial criteria for pathogenicity were fulfilled for these two mtDNA mutations, we sought direct evidence for their pathogenetic role.

Mutant mtDNAs are heteroplasmic, are present in high percentages in clinically affected tissues, and are undetectable in healthy maternal relatives. We analyzed mtDNA from muscle, white blood cells, and fibroblasts from patient 1. RFLP analysis of PCR fragments encompassing the tRNA^{Asn} gene digested with DdeI showed that the mutant mtDNA was heteroplasmic in all three tissues, and represented 69% (muscle), 4% (white blood cells), and 6% (first passage cultured fibroblasts) of total mtDNA (Fig. 4). We were unable to detect a population of mutant mtDNA in white blood cells from the healthy mother or from two maternal aunts of patient 1 (Fig. 4). These data are in agreement with the clinical observation that patient 1 had an isolated myopathy and no family history of neuromuscular disorders.

PCR-RFLP analysis using HinPI showed that mutant mtDNA was also heteroplasmic in patient 9, accounting for 64% (muscle), 8% (white blood cells), 48% (first passage cultured fibroblasts), and 18% (hair roots) of total mtDNA. The only maternal relative available was a healthy sister. We did not detect a mutant population in her white blood cells (Fig. 4).

Mitochondrial tRNA mutations cause different degrees of impairment in respiratory chain function and in mitochondrial protein synthesis. The activities of the respiratory complexes were measured in muscle homogenates from patients 1 and 9. The activities of complexes containing mtDNA-encoded subunits were slightly decreased when referred to muscle weight. However, these activities became significantly lower when corrected for abnormal mitochondrial proliferation, i.e., after normalizing respiratory complexes activities to the activity of citrate synthase (CS), a mitochondrial matrix enzyme which is a good index of mitochondrial volume (29; Fig. 5). All respiratory complexes (with the exception of complex II, which is encoded exclusively by nuclear genes) showed decreased activities in muscle homogenates from both patients, but more markedly in patient 1.

Cytochemical staining of muscle sections showed that both patients had a mosaic pattern of mitochondrial abnormalities at the cellular level (Fig. 6). Both patients had fibers with abnormal mitochondrial proliferation (RRF), as identified by intense staining for complex II (succinate dehydrogenase, or SDH; fibers marked "R" Fig. 6). However, whereas RRF lacked complex IV (cytochrome c oxidase, or COX) activity in patient 1, most RRF of patient 9 retained COX activity, either at normal or only partially reduced levels.

Immunodetection of two mitochondrial-encoded polypeptides (subunit I of NADH-ubiquinone oxidoreductase, or ND 1, and subunit II of COX, or COX II) and one nuclear-encoded polypeptide (subunit IV of COX, or COX IV) showed a pattern compatible with the cytochemical observations. In patient 1, RRF lacked ND 1 and COX II, while COX IV immunostaining was increased (Fig. 6). On the other hand, RRF from patient 9 had cross-reacting material for all three polypeptides. The nonquantitative nature of the immunocytochemical stainings does not allow the detection of partial decreases in protein levels in fibers with extensive mitochondrial proliferation.

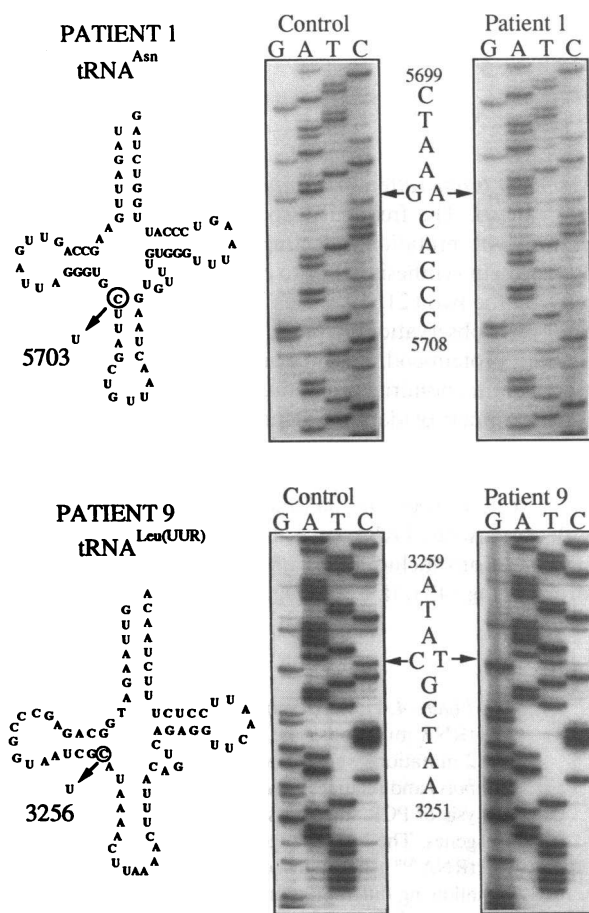


Figure 2. Detection of potentially pathogenic mtDNA mutations. The figure shows autoradiographs of sequencing ladders obtained from double-stranded PCR templates of control and patients. Two base changes with a potential pathogenic role observed in patients 1 and 9 are shown. Numbers refer to positions in the human mtDNA²⁸. The location of the point mutations in mature tRNA genes (circled nucleotides) are also illustrated.

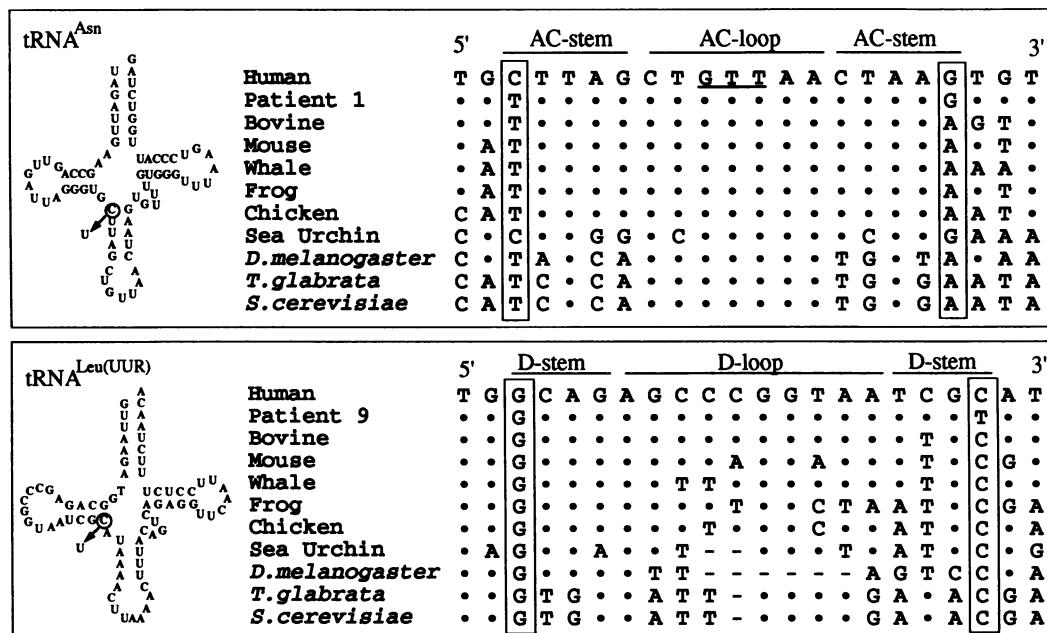


Figure 3. Location of tRNA mutations in primary or secondary tRNA structures. Evolutionary comparison of tRNA structures are shown. Relevant mitochondrial tRNA gene sequences from patients and other species are listed below the normal human sequence. Nucleotides in unboxed regions that do not differ from the human sequence are shown as dots (mtDNA sequences from various species were obtained from reference 47). The base change observed in patient 1 within the tRNA^{Asn} gene (top panel) disrupts the first base pair of the anticodon stem (boxed). The base change

observed in patient 9 within the tRNA^{Leu(UUR)} (bottom panel) disrupts not only a highly conserved secondary structure in the first base pair of the D-stem (boxed), but also a conserved primary structure.

Mutant mtDNAs are essentially homoplasmic in affected muscle cells. We used PCR amplification and RFLP analysis of mtDNA from individually microdissected muscle fibers in order to correlate the morphological observations with the relative levels of mutant and wild-type mitochondrial genomes. COX activity-negative (COX⁻) RRF from patient 1 had extremely high levels of mutant mtDNA (range 97–100%, Fig. 7), whereas normal muscle fibers (COX activity-positive, or COX⁺) had significantly lower levels (range 3–63%, Fig. 7). The proportion of mutant mtDNA observed in whole muscle was 69% (Fig. 7). As opposed to patient 1, RRF from patient 9 had only partially impaired COX activity. However, when analyzed for levels of mutant genomes, RRF were essentially homoplasmic for mutant mtDNAs (range 94–97%, Fig. 7). Normal, non-RRF had much lower relative levels of mutant genomes (34–75%). The percentage of mutant mtDNA in total muscle was 64%.

Discussion

Mitochondrial proliferation and impairment of mitochondrial protein synthesis. The frequent association of mitochondrial proliferation with mutations affecting genes involved in mitochondrial protein synthesis led us to propose a causal relationship between the two (21, 22). This hypothesis was bolstered by the converse observation; namely, that pathogenic mtDNA mutations in protein-coding genes are not usually associated with massive mitochondrial proliferation, even though they are also likely to impair oxidative phosphorylation and ATP production.

Muscle fibers with abnormal mitochondrial proliferation or RRF have long been the hallmark of mitochondrial disorders. Because isolated RRF have also been reported in many different conditions including inflammatory myopathies (41) and normal aging (42), it was thought to be a nonspecific ab-

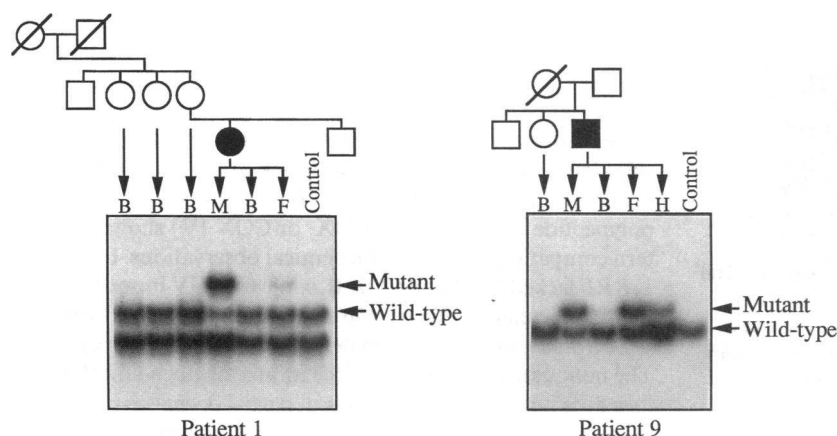


Figure 4. Heteroplasmy and sporadic nature of tRNA mutations. The heteroplasmic nature of the 2 mutations were studied in patients (filled symbols) and relatives (open symbols) by RFLP analysis of PCR fragments encompassing affected genes. The base change in patient 1, within the tRNA^{Asn} gene, abolished a DdeI restriction site, allowing differentiation of the mutant and wild-type mtDNAs. The mutation in patient 9 did not alter natural restriction sites, and a modified primer was used to create restriction sites for the enzyme HinPI, that could distinguish mutant and wild-type mtDNAs (see Methods for details). The following tissues were studied: skeletal muscle (M); white blood cells (B); fibroblasts (F); and hair roots (H).

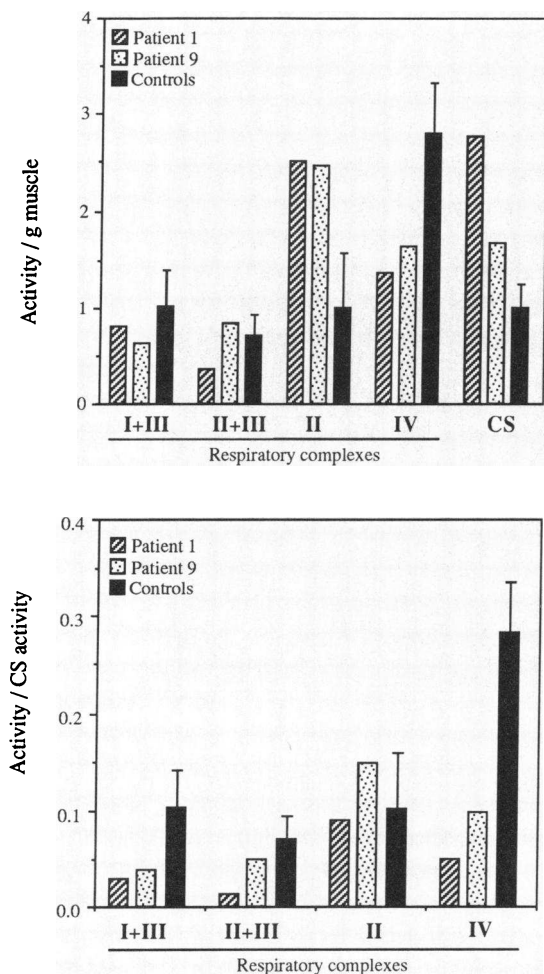


Figure 5. Mitochondrial enzyme activities in muscle homogenates. Muscle homogenates from patients 1 and 9 were used to measure respiratory chain complexes and citrate synthase (CS) activities. The upper panel shows enzyme activities (nmol product formed/min per gram of tissue) expressed as a function of tissue wet weight. The lower panel shows the activity of respiratory complexes normalized to CS. This correction gives a better estimate of enzyme activity per organelle since both patients had abnormal mitochondrial proliferation. The CS-corrected activities of complexes I + III and II + III observed in the two patients (with the exception of the activity of complex II + III in patient 9) were below 2 SD of the control group ($n = 50$). Both patients had complex IV (COX) activity below 3 SD of the control group. Error bars, 1 SD.

normality. However, low levels of mtDNA deletions have now been shown in both latter conditions (43, 44), suggesting that RRF may be specifically associated with a respiratory chain malfunction.

We screened 10 patients with abundant RRF for novel mtDNA mutations that might impair mitochondrial protein synthesis. Only two patients were found to have pathogenic mtDNA mutations, both in tRNA genes. We cannot exclude the possibility that one or more of the other polymorphisms observed could have contributed (possibly in a synergistic way) to the pathogenesis of the other eight patients, but more detailed studies are needed to clarify this issue. Alternatively, nuclear mutations affecting mitochondrial protein synthesis

could be responsible for some of these conditions, though examples of this pathogenic mechanism do not exist. Finally, it would be premature at this point to rule out that mtDNA mutations in protein-coding genes might also cause massive mitochondrial proliferation. This caution is warranted by the mitochondrial proliferation observed in a few cases of LHON (26) and in one patient with MELAS possibly associated with a missense mutation at position 11084 within the mitochondrial ND4 gene (25). It is also possible that mitochondrial proliferation is triggered only when oxidative phosphorylation is severely impaired. Although a defect in mitochondrial protein synthesis is likely to cause such a severe malfunction, certain mutations in structural genes might also have a similar consequence.

Origin and tissue distribution of mutant mtDNAs. The mtDNA mutation in patient 1 (G → A at position 5703) was not found in white blood cells from either the mother or two maternal aunts. However, because the patient herself had very low levels of mutant mtDNA in blood, the mutation may have arisen in maternal germline cells harboring a low proportion of mutant mtDNAs. We found the mutation in muscle, blood, and fibroblasts, indicating that it arose before or during early embryogenesis.

The mtDNA mutation in patient 9 (C → T at position 3256) was associated with a more generalized disorder, and there were higher levels of mutant mtDNA in blood and fibroblasts than in patient 1. We could not study this patient's mother, who had died without evidence of neuromuscular disease. However, white blood cells of an asymptomatic sister did not have the mutation.

The low levels of mutant mtDNA in white blood cells of these two patients contrasts with findings in patients with the MELAS-3243 or the MERRF-8344 point mutations (45). In those cases, white blood cells had high levels of mutant mtDNA and noninvasive molecular diagnosis was possible. However, blood tests may not be diagnostic in all cases. This concept has been previously illustrated by the complete absence of mutant mtDNA (a point mutation in the anticodon region of the tRNA^{Pro} gene; reference 22) in white blood cells of a young girl with a myopathy and high levels of mutant mtDNA in muscle.

The mitochondrial tRNA^{Leu(UUR)} gene appears to be an etiologic hot spot for mtDNA mutations. Of 16 pathogenic mtDNA point mutations in tRNA genes reported to date, including the two reported here, 9 are within the tRNA^{Leu(UUR)} gene (56%; Fig. 8). They are located at positions: 3243 (MELAS, PEO; reference 6); 3250 (myopathy; reference 9); 3251 (myopathy; reference 9a); 3252 (MELAS; reference 11); 3256 (MERRF/PEO; this work); 3260 (myopathy and cardiomyopathy; reference 8); 3271 (MELAS; reference 7); 3302 (myopathy; reference 10); and 3303 (myopathy and cardiomyopathy; reference 12). Moreover, the A → G transition at nt-3243 is probably the most common pathogenic mtDNA point mutation reported to date (Moraes, C. T., and S. DiMauro, unpublished observations), including the ones in protein-coding genes. The reason for this bias is not clear, but may be related to the heavy "transcriptional traffic" in this mtDNA region. The rate of transcription of mammalian mitochondrial rRNA genes is approximately 20-fold higher than that of other genes transcribed downstream from the heavy-strand promoter region (32). This difference has been attributed to a termination factor that binds downstream of the rRNA genes, within the

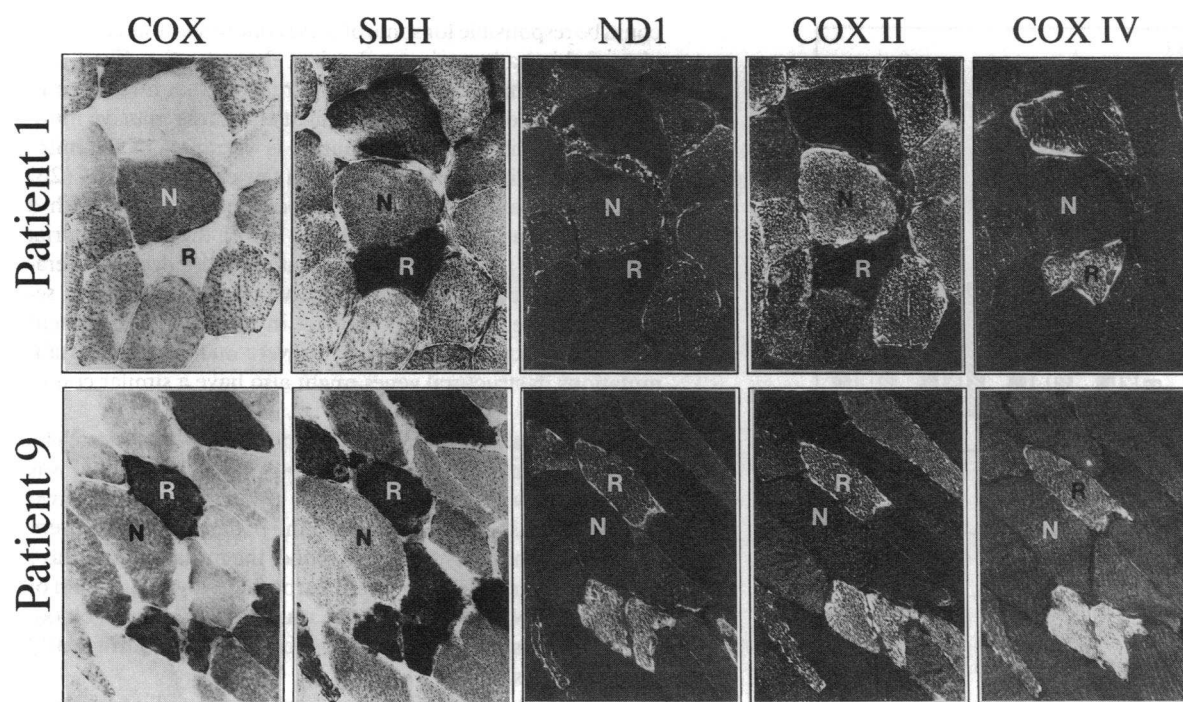


Figure 6. Correlations at the cellular level between enzyme activities and the presence of immunodetectable polypeptides. Serial muscle sections (8 μ m) from patients 1 and 9 were stained for cytochrome *c* oxidase (COX) and succinate dehydrogenase (SDH) enzyme activities, and immunostained for two mitochondrial-encoded proteins (ND 1 and COX II) and for a nuclear-encoded mitochondrial protein (COX IV). Representative RRFs are marked with "R" and normal fibers with "N". Note the severe reduction of mtDNA-encoded polypeptides in RRF from patient 1. RRF from patient 9 had a milder COX deficiency, and a milder decrease in crossreacting material after immunostaining with antibodies directed against mtDNA-encoded polypeptides.

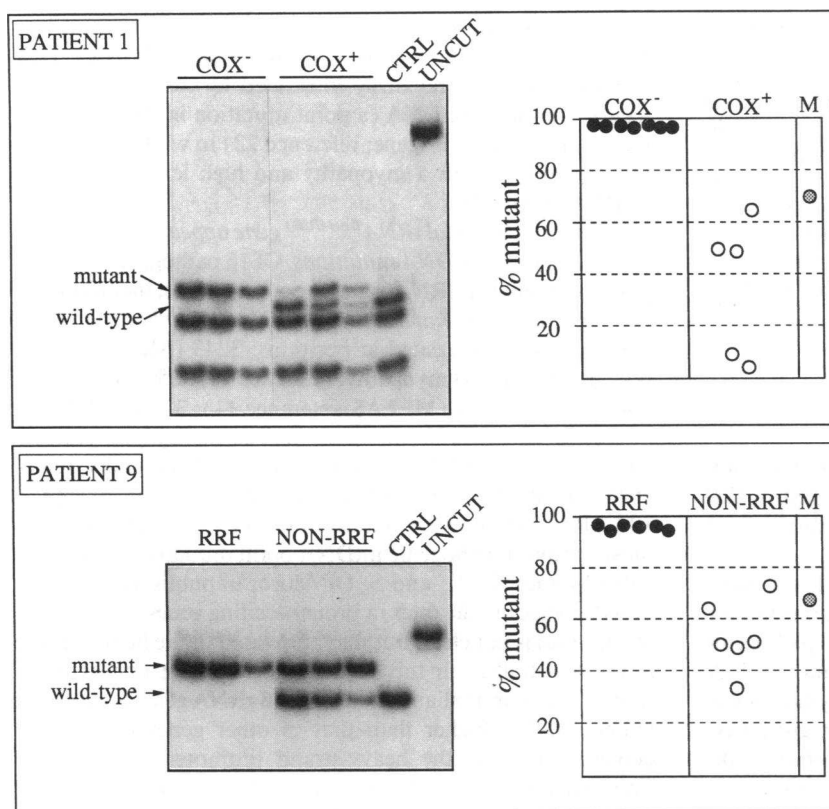


Figure 7. Mutant mtDNA levels in affected and normal muscle fibers. Muscle fibers from patients 1 and 9 were microdissected and selected regions of their mtDNA directly amplified by PCR (see Methods). PCR fragments were analyzed by RFLP after digestion with DdeI (patient 1), or HinPI (patient 9). Representative autoradiograms of non-denaturing polyacrylamide gels are shown in the left portion of each panel. COX⁻, COX-activity-negative (affected) fibers; COX⁺, COX-activity-positive (normal) fibers; RRF, ragged-red (affected) fibers; NON-RRF, weak SDH-staining (i.e., normal) fibers; CTRL, control fiber; UNCUT, PCR fragment not digested with restriction enzyme. The total determinations of % mutant mtDNA in affected (black circles) and normal (white circles) fibers are plotted on the right side of each panel. The levels of mutant mtDNA observed in total muscle DNA are also shown (M, shaded circles).

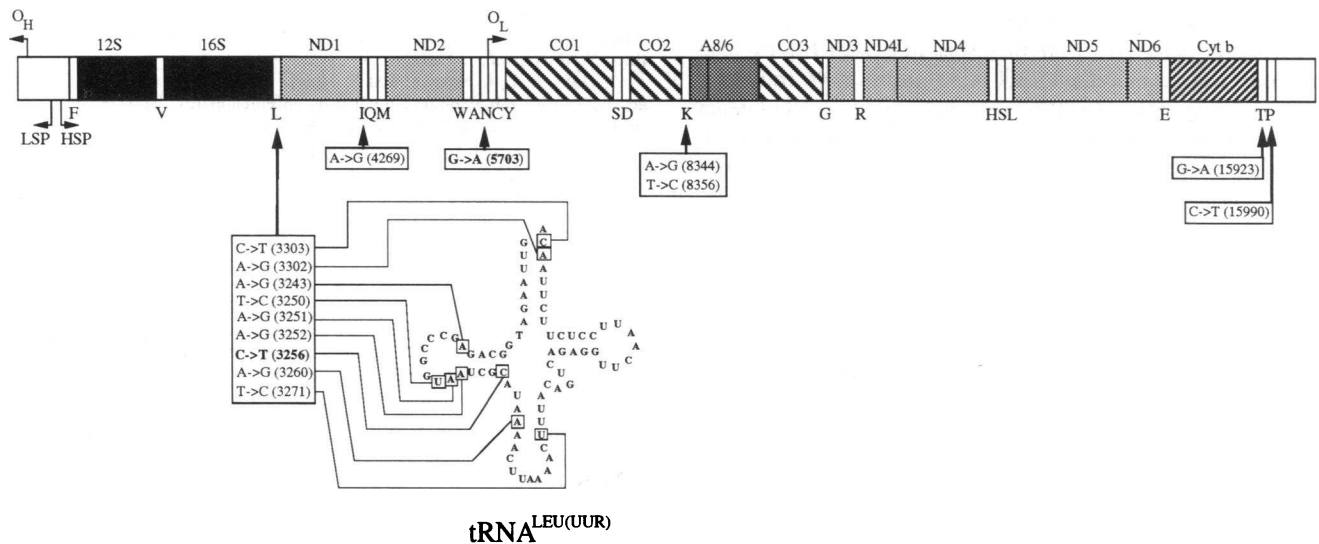


Figure 8. Pathogenic point mutations in human mitochondrial tRNA genes. The figure shows a linearized representation of the human mtDNA. Point mutations in tRNA genes (one letter aminoacid code) with strong evidence for pathogenicity are indicated. Mutations in tRNAs isoleucine and threonine were described in references 56 and 57, respectively. Note the relatively high incidence of point mutations in the tRNA^{Leu(UUR)} gene. Abbreviations for structural genes as in legend to Fig. 1.

tRNA^{Leu(UUR)} gene (46). Replication of the mtDNA heavy-strand proceeds in a direction opposite to that of its transcription (31), and may be disturbed by the high level of transcriptional activity. Furthermore, the binding of the termination factor to the tRNA^{Leu(UUR)} gene may interfere with the proof-reading activity of DNA polymerase γ , allowing misincorporated nucleotides to be fixed during mtDNA replication.

Cytochemical and immunocytochemical features of patient 9's skeletal muscle (harboring the 3256 mutation) are similar to the morphological features observed in association with the 3243 (21), 3271 (47), and 3250 (9) mutations in the tRNA^{Leu(UUR)} gene, that is, most RRF have normal or only mildly decreased COX activity and cross-reacting material in immunostaining using antibodies against mitochondrially encoded polypeptides. As discussed below, these similarities may not be coincidental and suggest that different mutations in the tRNA^{Leu(UUR)} may share a similar pathogenetic mechanism.

Pathogenesis of mitochondrial tRNA mutations. The two novel tRNA mutations described here act in a recessive manner, that is, 5–10% normal mtDNA seems to be sufficient to compensate for the deleterious effect of the mutant population. These "protective" wild-type percentages are similar to those described for the 3243-tRNA^{Leu(UUR)} mutation in MELAS (18, 21), the 8344-tRNA^{Lys} mutation in MERRF (19, 20), and the nt-15990 mutation in tRNA^{Pro} in a patient with isolated limb myopathy (22). Based on this protective effect, which is likely due to complementation between wild-type and mutant genomes, it seems reasonable to assume that mutant and wild-type mtDNA sequences coexist within the same organelle (intramitochondrial heteroplasmy).

The exact cause of the defect in mitochondrial function associated with these two novel mutations is not known. Because mitochondrial tRNAs also act as signals for processing of polycistronic primary transcripts (32), base changes that alter secondary structures may disturb processing. Other possible pathogenic mechanisms are discussed below for each mutation.

The nt-5703 mutation in tRNA^{Asn} causes severe focal COX deficiency and mitochondrial protein synthesis impairment. Because the mutation disrupts a highly conserved base pair at the "base" of the anticodon stem, it may influence the stability of the tRNA secondary structure. A 5-bp anticodon-stem is a common feature of most tRNAs (47), and the first base pair (nucleotides 27 and 43 in the tRNA nomenclature; reference 47) is particularly conserved. Experiments *in vivo* with *E. coli* tRNA^{Phe} suggested that the 27–43 base pair may contribute to tRNA^{Phe} identity (48). There is no information available on tRNA^{Asn} identity determinants, nuclear or mitochondrial, in any species.

The effect on protein synthesis of the nt-3256 mutation in the tRNA^{Leu(UUR)} gene could not be demonstrated unequivocally, because of the milder COX deficiency and the presence of cross-reacting ND 1 and COX II polypeptides in RRF of patient 9. Spectrophotometric assays for respiratory chain enzymes, however, showed a significant decrease in complex I, III and IV activities, though less severe than in patient 1. Base-pair disruptions in the D-stem of yeast tRNA^{Asp} (the same region of the 3256 mutation in the mitochondrial tRNA^{Leu(UUR)}) had several deleterious effects on tRNA maturation, including decreased aminoacylation (49) and decreased levels of physiologically normal chemical modifications (e.g., methylation or pseudouridine formation) in specific nucleotides (50).

The nt-3243 mutation, in the same tRNA^{Leu(UUR)} gene, has been studied in great detail and appears to be associated with a partial defect in mitochondrial protein synthesis. The mechanisms leading to this deficiency are also obscure. Studies *in vitro* showed that the nt-3243 mutation impairs binding of a transcription termination factor to mtDNA, potentially leading to unbalanced ratios of mitochondrial transcripts (18, 51). These *in vitro* observations however, were not confirmed *in vivo*, either in cultured cells (17, 18) or in muscle from patients harboring the mutation (21, 47, 52). The nt-3256 mutation resides within the last base footprinted by the mitochondrial termination factor (46), suggesting that this alteration should

not disturb protein binding significantly. The only molecular abnormality detected so far in association with the nt-3243 mutation was an increased level of an unprocessed transcript corresponding to the 16S rRNA + tRNA^{Leu(UUR)} + ND1 genes (termed RNA 19 in reference 17). This RNA processing impairment has been hypothesized to play a role in the observed partial translational defect (17, 53). High levels of this transcript have also been observed in a patient with the nt-3302 mutation (10). Detailed molecular studies of the nt-3256 mutation, as well as of other point mutations in tRNA^{Leu(UUR)}, may reveal similar processing abnormalities.

Genotype-phenotype correlations. Although particular mtDNA mutations are usually associated with specific phenotypes (e.g., deletions with PEO and Kearns-Sayre syndrome, the A → G transition at nt-8344 with MERRF, and the A → G transition at nt-3243 with MELAS), overlaps and atypical presentations are not rare and relatives of affected patients may have few or no symptoms. This variability is well exemplified by the A → G transition at nt-3243. This mutation has been associated with MELAS (6), PEO (6, 54), inherited diabetes and hearing loss (55), and other combinations of signs and symptoms commonly associated with mitochondrial disorders (e.g., retinopathy or seizures; reference 54).

Patient 1, with a mutation in the tRNA^{Asn} gene, had morphological (COX-negative RRF) and clinical features (PEO) resembling PEO patients with mtDNA deletions or with the nt-3243 mutation. Patient 9, with a mutation in the tRNA^{Leu(UUR)} gene, had an unusual clinical presentation with features of MERRF (myoclonus, epilepsy, and ataxia), PEO, and Leber's optic neuropathy. Collectively, the clinical features of patient 9 are distinct from the previously defined mitochondrial encephalomyopathies, and might represent a new phenotype. The basis for this clinical variability is not understood, but, besides the site of the mutation, tissue and cellular distribution of mutant and wild-type mtDNAs might influence the clinical phenotype.

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