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# Maternally Inherited Cardiomyopathy and Hearing Loss Associated with a Novel Mutation in the Mitochondrial tRNA<sup>Lys</sup> Gene (G8363A)

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## Summary

A novel G8363A mutation in the mtDNA tRNA<sup>Lys</sup> gene was associated, in two unrelated families, with a syndrome consisting of encephalomyopathy, sensorineural hearing loss, and hypertrophic cardiomyopathy. Muscle biopsies from the probands showed mitochondrial proliferation and partial defects of complexes I, III, and IV of the electron-transport chain. The G8363A mutation was very abundant (>95%) in muscle samples from the probands and was less copious in blood from 18 maternal relatives (mean 81.3% ± 8.5%). Single-muscle-fiber analysis showed significantly higher levels of mutant genomes in cytochrome *c* oxidase–negative fibers than in cytochrome *c* oxidase–positive fibers. The mutation was not found in >200 individuals, including normal controls and patients with other mitochondrial encephalomyopathies, thus fulfilling accepted criteria for pathogenicity.

## Introduction

Mitochondrial encephalomyopathies are a heterogeneous group of disorders that differ widely in their clinical features but have in common functional—and, less consistently, morphological—abnormalities of muscle mitochondria. Genetic analysis has revealed numerous pathogenic mtDNA mutations during the past 7 years, including large-scale rearrangements and >30 point mutations in tRNA or protein-coding genes (Schon et al. 1994).

Despite this remarkable progress, many patients with mtDNA mutations probably remain undiagnosed. Multisystem clinical involvement, maternal inheritance,

and “soft” signs in maternal relatives, such as short stature, diabetes, or migrainelike headache, are useful clues for recognizing diseases associated with mtDNA defects (Wallace 1992; DiMauro and Moraes 1993). In muscle biopsies, morphological evidence of mitochondrial proliferation (i.e., ragged red fibers [RRF]), often associated with scattered cytochrome *c* oxidase–negative (COX<sup>−</sup>) fibers, or combined biochemical defects of respiratory-chain complexes may further suggest that the mutations affect regions directly involved in mtDNA protein synthesis, such as the 22 tRNA genes (Moraes et al. 1993).

We have used SSCP analysis and direct DNA sequencing of all 22 tRNA genes to detect putative mtDNA mutations in nine patients who had clinical, biochemical, or morphological features of multiorgan involvement, affecting especially tissues that depend heavily on oxidative metabolism, such as brain and heart. Here, we describe two unrelated families with similar clinical presentations, characterized by maternally inherited cardiomyopathy (MICM) and hearing loss in multiple family members. Sequence analysis of mtDNA showed a novel G8363A transition in the tRNA<sup>Lys</sup> gene.

## Patients, Material, and Methods

### Family A

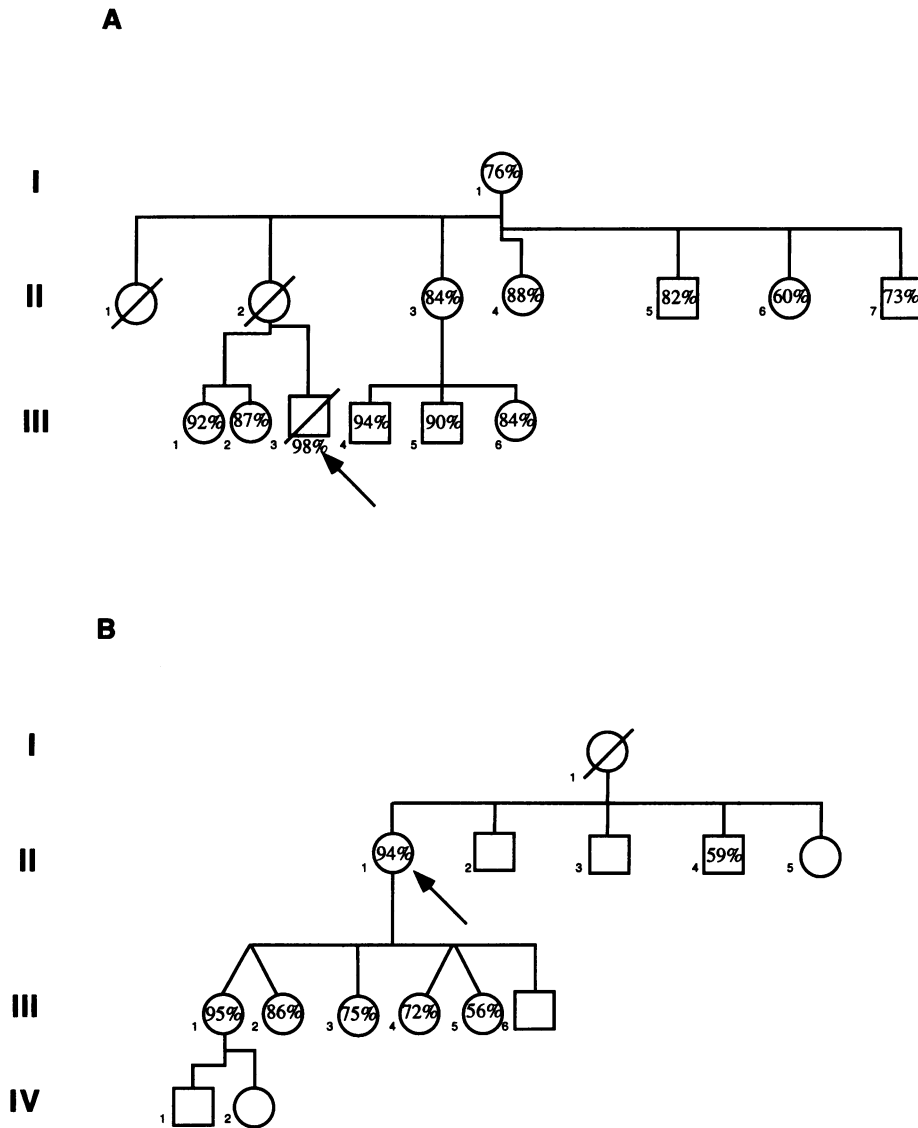
*Case III-3.*—Case III-3, the proband in family A (fig. 1A), was a Hispanic 16-year-old man who developed normally until age 8 years, when he presented with heart failure and cognitive regression. Cardiologic examination was compatible with dilated cardiomyopathy, and neurologic examination showed sensorineural hearing loss, ophthalmoparesis, ataxia, and muscle weakness. His clinical course worsened progressively, and he died at age 17 years, of cardiorespiratory arrest. Autopsy was not performed. Family history was remarkable for several maternal relatives who suffered from similar symptoms, as described below.

*Case I-1.*—Case I-1, the maternal grandmother, is in her 70s. Clinical examination revealed sensorineural hearing loss, mild truncal ataxia, and sensory loss suggestive of peripheral neuropathy.

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**Figure 1** Pedigrees of family A (panel A) and family B (panel B). Numbers are percentages of mutated genomes in blood samples in all subjects except the probands, in whom they represent means of muscle and blood mutant mtDNAs.

*Case II-1.*—Case II-1 was the proband's maternal aunt. She became symptomatic at age 7 years, with unspecified progressive heart complaints, and died suddenly at age 14 years.

*Case II-2.*—Case II-2 was the proband's mother. Cardiac complaints dated to her 20s, when she first noted exertional dyspnea and premature fatigue. At that time, she had increased levels of blood lactate and pyruvate. A muscle biopsy did not show RRF, but electron microscopy showed abnormal mitochondria with paracrystalline inclusions. She died at age 33 years, of cardiac failure.

*Case II-3.*—Case II-3, a 40-year-old woman, is asymptomatic.

*Case II-4.*—Case II-4, is a woman in her late 30s, in

whom clinical examination revealed sensorineural hearing loss and mild cerebellar ataxia.

*Case II-7.*—Case II-7, the proband's uncle, is in his 20s and has recently started complaining of hearing problems. He has not yet received full neurological or cardiological evaluation.

*Case III-1.*—Case III-1, the proband's half-sister, is a 14-year-old girl with progressive external ophthalmoparesis (PEO), sensory peripheral neuropathy, and unspecified "cardiac problems."

*Case III-2.*—Case III-2 is a 15-year-old girl with PEO and shortness of breath after mild physical exertion. She has not yet received complete cardiological evaluation.

*Case III-4.*—Case III-4 is a 5-year-old boy who developed normally until age 4 years, when weakness and

toe walking started. Neurological examination showed weakness of the pelvic girdle, hyperreflexia, ataxia, and mild developmental delay. Echocardiogram revealed hypertrophic cardiomyopathy. Ultrastructural examination of muscle showed both an increased number of mitochondria under the sarcolemma and increased lipid droplets in some muscle fibers.

*Case III-5.*—Case III-5 is mildly mentally retarded

*Case III-6.*—Case III-6 is apparently healthy.

#### Family B

*Case II-1.*—Case II-1, the 44-year-old African American proposita (fig. 1B), was healthy until age 35 years, when she first noted progressive hearing loss. At age 37 years she developed slurred speech, gait difficulties with leg pain and heaviness, and shortness of breath after mild exercise. Physical examination showed “horse collar” lipomas and increased fat tissue distributed in folds on the lateral aspects of the chest and abdomen. The thighs appeared thin, but the legs showed mild bilateral calf hypertrophy. Neurological examination revealed bilateral sensorineural hearing loss, mild proximal limb weakness, predominantly in the legs, and slight dysmetria. Neither seizures nor myoclonus was noted. Cardiac complaints dated to her 30s, but more recently she has complained of frequent chest pain, with mildly elevated blood pressure. Cardiologist examination, electrocardiogram, and echocardiogram were suggestive of cardiomyopathy. A thallium scintigraphy done in both vertical and horizontal long-axis and short-axis modes showed an irregular thallium uptake consistent with left-ventricular hypertrophy, involving especially the free wall. Serum creatine kinase (CK) levels were eight times normal, with a CK-MB fraction of 3.8%, consistent with myocardial involvement. Venous lactate was elevated (2.5 mM; normal <1.8 mM) and increased excessively after exercise (to four times normal). Electromyography showed myopathic features. Muscle biopsy revealed several RRF and COX<sup>-</sup> fibers, and electron microscopy showed an increased number of mitochondria under the sarcolemma and between myofibrils with frequent paracrystalline inclusions.

*Case I-1.*—Case I-1, the proband’s mother, had died at age 62 years, because of “intestinal pseudo-obstruction.” Past medical history had been remarkable for unspecified myocardial problems, gait difficulties, and hearing loss by age 50 years. The proposita’s brothers (II-2, II-3, and II-4) are asymptomatic, but the youngest sister (II-5) has a “large heart.”

*Case III-1.*—Case III-1, the eldest daughter of this family’s proband, developed seizures during a febrile episode at age 5 years. Around that time, she experienced left foot drop, with frequent falls, when she attempted to run. In subsequent years her symptoms worsened. At age 24 years, she complains of slurred speech, gait

instability, exercise intolerance, and myalgia during walking or climbing stairs. Blood venous lactate was mildly elevated. Psychological evaluation showed borderline mental retardation. Her 3-year-old son (IV-1) has a history of failure to thrive, developmental delay, congenital heart disease, and mental retardation. He recently started complaining of hearing impairment.

*Case III-2.*—Case III-2, the twin sister of III-1, has severe mental retardation and congenital hypertrophic cardiomyopathy. Blood venous lactate is 2.7 mM/liter, and serum CK levels are three times normal (MB fraction 2.8%, consistent with myocardial involvement). A recent audiometry showed severe neurosensory hearing loss.

*Case III-3.*—Case III-3 has hyperthyroidism.

*Case III-4.*—Case III-4 is mentally retarded, and parents report “abnormal behavior.”

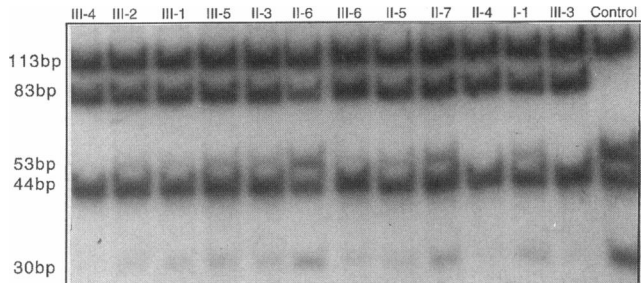
*Case III-5.*—Case III-5, the twin sister of III-4, is apparently healthy

#### Material and Methods

Respiratory-chain enzymes and citrate synthase activities were measured spectrophotometrically in 10% muscle extracts, as described elsewhere (DiMauro et al. 1987). Suitable RFLP and Southern blot analyses were used to rule out the presence of known pathogenic mtDNA mutations and large-scale rearrangements (Zeviani et al. 1988; Ciafaloni et al. 1992; Silvestri et al. 1993a; Santorelli et al. 1994). In particular, we excluded the typical mtDNA mutations associated with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes); MERRF (myoclonus epilepsy with RRF); maternally inherited Leigh syndrome; neurogenic muscle weakness, ataxia, and retinitis pigmentosa; and Leber hereditary optic neuropathy (Schon et al. 1994).

We used a combination of PCR-SSCP (Suomalainen et al. 1992) and direct DNA sequencing of the 22 tRNA genes to search for new mutations. Approximately 200 ng of total DNA were amplified by PCR (1 min at 94°C, 1 min at 55°C, 1 min at 72°C, for 25 cycles), and a last “hot-PCR cycle” was performed in the presence of [ $\alpha$ -<sup>32</sup>P] dATP. Oligonucleotide primers were as described elsewhere (Moraes et al. 1993). PCR products were boiled for 2 min, loaded onto a 0.5 × MDE 1% glycerol gel, and electrophoresed for 14 h at room temperature. The gel was dried and subjected to autoradiography for 18 h at room temperature. Direct sequencing of abnormal fragments, as well as of the two mtDNA-encoded ATPase genes (ATPase 6 and 8), was performed by use of the “ds DNA cycle sequencing kit” (Gibco BRL).

We employed a mispaired PCR method to screen for the novel G8363A mutation. A mismatched (5′-3′) 8341–8361 forward primer (mismatched G at np 8360) was used in combination with a backward primer (5′-3′) 8582–8561 to PCR amplify a 240-bp fragment,



**Figure 2** RFLP analysis of the G8363A mutation in members of family A. Subjects are as in fig. 1A. A 240-bp PCR-amplified fragment is normally cut by the endonuclease *HphI* (GGTGAN8/N7) into four fragments, of 113, 53, 44, and 30 bp. The G8363A mutation abolishes a site of cut, reducing the number of fragments to three (113, 83, and 44 bp).

which was later cleaved with the restriction enzyme *HphI* (GGTGA8/7). While the normal sequence yields four fragments, the presence of the mutant A8363 abolishes a site of cut, thus reducing the number of fragments to three (fig. 2).

To estimate the percentage of mutant mtDNAs, we added [ $\alpha$ - $^{32}$ P]dATP only in the last PCR cycle and electrophoresed the digestion products through a 12% non-denaturing polyacrylamide gel. Fragment intensities were measured by use of a Betascope 603 blot analyzer (Betagen).

Single muscle fibers were isolated and subjected to PCR amplification as described elsewhere (Moraes et al. 1992). RFLP analysis was performed as described above.

Previously described methods (Masucci et al. 1995) were employed for total RNA extraction from muscle and skin fibroblasts in subject III-4 from family A, RNA separation in 1.4% agarose gels, blotting onto nylon membrane, and hybridization to [ $\alpha$ - $^{32}$ P]dATP-labeled probes prepared by PCR for ND1, ND5, COX subunits I and II, and A 8/6 (ATP synthase subunits 8 and 6). Either an 18S rRNA or a  $\beta$ -actin probe was used to normalize the amount of RNA blotted for each sample.

## Results

Biochemical studies were performed in muscle extracts from the two probands and from case III-4 in family A. The activities of electron transport-chain complexes containing mtDNA-encoded subunits were decreased in all three specimens, suggesting a general impairment of mitochondrial protein synthesis (table 1).

Having ruled out large-scale rearrangements and the most common pathogenic point mutations in mtDNA, we used a combination of SSCP and direct sequencing to search for novel mtDNA mutations in the 22 tRNAs. We found a new tRNA<sup>Lys</sup> mutation, a G8363A substitu-

tion, in both families (fig. 3). RFLP analysis showed that the mutation was very abundant (>95%) in muscle from the probands and in muscle and skin fibroblasts from a severely affected maternal cousin but that it was also present, in lesser amounts (mean  $81.3 \pm 8.5\%$ , range 59%–94%), in blood from 17 maternal relatives (figs. 1 and 2). This newly recognized mutation was not present in >200 individuals of different ethnic backgrounds, including 40 controls with other neuromuscular disorders or mitochondrial encephalomyopathies.

To determine the threshold levels for phenotypic expression, we quantitated mutant and wild-type mtDNAs in single muscle fibers from the proband in family B, using a PCR-based method (Sciaccio et al. 1994). Fibers were divided into two groups, COX<sup>+</sup> ( $n = 34$ ) and COX<sup>-</sup> ( $n = 26$ ). There were no differences between the two group of fibers, in the total amount of amplified mtDNA. Although muscle tissue as a whole harbored high percentages of mutant genomes, mutant mtDNA was significantly more abundant in COX<sup>-</sup> fibers ( $93.8 \pm 2.6$ ) than in COX<sup>+</sup> fibers ( $90.8 \pm 4.1$ ) ( $t = 3.4$ ;  $df = 58$ ;  $P < .05$ ). Northern blots showed neither gross qualitative or quantitative abnormalities in the transcripts studied nor any extra bands (data not shown).

## Discussion

Mitochondrial diseases have been known for 30 years, since Luft's first description of a patient suffering from "nonthyroidal hypermetabolism" (Luft et al. 1962). However, the pathogenic importance of mtDNA mutations has been fully revealed only during the past 7 years (Schon et al. 1994). Awareness of the extreme clinical heterogeneity associated with mtDNA mutations, increased attention to maternal inheritance, and availability of effective molecular genetic screening contribute to explain the large number of pathogenic mtDNA mutations identified during the past few years (DiMauro and Moraes 1993). Nevertheless, there are cases in which specific mutations have not been found, even though clinical presentation or family history was suggestive of a mtDNA defect. Undoubtedly, additional pathogenic mutations remain to be identified, as documented by the present study.

We investigated nine patients from two unrelated families with heterogeneous clinical presentation: five had multiorgan involvement, including brain, muscle, and heart; maternally inherited hearing loss was the predominant feature in one patient; and cardiomyopathy and retinal degeneration dominated the clinical picture in another individual. Family history was positive for maternal inheritance, and both laboratory findings and morphological or biochemical studies of muscle were suggestive of disorders due to mtDNA mutations and of altered protein synthesis. We combined PCR-SSCP

**Table 1****Respiratory-Chain Enzyme Activities (mmol/min/mg Protein) in Muscle Homogenates**

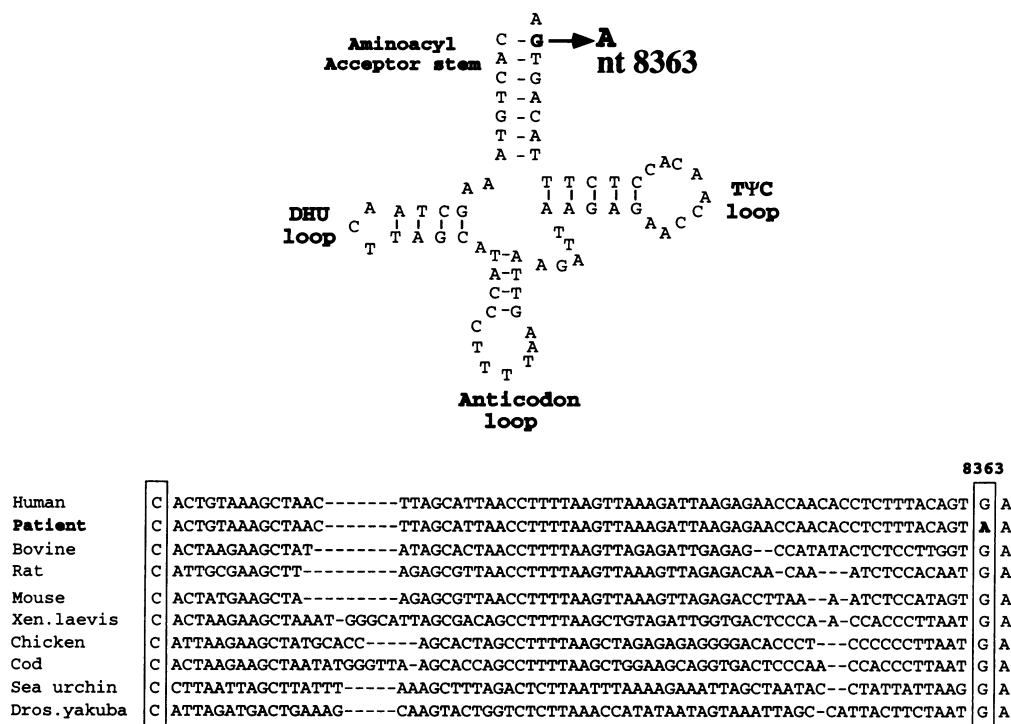
	Complex IV	Complexes I + III	Complexes II + III	Complex II	CS
Patient III-3 (family A)	1.32	.78	.53	1.4	18.92
Patient III-5 (family A)	1.76	.75	.45	1.2	21.45
Patient III-2 (family B)	1.30	.85	.67	2.4	15.83
Controls (n = 38)	2.8 ± .52	1.02 ± .38	.70 ± .23	1.00 ± .53	9.88 ± 2.55

and direct sequencing to search for novel mutations in mtDNA genes involved in protein synthesis, starting with the 22 tRNAs.

SSCP and sequence analysis revealed several novel mutations in tRNA genes. This was not surprising, because the mitochondrial genome has a high mutation rate, leading to the establishment of numerous neutral polymorphisms. Therefore, we evaluated the potential pathogenicity of each mutation, on the basis of the following criteria: the mutation should (i) affect an evolutionarily conserved nucleotide, (ii) be heteroplasmic, at least in some family members, and (iii) be absent in a large series of controls of different ethnic origin. These minimal criteria are generally accepted and were sufficient to exclude from further consideration all but one mutation: a G8363A transition in the tRNA<sup>Lys</sup> gene,

which affected a conserved residue, was present in high percentage (>95%) in the two propositi, occurred less abundantly in less affected relatives, and was not found in normal individuals. In addition, the same mutation caused similar clinical presentation in two apparently unrelated families. Sequence analysis of the highly polymorphic D-loop region of mtDNA failed to show any maternal relationship between the propositi of the two families, but we are performing complete haplotyping of the two families, to formally exclude any relationship.

The spectrum of clinical features observed in these two families is rather broad, including encephalomyopathy, PEO, sensorineural hearing loss, ataxia, cardiomyopathy, and hyperthyroidism. Although some of these features are seen in more common mtDNA disorders, such as MERRF and MELAS, the association of cardio-

**Figure 3** shown.

Cloverleaf of the mitochondrial tRNA<sup>Lys</sup>. The position of the novel mutation (arrow) and homology with other species are also shown.

myopathy and hearing loss in several generations in these two families distinguishes the syndrome from other mtDNA-associated disorders. Preferential involvement of these two organs is not surprising, because tissues with high oxidative metabolism, such as heart and cochlea, are particularly vulnerable to impaired mitochondrial function. Cognitive impairment was also present in several members of these pedigrees, probably because of impaired cerebral oxidative metabolism.

The G8363A mutation identified in these families represents the third putative pathogenic mutation found in the tRNA<sup>Lys</sup> gene; the other two are associated with MERRF (DiMauro and Moraes 1993). However, if one accepts strict clinical criteria for the diagnosis of MERRF (Silvestri et al. 1993a), neither one of our probands would fit this definition.

Additional evidence in favor of the pathogenic nature of the G8363A mutation emerges from the following considerations. First, this new mutation abolishes a highly conserved pairing in the aminoacyl stem of the tRNA<sup>Lys</sup>, which might disturb the double helix of the stem and alter the overall secondary structure of the molecule. In particular, the absence of a hydrogen-bonded base pair at the end of the acceptor stem is likely to impair both the activity of peptidyl-tRNA hydrolase, an enzyme of the translational apparatus (Dutka et al. 1993), and the correct attachment of amino acids to their cognate tRNA by aminoacyl-tRNA synthetase (Jahn et al. 1991; Saks et al. 1994). It is of interest that a change in a similar position occurs in the C3303T mutation in the tRNA<sup>Leu(UUR)</sup> gene, a mutation that is also characterized by maternally inherited cardiomyopathy (Silvestri et al. 1993b).

Second, single-fiber PCR analysis suggested a significant correlation between abundance of mutant mtDNA and biochemical abnormality, as has been found for other mtDNA mutations (Moraes et al. 1992). However, the threshold level of mutant mtDNA needed for biochemical and clinical phenotypes to become manifest was very high. It is, therefore, conceivable that other factors, including different nuclear and mitochondrial genetic backgrounds, might modulate phenotypic expression. These factors might contribute to explain the different clinical expression among different members in the two families.

The partial and multiple defects of oxidative phosphorylation (OXPHOS) metabolism observed in muscle biopsies from the probands suggest that the G8363A mutation causes a general impairment of mitochondrial protein synthesis. The relative content of lysine in different respiratory-chain subunits may explain the preferential involvement of complex I and complex IV, shown in MERRF patients and in this study, because lysine residues are more abundant in ND5, ND2, and COX I. However, in addition to decreases in the rates of synthe-

sis and in steady-state levels of mitochondrial polypeptides, the MERRF mutations have been also associated with abnormal translation products (Yoneda et al. 1994; Masucci et al. 1995). We are now investigating the presence of similar abnormal peptides in skin fibroblasts from a patient with the G8363A mutation.

The same mutation produced similar clinical manifestations in two unrelated families, and there was a reasonable correlation between percentage of mutant mtDNA and clinical severity, though not as striking as we have observed in MELAS (Hirano and Pavlakis 1994). For example, patients II-3 and II-7 in family A and patient III-5 in family B, who harbored lower amounts of mutant genomes, were less severely affected than the probands. However, we did not study tissues other than blood, in most maternal relatives; therefore, it is possible that higher percentages of mutant genome are present in affected tissues, such as heart in case III-2 in family B. Finally, pathogenicity is supported by the simultaneous report, by an independent group (Ozawa et al. 1995), of the G8363A mutation in affected families of different ethnic origin.

Clinical examination of additional affected and asymptomatic individuals from our families, as well as generation of transmitochondrial cybrids (King and Attardi 1989), may give us better insight on the preferential tissue involvement and may clarify the deleterious effects of the G8363A mutation on OXPHOS metabolism. Our findings add to the evidence of genetic heterogeneity underlying familial deafness syndromes and MICM (Casali et al. 1995). Whether these syndromes occur independently or in combination, mtDNA mutations should be considered in differential diagnosis, particularly when there is evidence of maternal inheritance. Although accurate molecular diagnosis may not immediately affect prognosis or treatment, it is of crucial importance for genetic counseling.

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