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Mitochondrial Encephalomyopathy and Complex III Deficiency Associated with a Stop-Codon Mutation in the Cytochrome *b* Gene

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We have reinvestigated a young woman, originally reported by us in 1983, who presented with exercise intolerance and lactic acidosis associated with severe deficiency of complex III and who responded to therapy with menadione and ascorbate. Gradually, she developed symptoms of a mitochondrial encephalomyopathy. Immunocytochemistry of serial sections of muscle showed a mosaic of fibers that reacted poorly with antibodies to subunits of complex III but reacted normally with antibodies to subunits of complexes I, II, or IV, suggesting a mutation of mtDNA. These findings demonstrate the diagnostic value of immunocytochemistry in identifying specific respiratory-chain deficiencies and, potentially, distinguishing between nuclear- or mtDNA-encoded defects. Sequence analysis revealed a stop-codon mutation (G15242A) in the mtDNA-encoded cytochrome *b* gene, resulting in loss of the last 215 amino acids of cytochrome *b*. PCR-RFLP analysis indicated that the G15242A mutation was heteroplasmic and was present in a high percentage (87%) of affected tissue (skeletal muscle) and a low percentage (0.7%) of unaffected tissue (blood) but was not detected in controls. Analysis of microdissected muscle fibers showed a significant correlation between the immunoreactivity toward the Rieske protein of complex III and the percentage of mutant mtDNA: immunopositive fibers had a median value of 33% of the G15242A mutation, whereas immunonegative, ragged-red fibers had a median value of 89%, indicating that the stop-codon mutation was pathogenic in this patient. The G15242A mutation was also present in several other tissues, including hair roots, indicating that it must have arisen either very early in embryogenesis, before separation of the primary germ layers, or in the maternal germ line. The findings in this patient are contrasted with other recently described patients who have mutations in the cytochrome *b* gene.

Introduction

The mitochondrial myopathies and encephalomyopathies (MIM 251900) are an increasingly recognized and diverse group of disorders of mitochondrial function that are frequently associated with defects of the respiratory chain (DiMauro and Bonilla 1997; DiMauro et al. 1998). The diversity of these disorders reflects not only the complexity of the respiratory chain itself, made up of >80 individual proteins that are organized into five major complexes (I–V), but also the unique properties of mtDNA, which encodes 13 of these polypeptides: 7 (ND1–6 and 4L) in complex I (NADH:ubiquinone oxidoreductase); 1 (cytochrome *b*) in complex III (ubiquinol:cytochrome *c* reductase); 3 (COX I–III) in complex IV (cytochrome *c* oxidase); and 2 (A6 and A8) in complex V (ATP synthase). Because of the small size and known sequence of human mtDNA (Anderson et al. 1981), many mutations have now been recognized (Chinnery and Turnbull 1999). These disorders are clinically and biochemically heterogeneous, most often being associated with deficient activity of complex I, complex IV, or both. Disorders of complex III are relatively rare. Because of the overlap of clinical findings and frequent lack of correlation among the biochemical, histochemical, and molecular data, these disorders are best classified on the basis of the pathogenic mtDNA mutation. Hundreds of large-scale rearrangements of mtDNA (deletions and/or duplications) and >50 pathogenic point mutations in tRNA genes have been recognized (Chinnery et al. 1999; Wallace 1999). Pathogenic mutations in the rRNA or protein-coding genes are less frequent. Most disorders of mtDNA are heteroplasmic, meaning that both normal and mutant mtDNA species are present. They are frequently asso-

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ciated with so-called ragged red fibers (RRFs) seen on muscle biopsies, reflecting the proliferation of mutant mitochondria in energy-deficient fibers. In contrast to the major rearrangements of mtDNA, which are most often associated with progressive external ophthalmoplegia or Kearns-Sayre syndrome (MIM 530000) and which are almost always sporadic, many mutations in the tRNA, rRNA, or protein-coding genes are inherited from the mother, reflecting the maternal inheritance of the mitochondrial genome. Well-known examples are the A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene, which is associated with mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS [MIM 540000]); the A8344G mutation in the $tRNA^{Lys}$ gene, which is associated with myoclonic epilepsy with RRFs (MERRF [MIM 545000]); and the G11778A mutation in ND4 (subunit 4 of complex I), which is associated with Lebers hereditary optic neuropathy (LHON [MIM 535000]).

Until recently, relatively few patients with isolated complex III deficiency (MIM 124000) had been reported, and only in one of these had a mutation in the cytochrome *b* gene (MIM 516020) been shown to be pathogenic (Dumoulin et al. 1996). In 1998, we published a preliminary abstract (Kennaway et al. 1998) documenting a stop-codon mutation in the cytochrome *b* gene in muscle from a patient with severe exercise intolerance and lactic acidosis, associated with complex III deficiency, who responded to menadione and ascorbate (Darley-Usmar et al. 1983; Kennaway et al. 1984; Argov et al. 1986). Since then, 12 additional pathogenic mutations in the cytochrome *b* gene have been described (Andreu et al. 1998, 1999*a*, 1999*c*, 2000; De Coo et al. 1999; Legros et al. 1999; Pulkes et al. 1999; Valnot et al. 1999). Interestingly, most of these patients have presented with the predominant feature of severe exercise intolerance, sometimes including muscle weakness and/or myoglobinuria. Two had hypertrophic cardiomyopathy. We now present the characterization of the cytochrome *b* mutation in our patient, provide an update of her disease progression over the past 15 years, and contrast our findings regarding her with those of other reported patients.

Patient and Methods

Patient

The proband is a 34-year-old woman who was first seen by us at age 17 years. She had a history of progressive exercise intolerance and lactic acidosis starting at age ~9 years. Muscle biopsy revealed many RRFs and severe deficiency of complex III activity (5% of control) in isolated mitochondria (Darley-Usmar et al. 1983; Kennaway et al. 1984). Other respiratory-chain activities were normal. Western blot analysis revealed low levels of multiple subunits of complex III, and cytochrome spectra showed severe reduction of cytochrome *b* with normal levels of cytochrome aa_3. Complex III activity was normal in peripheral blood leukocytes, transformed lymphocytes, and cultured skin fibroblasts (Darley-Usmar et al. 1986). Exercise testing at this time showed an early anaerobic threshold and severe reduction in maximal oxygen consumption (Elliot et al. 1989). Evaluation of muscle bioenergetic capacity by [^{31}P]–magnetic resonance spectroscopy (MRS) revealed low levels of phosphocreatine relative to inorganic phosphate, with slow recovery following exercise (Eleff et al. 1984). After treatment with menadione and ascorbate, the patient showed improvement in clinical parameters, as well as in the bioenergetic state of her skeletal muscle, as documented by [^{31}P]-MRS (Eleff et al. 1984; Argov et al. 1986).

By age 19 years, the patient had clear evidence of encephalopathy, with emotional lability, seizures, abnormal EEG, intermittent visual hallucinations, depression, and psychiatric problems. At age 23 years, a needle muscle biopsy was performed, with informed consent, during elective bilateral tubal ligation. Menadione had to be discontinued because of its withdrawal by the FDA. The following year, she developed increasing episodes of encephalopathy, with poor balance, diplopia, confusion, hallucinations, and occasional myoclonic twitches. These continued over the next few years, accompanied by nausea, vomiting, and complex partial seizures. She suffered a left-sided stroke from which she recovered completely. By age 31 years, emotional problems with memory loss, dizziness, vomiting, and weight loss were evident. Echocardiogram and retinal examination were normal. Because of excessive menstrual bleeding, an elective hysterectomy was performed, and samples of uterus were frozen for subsequent analysis. Over the past 2–3 years, the patient has been remarkably stable, with no major encephalopathic episodes. Recently, she developed ovarian cysts, which necessitated surgical resection. These were identified as benign mucinous cystadenomas. Samples of ovarian tissue and rectus abdominis muscle were obtained at the same time, with informed consent, and frozen for subsequent DNA analysis. These studies were approved by our Institutional Review Board.

Fibroblast Culture

Cultured skin fibroblasts were grown in alpha MEM (Gibco BRL) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. Uridine (50 μg/ml) was added where indicated.

Muscle Histochemistry and Immunocytochemistry

Skeletal muscle, obtained by open biopsy, was immediately frozen in liquid nitrogen–cooled isopentane and stored at −80°C. Muscle sections (8 μm) were stained for cytochrome *c* oxidase (COX), succinate dehydrogenase (SDH), and myofibrillar actomyosin ATPase, as described elsewhere (Taanman et al. 1996). Fiber type was determined by the ATPase activity at pH 9.4, following preincubation at pH 4.5. RRFs were identified with the modified Gomori trichrome stain or by increased SDH activity. Immunocytochemistry was performed as described elsewhere (Keightley et al. 1996; Taanman et al. 1996), using a monoclonal antibody to COX subunit I (COXI) and polyclonal antibodies to subunit 1 of complex I (ND1, a kind gift from Dr. R.F. Doolittle), holocomplex II (holoII, kindly provided by Dr. B. Akrell), and to three subunits of complex III (the Rieske protein, 13.4-kDa protein, and cytochrome *b,* generously provided by Dr. L. Yu).

Isolation, Cloning, and Sequencing of mtDNA

DNA was isolated from tissues as described elsewhere (Keightley et al. 1996). For hair roots, a modification of a standard method (Bing and Bieber 1998) was used. Briefly, a 1-cm portion containing the root was rinsed in sterile deionized water. Cells were lysed overnight at 37°C in 0.01 M Tris-Cl, pH 7.4, 0.01 M EDTA, 0.01 M NaCl, 1% SDS, and 0.15 mg/ml proteinase K and DNA purified by organic extraction and ethanol precipitation (Sambrook et al. 1989). The cytochrome *b* gene was amplified by PCR using a mismatched (italicized nucleotides) forward primer 5′-AGGAGAA*TT*CTTAGAAGAAAACCCCACAAAC-3′ and the reverse primer 5′-ATAGCGGCCGTTGATGGGTGAGTCAATAC-3′, which bind to nt 14599–14629 and 16088–16060 of mtDNA, respectively. Sequences are numbered according to the L strand of the Cambridge reference sequence (Anderson et al. 1981). The PCR reaction (75 μl) contained 10 pmol of each primer, 10 mM Tris-Cl (pH8.3), 50 mM KCl, 1.5 mM $MgSO_4$, 0.01% (w/v) gelatin, 160 μM dNTPs, and *Taq* polymerase. Reactions were cycled 32 times for 20 s at 94°C, 30 s at 64°C, and 60 s at 72°C. Amplified products were purified from agarose gels using Wizard PCR Preps (Promega), digested with *Sau*3AI, and cloned by ligation into *Bam*HI-digested pBSII-SK$^+$ (Stratagene). After transformation of *Escherichia coli* XL-1 Blue (Stratagene) (Hanahan 1985), colonies were selected and plasmid DNA screened to identify clones representing each of the *Sau*3AI fragments (mtDNA nt 14871–15061, 15062–15358, and 15359–15592). The 5′ end of the cytochrome *b* gene was cloned using the *Eco*RI site introduced by the mismatched forward PCR primer (italicized bases in sequence above) and a *Xho*I site at nt 14955; directional clones were generated by digesting agarose purified PCR product with *Eco*RI and *Xho*I and ligating the resulting 352-bp fragment into pBSII-SK$^+$. The directional (*Eco*RI/*Xho*I) clone and internal *Sau*3AI clones were sequenced from plasmid DNA (both strands, on a minimum of two clones each), using the Sequenase deaza-dGTP sequencing kit (USB). The 3′ end of the gene resisted cloning and was therefore cycle sequenced (both strands) on the full-length PCR product, using fluorescently labeled dideoxynucleotide terminators and an Applied Biosystems 373A automated sequencer.

Detection and Quantitation of the Cytochrome b G15242A Mutation by PCR-RFLP

The G15242A mutation introduces an *Eam*1104I restriction site, which was used to develop a PCR-based RFLP assay for mutation detection. A 769-bp fragment, which spanned nt 14976–15744 of mtDNA and also contained two wild-type *Eam*1104I sites (nt 15031 and 15451), was generated by PCR (50-μl reaction volume) as above, except that (1) the forward primer was 5′-GAATCATCCGCTACCTTCACGCC-3′ (nt 14976–14998) and the reverse primer was 5′-AGGAGGTCTGCGGCTAGGAGTCA-3′ (nt 15744–15722) and (2) 30 μM dATP (or dCTP) was used instead of 160 μM. Reactions were cycled 24 times for 20 s at 94°C, 30 s at 68°C, and 60 s at 72°C, followed by an additional cycle (using a 2-min denaturation) in the presence of 5 μCi (3,000 Ci/mmol) of [α^{32}P]-dATP (or dCTP). Labeled PCR products were digested with *Eam*1104I (Stratagene) and resolved by nondenaturing PAGE (6% polyacrylamide). Fragments of 420, 291, and 58 bp were produced in wild-type mtDNA. In the mutant, the 420-bp fragment was cleaved into fragments of 217 and 203 bp. The proportion of mutant mtDNA was quantitated from the 420-, 217-, and 203-bp fragments, using a phosphorimaging system and IMAGEQUANT software (Molecular Dynamics), with local area background subtraction.

Analysis of Microdissected Muscle Fibers

For single-muscle-fiber analysis, alternating 40- and 8-μm-thick serial sections of muscle were prepared. The 40-μm sections were stained immunocytochemically with antisera to the Rieske protein of complex III and placed in 50% ethanol. The 8-μm-thick flanking sections were also stained immunocytochemically for the Rieske protein, and these, which stained more intensely than the 40-μm sections, were used as references for scoring the adjacent 40-μm sections as complex III positive or negative. Additional 8-μm flanking sections were stained histochemically for SDH, to identify RRFs. Single fibers were microdissected from the 40-μm sections using stainless steel microelectrodes and were prepared as described elsewhere (Keightley et al. 1996). PCR-RFLP analysis was conducted as described above, except that (1) 40

pmol of each primer was used, (2) hot-start PCR was used, and (3) reactions were cycled 30 times for 40 s at 95°C, 40 s at 60°C, and 40 s at 72°C. Last-cycle labeling (95°C for 1 min, 60°C for 45 s, and 72°C for 2 min) was performed on 35 μl of the PCR reaction, after addition of a further 15 pmol of each primer, 2 U of *Taq* polymerase, and 10 μCi of [α^{32}P]-dCTP (6,000 Ci/mmol). PCR products were purified over Microspin S-200 HR columns (Pharmacia), were vacuum dried, and were subjected to RFLP analysis as described above. Statistical analysis used Welsh's alternative *t* test (Instat Program, version 2.01).

Results

Histochemistry and Immunocytochemistry of Muscle Sections

Previous studies of muscle from our patient had identified numerous RRFs, the complete absence of COX-negative fibers, and deficient activity of complex III associated with multiple subunit deficiencies (Darley-Usmar et al. 1983; Kennaway et al. 1984). We therefore investigated whether we could use an immunocytochemical method to confirm an isolated complex III deficiency in serial sections of muscle (fig. 1). The results showed that individual type I fibers (black arrows) or type II fibers (white arrows) could be identified as complex III positive (filled-in arrows) or negative (outlined arrows), on the basis of their punctate (mitochondrial) intermyofibrillar immunoreactivity toward antisera against complex III subunits (cytochrome *b,* the Rieske protein, and the 13.4-kDa protein), compared with their immunoreactivity to antibodies against complex I (NDI), complex II (holoII), or complex IV (COXI). The heavier staining with antisera against the Rieske protein, compared with that of antisera against cytochrome *b* or the 13.4-kDa protein, reflects the stronger immunoreactivity of the Rieske protein antiserum; the heavier labeling in subsarcolemmal regions of RRFs appeared to be predominantly nonspecific. These results not only confirmed the specificity of the complex III deficiency but, additionally, identified a mosaic of complex III–positive and –negative type I and type II fibers, suggesting a mutation in the mtDNA-encoded cytochrome *b* gene in this patient.

Detection of a Stop-Codon Mutation in the Cytochrome b *Gene*

In view of the severe and specific deficiency of complex III in this patient and the mosaic of complex III–positive and –negative muscle fibers, we next sequenced the cytochrome *b* gene in muscle. This identified a G→A transition at nt 15242 (G15242A), which converts a glycine codon to a stop codon (G166X; fig. 2; GenBank accession number AF254896). This change, which has not been reported previously, results in the loss of 215 amino acids, representing 57% of the protein at the C terminus of cytochrome *b*. The only other change in the patient's cytochrome *b* gene, compared with the Cambridge reference sequence, was the reported A15326G polymorphism (Andreu et al. 1999*b*). We also ruled out the presence of the common MELAS A3243G and MERRF A8344G mtDNA mutations (data not shown).

Quantitation of the Cytochrome b *Stop-Codon Mutation in Different Tissues*

The G15242A mutation creates an *Eam*1104I restriction site, which allowed us to develop a simple PCR-RFLP method for detection and quantitation of the proportion of mutant mtDNA. PCR-RFLP analysis of skeletal muscle and blood from the patient and her relatives (fig. 3) revealed very high levels of the G15242A mutation (87%) in the patient's skeletal muscle and very low, but detectable, levels in blood (0.7%). Very faint mutant bands suggested the presence of trace amounts of the mutation (<0.2%) in blood from the patient's sister, but this was at the limit of detection. The mutation was not detected in her mother's blood nor in >80 control samples (58 blood and 28 muscle samples from controls and patients with a variety of neuromuscular diseases). Mutation analysis in other tissues from the patient (fig. 3) showed very high levels of mutant mtDNA in rectus abdominis muscle (92%), high levels in uterus (61%), and low levels in ovarian tissue, both from the cyst itself and from surrounding tissue that contained cyst contamination (4%–5%). The proportion of mutant mtDNA in fibroblasts varied with the length of time in culture, increasing steadily from 29% to 75% during the course of 10 population doublings over a period of 7 weeks (fig. 3). A similar trend was seen regardless of whether the culture medium was supplemented with uridine, which is essential for the growth of respiratory-deficient cells (King and Attardi 1989) (data not shown). In a series of 22 individual hair roots from the patient, the G15242A mutation was detected in 8; the proportion of the mutation varied widely, ranging from <0.4% to 66% (mean 4.6%).

Proportion of the Cytochrome b *G15242A Mutation in Single Muscle Fibers*

Individual fibers were classified as complex III positive or negative on the basis of immunostaining of flanking sections with antisera against the Rieske protein of complex III (see fig. 1) then analyzed by PCR-RFLP. The results (fig. 4) showed a significant correlation between the proportion of the G15242A mutation in complex III–positive fibers (32% ± 29%; mean ± SD), compared with that in complex III–negative fibers (66% ± 26%) ($P < .0001$), and especially in complex III–negative RRFs (89% ± 8%) ($P < .0001$). The cor-

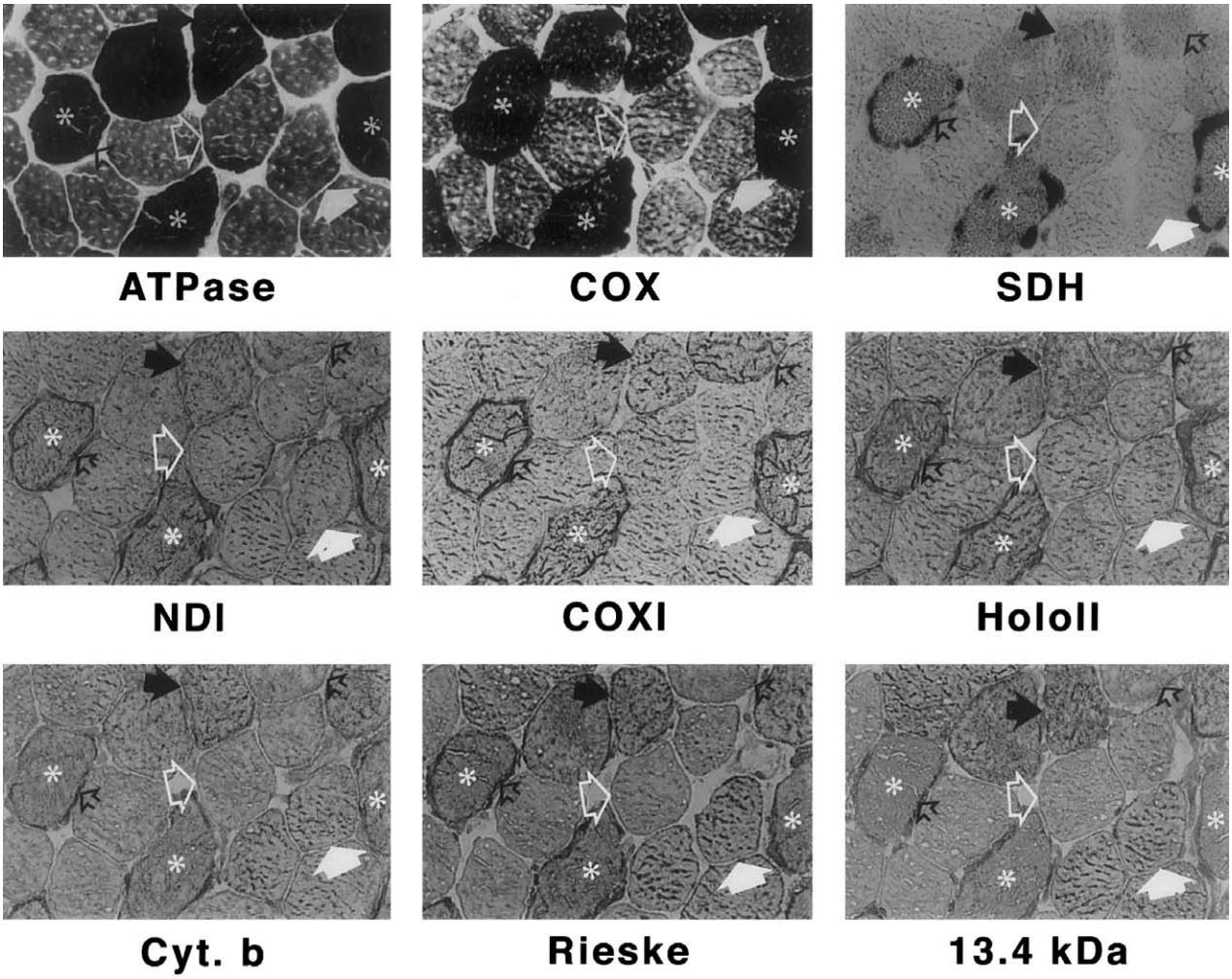

Figure 1 Histochemistry and immunocytochemistry of serial sections of skeletal muscle from the patient. Type I (heavy) and type II (light) fibers are distinguished on the basis of their intensity of staining for ATPase, COX, and SDH activities (*top*). The black arrows (two outlined and one filled in), most easily seen with the SDH reaction, identify three type I fibers, one of which is an RRF (*). Two other RRFs (*) are also identified. The white arrows (one outlined and one filled in) identify two type II fibers. The center panel shows immunoreactivity with antibodies to ND1, COXI, and holoII. Similar intensity of staining is seen in the three type I fibers and in the two type II fibers. The bottom panels show immunoreactivity with antibodies to three subunits of complex III (cytochrome *b,* the Rieske protein, and the 13.4-kDa protein). In these cases, a clear difference can be seen between two of the type I fibers (*outlined black arrows*), which react poorly with all complex III antibodies, compared with the third type I fiber (*filled-in black arrow*). Similarly, one of the type II fibers (*outlined white arrow*) reacts poorly with all three complex III antibodies, compared with the other type II fiber (*filled-in white arrow*).

responding median values were 33%, 76%, and 89%, respectively. The immunopositive reaction in 3 of 33 fibers with >70% of the G15242A mutation and the immunonegative reaction in 6 of 31 fibers with <50% of the mutation most likely reflect the fact that the immunostaining was performed in 8-μm sections immediately adjacent to the 40-μm sections used for mutation analysis, and there may be some variability in the distribution of the mutant mtDNA, even between adjacent sections. Another possibility is that there is not an exact correlation between the levels of Rieske protein and the proportion of mutant mtDNA, since deficiency of the Rieske protein is a secondary phenomenon, reflecting lack of assembly or increased degradation of nuclear-encoded subunits of complex III, as a consequence of deficient functional cytochrome *b*.

Discussion

Our patient, who was diagnosed many years ago with complex III deficiency, has been of particular interest because of her well-documented clinical and bioenergetic response to treatment with menadione and ascorbate, which were proposed to bypass the defect of electron

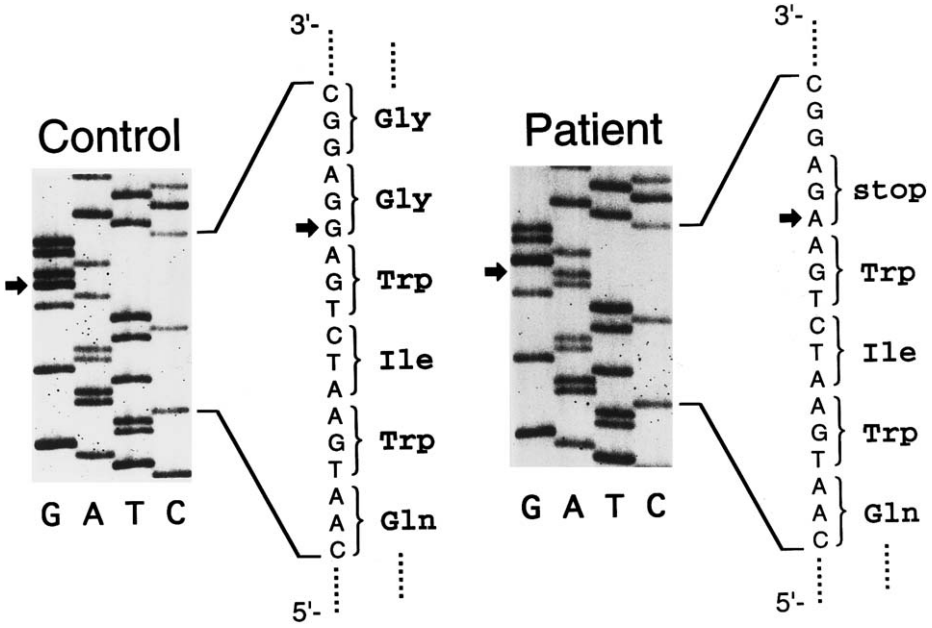

Figure 2 Identification of a nonsense mutation in the cytochrome *b* gene. Autoradiogram of a DNA sequencing gel spanning nt 15230–15247 (5′→3′) of human mtDNA, showing sequence from a control (*left panel*) and the patient's muscle (*right panel*). The G→A transition in the patient's sequence (nt 15242; *arrow*) converts a glycine codon (GGA) to a stop codon (AGA).

transport between ubiquinol and cytochrome *c* (Eleff et al. 1984; Argov et al. 1986). Although the original studies of muscle—including enzymatic, spectral, and western blot analyses—all indicated a severe and specific deficiency of complex III, the molecular basis was not known. Complex III of the mitochondrial respiratory-chain transfers electrons from ubiquinol to cytochrome *c,* coupled with the transfer of electrons across the mitochondrial inner membrane. It contains three redox-active centers (cytochrome *b,* cytochrome c_1, and the Rieske FeS protein) and is composed of 11 polypeptide subunits, 1 of which (cytochrome *b*) is encoded by mtDNA. Disorders of complex III are relatively rare, comprising only 7% of patients with respiratory-chain disorders in a recent series of 157 patients (von Kleist-Retzow et al. 1998). A deficiency of complex III could be of either nuclear or mitochondrial origin. The immunocytochemical studies of skeletal muscle, presented here, confirmed the specificity of the complex III deficiency in our patient and, more importantly, identified a mosaic of complex III–immunopositive and –immunonegative fibers, strongly suggesting a mutation of mtDNA. The ability to utilize antisera to the Rieske or 13.4-kDa proteins for these experiments presumably reflects a lack of assembly or increased degradation of nuclear-encoded subunits of complex III in the absence of a functional cytochrome *b,* as also occurs in yeast (Sen and Beattie 1985). A mosaic of COX-positive and -negative fibers is readily recognized histochemically in patients with COX deficiency associated with mutations of mtDNA (Keightley et al. 1996; DiMauro and Bonilla 1997). In contrast, no histochemical stain is available to document similar mosaicism in patients with complex I or complex III deficiency. Our results suggest that immunocytochemistry, using serial sections of muscle and specific antibodies to complex I or complex III, may be a valuable diagnostic technique to localize a specific respiratory-chain deficiency, especially in small muscle samples. This technique can also provide direction for molecular analyses focused on either the nuclear or mitochondrial genome. Moreover, for mutations of mtDNA, it can be used to correlate the proportion of mutant mtDNA with the phenotype in single muscle fibers, a critical step in establishing the pathogenicity of an mtDNA mutation, as has been demonstrated here.

The G15242A mutation in our patient is almost certainly pathogenic, resulting in a protein truncated by >50% at the C terminus. Additional evidence for pathogenicity is as follows: (1) the mutation is heteroplasmic and occurs at higher proportions in affected tissue (muscle) than in unaffected tissue (blood); (2) it has not been described elsewhere and was not identified in control samples; (3) the mutation is consistent with the biochemical and immunocytochemical phenotype that indicated isolated complex III deficiency (furthermore, the pattern of subunit deficiencies, documented elsewhere in western blot analyses, included core proteins 1 and 2 and the Rieske protein, with sparing of cytochrome

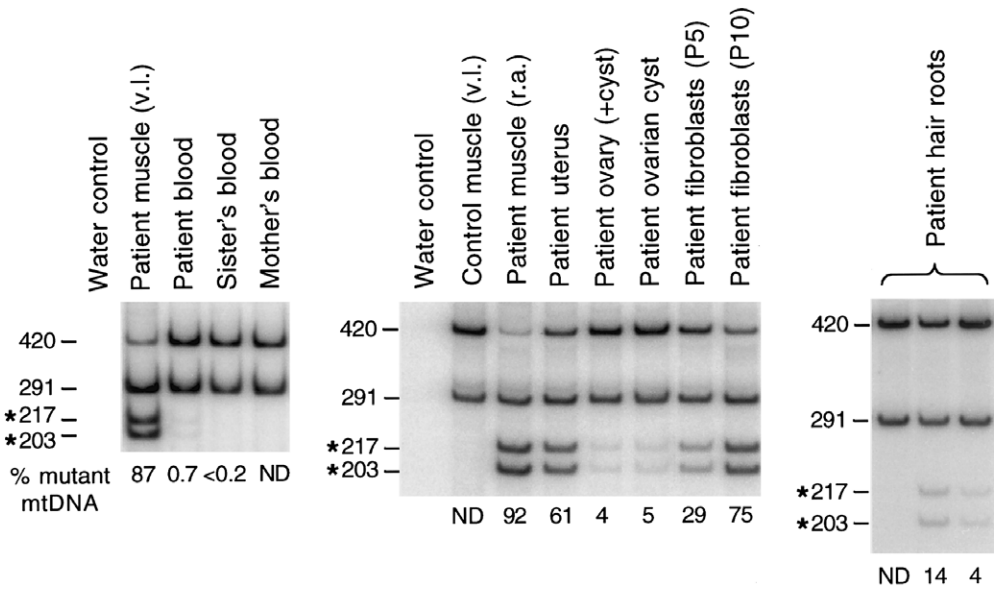

Figure 3 Distribution and quantitation of the cytochrome *b* G15242A mutation in different tissues. MtDNA was amplified by PCR, with last-cycle labeling, to generate a 769-bp fragment, which was digested with *Eam* 1104I, and fragments were separated by nondenaturing polyacrylamide electrophoresis (6%). In wild-type mtDNA, fragments of 420, 291, and 58 bp were produced (the last is not shown). In mutant mtDNA, the 420-bp fragment was cleaved into products of 217 and 203 bp (*). Cultured skin fibroblasts at passage 5 (P5) were compared with those at passage 10 (P10). Also shown are three representative hair roots. The proportion of the G15242A mutation in each sample is indicated. ND = not detected; v.l. = vastus lateralis; r.a. = rectus abdominis.

c_1, a pattern identical to that seen in yeast mutants deficient in cytochrome *b* [Sen and Beattie 1985]); and (4) there is a genotype:phenotype correlation in individual muscle fibers, with complex III–immunodeficient fibers having a significantly higher proportion of mutant mtDNA than complex III–immunopositive fibers.

The G15242A mutation in our patient was the second pathogenic cytochrome *b* mutation to have been identified (Dumoulin et al. 1996; Kennaway et al. 1998). Since then, 12 additional patients with cytochrome *b* mutations have been described (table 1). Remarkably, five of these also had stop-codon mutations, and one other had a 4-bp deletion that also led to premature termination of translation. Many of the others had mutations within or close to the ubiquinone-binding sites of cytochrome *b* (Iwata et al. 1998; Andreu et al. 1999*c*), suggesting that most, if not all, of the cytochrome *b* mutations are likely to have a severe effect on mitochondrial energy production.

The phenotypes of the 14 patients with cytochrome *b* mutations are surprisingly similar, 10/14 presenting with isolated exercise intolerance and lactic acidosis, sometimes accompanied by myoglobinuria (4/14). None had ophthalmoplegia. In at least nine patients, including ours, RRFs were identified in muscle. RRFs are commonly found in patients who have major rearrangements of mtDNA or mutations of mtDNA-encoded tRNA genes and have also been described in at least three patients with mutations of mtDNA-encoded COX genes (Keightley et al. 1996; Comi et al. 1998; Clark et al. 1999) (MIM 516050.0003, MIM 516030, and MIM 516040, respectively). However, they are not normally found in patients with mutations of other protein-encoding genes, such as those in ND1 (MIM 516000), ND5 (MIM 516005), or ND6 (MIM 516006), commonly associated with LHON, or the T8993G mutation in the A6 gene associated with Leigh syndrome or neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP [MIM 551500]). The factors that trigger the proliferation of mitochondria in some but not other disorders of mtDNA protein-encoding genes are not known. However, our results and the above data indicate that the presence of RRFs does not necessarily reflect a generalized defect of mitochondrial protein synthesis, as previously thought, but more likely represents a severe energy deficit in cells with high levels of mutant mtDNA.

The consistency of phenotype in most patients with mutations in the cytochrome *b* gene contrasts with other patients with complex III deficiency, in whom the age at onset and clinical manifestations vary widely; in many cases, this may reflect mutations in nuclear- rather than in mtDNA-encoded genes (Mourmans et al. 1997). There is a similar contrast between the majority of patients with cytochrome *b* mutations and most patients with mutations of mtDNA-encoded tRNA genes in

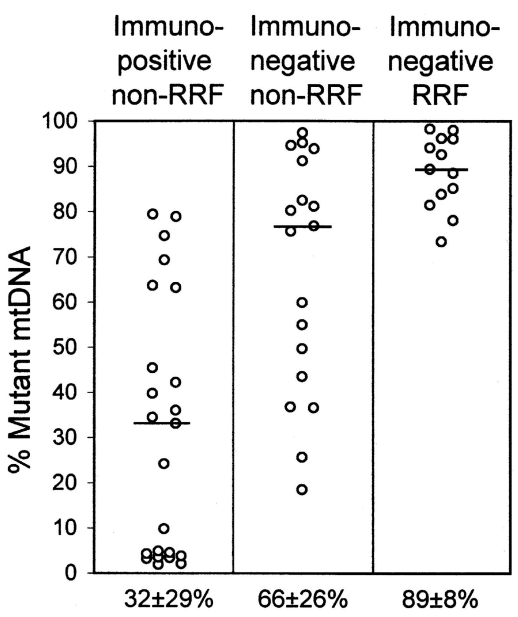

Figure 4 Proportion of the cytochrome *b* G15242A mutation in single muscle fibers. Alternating 40- and 8-μm-thick serial sections of muscle were obtained. The 40-μm sections and flanking 8-μm sections were stained immunocytochemically for the Rieske protein of complex III and were scored as complex III positive or complex III negative on the basis of the more intense staining of the 8-μm sections. Additional 8-μm flanking sections were stained histochemically for SDH, to identify RRFs. The 40-μm sections were microdissected and subjected to PCR-RFLP analysis. The median proportion of mutant mtDNA in each fiber type is indicated by a horizontal line. The mean $\pm$ SD is shown below. The differences between immunopositive and immunonegative non-RRFs (*columns 1 and 2*), and between immunopositive and immunonegative RRFs (*columns 1 and 3*), were highly significant ($P < .0001$).

which the variability of clinical manifestations is enormous. Also in contrast to patients with tRNA mutations, which are frequently maternally inherited, all of the patients with cytochrome *b* mutations, with the possible exception of ours, have been sporadic cases, suggesting that the cytochrome *b* mutation, if present in the maternal germ line, may frequently be lethal during early fetal development. Mutations in other mtDNA protein-encoding genes, such as those in the A6 gene associated with Leigh syndrome or NARP, or the mutations in complex I genes commonly associated with LHON, however, are almost always maternally inherited. The fact that the LHON mutations are usually homoplasmic, are not associated with RRFs, and are maternally inherited suggests that they may have a less severe effect on ATP synthesis than the mutations in the cytochrome *b* gene, discussed above. Studies in transmitochondrial cell lines should help answer this question.

Our patient differs from most other patients with cytochrome *b* mutations in two important respects. First, although her presentation was dominated by severe exercise intolerance and lactic acidosis between the ages of 9 and 19 years, she did have seizures from her early teenage years, and she has developed significant encephalopathy over the last 15 years. This raises the possibility that, given more time, some of the other patients may also progress to a more severe phenotype. One other patient with a cytochrome *b* mutation had a predominantly neurological presentation, described as juvenile Parkinson/MELAS overlap syndrome (patient 5, table 1), and two others had severe hypertrophic cardiomyopathy (patients 11 and 14). These three patients also had an earlier age at onset and more severe presentation, compared with many other patients. Notably, at least two of them had detectable levels of mutation in blood and/or fibroblasts.

The second difference between our patient and most of the other cases concerns the origin and tissue distribution of the mutation. In the patients with an isolated skeletal myopathy, the mutant mtDNA was absent in blood and/or fibroblasts in all cases examined. This is consistent with the restriction of the clinical phenotype to skeletal muscle, the spontaneous occurrence of these mutations, and the likelihood that they would not be transmitted to the patients' offspring (Griggs and Karpati 1999). The highly skewed distribution of the mutation between blood or fibroblasts, compared with muscle, and the restriction of the clinical phenotype to muscle, have led to the suggestion that the mutation in these patients may have arisen in muscle progenitor cells, after differentiation of the primary germ layers (Andreu et al. 1999*c*). However, another possibility is the occurrence of a sporadic mutation early during embryogenesis or in the maternal germ line, with a highly skewed distribution in tissues of the patient, arising as a result of either mitotic segregation or selection against the mutation by some unknown mechanism in most tissues. Certainly, the absence of the mtDNA mutation in blood and fibroblasts does not preclude its presence in a variety of other tissues, as illustrated by the patient with a heteroplasmic $tRNA^{Leu(CUN)}$ mutation who had sensorineural deafness and retinopathy in addition to a severe skeletal myopathy (Fu et al. 1996). In our patient, the G15242A mutation was relatively widely distributed, with very low levels in blood, low levels in ovarian tissue, and moderate to high levels in uterus and cultured skin fibroblasts. More importantly, the mutation was also present in hair roots. In contrast to all other tissues examined, which are of mesodermal origin, hair roots are derived from primitive ectoderm, indicating that the mutation in our patient must have arisen very early in embryogenesis, before separation of the primary germ layers, or in maternal germ line cells. Analysis of blood from the patient's mother and sister did not differentiate between these two possibilities.

Table 1

Summary of Patients with Mutations in the mtDNA-encoded Cytochrome *b* Gene

Patient	Sex/Age	Age at Onset (years)	Phenotype	RRF/ Lactic Acidosis	Cytochrome *b* Mutation	Amino Acid Change	% Mutation in Muscle	Mutation Present in Blood/ Fibroblasts	Reference
1[a]	F/34 y	9	EI, LE	+/+	G15242A	G166X	87	+/+	Kennaway et al. (1998)
2	M/25 y	10	EI, P	+/+	G15615A	G290D	80	−/NA	Dumoulin et al. (1996)
3	F/38 y	25	EI, W	−/+	G15762A	G339E	85	−/NA	Andreu et al. (1998)
4	F/27 y	12	EI, W, M	+/+	G15059A	G105X[b]	63	−/NA	Andreu et al. (1999*a*)
5	M/20 y	6	JP/MELAS	−/+	14787del4	Del14fs/tr49	>95	+/+	DeCoo et al. (1999)
6	M/43 y	30	EI, W, M	+/−	15498del24	Del251-258	50	−/−	Andreu et al. (1999*c*)
7	F/52 y	Child	EI, W	+/+	G14846A	G34S	85	−/−	Andreu et al. (1999*c*)
8	F/38 y	Child	EI	+/+	G15168A	W141X	70	−/NA	Andreu et al. (1999*c*)
9	M/32 y	Child	EI	+/+	G15084A	W113X	87	−/NA	Andreu et al. (1999*c*)
10	M/51 y	Child	EI, M	NA/+	G15723A	W326X	87	NA/NA	Andreu et al. (1999*c*)
11	F/8 y	3	SHC	NA/+	G15243A	G166E	90[c]	NA/+	Valnot et al. (1999)
12	M/18 y	8	EI, M	+/+	G15150A	W135X	60	−/−	Legros et al. (1999)[d]
13	M/51 y	Teen	EI, W	+/+	T15197C	S151P	70	−/−	Legros et al. (1999)[d]
14	F/4 w	Infant	SHC	NA/NA	G15498A	G251D	NA	NA/NA	Andreu et al. (2000)

NOTE.—EI = exercise intolerance; W = weakness; M = myoglobinuria; SHC = severe hypertrophic cardiomyopathy; JP = juvenile Parkinsons; P = muscle pain; LE = late-onset encephalopathy; y = years; w = weeks; Del = deletion; fs = frameshift; tr = termination; NA = not available or not analyzed.

[a] Present case.

[b] Corrected from Andreu et al. 1999*b*.

[c] Cardiac muscle.

[d] Also includes data from A. Lombes, personal communication.

Although the average proportion of the G15242A mutation in hair roots from our patient was only 4.6%, the finding of much higher proportions in some individual hair roots (up to 66%) than in blood (0.7%) suggests that hair roots may provide a more sensitive method for detecting heteroplasmic mtDNA mutations, as has been suggested elsewhere, in studies of a family harboring a mitochondrial $tRNA^{Glu}$ mutation (MIM 590025) (Hao et al. 1995). Examination of mutant mtDNA in patients with MELAS, MERRF, and NARP showed a good correlation between the percentage of mutant mtDNA in blood, compared with muscle, buccal cells, and hair roots (Wong and Lam 1997). These authors stress that pooling of hair cells minimizes the discrepancies obtained with single-follicle analysis. However, in patients such as ours, in whom the mutant mtDNA in blood is barely detectable, the wide variability in hair roots suggests that measurement of mutant mtDNA in individual follicles may be more sensitive for detecting asymptomatic carriers of the mutation in whom the level of mutant mtDNA in pooled follicles may be at the limit of detection.

The marked variability in the distribution of mutant mtDNA between different tissues (e.g., muscle and blood) and even within individual tissues (e.g., muscle fibers or hair roots) presumably reflects replicative segregation. In cultured skin fibroblasts, the increasing proportion of the G15242A mutation with increasing time in culture is harder to understand, since there is no obvious selective advantage to cells or mitochondria harboring high levels of a stop-codon mutation. However, in fibroblast cultures from one patient with intracellular mtDNA triplasmy, the proportion of one mutation (A5656G) was also found to increase with increasing time in culture (Bidooki et al. 1997). Moreover, increasing proportions of mutant mtDNA have been reported over time in vivo in patients harboring pathogenic mtDNA deletions (Larsson et al. 1990) or point mutations (Weber et al. 1997). In one family with a heteroplasmic $tRNA^{Glu}$ mutation, the proportion of mutant mtDNA (studied in individual hair roots) tended to increase in successive generations (Hao et al. 1995). Finally, in transmitochondrial cell lines harboring the MELAS 3243 mutation, different levels of heteroplasmy were found to either increase, decrease, or remain stable over time in culture; although the mechanisms responsible for these changes are not understood, they appear to depend on the nuclear background of the cells (Yoneda et al. 1992; Dunbar et al. 1995).

The presence of the G15242A mutation in hair roots from our patient suggests that the mutation may also be present in the CNS, which is also derived from primitive ectoderm. This would be consistent with her neurological symptoms. In this regard, it should be noted that the cytochrome *b* microdeletion in the patient with a more severe neurological disorder described as juve-

nile Parkinson/MELAS overlap syndrome (table 1) was also present in hair roots. The occurrence of heteroplasmic cytochrome *b* mutations in hair roots from the two patients with encephalopathic manifestations suggests that hair-root analysis can provide a useful reflection of the tissue distribution of the mutation, the potential clinical phenotype, and, perhaps, the risk of transmitting the mutation to their offspring. These questions can only be answered by further long-term studies of additional patients.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank (for cytochrome *b* sequence in our patient [accession number AF254896])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for complex I subunit ND1 [MIM 516000]; ND5 [MIM 516005] and ND6 [MIM 516006] mutations; complex III deficiency [MIM 124000]; complex III cytochrome *b* mutation [MIM 516020]; COX deficiency [MIM 220110]; COX subunit I [MIM 516030], II [MIM 516040], and III 15BP DEL [MIM 516050.0003] mutations; Kearns-Sayre syndrome [MIM 530000]; LHON [MIM 535000]; MELAS [MIM 540000]; MERRF [MIM 545000]; mitochondrial myopathy [MIM 251900]; NARP [MIM 551500]; and $tRNA^{Glu}$ mutation [MIM 590025].

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