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An mtDNA Mutation in the Initiation Codon of the Cytochrome *C* Oxidase Subunit II Gene Results in Lower Levels of the Protein and a Mitochondrial Encephalomyopathy

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Summary

A novel heteroplasmic 7587T→C mutation in the mitochondrial genome which changes the initiation codon of the gene encoding cytochrome *c* oxidase subunit II (COX II), was found in a family with mitochondrial disease. This T→C transition is predicted to change the initiating methionine to threonine. The mutation load was present at 67% in muscle from the index case and at 91% in muscle from the patient's clinically affected son. Muscle biopsy samples revealed isolated COX deficiency and mitochondrial proliferation. Single-muscle-fiber analysis revealed that the 7587C copy was at much higher load in COX-negative fibers than in COX-positive fibers. After microphotometric enzyme analysis, the mutation was shown to cause a decrease in COX activity when the mutant load was >55%–65%. In fibroblasts from one family member, which contained >95% mutated mtDNA, there was no detectable synthesis or any steady-state level of COX II. This new mutation constitutes a new mechanism by which mtDNA mutations can cause disease-defective initiation of translation.

Introduction

Mitochondrial cytopathies are a heterogeneous group of clinical disorders affecting predominantly muscle and nerve but can cause a variety of different symptoms and signs (Johns 1995; Chinnery and Turnbull 1997; Schon et al. 1997). In many patients the genetic defect involves mtDNA and takes the form of rearrangements or point mutations (Wallace 1992; DiMauro and Moraes 1993). Point mutations may involve either RNA or protein-encoding genes, the former group being much more prevalent, with >40 separate mutations already identified (Johns 1995). Much less common are point mutations that alter the protein-coding genes, such as complex I–gene mutations that result in Leber hereditary optic neuropathy (LHON; Wallace et al. 1988; Howell et al. 1991*a*), or point mutations in the ATPase 6 gene that results in neurogenic muscular weakness, ataxia, and retinitis pigmentosa (Holt et al. 1990).

The site of an mtDNA mutation provides key information about the mechanism by which the mutation causes a biochemical defect. Pathogenic point mutations involving tRNA genes impair mitochondrial protein synthesis in a general fashion; those in protein-coding genes may affect the catalytic activity or stability of a respiratory-chain complex. In a family in which affected members have a mitochondrial encephalomyopathy, we have identified a mutation in the initiation codon of the cytochrome *c* oxidase subunit II (COX II) gene, in which a T→C substitution is predicted to change methionine to threonine. The mutation was shown to be present at 67% in muscle from the index case and at 91% in muscle and at 36% in blood from her son, who is more severely affected. We suggest that this mutation may represent a novel disease mechanism for mtDNA defects—the impairment of translation initiation of this integral subunit of COX.

Patients and Methods

Patients

Index case I-1, the mother of the family, is a 57-year-old woman of normal intellect with a 5–10-year history

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Table 1

Respiratory Chain Complex–Activity in Muscle Mitochondria

	Activity in Muscle Mitochondria[a]	
Complex	Control[b] ($n = 10$)	Patients
Complex I	229.3 ± 76.4	308.1
Complex II	351.5 ± 113.2	425.5
Complex III	1268.3 ± 355.5	1,330.0
Complex IV	1.11 ± .33	.42
Citrate synthase	907.8 ± 255.1	1,147.8

Note.—Values for control are mean and standard deviation of 10 control subjects.

[a] Activities and substrates are as follows: citrate synthase, activity expressed as mmol 5-thio-2-nitrobenzoate produced/min/mg mitochondrial protein; complex I, rotenone-sensitive NADH ubiquinone oxido-reductase activity expressed as NADH oxidized/min/mg mitochondrial protein; complex II, activity expressed as nmol 2,6-DCPIP (dichlorophenolindophenol) reduced/min/mg mitochondrial protein; and complex III and complex IV, activity expressed as an apparent first-order rate constant (s/mg mitochondrial protein) for COX reduction or oxidation, respectively.

[b] Data are mean ± SD.

of fatigue and unsteadiness of gait. There was no clinical evidence of retinal disease, deafness, muscle weakness, or cardiac disease. Case II-1 is a 30-year-old asymptomatic man. This patient declined any investigation. Case II-2 is a 34-year-old man who is severely affected. Although normal at birth and in early childhood, at age 5 years he developed progressive gait ataxia. This progressed so that he became wheelchair bound by age 25 years. Neuropsychometric testing showed that he was severely cognitively impaired. Clinical examination revealed bilateral optic atrophy, pigmentary retinopathy, a marked decrease in color vision, and mild distal-muscle wasting. Results of routine biochemical and hematologic evaluations were normal.

Respiratory Chain–Complex Analysis

A mitochondrial fraction was isolated from fresh muscle, obtained by needle biopsy from the index case. The activities of the individual respiratory-chain complexes and citrate synthase were determined spectrophotometrically as described elsewhere (Birch-Machin et al. 1994).

Histological and Histochemical Analysis

Transversely orientated blocks were frozen in isopentane and were cooled to −150°C with liquid nitrogen. Sections (10 μm) were cut for hematoxylin and eosin labeling. Histochemical studies were done on either 8-μm or 30-μm sections (for use in subsequent single-fiber PCR analysis). We assayed COX activity by using a medium containing 4 mM 3,3′-diaminobenzidine tetrahydrochloride and 100 μM cytochrome *c* in 0.1 mM phosphate (pH 7.0) at 25°C. Succinate dehydrogenase activity was assayed with 1.5 mM nitroblue tetrazolium, 130 mM sodium succinate, 0.2 mM phenazine methosulfate, and 0.1 mM sodium azide in 0.1 mM phosphate (pH 7.0) at 25°C. Sections were stained with the Gomori trichrome stain, as described by Engel and Cunningham (1963).

Microphotometric Enzyme Analysis

Microphotometric enzyme analysis was done on 8-μm sections as described elsewhere (Johnson et al. 1993); COX-deficient fibers were those with COX activity below the lower limit of the normal range for that particular fiber type (Johnson et al. 1993). Zero activity was defined as increase in absorbance (ΔA_{450}/s) in the presence of the COX inhibitor (sodium azide [2.5 mM]).

Myoblast and Fibroblast Culture

Myoblast cultures were obtained as described elsewhere (Clark et al. 1997). Fibroblast cultures were obtained by explant culture from skin biopsy samples. Myoblasts were grown in Hams F10 (Gibco BRL Life Technologies), supplemented with 20% FCS and 1% chick-embryo extract (ICN Flow Biomedicals). Fibroblasts were grown in EMEM (Sigma), supplemented with 10% FCS. Both growth media were supplemented with sodium pyruvate (110 μg/ml) and uridine (50 μg/ml), which are required for growth of respiration-deficient cells (King and Attardi 1989).

Isolation and Sequencing of mtDNA

Total DNA was isolated from muscle (frozen in liquid nitrogen and ground into a fine powder), leukocytes (iso-

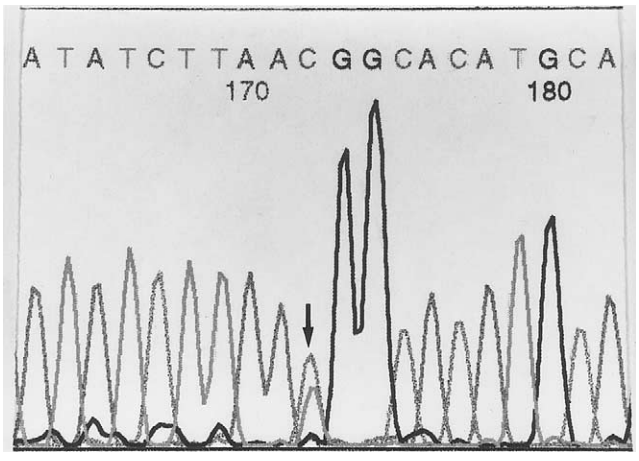

Figure 1 Chromatogram from fluorescent M13 sequencing. Sequence shows nt 7578–7597. An arrow represents the heteroplasmic T→C transition at position 7587.

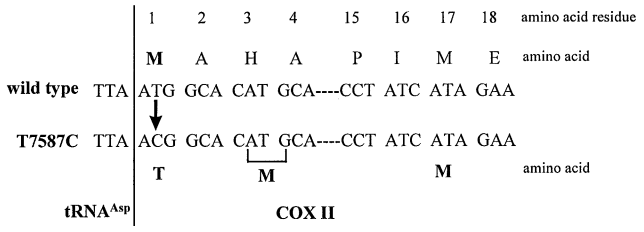

Figure 2 Schematic illustrating the position of the 7587C mutation within the mitochondrial genome, as well as the associated amino acid change. The initiation codon of COX II is changed to threonine. Initiation of translation could occur either at the next initiation site (AUG), which is "out of frame" and would yield a five-residue polypeptide, or at the next "in-frame" initiation site (AUA), 16 codons downstream.

lated by centrifugation of the plasma layer), and cultured cells. Muscle homogenate or cell pellets were resuspended in 10 mM Tris HCl (pH 8.0), 1 mM EDTA, 1% SDS, and 20 mg proteinase K/ml and were left at 37°C overnight. Standard phenol/chloroform extraction and ethanol precipitation were then done. We performed sequencing using M13-tailed primers and dye-primer sequencing with an automated DNA sequencer (model ABI 373; Perkin Elmer/ABI).

Quantification of Mutated mtDNA

Sections of 30 μm or 8 μm were dual stained for succinate dehydrogenase and COX activities. Single-fiber PCR was then done as described elsewhere (Sciacco et al. 1994). Either COX-positive (stained brown) or COX deficient (stained blue) fibers were isolated and placed in an individual microfuge tube containing 10 μl dH_2O. After centrifugation and removal of the water, each fiber was lysed by the addition of 10 μl 200 mM KOH, and 50 mM DTT and was heated to 65°C for 1 h. Ten microliters of neutralizing buffer (900 mM Tris-base, 200 mM HCl) were then added to each tube. PCR analysis was done directly with 10-μl samples.

Quantification of mutated mtDNA from blood, muscle, cultured cells, and individual muscle fibers was performed as follows: a 385-bp fragment was amplified with the forward mismatch primer (positions 7565–7585) 5′ GGCTAAATCCTATATATGTTA 3′ and the reverse primer (positions 7949–7931) 5′ TGTAGGAGTTGAAGATTAG 3′. PCR cycling conditions were 94°C for 5 min (1 cycle); 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min (30 cycles); and 72°C for 8 min (1 cycle). After further addition of 30 pmol of each primer, 1 U *Taq* polymerase, and 2.5 μCi $\alpha[^{32}P]$-dCTP (3,000 Ci/mmol; Amersham), the products were submitted to a single cycle of 94°C for 8 min, 50°C for 2 min, and 72°C for 12 min. Samples were then extracted with phenol/chloroform and diluted to 100 cpm/μl. Ten microliters were digested with 10 U *Hin*cII. Digests were electrophoresed through a 3% Metaphor agarose gel (FMC Bioproducts), dried onto a support, and analyzed on a PhosphorImager system with ImageQuant software (Molecular Dynamics). The mismatch primer creates an additional *Hin*cII site in the PCR product in the presence of the mutation. On digestion, wild-type mtDNA will cut only once, to produce 94-bp and 291-bp fragments. In the presence of the mutation, however, the 291-bp fragment is further cleaved, yielding fragments of 19 bp and 272 bp. Because the 19-bp fragment is lost from the gel, final calculations of mutant load were normalized for relative levels of cytosine in each visible fragment. The use of a mismatch primer is a widely used and accepted method of generating a mismatch when no suitable restriction enzyme is available. Results from a mismatch primer have been shown to be consistent with other methods of quantification (Shoffner et al. 1990).

Quantification of mutated mtDNA in muscle, blood, and fibroblasts of patient II-2 was done by fluorescent dUTP added to the last PCR cycle. The digested PCR products were then separated onto a 6% nondenaturing polyacrylamide gel and were analyzed with GeneScan software (Perkin Elmer/ABI) (Chalmers et al. 1997).

Mitochondrial Protein Translation

Protein translation was performed according to the method described by Chomyn (1996). Fibroblasts were grown in a 25-cm^2 flask until 80% confluent and incubated at 37°C for 2 h with 1 ml labeling medium (methionine/cysteine-free DMEM, 10% dialyzed FBS, 10 μg emetine/ml, and 500 μCi $[^{35}S]$-methionine/cysteine [3,000 Ci/mmol; Amersham]). After removal of the incubation medium, the cells were washed in PBS and a cell pellet was prepared. Protein samples containing 50,000 disintegrations per minute were separated onto a 12% polyacrylamide (Prosieve 50; Flowgen) SDS gel. Products were visualized with a PhosphorImager system (Molecular Dynamics).

Immunoblot Analysis of Cultured Skin–Fibroblast Protein

Total cellular protein (50 μg) was separated onto a 15% SDS polyacrylamide gel and was transferred to Immobilon polyvinylidene difluoride membrane (Millipore) by electroblotting, with use of a minitransfer system (Hoefer Scientific). The membrane was blocked with a 5% milk solution prior to addition of antibodies. Incubations with primary antibodies (monoclonal to COX II, polyclonal to COX subunit IV [COX IV], and crotonase) were done for 2 h at room temperature. Rabbit

Table 2

mtDNA Changes from the Index Case Compared with the Reference Sequence

Base Change	Gene [a]	State	Amino Acid Change	Previous Reports[b]
7028C→T	COX I	Homoplasmic	None	Previously identified polymorphism
14798T→C	cyt b	Homoplasmic	Phenylalanine to leucine	Previously identified polymorphism
10084T→C	ND3	Homoplasmic	Isoleucine to threonine	Not previously identified
9655G→A	COX III	Homoplasmic	Serine to asparagine	Not previously identified
7587T→C	COX II	Heteroplasmic	Methionine to threonine	Not previously identified

[a] ND = NADH ubiquinone oxido-reductase; cyt b = apo cytochrome-*b*.
[b] Previous identification was made on the basis of data obtained from the MITOMAP database.

antimouse IgG horseradish peroxidase (HRP) and goat antirabbit IgG HRP–conjugated secondary antibodies were used. The proteins were detected with an enhanced chemiluminescence kit (Amersham).

Northern Blot Analysis

We isolated total cytosolic RNA directly from 2–3 × 10^6 cells by using the total RNA isolation kit, Ultraspec RNA (Biotecx). RNA (18 μg) was separated onto a 1.2% agarose gel containing 2.5% formaldehyde and was transferred to GeneScreen-plus (NEN DuPont) membrane by standard capillary technique. Regions of mtDNA were amplified by PCR to produce probes for northern blots. We generated the COX II probe by amplifying a region spanning nucleotides (nt) 7405–8364, digesting the product with *Mbo*II, and extracting the 280-bp fragment from agarose gels. We generated the NADH ubiquinone oxido-reductase subunit 4 (ND4) probe by amplifying the region that spanned nt 11579–12419, cleaving it with *Spe*I, and extracting the 380-bp fragment from agarose gels. A 1.2-kb β-actin probe was generated as described by Gunning et al. (1983). Radiolabeling was performed by the random-primer method, supplemented with 100 μM of each nucleotide, 25 μCi[^{32}P]-dCTP (3,000 Ci/mmol; Amersham), and 1 U Klenow fragment (Boehringer-Mannheim). Unincorporated nucleotides were removed from the sample by gel filtration (Sepadex G-50 DNA grade; Pharmacia). All incubations were done in a rotating Hybaid tube (Hybaid). Prehybridization was done at 42°C for ⩾2 h by incubation of the membrane with hybridization solution (5 × SSPE, 50% formamide, 5 × Denhardt's solution, 1% SDS). A 1–2 × 10^6-cpm radiolabeled probe was used for each 10 ml of hybridization solution, which we denatured by boiling it for 2–5 min in a water bath before adding it directly to the hybridization solution. Hybridization was then done at 42°C overnight. Two 15-min washes were initially done at room temperature with 2 × SSPE, followed by a 15-min wash at 65°C with 2 × SSPE and 2% SDS. The blots subsequently were subjected to PhosphorImager analysis.

Results

Activity of Respiratory-Chain Complexes

COX complex IV activity was 35% of control values in the mitochondrial fraction from muscle from the index case. Activity of the other complexes was within the normal range (table 1).

Muscle Histochemistry

For the index case, ~l5% of fibers contained subsarcolemmal accumulation of mitochondria (ragged red fibers), as determined by intense succinate dehydrogenase reactivity at the periphery of the fiber. Microphotometric analysis revealed that only 30% of fibers had COX activity within the normal range, 30%–40% of fibers were COX negative (activity could not be distinguished from background levels), and the others had intermediate activity (i.e., were COX deficient). The clinically affected son was shown to have 80% COX-negative fibers, and, on Gomori trichrome staining, ~50% of fibers showed subsarcolemmal accumulation of mitochondria.

Detection of a Heteroplasmic T→C Mutation by Automated DNA Sequencing

No mtDNA rearrangement was observed on Southern blot analysis (data not shown). The presence of ragged red fibers and a mosaic of COX-positive and -negative fibers on histochemical analysis of the muscle were consistent with a tRNA mutation. On direct sequencing, however, no base change from the reference sequence (Anderson et al. 1981) was found in any of the 22 tRNA genes. Because of the compact nature of the mitochon-

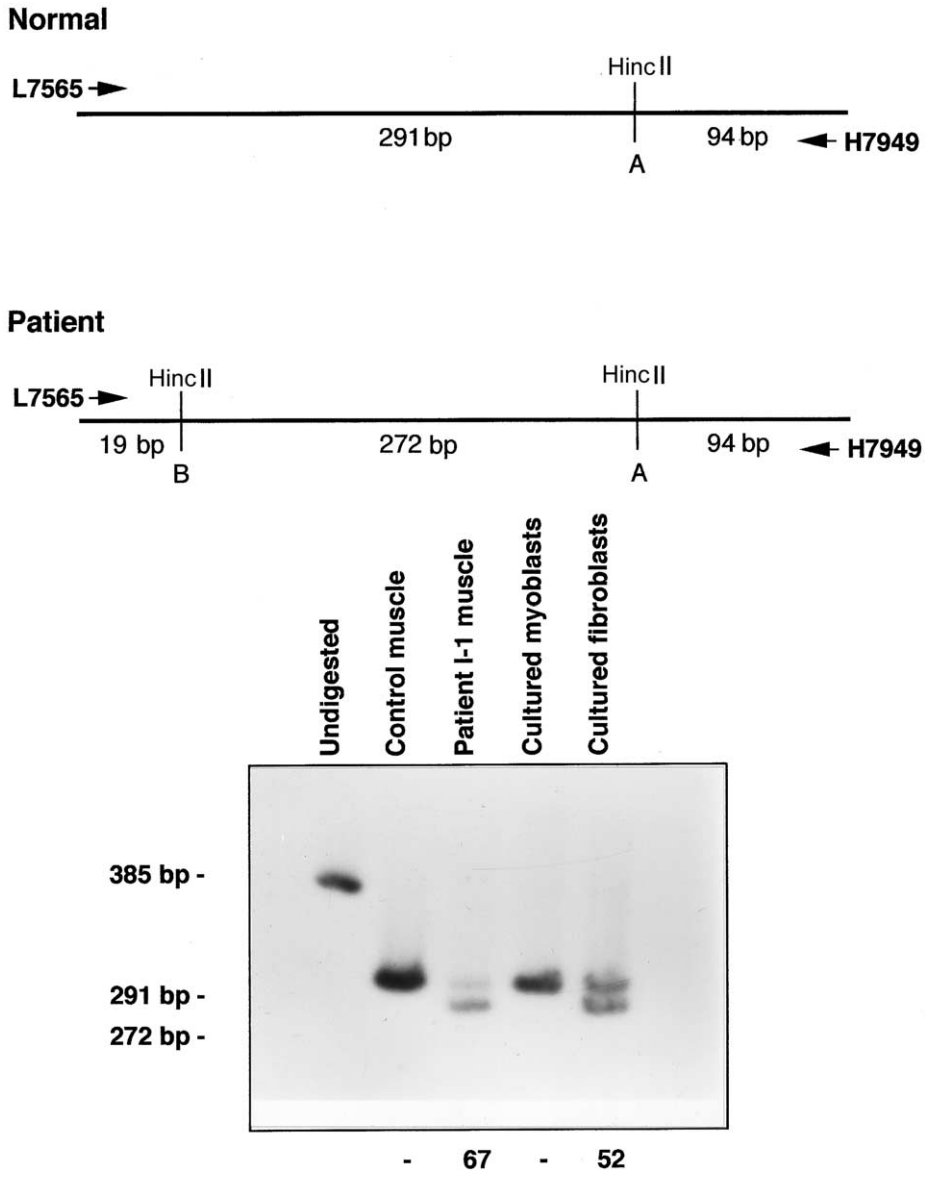

Figure 3 Quantification of the 7587C mutation in muscle, cultured fibroblasts, and cultured myoblasts from the index case. PCR and RFLP were done as described in Patients and Methods, and products were electrophoresed through a 3% Metaphor agarose gel. The levels of mutated mtDNA are represented as percentages of total mtDNA and are shown (*bottom*). To separate the 291-bp and 272-bp bands, it was necessary to run the 94-bp and 19-bp fragments off the bottom of the gel.

drial genome and the absence of introns, the protein-coding genes are not markedly separated from the tRNA/rRNA genes. Thus we obtained the sequence of the start of the COX II gene when analysis of the $tRNA^{Ser(UCN)}$ and $tRNA^{Asp}$ genes was undertaken. A heteroplasmic T→C transition was found at nt position 7587 (fig. 1), in the second base of the first codon of the gene encoding COX II (fig. 2). This mutation is predicted to change the initiating methionine to threonine. No other heteroplasmic base changes were found in this gene or in any of the other mitochondrial-encoded COX genes. Four homoplasmic base changes, compared with the standard sequence (Anderson et al. 1981)—14798T→C, 9655G→A, 7028C→T, and 10084T→C—were found within the protein-coding genes. The predicted outcome of these base changes is summarized in table 2.

Quantification of the 7587C Mutation in the Index Case and Her Affected Son

Although, on the basis of the sequence data, it appeared that the mutation was heteroplasmic, we wanted

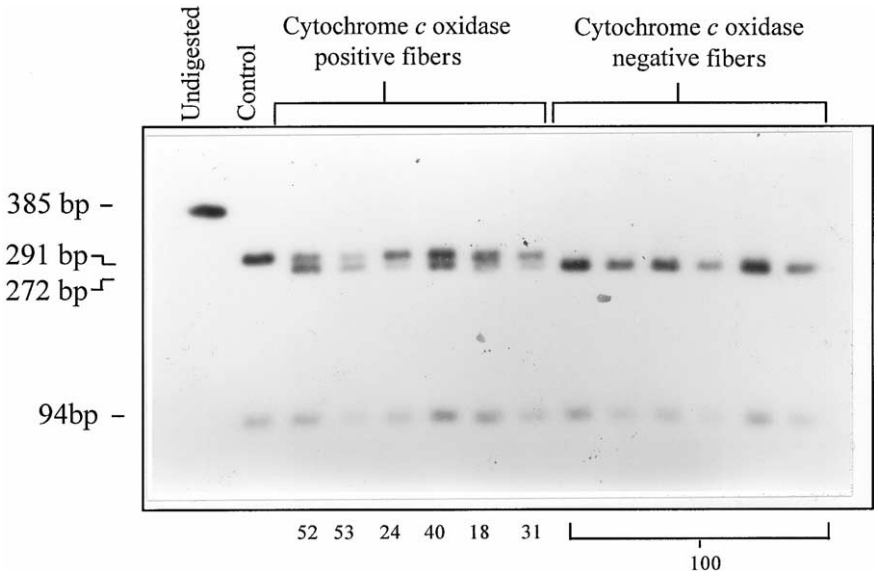

Figure 4 Distribution of the 7587C mutation in single fibers expressing either a COX-positive or -negative phenotype. The levels of mutated mtDNA are represented as percentages of total mtDNA and are shown underneath. To separate the 291-bp and 272-bp bands, it was necessary to run the 19-bp fragment off the bottom of the gel.

to determine the level of mutated and wild-type mtDNA. The level of the mutation was shown to be 67% in muscle from the index case and 52% in fibroblasts but was undetectable in blood and cultured myoblasts (fig. 3). Subsequent investigation of patient II-2 revealed the presence of the 7587C allele at 91% in muscle, 36% in blood, and 89% in cultured fibroblasts. The 7587C allele, however, increased to homoplasmic levels in fibroblasts after substantial culturing.

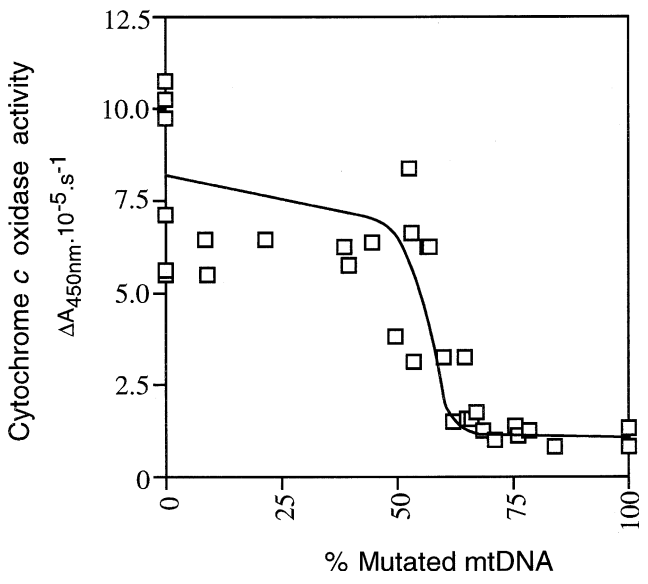

Figure 5 Threshold of the 7587C mutation. Microphotometric enzyme analysis and single-fiber PCR were performed on adjacent 8-μm serial sections as described in Patients and Methods. Values for COX activity are represented as $\Delta A_{450nm} \times 10^{-5}$/s.

Correlation of the Mutation with a Biochemical Defect

To investigate whether the 7587C mutation correlated with COX deficiency, COX-positive and -negative single fibers were isolated from a 30-μm section of muscle from the index case (I-1). In COX-positive fibers, 17%–52% of alleles were mutant; in COX-negative fibers, wild-type alleles were undetectable (fig. 4). The COX-negative fibers picked at this stage of investigation were the most intensely blue stained (ragged red and non–ragged red fibers) and thus the most severely affected biochemically.

Determination of the Biochemical Threshold for the 7587C Mutation

Because it is possible that the 7587C allele results in a novel disease mechanism, we wanted to investigate whether there was either a relationship between mutant load and biochemical activity or a threshold. To determine the level at which the 7587C mutation induced a biochemical defect, COX activity was measured and single-fiber PCR was performed on serial 8-μm muscle sections. COX activity was severely impaired when 55%–65% of the mtDNA was mutated (fig. 5).

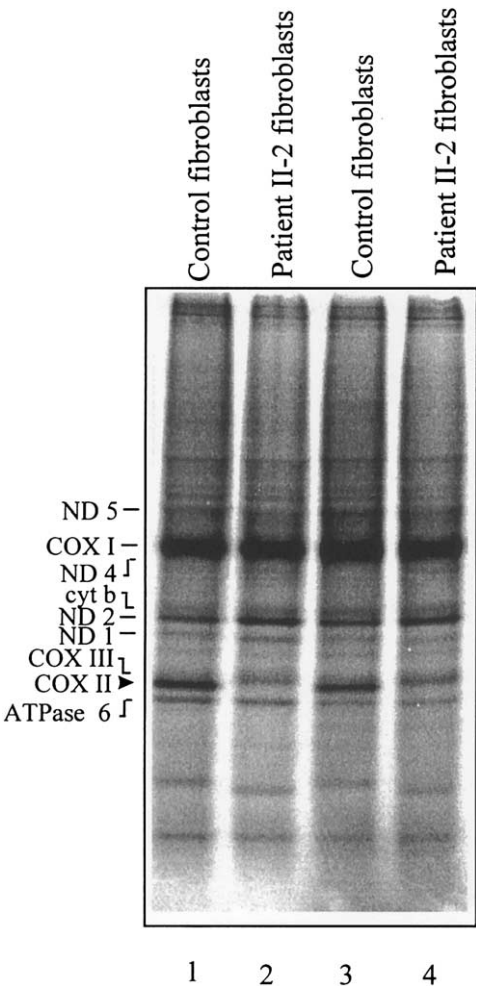

Figure 6 Mitochondrial protein synthesis in fibroblasts. Fibroblasts were cultured in the presence of 10 μg emetine/ml and were pulse labeled for 30 min (*lanes 1 and 2*) or 2 h (*lanes 3 and 4*) with 500 μCi ^{35}S methionine/cysteine. Products were separated through a 1.2% polyacrylamide gel as described in Patients and Methods. Products were labeled according to molecular weight and findings from products from previously reported in vivo translation assays (Chomyn 1996). ND = NADH ubiquinone oxido-reductase; cyt b = apo cytochrome-*b*; and ATPase = ATP synthase.

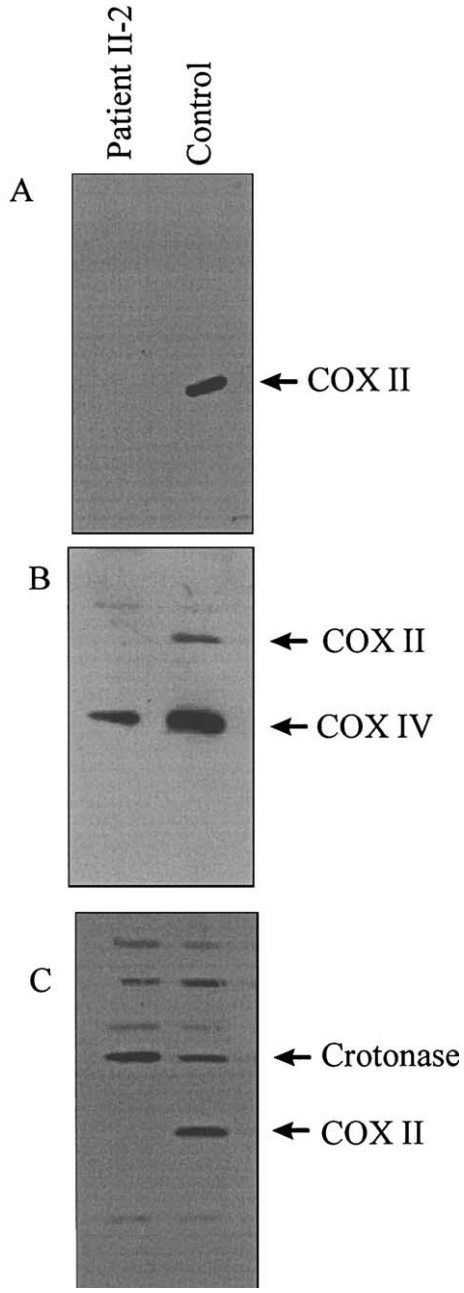

Figure 7 Immunoblotting of whole-cell lysate. Equal amounts (15 μg) of protein were subjected to SDS PAGE and were blotted onto a polyvinylidene difluoride membrane as described in Patients and Methods. *A*, Incubation with a monoclonal COX II antibody, demonstrating absence of COX II in patient II-2 fibroblasts. *B*, Incubation with a polyclonal antibody to COX IV. Patient II-2 fibroblasts contain half the amount of COX IV, compared with that in the control. *C*, Incubation with a polyclonal antibody to crotonase, demonstrating that the results shown in *A* are not due to a variation in protein loading. The more intense signal obtained from patient II-2 fibroblasts suggests that there is either up-regulation of crotonase or slightly more protein in this lane compared with that in the control fibroblasts. High-molecular-weight species represent nonspecific proteins recognized by the crotonase antisera. Molecular-weight markers were included on the gel to check product sizes.

Mitochondrial Translation Products

Two methods were employed to investigate the effect of the 7587C mutation on expression of the COX II protein. For these studies, we used fibroblasts from patient II-2 that had, on continued culture, become virtually homoplasmic for mutated mtDNA. Translation of mitochondrial-encoded proteins demonstrated that no full-length COX II protein was present (fig. 6). Levels of all other translation products were similar in patient and control fibroblasts. Immunoblotting with a monoclonal antibody to the COX II did not detect this subunit in fibroblasts from patient II-2 (fig. 7*A*). Immunoblotting

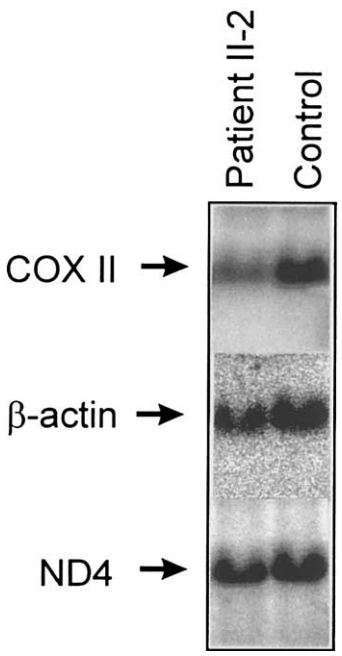

Figure 8 Northern blot hybridization analysis. Equal amounts (18 μg) of RNA were separated onto a 1.2% agarose gel containing 2.5% formaldehyde and were transferred to a nitrocellulose membrane, and mitochondrial and nuclear probes were constructed and radiolabeled as described in Patients and Methods. Hybridization was done with a 280-bp COX II probe, a 1.2-kb β-actin probe, and a 380-bp ND4-specific probe. The steady-state level of COX II mRNA in patient II-2 is one-third of that in controls.

with an anti–COX IV antibody demonstrated that half the amount of this subunit is present in patient II-2 fibroblasts, compared with control fibroblasts (fig. 7*B*).

Determination of Steady-State mRNA Levels

Northern blot hybridization with specific probes to transcripts encoding COX II, ND4, and β-actin was used to determine the steady-state levels of the respective mRNAs in patient II-2's fibroblasts (fig. 8). Using β-actin as a guide, we determined that there was slightly more mRNA present in the control lane than in the patient lane (fig. 8). The ratio of ND4 transcript to β-actin was the same in the two cell lines, whereas there was a 2.3-fold decrease in the level of COX II mRNA (fig. 8). This result was confirmed by RNA prepared from cells grown on two separate occasions.

Discussion

We have described a family with an mtDNA mutation within the start codon of the gene encoding COX II. The mother sought medical opinion because she was aware that mitochondrial disease may be transmitted maternally and because of concern about her unaffected son. She was not aware of her own neurological difficulties at the time of her presentation. The family demonstrates the extreme variability in symptoms seen in patients with heteroplasmic mtDNA mutations. In addition, it confirms the difficulty in giving genetic advice to women with heteroplasmic mtDNA point mutations. Our patient has one child who developed severe neurological symptoms starting at age 5 years, whereas the other son is clinically unaffected at age 30 years. We suggest that this mutation is pathological for the following reasons: (1) it is heteroplasmic; (2) it results in an animo acid change at a functionally important site; (3) it segregates with a biochemical defect; and (4) the molecular nature of the mutation is consistent with the findings of biochemical, histochemical, protein translation, and mRNA analysis, in which translation initiation is impaired.

We sequenced the majority of the mitochondrial genome from the index case and found a number of other changes, compared with the Cambridge reference sequence (Anderson et al. 1981). 7028C/T (Torroni et al. 1996) and 14798T/C (Brown et al. 1992) are recognized polymorphisms. A previously unreported homoplasmic change was detected in ND3 (10084T→C), which is predicted to alter an amino acid but could not account for the biochemical defect. The base change in the gene encoding COX subunit III (COX III; 9655G→A) has not been reported. This mutation is more difficult to explain, because it is not only predicted to change an amino acid, but it also involves a conserved residue. The base change, however, is homoplasmic in blood and muscle, and, although this certainly does not exclude pathogenicity, it makes it less likely. A homoplasmic change could not explain the muscle-histochemistry findings with the presence of COX-positive and -negative fibers. The correlation of the 7587C mutant load with the loss of COX activity in individual muscle fibers is very important in the determination of whether a mutation is pathogenic.

The presence of the 9655A mutation calls attention to two issues. First, it stresses how difficult it is to define the pathogenic basis of mtDNA mutations, even if they apparently occur at important sites. The criteria that can be used to confirm pathogenicity are evidence of segregation of the genetic defect with disease within a pedigree, such as in the case of LHON (Wallace et al. 1988; Howell et al. 1991*a*; Johns et al. 1992), or correlation with a biochemical defect, such as in the case of COX-negative fibers (Weber et al. 1996). Second, others have suggested that secondary mutations may influence the expression of a primary mutation (Howell et al. 1991*b;* Torroni et al. 1997). It seems unlikely in this case that 9655A could be influencing the expression of 7587C, because the latter mutation occurs in the initiation codon of an open reading frame; however, this possibility cannot be discarded.

We have been unable to detect mature COX II polypeptide in patient II-2 fibroblasts with a near-homoplasmic level of the 7587C mutation. The implication for the absence of a start codon at this position is either that translation begins at another initiation site or that no translation occurs at all. If translation initiates from the next initiation site, a short polypeptide of five residues is predicted to be generated. If translation initiates at the next "in-frame" initiation site, a truncated polypeptide lacking the N-terminal 16 residues would be produced. Since the epitope recognized by the COX II antibody has not been mapped, it is a formal possibility that the absence of reactivity on the western blot could be a result of the epitope being within this N-terminal sequence. No truncated protein was detected, either by immunoblotting or by comparison of total mitochondrial protein synthesis in control versus patient cells. Therefore, we believe that the results are consistent with the absence of translation of COX II mRNA carrying the 7587C mutation in the initiation codon.

COX II mRNA levels were shown to be substantially lower in patient II-2 fibroblasts. In the absence of translation, the mutated mRNA species will presumably be vulnerable to degradation. We therefore propose that the inability to translate the COX II transcript at the correct initiation codon results in more-rapid degradation of mRNA.

An alternative disease mechanism for the T7857C mutation is impaired cleavage of the $tRNA^{Asp}$. This would result in a relative tRNA deficiency and thus impair the translation of all mitochondrial-encoded proteins. For the reasons highlighted above, and since the activity of complex I and complex III was normal in the index case, we think that this is highly unlikely.

Measurement of the threshold for specific mtDNA mutations has been performed in cultured cells elsewhere (Boulet et al. 1992; Bentlage and Attardi 1996; Villani and Attardi 1997). The microphotometric enzyme–assay system for measurement of COX activity within individual muscle fibers (Johnson et al. 1993) is used in conjunction with demonstration of myofibrillar ATPase in serial sections, so that a normal range of COX activity in individual muscle fibers of each fiber type is defined in control samples. In previous studies, a good correlation between microphotometric enzyme analysis and biochemical studies has been shown in patients with Leigh syndrome (Johnson et al. 1993). We measured COX activity and performed single-fiber PCR on adjacent muscle sections to determine the biochemical threshold (the percentage of mutated mtDNA required to cause a biochemical defect) for the 7587C mutation. Analysis of affected tissue will reflect the situation in vivo. The relative level of mutated versus wild-type mtDNA required to produce a COX-deficient phenotype was 55%–65%. No fiber that had a high mutant load demonstrated normal activity; this was also shown in the initial single-fiber analysis, in which the highest level of mutated mtDNA found in COX-positive fibers was 52%. The threshold phenomenon is common in heteroplasmic mtDNA mutations, with the level of mutated mtDNA required to induce a biochemical deficit varying for different mutations. For tRNA mutations, this level is usually >85% (Bentlage and Attardi 1996), because the mutated tRNA is partially active. Enriquez et al. (1995) have reported that cybrids homoplasmic for the 8344G mutation still have aminoacylated $tRNA^{Lys}$ molecules, albeit at reduced levels. Thus, partial activity is believed to explain the remarkably recessive nature of tRNA mutations. The newly reported 7587C mutation appears to prevent translation of COX II, ruling out the possibility of generation of a partially active product and possibly explaining why this biochemical threshold occurs at lower mutant loads.

It is not known exactly how COX is assembled, but there is evidence that mitochondrial-encoded subunits II and III are involved initially (Wielburski et al. 1982; Wielburski and Nelson 1983). It is therefore possible that a critical level of these subunits is required before any holoenzyme is assembled. This would account for the absence of COX activity, even in the presence of 35%–45% wild-type mtDNA.

To conclude, we have identified a novel pathogenic mtDNA point mutation in the initiation codon of COX II. We have demonstrated that this mutation results in an inability to express COX II protein, causing a specific decrease in COX activity.

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Electronic-Database Information

URL for data in this article is as follows:

MITOMAP, http://www.gen.emory.edu/MITOMAP (for polymorphism identification)

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