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Mutations in *COX10* result in a defect in mitochondrial heme A biosynthesis and account for multiple, early-onset clinical phenotypes associated with isolated COX deficiency

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Deficiencies in the activity of cytochrome *c* oxidase (COX) are an important cause of autosomal recessive respiratory chain disorders. Patients with isolated COX deficiency are clinically and genetically heterogeneous, and mutations in several different assembly factors have been found to cause specific clinical phenotypes. Two of the most common clinical presentations, Leigh Syndrome and hypertrophic cardiomyopathy, have so far only been associated with mutations in *SURF1* or *SCO2* and *COX15*, respectively. Here we show that expression of *COX10* from a retroviral vector complements the COX deficiency in a patient with anemia and Leigh Syndrome, and in a patient with anemia, sensorineural deafness and fatal infantile hypertrophic cardiomyopathy. A partial rescue was also obtained following microcell-mediated transfer of mouse chromosomes into patient fibroblasts. COX10 functions in the first step of the mitochondrial heme A biosynthetic pathway, catalyzing the conversion of protoheme (heme B) to heme O via the farnesylation of a vinyl group at position C2. Heme A content was reduced in mitochondria from patient muscle and fibroblasts in proportion to the reduction in COX enzyme activity and the amount of fully assembled enzyme. Mutation analysis of *COX10* identified four different missense alleles, predicting amino acid substitutions at evolutionarily conserved residues. A topological model places these residues in regions of the protein shown to have important catalytic functions by mutation analysis of a prokaryotic ortholog. Mutations in *COX10* have previously been reported in a single family with tubulopathy and leukodystrophy. This study shows that mutations in this gene can cause nearly the full range of clinical phenotypes associated with early onset isolated COX deficiency.

INTRODUCTION

Cytochrome *c* oxidase (COX) deficiency is one of the most common metabolic diagnoses associated with respiratory chain defects in humans. Patients with COX deficiencies can present with a broad and heterogeneous range of clinical phenotypes, including Leigh syndrome, a French Canadian form of Leigh syndrome, fatal infantile hypertrophic cardiomyopathy and myopathy, fatal infantile lactic acidosis and reversible COX deficiency in skeletal muscle (1–4).

COX, the terminal enzyme of the electron transport chain, is embedded in the inner mitochondrial membrane where it catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen. In mammals, it is a multimeric protein

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composed of 13 subunits, three of which form the catalytic core of the enzyme and are encoded in mitochondrial DNA (subunits I–III). Highly conserved domains within subunits I and II contain two copper binding sites (Cu_A and Cu_B) and two heme moieties (heme a and a_3) essential to catalytic function (5,6). The remaining 10 less-conserved subunits are of nuclear origin, and are believed to confer structural stability and modulate enzyme activity. Additional, nuclear-encoded proteins are also required for the maturation and assembly of individual subunits into a functional holoenzyme complex (7). While these ancillary proteins themselves do not form part of the COX complex, they function at different stages of holoenzyme biogenesis, including transcription, translation, membrane insertion of structural subunits, as well as the synthesis, chaperoning and addition of the prosthetic groups (4). The total number of proteins required for the assembly process remains unknown; however, more than 30 different genetic complementation groups for COX assembly have been identified in the yeast *Saccharomyces cerevisiae* (8,9). At least 20 of these have been assigned to accessory proteins (4), and more than half of these have known human orthologs.

Although mutations in the three mtDNA-encoded COX subunits have been reported in several patients in association with a number of different, mostly encephalomyopathic, clinical phenotypes (2), pedigree studies suggest that the majority of gene defects associated with fatal infantile COX deficiency are of nuclear origin and inherited as autosomal recessive traits (10). Despite the concerted efforts of a number of groups (11,12), mutations in the nuclear-encoded COX subunits have not been found. In contrast, defects in six nuclear genes whose protein products are critical to COX biogenesis have been identified. Although all of these genes are ubiquitously expressed, each has so far been associated with a particular clinical phenotype: *SURF1* [Leigh syndrome (13,14)], *SCO2* and *COX15* [fatal infantile hypertrophic cardiomyopathy (10,15,16)], *SCO1* [ketoacidotic coma and hepatopathy (17)], *COX10* [tubulopathy and leukodystrophy (18)] and *LRPPRC* [French-Canadian Leigh syndrome (19)]. The molecular basis for the tissue-specificity of the observed clinical phenotypes remains unknown.

COX10, an assembly factor essential for the biogenesis of COX that was initially identified in yeast (20), plays a critical role in the mitochondrial heme biosynthetic pathway by catalyzing the conversion of protoheme (heme B) to heme O, via the farnesylation of a vinyl group at position C2. Heme O is in turn converted to heme A, one of the prosthetic groups critical to COX function, by Cox15p, ferredoxin, ferredoxin reductase (21–23), and possibly an as yet uncharacterized enzyme (24). Here, we report pathogenic mutations in *COX10* in two patients with isolated COX deficiency, one presenting with anemia and classical Leigh syndrome, and the other with anemia, sensorineural hearing loss and fatal infantile hypertrophic cardiomyopathy. Mutations in *COX10* have previously been reported to cause tubulopathy and leukodystrophy, based on results from a single family (18). We show that overexpression of *COX10* increases the synthesis of heme A, which, in turn, influences the assembly of the COX complex and rescues COX activity. These data demonstrate that mutations in a single COX assembly gene can result in multiple clinical phenotypes.

RESULTS

Clinical presentation and characterization of the COX defect in patients D and S

Patient D, a white male infant born at term, presented in the first week of life with hypoglycemia, metabolic acidosis, hypotonia and poor feeding. Metabolic laboratory evaluation showed persistent lactic acidosis, elevated plasma alanine and proline, and no evidence of an organic acidemia. Although the acidosis was initially controlled on oral citrate therapy, poor feeding behavior and poor weight gain persisted. Profound sensorineural hearing loss was demonstrated by brainstem evoked response testing at 2 months age. Severe transfusion-dependent macrocytic anemia developed by 3 months. He was hospitalized recurrently with acute episodes of dehydration, increased acidosis and hypotension complicated by anemia. Cardiovascular evaluation including ECG and echocardiogram revealed severe biventricular hypertrophic cardiomyopathy at age 4 months. Diagnostic muscle biopsy showed normal structural histology but severely reduced or absent COX staining in most fibers. COX activity measured in mitochondria isolated from frozen muscle was less than 5% of control values; all other respiratory chain activities were normal. The infant died at 5 months of age.

In-gel activity assays and immunoblot analysis of muscle mitochondria from patient D showed virtually undetectable levels of fully assembled COX (Fig. 1), in agreement with the very low COX activity measured in muscle mitochondria (Table 2). Immunoblot analysis with antibodies against COX subunit I (Fig. 1) and COX subunit IV (data not shown) did not, however, show any COX subcomplexes, such as those typically found in patients with *SURF1* mutations. The levels of other respiratory enzymes, measured as the amount of immuno-detectable holoenzyme complex, were normal, as was the in-gel activity of complex I. COX activity was decreased to 40% of control in patient D fibroblasts (Table 2), consistent with the decreased amount of the fully assembled holoenzyme observed by BN-PAGE analysis (Fig. 4).

Patient S was the third child of healthy, non-consanguinous German parents. Two siblings are healthy. Her development stopped suddenly after one and a half months and she presented with hypotrophy, transfusion-dependent anemia, slight splenomegaly, severe hypotonia and high lactate/pyruvate levels in both blood and CSF. MRI analysis showed severe symmetrical lesions in the putamen and pallidum typical of Leigh syndrome. She died at the age of 4 months due to a central respiratory failure. COX activity in a muscle biopsy was decreased to 16% of the lowest control values, while the other respiratory enzymes activities were normal. COX activity in patient fibroblasts was 18% of control levels (Table 2). To characterize the assembly defect of COX in this patient we analyzed patient fibroblasts using two-dimensional BN-PAGE (Fig. 2). Antibodies against COX subunit IV and COX subunit I showed a marked decrease in the amount of fully assembled COX complex (about 15% of control); however no COX subcomplexes could be detected. COX subunit II was practically undetectable in the fully assembled complex (Fig. 2); over-exposure of the blot showed marginal levels of this subunit (<5% of control; data not shown).

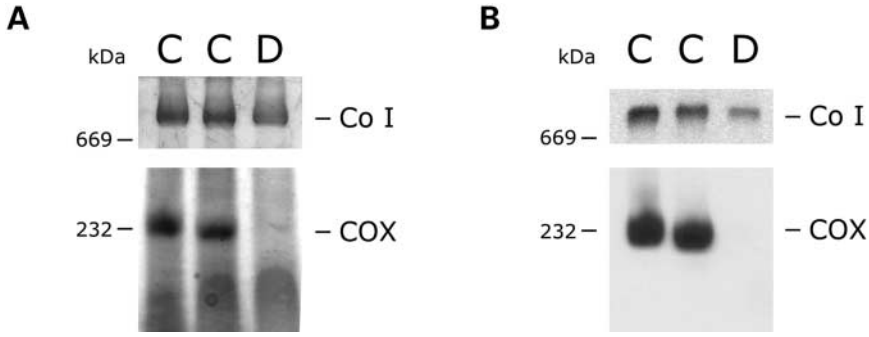

Figure 1. Blue-native PAGE analysis of COX levels in patient D muscle mitochondria. Muscle mitochondria (10 μg protein) from two controls (C) and patient D were separated by BN-PAGE and the in-gel activity (**A**) and the content (**B**) of COX and Complex I (Co I) were determined. Antibodies against COX subunit I and the ND1 subunit of complex I were used for the immuno-blot in (B). The patient muscle mitochondria show a severe reduction in the total amount of fully assembled COX. The migration of molecular mass standards is indicated on the left.

Strategies for complementation cloning of the molecular defect

It is generally not possible to use linkage analysis for the identification of the genetic defect in patients with early-onset autosomal recessive respiratory chain disorders because of small family size and the large amount of molecular heterogeneity associated with the same biochemical deficiency. We have previously used two different functional complementation strategies in patient cell lines to circumvent this problem: microcell-mediated chromosome transfer of normal human chromosomes (10,14), and expression of candidate cDNAs from retroviral vectors (16). We reasoned that the chromosome transfer method might be made more efficient if mouse chromosomes were used instead of human chromosomes, as all chromosomes transferred in this case would be informative. To test this idea we randomly tagged chromosomes in mouse A9 cells with a retroviral vector expressing the hygromycin resistance gene as a source of donor chromosomes. In a parallel experiment we created retroviral expression vectors for the human orthologues of 10 COX assembly factors identified in yeast, and tested these individually as candidate genes in 20 different fibroblast cell lines from patients with isolated COX deficiency.

Complementation of the COX defect by microcell-mediated transfer of mouse chromosomes

The hygromycin-tagged mouse A9 cell line was micronucleated, and the resulting micronuclei were fused with fibroblasts from patient S. Of the 48 hygromycin-resistant clones that were recovered in this experiment, 23 were analyzed biochemically and genotyped using microsatellite markers 7–9 weeks after the monochromosomal transfer. COX activity recovered to 44% of control in one of these clones (Table 2). BN–PAGE and 2D–PAGE showed a significant increase in the amount of the immunodetectable COX complex in this clone (Fig. 3), indicating a partial rescue of the defect. Non-rescued clones had a similar or lower COX activity and content than the original patient cells (Fig. 3). Microsatellite analysis showed that 13 mouse chromosomes were present in the rescued clone (chromosomes 3, 5, 8, 9, 10, 11, 12, 14, 15, 16, 17, 18 and 19). Analysis of this clone following an additional 4 weeks in culture showed a loss of parts of chromosomes 8 (14–23 cM), 11 (20–43 cM), 12 (29–55 cM), 15 (15–48.5 cM), 17 (18.7–29.5 cM), and 19 (24–51 cM), and loss of the phenotypic rescue (data not shown). Several known COX assembly factors are present in the chromosomal regions lost in this experiment: *COX10* and *SCO1* on chromosome 11; *COX15* on chromosome 19; and *COX16* on chromsome 12. Analysis of the mouse *COX10 (mCOX10)* gene using two different primer pairs (Table 1) showed a perfect correlation between the presence of the gene and rescue of the COX phenotype, both in the clone in which complementation was lost on extended growth, and in all other non-complementing clones.

Table 1. COX10 primers

Species	Name	5′–3′ sequence
Human	Full-length forward Gateway compatible	aaaagcaggctaccatggccgcatctccgcacactctc
	Full-length reverse Gateway compatible	agaaagctgggttcagctgggaggggggccctggat
	Internal forward	gttccgggagttgccattctg
	Internal reverse 1	caaccacagctccgacccatg
	Internal reverse 2	caaagataggctgggcggtgag
Mouse	Full-length forward Gateway compatible	aaaaagcaggctaccatggccgcgtccccgcacac
	Full-length reverse Gateway compatible	agaaagctgggttcagctcggagcctctcccttg
	Exon 1 forward	gagctcacctagtctacatctgga
	Exon 1 reverse	catcttgctagcccgccctctga
	Exon 7 forward	ggtcttccctgtcatctcc
	Exon 7 reverse	agggcagtgctgtccgtctg

Overexpression of COX assembly factors

COX activity was restored to control levels when immortalized fibroblasts from patients S and D were transduced with a retroviral vector overexpressing the *COX10* cDNA (Table 2). Constructs expressing other COX assembly factors (*SURF1*, *SCO1*, *SCO2*, *COX17*, *COX11*, *COX15.1*, *COX18*, *OXA1* or

Table 2. COX activities and levels of heme A/B in patient skeletal muscle and fibroblasts. Mitochondrial heme content, COX and citrate synthase (CS) activities were determined as described in Materials and methods. The numbers in parentheses indicate the number of independent measurements

	Patient D				Patient S			
	Muscle	Fibroblasts			Fibroblasts			
			+*COX10*	+*mCOX10*		+*COX10*	+*mCOX10*	+A9
COX/CS	4.1	40.0 ± 2.1	109.6	51.9	17.9 ± 5.4	99.9 ± 24.6	48.6	44.0
(% control)		($n = 10$)	($n = 2$)	($n = 2$)	($n = 34$)	($n = 4$)	($n = 2$)	($n = 2$)
heme A/B	6.6	47.6	107.2	ND	20.4	80.5	ND	ND
(% control)								

+*COX10*, cells overexpressing *COX10* cDNA; +*mCOX10*, cells overexpressing mouse *COX10* cDNA; +A9, rescuing A9/patient S clone. ND, not determined.

PET191) failed to complement the defect in either patient. The normalization of COX activity was accompanied by an increase in the amount of the COX holoenzyme complex (Fig. 4), showing that rescue of the assembly defect was responsible for the increased enzyme activity. Overexpression of *mCOX10* in patient fibroblasts increased the COX activity to about 50% of control (Table 2), in agreement with a partial rescue of the defect seen after microcell-mediated transfer of mouse chromosomes.

Heme A biosynthesis

Yeast Cox10p is known to be involved in the biosynthesis of heme A, the heme prosthetic group associated with COX subunit I, and a mutation in human *COX10* has previously been shown to result in an absence of spectroscopically detectable cytochrome aa_3 in a patient with tubulopathy and leukodystrophy (18). To evaluate the biosynthesis of heme A in our patients, we isolated total heme from muscle and fibroblast mitochondria and analyzed it by HPLC. Muscle mitochondria of patient D contained 7% of control levels of heme A (Fig. 5, Table 2), consistent with the decrease in COX activity and holoenzyme protein levels. COX10 catalyzes conversion of protoheme into heme O, which is very rapidly converted into heme A by a three-component mono-oxygenase. No heme O could be detected in patient muscle mitochondria, suggesting that the second step of the heme A biosynthesis functions normally.

The low COX activity in patient D and S fibroblasts was also paralleled by reduced levels of heme A in these samples. The heme A content was decreased to 48 and 20% in patients D and S fibroblasts, respectively (Table 2). Overexpression of *COX10* in patient fibroblasts increased the heme A levels to near control levels (107% in D and 81% S fibroblasts), indicating that over-expression of *COX10* corrects the observed defect in the heme A biosynthetic pathway.

Mutation analysis of the COX10 gene

We amplified and sequenced the 1332 bp RT–PCR cDNA, and all seven exons of the *COX10* gene in fibroblasts from patients D and S to search for mutations in the *COX10* gene. DNA sequence analysis showed two heterozygous missense mutations in patient D fibroblasts; a C791A transversion in exon 4, and a C878T transition in exon 5, predicting amino acid substitutions at T196K and P225L, both evolutionarily conserved positions in the protein. The presence of the mutations was confirmed by restriction endonuclease digestion with *BsmF*I and *MspA1*I (Fig. 6). The patient's mother was heterozygous for the C791A mutation, and the father was heterozygous for the C878T mutation. Patient D was also found to be homozygous for previously identified SNPs at G708A, A903G, and heterozygous for G680A.

Patient S was heterozygous for two missense mutations in exon 7, an A1211T transversion and an A1211G transition, predicting two different amino acid substitutions at the same evolutionarily conserved site in the protein: D336V and D336G (Fig. 6). Endonuclease restriction analysis with *Acc*I, which detects the A1211T transversion, showed that both patient S and her mother were heterozygous for this mutation, while the father and two unaffected siblings were homozygous for the wild-type allele. Endonuclease digestion with *Bbs*I, which detects both the A1211T and the A1211G transition, indicated that patient S appeared homozygous for the two mutations, while father, mother and sister appeared heterozygous for these two mutations. Taken together, these data show that patient S is heterozygous for both mutations, the patient's father and sister are heterozygous for A1211G, the mother is heterozygous for A1211T, and the brother is homozygous for the wild-type alleles. The mutations present in the two index patients were absent in 50 control individuals tested by RFLP analysis.

DISCUSSION

This study establishes that mutations in a single COX assembly gene, *COX10*, cause isolated COX deficiency associated with multiple clinical phenotypes. In addition to the previously characterized tubulopathy and leukodystrophy (18), two further clinical presentations resulting from mutations in *COX10* were identified: Leigh syndrome and fatal infantile hypertrophic cardiomyopathy. Both patients also presented with transfusion-dependent anemia.

The underlying genetic defect in both patients was identified by functional complementation using a panel of retroviral vectors expressing individual human COX assembly genes (16). COX activity in cultured fibroblasts derived from patients D and S was specifically restored by overexpression of a cDNA coding for *COX10*. Overexpression of cDNAs of other COX assembly factors, including *COX15.1*, which is also involved in mitochondrial heme biosynthesis, failed to complement the COX deficiency. Moreover, transduction of fibroblast lines

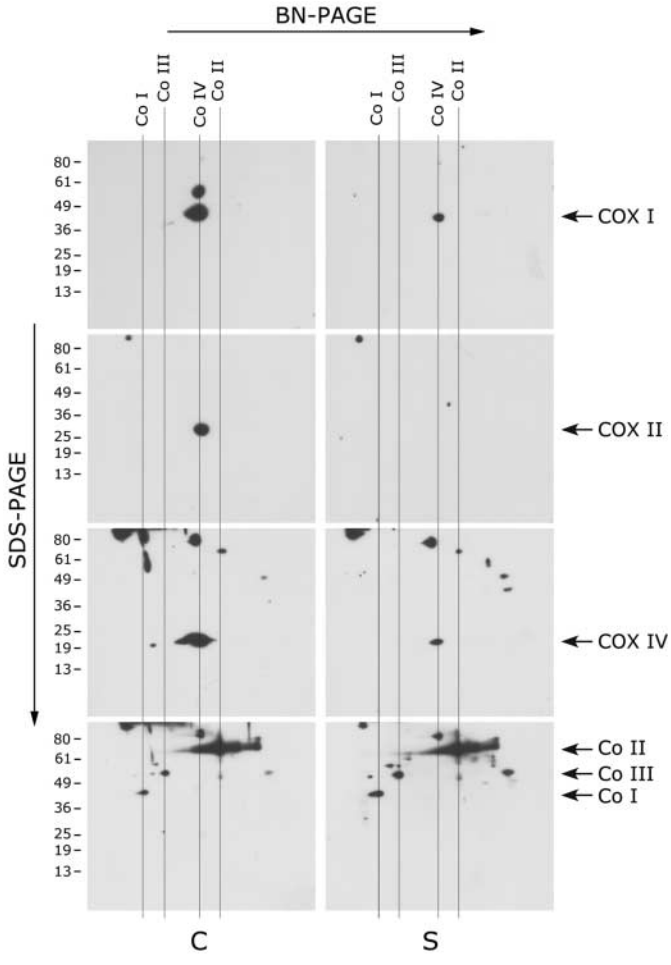

Figure 2. COX assembly defect in patient S fibroblasts. Mitoplasts (20 μg protein) isolated from patient S and control (C) fibroblasts were separated using 2D-BN/SDS–PAGE. Immunoblot analysis was performed using antibodies directed against COX subunits I, II and IV, against the 39 kDa subunit of complex I (Co I), the 70 kDa subunit of complex II (CO II) and the Core 1 protein of complex III (Co III). The levels of COX subunits were markedly decreased in patient S fibroblasts, however no subcomplexes were present. The migration of molecular mass standards (in kDa) is indicated on the left.

from *SURF1* and *SCO2* patients, or from COX-deficient patients with unknown genetic defects, with *COX10* was unable to restore residual COX activity to wild-type levels. These observations indicate that little if any functional redundancy exists among the COX assembly factors investigated in this study, and strongly suggest that mutations in *COX10* account for the observed COX deficiency in patients D and S.

Additional support for a *COX10* gene defect was obtained from microcell-mediated transfer of mouse chromosomes in patient S fibroblasts, and by expression of *mCOX10*. The incomplete rescue of the human phenotype by the mouse ortholog is somewhat surprising, given the evolutionary conservation of these assembly factors, and the fact that the mouse and human *COX10* cDNAs are 81% identical and 87% similar. Several structural subunits of COX have, however, undergone a rapid evolutionary change, thought to be functionally adaptive, in the primate lineages leading to humans (25). It is thus possible that similar changes have occurred in some of the COX assembly proteins during primate evolution. If so this could limit the strategy of using mouse chromosomes to map defective human genes in these disorders.

Sequence analysis of the *COX10* gene confirmed that both patients were compound heterozygotes for missense mutations that predict amino acid substitutions at evolutionarily conserved residues (Fig. 6). Human COX10 is predicted to have nine transmembrane domains (HMMTOP; Fig. 6C). The hydrophilic loops between transmembrane domains II/III and VI/VII (transmembrane domains II/III and IV/V of the bacterial homolog CyoE) (26) have previously been shown to be critical for the catalytic function of the bacterial enzyme (27). In patient D, the C791A mutation predicts a T196K substitution. This mutation localizes to a conserved site in the predicted second transmembrane domain, as does the homozygous N204K missense mutation identified in the original *COX10* patient (18). Amino acid changes within this domain may affect COX10 function by altering the conformation of hydrophilic loop II/III, which, in the *E. coli* ortholog, has been shown to contain a highly conserved motif critical to the efficient transfer of the farnesyl moiety to position C2 of heme B (27). The C878T mutation in patient D predicts a P225L substitution, a residue that is conserved in five of eight species shown in Figure 6. Although at least three species have a valine at this position, the presence of either of these two residues, which are contained within the motif 'RTKXR(P/V)', is predicted to be critical for direct binding of the farnesyl diphosphate moiety (27). Both mutations in patient S occur at the same nucleotide, predicting D336G and D336V substitutions. The presence of either aspartate or glutamate at position 336, deduced by alignment of COX10 across species, suggests that conservation of an acidic residue at this position is important to the structure of hydrophilic loop VI/VII. Mutational analyses of residues within this loop implicate this structure in the stabilization of heme B prior to its farnesylation (28).

The presence of some residual heme A:farnesyltransferase activity in both patients is supported by our HPLC analyses of mitochondrial heme content. Total heme A content in mitochondria from both patients was comparable to residual COX activity, and to the amount of fully assembled COX complex. Heme O was not detectable. Similar results were obtained in COX-deficient fibroblast lines from *SURF1* and *SCO1* patients (data not shown). This suggests that, while measuring total heme A content will identify patients with COX deficiency, it cannot distinguish between specific defects in mitochondrial heme biosynthesis or other, unrelated defects in COX holoenzyme assembly. A decrease in heme A content, coupled with an increased heme O content in heart (but not fibroblast) mitochondria does, however, characterize patients with a defect in COX15 (16).

The observation that the amounts of the fully assembled holoenzyme, heme A and residual COX activity were concordant in both patients suggests that COX is catalytically competent, and that the inability to maintain wild-type levels of the holoenzyme is due to a specific defect in heme A biosynthesis. Although three intermediates in COX assembly (termed S1–S3) have been identified from studies in a human leukemia cell line (29), the stage at which the heme prosthetic groups are added during enzyme assembly remains unclear. Studies in *Rhodobacter sphaeroides* have demonstrated that

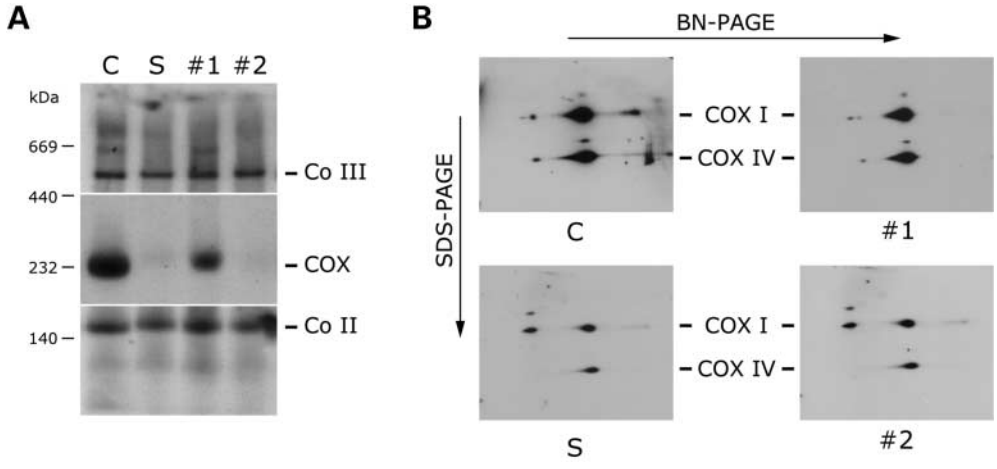

Figure 3. Complementation of the COX defect by mouse chromosomes. Mitoplasts (20 μg protein) isolated from patient S and control (C) fibroblasts and two A9/patient S clones were analyzed by BN-PAGE (**A**) and 2D-BN/SDS-PAGE (**B**). Immunoblot analysis was carried out using antibodies directed against COX subunits I and IV, and against the 70 kDa subunit of complex II (Co II) and the Core 1 protein of complex III (Co III). The COX defect was partially complemented in clone 1. Clone 2 is non-complementing. The migration of molecular mass standards is indicated on the left in (A).

COX subunit I (S1) containing one molecule of heme A accumulates in the membrane in the absence of COX subunits II and III; however, insertion of a full complement of heme A and formation of the binuclear center was shown to require the association of COX subunit I and COX subunit II (30). Unassembled COX subunit I (S1) did not accumulate in patient S. Moreover, patient S lacked any assembly intermediates, as did a patient with a deficiency in heme A biosynthesis due to mutations in *COX15* (16). This COX assembly phenotype contrasts with that seen in patients with mutations in the assembly factor *SURF1*, where the S2 assembly intermediate (containing COX subunits I and IV) accumulates relative to fully assembled COX (31,32). These data argue that the stabilization of COX subunit I within the inner mitochondrial membrane requires its relatively rapid association with COX subunit IV, an event that probably involves the insertion of at least a portion of the total heme A complement. It is not, however, clear why compromised heme A biosynthesis leads to a greater reduction of COX subunit II relative to COX subunits I and IV (18,33).

The tissue specificity of the clinical phenotypes produced by mutations in different COX assembly factors remains one of the most important unanswered questions in these disorders. As far as is known, these factors are encoded in housekeeping genes whose expression in different tissues simply reflects differences in mitochondrial content. How this translates into tissue-specific effects remains unknown; however, it is reasonable to assume that the clinical phenotype is determined by the severity and pattern of the COX deficiency. For instance patients who present early with hypertrophic cardiomyopathy, like patient D described here, or patients with reported *SCO2* (10,15) or *COX15* (16) mutations, appear to have very low COX activities in skeletal muscle/heart relative to other tissues. Residual COX activity in striated muscle in these cases is generally also less than that observed in patients with Leigh syndrome. While this might explain why Leigh syndrome patients do not develop cardiomyopathy, it does not explain the range of COX deficiencies often seen in different cell types carrying the same pathogenic allele. What does seem clear from the data we present here and in Valnot *et al.* (18) is that different clinical phenotypes can be caused by allelic variants in the same COX assembly gene, indicating that it will be necessary to consider multiple candidate genes in the analysis of patients with early-onset isolated COX deficiency.

The early-onset severe anemia seen in both patients in this study is particularly interesting, as this symptom is uncommon in patients with respiratory chain deficiencies except Pearson syndrome, which is characterized by refractory sideroblastic anemia and pancytopenia (34). Mutations in *NDUFS1*, a nuclear-encoded Fe-S subunit of complex I, have been reported in two siblings with macrocytic anemia (35), and heteroplasmic mtDNA mutations in the gene coding for COX subunit I have been suggested to cause acquired idiopathic sideroblastic anemia (36), but to our knowledge anemia has not been reported in patients with isolated autosomal recessive COX deficiency. These observations at least raise the possibility that a deficiency in heme O biosynthesis *per se* can interfere with normal erythropoiesis.

MATERIALS AND METHODS

Cell lines

Primary cell lines were established from patient skin fibroblasts. The patient and the control cell lines were immortalized by transduction with retroviral vectors expressing the E6E7 genes of type 16 human papilloma virus (HPV-16; patient D), or the HPV-16 E7 gene plus a retroviral vector expressing the catalytic component of human telomerase (htert; patient S) (37). The fibroblasts were grown at 37°C in an atmosphere of 5% CO_2 in high glucose DMEM supplemented with 10% fetal bovine serum.

The mouse A9 cell line was transduced with a retroviral vector expressing a hygromycin resistance gene, and was grown at 37°C in an atmosphere of 5% CO_2 in high glucose DMEM

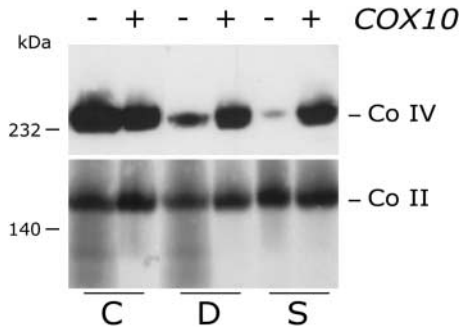

Figure 4. Complementation of the COX assembly defect in patient fibroblasts. Mitoplasts (20 µg protein) isolated from control (C) and patient D and S fibroblasts, and from the same cells overexpressing *COX10* were analyzed by BN–PAGE. Immunoblot analysis was carried out using antibodies against COX subunit IV and against the 70 kDa subunit of complex II (Co II). Overexpression of *COX10* rescues COX levels in the patients' mitochondria. The migration of molecular mass standards is indicated on the left.

supplemented with 10% fetal bovine serum and 800 U/ml of hygromycin B.

Muscle tissue, preparation of mitochondria

Open skeletal muscle biopsy specimens from patient D and controls were obtained with informed consent, and research studies were approved by the appropriate Institutional Review Boards. Biopsy muscle samples were frozen immediately after removal. Diagnostic studies on muscle mitochondria were performed as described previously (38). Muscle and fibroblast homogenates (5% w/v) prepared in 250 mM sucrose/10 mM Tris–HCl/1 mM EDTA (pH 7.4) were centrifuged twice for 10 min at 600*g* to obtain post-nuclear supernatant. Mitochondria were pelleted by centrifugation for 20 min at 10 000*g*.

Electrophoretic methods

Blue-Native PAGE (BN-PAGE) (39) was used for separation of samples in the first dimension on 6–15% polyacrylamide gradient gels as previously described (40). Mitoplasts were prepared from fibroblasts by treatment with 0.8 mg of digitonin/mg of protein, as described previously (40). Mitoplasts or mitochondria were solubilized with 1% lauryl maltoside, and 10–20 µg of the solubilized protein was used for electrophoresis. In-gel qualitative assays for complex I and COX activity were performed as described (41). For the two-dimensional analysis the BN–PAGE gel strips were incubated in 1% SDS and 1% β-mercaptoethanol for 45 min and subsequently separated on 10% Tricine/SDS–PAGE (42). COX subunits and complexes I–III were detected by immunoblot analysis using monoclonal antibodies (Molecular Probes) except for the anti-ND1 antibody, which was a kind gift of A. Lombes (Paris).

Enzyme activity

COX and citrate synthase activities were measured in fibroblast cell extracts as described (43,44). Protein concentration was measured by the Bradford method (45).

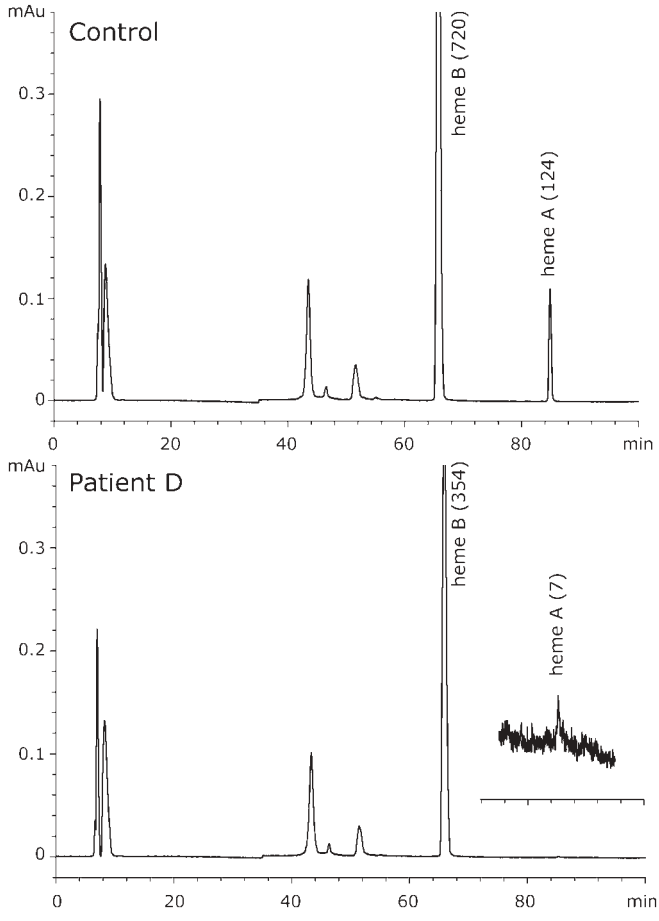

Figure 5. Analysis of mitochondrial hemes in skeletal muscle tissue. Total hemes were extracted from skeletal muscle mitochondria isolated from two controls and patient D with acidified acetone and separated by reverse phase HPLC. The heme B and heme A peaks were identified from the elution times of known standards, and the areas under the peaks were quantified and are reported in arbitrary units (parentheses). A severe reduction in the ratio of heme A to heme B was observed in mitochondria from patient D when expressed relative to the controls (~7%).

Microcell-mediated chromosome transfer

The hygromycin resistant mouse A9 cell line was used as a donor line for the microcell-mediated chromosome transfer. Mouse chromosomes were transferred into the patient S fibroblast cell line as described previously (14). Briefly, the donor cell line was exposed to colchicine (0.06 µg/ml in DMEM containing 10% FBS and 800 U/ml hygromycin B) for 48 h to induce micronucleation. The cells were then collected by trypsinization and plated on plastic bullets coated with concanavalin A. Microcells were prepared by centrifugation for 30 min at 34°C and 22 000*g* in media containing 10 µg/ml cytochalasin B, filtered through 8 and 5 µm filters, pelleted at 2 800*g* and resuspended in serum-free medium. The microcell suspension was added to the plate containing the recipient cells together with phytohaemagglutinin (100 µg/ml), incubated for 45 min and fused with PEG-1500:DMSO (40:10%). After 72 h, fused cells were selected in 100 U/ml of hygromycin B. Colonies were picked, expanded and analyzed 2–4 weeks later.

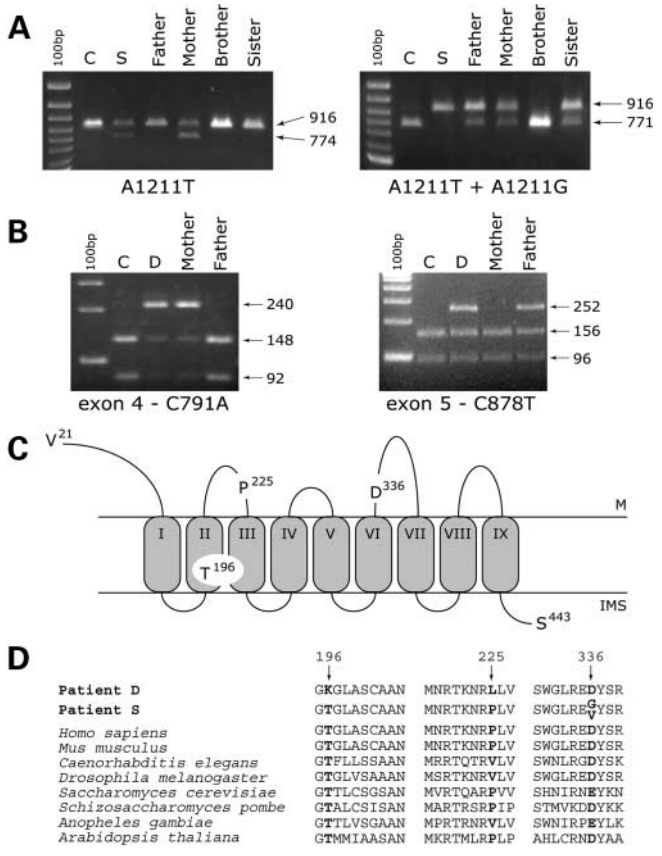

Figure 6. Molecular analysis of the *COX10* gene and protein. RFLP analysis of *COX10* mutations in patients S (**A**) and D (**B**) and in a control (C). Predicted topological model of the mature COX10 protein (**C**). The nine transmembrane domains (predicted by HMMTOP) are indicated by roman numerals. The positions and identities of the amino acid residues affected in the patients are indicated on the model. IMS, intermembrane space; M, matrix (**D**). Sequence alignment of segments of the COX10 protein showing the short evolutionarily conserved sequences containing the missense mutations. Substituted residues predicted from the *COX10* mutations are shown in bold.

Genome scan

One-hundred and sixty fluorescent-labeled oligonucleotide primers for polymorphic microsatellite mouse markers were obtained (Research Genetics; Invitrogen). Genotyping was done on DNA isolated from A9/patient S clones using AmpliTaq Gold® DNA polymerase on an ABI Prism® 3700 DNA analyzer (Applied Biosystems). The presence of individual mouse marker was identified using GeneScan® and Genotyper® Analysis Software (Applied Biosystems).

Cloning and analysis of mouse *COX10* gene

Mouse *COX10* (*mCOX10*) cDNA was amplified by OneStep RT-PCR (Qiagen) using specific primers (Table 1) modified for cloning into GATEWAY (Invitrogen) vectors. The PCR construct was cloned into the GATEWAY-modified retroviral expression vector, pLXSH. The presence of *mCOX10* gene in A9/patient S clones was verified by PCR using mouse specific primers for exon 1 and exon 7 (Table 1).

Virus production and infection

Stable virus-producing cell lines were generated using procedures described previously (46). Briefly, 10 retroviral vectors (*SURF1*, *SCO1*, *SCO2*, *COX17*, *COX11*, *COX15.1*, *COX18*, *COX10*, *OXA1* or *PET191*), described previously (16), and *mCOX10* retroviral vector were used to transfect a GP + E86 ecotropic packaging cell line (47) and the virus produced was used to infect the amphotropic packaging cell line PA317 (48). Fibroblasts were infected by exposure to virus-containing medium in the presence of 4 μg/ml of polybrene as described (49).

Extraction and separation of mitochondrial hemes and HPLC

Mitochondrial hemes were prepared and analyzed by HPLC, with the following modifications to the previously described protocol (16). Briefly, total heme was extracted from skeletal muscle (20 μg protein) and fibroblast (100 μg protein) mitochondria with acetone containing 2.5% HCl. The mixture was vortexed, centrifuged for 5 min at 14 000*g* and mixed with 50% acetonitrile. Insoluble material was removed by a second centrifugation, the extract adjusted to approximately pH 3.5 with 1.65 M ammonium hydroxide, and clarified by centrifugation. The extracts were applied to a 1 × 150 mm Luna™ 3 μm C18(2) column (Phenomenex, CA, USA). Hemes were eluted at a flow rate of 50 μl/min using a 0–40% acetonitrile gradient over the first 0.75 ml, followed by a 40–75% linear acetonitrile gradient over the subsequent 2.1 ml. All gradient solutions contained 0.05% trifluoroacetic acid. The elution and identification of heme compounds were monitored at 400 nm against the elution profile of known standards (hemes B + O from *E. coli* spiked with heme A derived from purified bovine COX). The heme content was calculated from the integrals of heme A and B peaks. The values in control muscle and in control fibroblasts were means of two and five independent measurements, respectively.

COX10 mutation analysis

DNA was isolated from patient and control fibroblasts, from blood samples obtained from the parents of patient D, and from the parents and siblings of patient S. Primer pairs described previously (18), were used for the PCR amplification and sequencing of the *COX10* exons. Primer pairs shown in Table 1 were used for the RT–PCR amplification and sequencing of fibroblast *COX10* cDNA. The presence of the C791A mutation was verified by *BsmF*I digestion of a 240 bp exon 4 PCR product, and the presence of the C878T mutation was verified by *MspA1*I digestion of a 252 bp exon 5 product. The exon 7 mutations A1211T and A1211G were confirmed by digestion with *Bbs*I, and the presence of A1211T mutation was verified by digestion with *Acc*I.

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