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The First Nuclear-Encoded Complex I Mutation in a Patient with Leigh Syndrome

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Summary

Nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase (complex I) is the largest multiprotein enzyme complex of the respiratory chain. The nuclear-encoded NDUFS8 (TYKY) subunit of complex I is highly conserved among eukaryotes and prokaryotes and contains two 4Fe4S ferredoxin consensus patterns, which have long been thought to provide the binding site for the iron-sulfur cluster N-2. The NDUFS8 cDNA contains an open reading frame of 633 bp, coding for 210 amino acids. Cycle sequencing of amplified NDUFS8 cDNA of 20 patients with isolated enzymatic complex I deficiency revealed two compound heterozygous transitions in a patient with neuropathologically proven Leigh syndrome. The first mutation was a C236T (P79L), and the second mutation was a G305A (R102H). Both mutations were absent in 70 control alleles and cosegregated within the family. A progressive clinical phenotype proceeding to death in the first months of life was expressed in the patient. In the 19 other patients with enzymatic complex I deficiency, no mutations were found in the NDUFS8 cDNA. This article describes the first molecular genetic link between a nuclear-encoded subunit of complex I and Leigh syndrome.

Introduction

The oxidative phosphorylation (OXPHOS) system of mammals consists of five complicated multiprotein complexes (complexes I–V). Complexes I and II accept electrons from the reduced coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), respectively. These electrons are transported along the electron transport chain, and coupled to this transport a proton gradient is created by complexes I, III, and IV across the inner mitochondrial membrane. ATP synthetase (complex V) couples proton movement down its electrochemical gradient with the synthesis of ATP from adenosine diphosphate and P_i.

Complex I has been widely studied in prokaryotes as well as eukaryotes. Genetic characteristics of NADH: ubiquinone oxidoreductase are best documented in *Bos taurus* (Walker 1992), *Neurospora crassa* (Weiss et al. 1991), and *Escherichia coli* (Weidner et al. 1993; Friedrich 1998). *E. coli* complex I consists of the 14 subunits that together are considered to be the minimal structural unit for enzymatic activity. In humans, the exact number of complex I subunits is unknown (for a review see Smeitink et al. 1998*b*). *B. taurus* is the mammal nearest to humans whose complex I has been structurally elucidated (Walker 1992; Grigorieff 1998); it contains ~41 subunits (Walker 1995; Walker et al. 1995), 34 of which are encoded by the nucleus and the remaining 7 by mtDNA (Chomyn et al. 1985, 1986). Complex I contains several redox centers to facilitate electron transport. These are hypothesized to be one non–covalently bound flavin mononucleotide and 6–8 iron-sulfur clusters (Yamaguchi and Hatefi 1993; Albracht et al. 1997; Ohnishi 1998).

One of the most frequently encountered syndromes associated with defects of the OXPHOS system was named after the man who first described a young patient suffering from a progressive neurodegenerative disorder with characteristic neuropathology, namely, Leigh syndrome (MIM 256000; Leigh 1951). The estimated incidence of Leigh syndrome is ~1:40,000 live births (Rahman et al. 1996). Several inheritance patterns can be differentiated in Leigh syndrome (DiMauro and De Vivo 1996): autosomal recessive, with defects described in biotinidase (Baumgartner et al. 1989), pyruvate dehydrogenase complex (PDHc), OXPHOS complex II (Bourgeron et al. 1995), and complex IV (Willems et al.

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1977); X-linked recessive, with defects in the $E_{1\alpha}$ subunit of PDHc (Matthews et al. 1993); and maternal, with defects in subunit 6 of ATP synthetase (Shoffner et al. 1992; Tatuch et al. 1992), tRNA(Lys) (Hammans et al. 1993), tRNA(Trp) (Santorelli et al. 1997), tRNA(Val) (Chalmers et al. 1997), and tRNA(Leu) (Graeber and Muller 1998), MERFF mutation A8344G (Berkovic et al. 1991), and A3243G (Rahman et al. 1996). Recently, respiratory-chain enzyme assays performed in patients with Leigh syndrome revealed that complex I deficiency (MIM 252010) is a major contributor to this disease entity (Morris et al. 1996; Rahman et al. 1996), but until now molecular genetic defects have not been described.

The NDUFS8 (TYKY) is a highly conserved nuclear-encoded complex I subunit with equivalents present in many species, best documented in humans (Procaccio et al. 1997), *B. taurus* (Walker 1992), *N. crassa* (Duarte et al. 1996), *E. coli* (Weidner et al. 1993), and *Rhodobacter capsulatus* (Chevallet et al. 1997). The position of the NDUFS8 subunit in different fractions of complex I (flavoprotein [FP] fraction, iron-sulfur protein [IP] fraction, and hydrophobic protein [HP] fraction) has not been firmly established. Masui et al. (1991) located the bovine TYKY subunit in the IP fraction, but generally it is considered to belong to the HP fraction (Walker 1992). The amino acid sequence contains two 4Fe4S ferredoxin consensus patterns (PROSITE pattern PS00198: CIACKLCEAICP and CIYCGFCQEACP). Whether these consensus patterns provide the binding site for iron-sulfur cluster N-2 is still under debate (Dupuis et al. 1991; Albracht et al. 1997; Duarte et al. 1997). Albracht and coworkers proposed in 1997 that the NDUFS8 subunit has functional significance in the reduction of ubiquinone coupled to the pumping of protons. Recently, homologous recombination studies performed with the NUOI subunit of *R. capsulatus* (equivalent to human NDUFS8) showed that NUOI plays an important role in the assembling of complex I, which emphasizes the importance of this subunit (Chevallet et al. 1997).

Herein we report the results of a mutational analysis study of the NDUFS8 subunit performed in 20 patients with isolated complex I deficiency. Our study revealed two new compound heterozygous transition mutations in one patient with Leigh syndrome. Detailed information concerning the clinical characteristics of this patient is presented. This is the first molecular genetic link between Leigh syndrome and a nuclear-encoded subunit of NADH:ubiquinone oxidoreductase. In addition, we study the mRNA tissue distribution of the NDUFS8 subunit and discuss possible connections between tissue expression and the clinical phenotype of the patient.

Patients, Material, and Methods

Case Report

In May 1990, a 5-wk-old male infant presented with poor feeding and episodes of apnea and cyanosis. The symptoms, which had existed since the first day of life, escalated in the course of an acute gastroenteritis. He was the second child of healthy, nonconsanguineous parents of Dutch origin. Pregnancy, birth, and first hours of life were uneventful. The family history was negative for major neurological disorders. At admission, the main symptoms were mild cyanosis, severe hypercarbia, a cardiac murmur (grade 2/4), drowsiness with absent optical and acoustical blink, eye flutter, intense hypotonia, brisk tendon reflexes with ankle clonus, and erratic seizures. A weak cry, rudimentary sucking, and primitive reflexes were still present. The occipitofrontal circumference was normal. Dysmorphic features or hepatomegaly were absent. Cardiac investigation revealed a moderate hypertrophic obstructive cardiomyopathy. Computed tomography of the brain showed extensive white-matter hypodensity, mild ventricular enlargement, and, at a later occasion, also hypodense symmetric lesions in putamen and mesencephalon. Evoked potentials were delayed for all modalities tested.

Blood tests showed increased lactate (3.4 mmol/l; normal range, 0.6–2.1 mmol/l) and pyruvate (167 μmol/l; normal range, 30–80 μmol/l) levels. Cerebrospinal fluid (CSF) lactate and pyruvate levels were elevated to 5.6 mmol/l (normal range, 1.4–1.9 mmol/l) and 193 μmol/l (normal range, 96–145 μmol/l), respectively. The CSF lactate/pyruvate ratio was also increased (29.0; normal range, 11.7–16.5), and the CSF protein content was slightly increased (504 mg/l; normal range, 245–460 mg/l).

On the basis of these findings, a clinical diagnosis of subacute necrotizing encephalomyelopathy, or Leigh syndrome, was made. Light microscopy studies of a quadriceps femoris muscle biopsy sample obtained when the infant was 10 wk of age showed a reduced number of small type I fibers (16%; normal, 50%); ragged red fibers were not seen. Electron microscopy (EM) studies were not performed. At the age of 11 wk, the infant died of cardiorespiratory failure. Biochemical examinations performed on skin fibroblasts, skeletal muscle, heart muscle, and liver and brain tissue, obtained at autopsy performed<1 h after death, revealed severely reduced activity of NADH:ubiquinone oxidoreductase (39% residual activity of the lowest reference value in muscle tissue; see table 1), whereas all other complexes of the respiratory chain expressed normal activity. PDHc activity was slightly reduced in muscle. In cultured skin fibroblasts, an isolated NADH:ubiquinone oxidoreductase deficiency was also observed (69% residual activity

Table 1

Respiratory-Chain Enzyme Activities in Index Patient's Tissue Specimens

	Muscle Tissue		Skin Fibroblasts		Heart Tissue		Liver Tissue		Brain Tissue	
Enzyme	Patient	Controls	Patient	Controls	Patient	Controls	Patient	Controls	Patient	Controls
NADH:Q1 oxidoreductase	1.7[a]	4.4–19 (31)	.069[b]	.10–.26 (14)	±0[a]	4.5–41 (6)	2.5[a]	4.7–9.2 (7)	.16[a]	5.1–19 (5)
Cytochrome c oxidase	170[a]	73–284 (21)	181[a]	147–252 (14)	174[a]	73–412 (7)	53[a]	13.9–108 (11)	58[a]	26–181 (14)
Succinate:cyt c oxidoreductase	24[a]	22–78 (39)	.33[b]	.21–.44 (14)	26.3[a]	11.2–62 (7)	14[a]	6.0–51 (9)	14[a]	11–40 (6)
Citrate synthase	87[a]	48–146 (43)	154[a]	144–257 (14)	316[a]	208–490 (7)	37[a]	13.7–96 (10)	216[a]	53–207 (10)
PDHc	2.5[a]	2.8–8.0 (15)	…	…	9.1[a]	2.3–21 (6)	1.1[a]	.38–7.4 (24)	…	…

Note.—Patient's individual respiratory-chain enzyme and PDHc activities measured in skeletal muscle tissue, cultured skin fibroblasts, heart tissue, liver tissue, and brain tissue. Complex I activity is severely reduced in all tissue specimens. Numbers in parentheses denote how many controls were used for each enzyme assay.

[a] mU/mg protein.

[b] mU/mU cytochrome c oxidase.

of the lowest reference value; see table 1). Biochemical studies of heart, liver, and brain tissue (also obtained at autopsy) revealed an overall reduced activity of NADH: ubiquinone oxidoreductase, with, respectively, virtually 0%, 53%, and 3% residual activity of the lowest reference value (table 1). The completely deficient complex I activity in heart tissue was repeatedly confirmed. Macroscopically, the brain had a normal appearance. Microscopic investigation of the brain showed extensive bilateral symmetrical degeneration, predominantly in the rostral and caudal brain stem, diencephalon, and central nuclei, but also elsewhere, including the spinal cord and semioval center. The lesions consisted of spongiform degeneration, capillary proliferation with endothelial swelling, demyelinization, and gliosis. Neuronal damage was variable in different brain slides, partly because there was a relative sparing of neurons in comparison to the surrounding tissue. EM studies of heart and liver tissue were not performed. On the basis of data obtained at autopsy, the patient was neuropathologically diagnosed with Leigh syndrome.

Patient Group

Twenty patients with isolated complex I deficiency (4 females and 16 males) were included in this study. Concise clinical characteristics have been published elsewhere (Loeffen et al. 1998), and a detailed report of our complex I–deficient patient group will be published elsewhere (Loeffen et al. unpublished data). In this patient group the NDUFA1, NDUFS4, NDUFS5, and NDUFB6 cDNA were previously examined by direct DNA sequencing (Loeffen et al. 1998, Loeffen et al. in press; Smeitink et al. 1998*a*; van den Heuvel et al. 1998). In one patient a mutation was found in the NDUFS4 subunit. Neither major mitochondrial DNA rearrangements nor common pathogenic mtDNA mutations (A3243G [MELAS], T3271C [MELAS], A4317G [MELAS], A8344G [MERRF], T8993G/C [NARP/Leigh syndrome]) were present in this patient group.

Commercially Purchased Materials

Molecular size markers, *Taq* DNA polymerase, Superscript II RNAse H^- reverse transcriptase, *Bal*I restriction endonuclease, and custom-made oligonucleotide primers were obtained from Life Technologies; *Pin*AI restriction endonuclease from Promega; a d-Rhodamine DyeDeoxy Terminator Sequencing Kit from Perkin Elmer; gel purification systems from Qiagen; a poly A^+ RNA pool and a Poly A^+ RNA master blot from Clontech; and RNAzol from Biosolve BV.

Software

Consensus-pattern searches in the NDUFS8 amino acid sequence were performed with PROSITE. Sequence alignments were performed with Sequence Navigator (Perkin Elmer). Hydropathy and secondary structure were plotted by means of methods based on the procedures described by Chou and Fasman (1978), Kyte and Doolittle (1982), and Nishikawa (1983). Computer software was obtained from the National Center for Computer-Aided Chemistry and Bioinformatics (CAOS/CAMM).

Tissue Culture and Enzyme Measurements

In our patient group, biochemical enzyme measurements of respiratory-chain complexes were performed on skin fibroblasts cultured according to standard procedures. Enzyme assay procedures were slightly modified from those used for muscle tissue (Bentlage et al. 1996).

The autopsy tissue materials of the patient described in this article were obtained <1 h after death and immediately deep frozen in liquid nitrogen and kept at −80°C until enzyme assays were performed. The enzyme

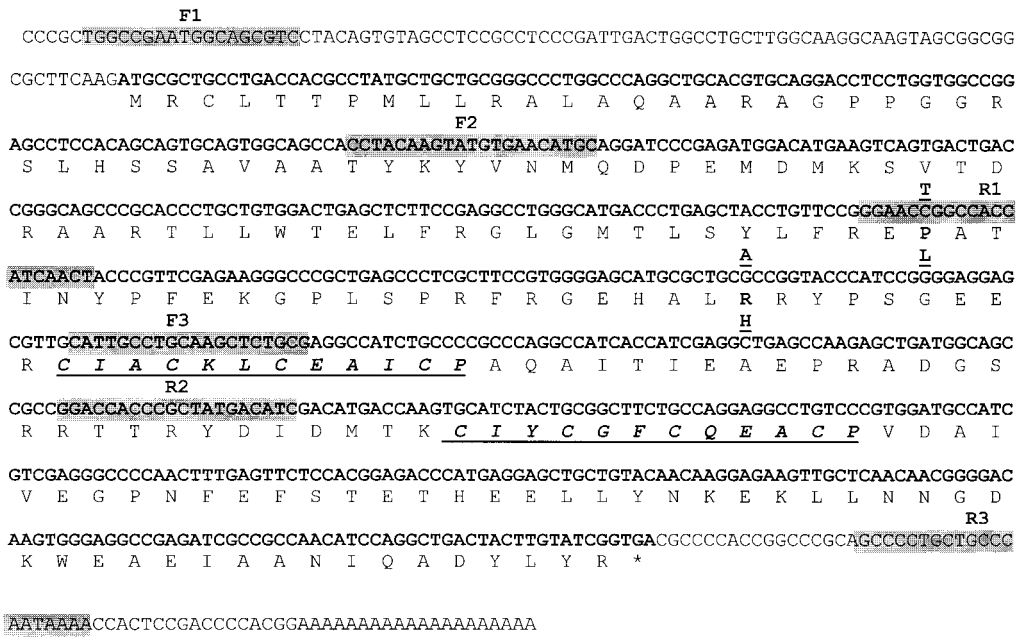

Figure 1 Human NDUFS8 cDNA and amino acid sequence (Procaccio et al. 1997). Primers used for amplification of the ORF were F1 and R3; all primers were used in DNA sequence reactions. The ORF is printed bold, and both consensus 4Fe4S ferredoxin clusters are in italic and underlined. Mutations are shown above the cDNA sequence, substitutions below the amino acid sequence.

assays described in this paper were based on the following methods (slightly modified): NADH:ubiquinone oxidoreductase according to Fischer et al. (1986); succinate cyt c oxidoreductase according to Fischer et al. (1985); cytochrome c oxidase according to Cooperstein and Lazarow (1951); citrate synthase according to Srere (1969); and PDHc according to van Laack et al. (1988). Control tissue specimens were handled and stored under conditions similar to those of the patient. In our experience, loss of respiratory-chain enzyme activities in deep-frozen tissue samples obtained <2 h after death is negligible. Control values and number of controls are listed in table 1. Values in fibroblasts are expressed on a cytochrome c oxidase base (cytochrome c oxidase and citrate synthase are expressed on a protein base). We chose cytochrome c oxidase as a marker instead of citrate synthase, because in the presented patient enzyme activities were measured in deep-frozen fibroblasts ($600 \times g$ supernatant). In our experience, citrate synthase can be artificially decreased in deep-frozen tissue because of leakage from the mitochondria, since it is a mitochondrial matrix enzyme.

Mutational Analysis of the NDUFS8 cDNA

Patient RNA was extracted from cultured skin fibroblasts according to methods described by Chomczynski and Sacchi (1987). Wild-type RNA was commercially obtained and consisted of pooled poly A^+ RNA from 12 healthy Caucasian males and females, age range 12–78 years. The RNA was reverse transcribed to cDNA by superscript II RNAse H^- reverse transcriptase with oligo(dT) and random hexamer primers (Ploos van Amstel et al. 1996).

Oligonucleotide primers were designed according to the NDUFS8 cDNA sequence published by Procaccio et al. (1997) (GenBank accession number U65579). The primer oligonucleotides F(orward)1 (5′-TGGCCGAATGGCAGCGTC-3′) and R(everse)3 (5′-TTTTATTGGGCAGCAGGGGC-3′) were applied in PCR to amplify the complete open reading frame (ORF). PCR was performed in a reaction volume of 25 μl containing 1 μl of cDNA template, 0.5 unit of *Taq* DNA polymerase, 2.5 μl of 10 × PCR buffer, 1.5 mM of $MgCl_2$, 50 ng of F1 and R3, and 2.5 mM of dNTPs. PCR parameters were 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min extension at 72°C. The cycles were preceded by an initial denaturation step of 1 min at 94°C and were followed by a final extension step of 10 min. The amplification product was purified from a 2% agarose gel. Sequence reactions using the DyeDeoxy Terminator Sequencing Kit were performed with oligonucleotide primers F1, F2 (5′-CCTACAAGTATGTGAACATGC-3′), F3 (5′-CATTGCCTGCAAGCTCTGCG-3′), R1 (5′-AGTTGATGGTGGCCGGTTCC-3′), R2 (5′-GATGTCATAGCGGGTGGTCC-3′), and R3 (fig. 1). The cDNA sequence analysis was performed on the

automated ABI 377 sequencer according to the protocol provided by the manufacturer.

Restriction Enzyme Endonuclease Analysis

Both mutations were confirmed by restriction endonuclease analysis of the NDUFS8 cDNA fragment obtained with oligonucleotide primers F1 and R3. C236T was cleaved with restriction enzyme *Bal*I (recognition site 5′-TGG‡CCA-3′) and G305A with *Pin*AI (recognition site 5′-A‡CCGGT-3′). (The double daggers [‡] indicate the break point as created by the restriction enzymes.) The transition mutations present in our patient (position underlined in the digestion recognition site) introduce the restriction sites. For both digestions, conditions were used as recommended by the manufacturer.

Tissue Distribution of the NDUFS8 mRNA

A human RNA master blot was used to quantify the expression of NDUFS8 mRNA in a range of human adult and fetal tissues. The quantity of RNA spotted for each tissue onto the master blot was normalized using eight different housekeeping gene transcripts as probes (ribosomal protein S9, ubiquitin, 23 kDa highly basic protein, hypoxanthine guanine phosphoribosyl transferase, tubulin, β-actin, phospholipase, and glyceraldehyde triphosphate dehydrogenase). Using a standard hybridization solution (Sambrook et al. 1989), we hybridized the master blot with an $\alpha[^{32}P]$-dCTP–labeled cDNA probe. The blot was washed twice with 2 × SSC, 1% SDS, at 65°C and was subjected to autoradiography.

Results

Biochemical Measurements

In our patient group, residual complex I activity measured in cultured skin fibroblasts was 10%–80% of the lowest reference value (expressed on a cytochrome c oxidase base). The other respiratory-chain complexes showed normal activities in all patients. In most patients, respiratory-chain complex activities were also measured in other tissues apart from fibroblasts. In the case of the patient with two compound heterozygous transition mutations in the NDUFS8 gene, enzyme activities were measured in a quadriceps muscle biopsy specimen, in cultured skin fibroblasts, and in liver, brain, and heart tissue. In all of these tissues complex I activity was markedly reduced, whereas other complexes of the electron transport chain were essentially within the normal range (table 1).

Mutational Analysis of the NDUFS8 cDNA

PCR with oligonucleotide primers F1 and R3 produced a cDNA fragment of 757 bp, including the complete ORF of the NDUFS8 subunit. Direct DNA sequence analysis of amplified pooled control NDUFS8 cDNA confirmed the published wild-type cDNA sequence. Amplified NDUFS8 patient cDNA was then cycle sequenced and analyzed. In one patient this revealed two heterozygous transitions. The first mutation, a C→T at bp position 236, resulted in an amino acid change of a proline into a leucine (amino acid position 79). In the second mutation, a G→A at bp position 305, an arginine had been replaced by a histidine (amino acid position 102). The presence of the first and second mutations in the common population was checked by restriction endonuclease analysis. In 35 controls (70 alleles), C236T and/or G305A were not present. The other 19 patients with enzymatic complex I deficiency all displayed the cDNA sequence corresponding to the human NDUFS8 cDNA described elsewhere (Procaccio et al. 1997).

Confirmation of Mutations by Restriction Endonuclease Analyses

RNA isolated from whole blood of both parents and three healthy siblings (two boys, one girl) was reverse transcribed to cDNA. Oligonucleotide primers used for cloning of the complete ORF of the NDUFS8 subunit were applied in PCR. The PCR fragment was digested with restriction enzymes *Bal*I (C236T) and *Pin*AI (G305A). The results were visualized on a 4% agarose gel. Since both restriction endonucleases cut in the region containing the mutation, the wild-type NDUFS8 cDNA fragment remained intact. In the patient both mutations were heterozygous, and after cleavage with *Bal*I they expressed three bands (a wild-type cDNA fragment, one fragment of 325 bp, and one fragment of 432 bp). Incubation with *Pin*AI again revealed three fragments (a wild-type cDNA fragment, one band of 365 bp, and one band of 392 bp). When *Bal*I and *Pin*AI were added simultaneously, the corresponding four digestion fragments were seen, which implies that the two mutations are on different alleles (data not shown). Three bands corresponding to the patient's were expressed in the father's cDNA after incubation with *Bal*I, and a single wild-type band was expressed when *Pin*AI was applied. The mother's cDNA showed an undigested wild-type band after incubation with *Bal*I and three bands (similar to the patient's) when *Pin*AI was used. An older brother had the same digestion pattern as the father, a younger sister the same fragment pattern as the mother, and the youngest son showed only wild-type bands after digestion with *Bal*I and *Pin*AI (fig. 2).

Tissue Distribution of the NDUFS8 mRNA

Hybridization of the $[^{32}P]$-dCTP–labeled NDUFS8 cDNA probe to the RNA master blot revealed a ubiquitous expression pattern in human adult and fetal tissues. Adult tissue dots with relatively higher intensities are, in decreasing order, heart, skeletal muscle, pituitary

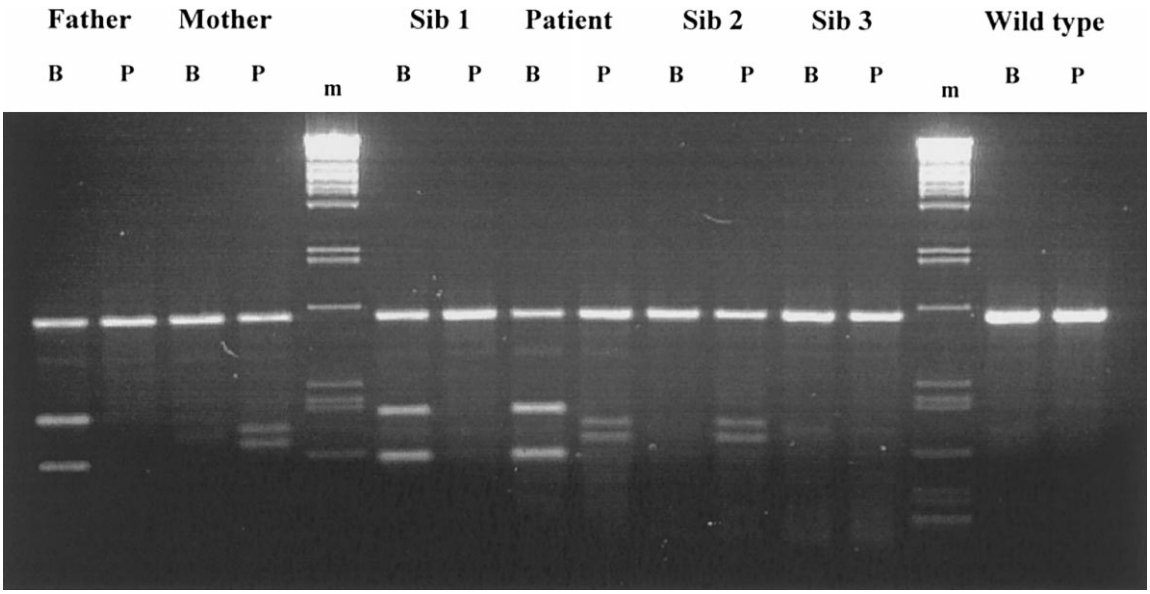

Figure 2 Restriction endonuclease analysis pattern. Wild-type and Sib 3 NDUFS8 cDNA is undigested; patient is a carrier of C236T and G305A, father and sib 1 are carriers of C236T, and mother and sib 2 are carriers of G305A. The marker (m) is Lambda PSTI. For each person, the first lane contains the fragments after digestion with *Bal*I (B), and the second lane the fragments after *Pin*AI (P) was applied.

gland, adrenal gland, kidney, caudate nucleus, putamen, substantia nigra, and lung. Among fetal tissues, heart expressed the highest dot intensity (fig. 3).

Discussion

The clinically, biochemically, and genetically heterogeneous group of OXPHOS disorders (estimated incidence 1:10,000 live births; Bourgeron et al. 1995) has a main contributor in Leigh syndrome combined with isolated complex I deficiency. In the late 1980s and early 1990s, several mtDNA mutations associated with complex I deficiency were identified (Wallace et al. 1988; Holt et al. 1989; Poulton and Gardiner 1989; Zeviani et al. 1989; Goto et al. 1990; Holt et al. 1990; Tanaka et al. 1990; Zeviani et al. 1991; Shoffner et al. 1992). Yet, in many patients with isolated complex I deficiency, mtDNA mutations cannot be found (Buddiger et al. 1997; Loeffen et al. in press). Recently, we found the first mutation—a 5-bp duplication in the NDUFS4 (18 kDa) subunit—to be identified in a nuclear-encoded subunit of complex I (van den Heuvel et al. 1998). These findings make further studies of mutations in nuclear-encoded subunits of complex I necessary.

We chose the NDUFS8 subunit for mutational analysis studies for several reasons. In the first place, the NDUFS8 subunit is highly conserved among species, with, for example, 93% amino acid identity between humans and *B. taurus*. It is also one of the 14 subunits that together constitute the minimal structural unit for enzymatic activity, as found in *E. coli*, which stresses its possible functional significance. Finally, it contains two consensus 4Fe4S ferredoxin patterns, which have long been considered to be binding sites for iron-sulfur cluster N-2. These findings make this protein a strong candidate gene for isolated complex I deficiency.

Cycle sequencing of the NDUFS8 cDNA in our complex I–deficient patient group revealed two compound heterozygous mutations in a patient neuropathologically diagnosed with Leigh syndrome. The clinical phenotype of this patient and the results of additional laboratory and imaging studies are highly suggestive of an OXPHOS disorder, and diagnosis (isolated complex I deficiency) was biochemically confirmed in muscle tissue; cultured skin fibroblasts; and heart, liver, and brain tissue. The first transition (C236T) resulted in an amino acid substitution of a leucine for a proline. It is well known that proline markedly influences protein architecture. A Chou-Fasman plot comparison of NDUFS8 wild types and NDUFS8 containing this specific mutation clearly revealed the disturbance of an α-helix by a β-sheet. Although it remains purely speculative which conformation the NDUFS8 protein will have when incorporated in the entire complex, proline is very important for secondary and tertiary structure, and thus the disappearance of this amino acid will likely disturb the protein structure. The second mutation (G305A) replaced an arginine with a histidine. Histidine contains an imidazole side chain that can readily switch between uncharged and positively charged to catalyze the making and breaking of bonds. A Basic Local Alignment Search Tool (BLAST) search at the server of the National Cen-

	1	2	3	4	5	6	7	8
a	whole brain	amygdala	caudate nucleus	cerebellum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
b	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	subthala-mic nucleus	spinal cord	
c	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
d	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
e	kidney	liver	small intestine	spleen	thymus	peripheral leucocyte	lymph node	bone marrow
f	appendix	lung	trachea	placenta				
g	fetal brain	fetal heart	fetal kidney	fetal liver	fetal spleen	fetal thymus	fetal lung	
h	yeast total RNA 100 ng	yeast tRNA 100 ng	E.coli rRNA 100 ng	E.coli DNA 100 ng	Poly r(A) 100 ng	human C0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

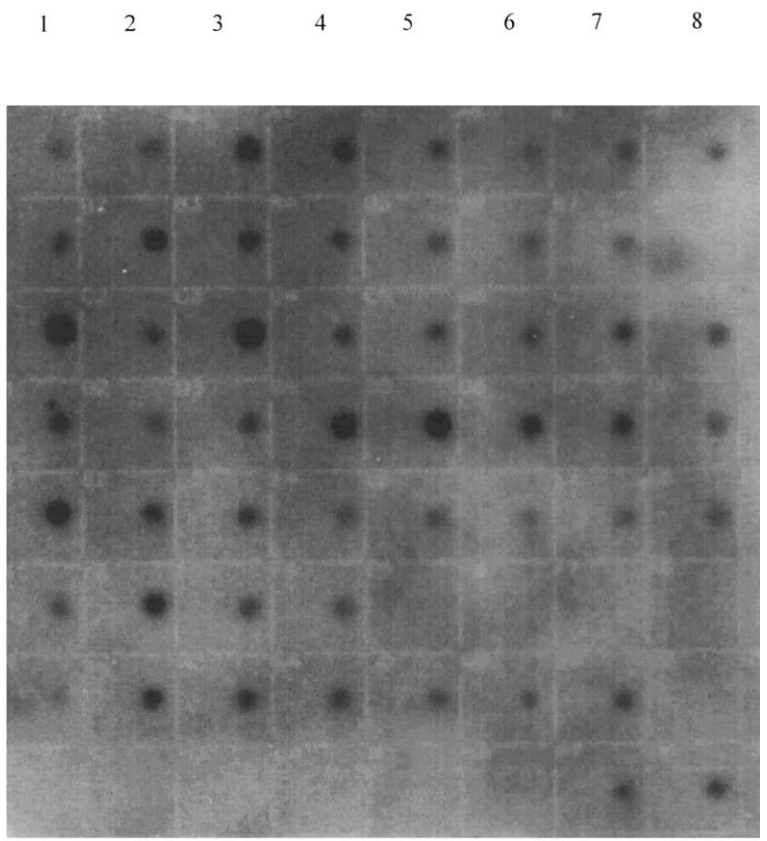

Figure 3 Tissue expression of NDUFS8 mRNA with a poly A^+ RNA master blot. NDUFS8 is ubiquitously expressed in human tissues, with a relatively higher expression in the human heart, skeletal muscle, gland tissue, kidney, caudate nucleus, putamen, and substantia nigra.

ter for Biotechnology Information with the human NDUFS8 amino acid sequence revealed an extremely strong conservation of the region containing the arginine, which is mutated in the patient. This region of 24 amino acids is ±100% conserved in, among others, *B. taurus, N. crassa, Paracoccus denitrificans, Rhodobacter capsulatus,* and *Nicotiana tabacum*. This stresses the possible significance of this region, which is located just prior to the first cysteine-rich 4Fe-4S ferredoxin consensus pattern (eight amino acid residues between the mutated arginine and the consensus cluster). An arginine-to-histidine mutation is the main genetic cause in another disease associated with complex I deficiency, namely, Leber hereditary optic neuropathy (Wallace et al. 1988; Singh et al. 1989). Although both amino acids are basic, they are clearly not, in all cases, exchangeable. Since neither mutation is present in 70 control alleles and in the remaining 19 complex I–deficient patients, it is unlikely that these mutations represent population polymorphisms. These two mutations with an autosomal recessive inheritance pattern, combined with the lethal clinical course within the early months of life, provide further evidence for the functional importance of this iron-sulfur cluster containing the nuclear-encoded complex I subunit.

Restriction endonuclease analysis performed on NDUFS8 cDNA of all members of the patient's family showed that mutation C236T was inherited from the father and mutation G305A from the mother. In the oldest son (carrier of C236T), the activity of respiratory-chain enzyme complexes was measured in a skeletal muscle biopsy specimen, and that of each complex was within the normal range. While the mother was pregnant with the younger sister (carrier of G305A), chorionic villi were obtained for biochemical measurements of the respiratory-chain complex activities, which were again within normal limits (no muscle biopsy was performed in the younger sister). It seems likely, therefore, that both mutations are necessary to reduce the enzymatic activity of NADH:ubiquinone oxidoreductase.

Leigh syndrome has been linked, by biochemical enzyme assays, to several single-enzyme and enzyme-complex deficiencies, including deficiencies in biotinidase, PDHc, NADH:ubiquinone oxidoreductase, succinate dehydrogenase, cytochrome c oxidase, and ATP synthetase. However, molecular genetic defects have been described in only a few subunits of PDHc: the FP subunit of succinate dehydrogenase, subunit 6 of ATP synthetase, and some mitochondrially encoded tRNAs. Recent mutational analysis of all 10 nuclear-encoded subunits of cytochrome c oxidase (complex IV of the OXPHOS system) in a Leigh syndrome patient with cytochrome c oxidase deficiency revealed no mutations (Adams et al. 1997). The patient described in the present article provides the first genetic link between a nuclear-encoded subunit of complex I and Leigh syndrome. Since complex I deficiency is a large contributor to Leigh syndrome, other patients with isolated complex I deficiency in whom a similar clinical phenotype is expressed should be screened for NDUFS8 mutations as well.

The tissue expression of the NDUFS8 cDNA showed dots with relatively high intensity in heart, skeletal muscle, kidney, pituitary gland, adrenal gland, caudate nucleus, putamen, and lung. All of these tissues have a relatively high energy demand and are therefore likely to be affected by reduced energy availability. The patient with Leigh syndrome reported here had symptoms that strongly correlate with the high-intensity dots present on the NDUFS8 master blot, such as hypertrophic cardiomyopathy, hypotonia, convulsions, and episodes of respiratory distress. The specific neuropathology seen in Leigh syndrome can be hypothesized to be the result of oxidative stress caused by a diminished activity of complex I. It would be reasonable to assume that oxygen radicals are generated in the vicinity of an iron-sulfur cluster. A defective NDUFS8 subunit, as seen in our patient, is a likely source of malfunctioning electron transport and therefore a possible source of free radicals. The review by Robinson (1998) concerning oxygen free radicals and human complex I deficiency states that NDUFS8 would be a prime candidate for an electron donor for superoxide formation. Future research is necessary to investigate the possible relationship between the specific neuropathology present in Leigh syndrome and malfunction of the NDUFS8 subunit.

In conclusion, we have described herein the first mutations identified in the nuclear-encoded NDUFS8 subunit of complex I (possible binding site for iron-sulfur cluster N-2) in a patient with isolated complex I deficiency whose condition was neuropathologically diagnosed as Leigh syndrome. Our analysis of these compound heterozygous transitions is the second report of genetic defects in nuclear-encoded subunits of complex I. The frequency of these mutations in complex I–deficient patients in general and, more specifically, in patients diagnosed with Leigh syndrome must be further established. These findings contribute to possibilities for genetic counseling and prenatal diagnosis, which are of utmost importance since therapeutic intervention in complex I disease is very unsatisfactory. We will continue to search for other candidate genes for complex I deficiency.

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Electronic-Database Information

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

National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/genbank/query_form.html (for human NDUFS8 [TYKY] cDNA sequence [U65579])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for Leigh syndrome [MIM 256000] and complex I deficiency [MIM 252010])
PROSITE, http://www.ebi.ac.uk/searches/prosite_input.html (for 4Fe4S ferredoxin consensus pattern searches in the NDUFS8 amino acid sequence)

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