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ORIGINAL INVESTIGATION

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Compound heterozygous mutations in the flavoprotein gene of the respiratory chain complex II in a patient with Leigh syndrome

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Abstract Succinate dehydrogenase (SDH) deficiency represents a minor cause of Leigh syndrome (LS). Noticeably, the first mutation in a nuclear-encoded respiratory chain component, a mutation in the 5p15 copy of the flavoprotein (*Fp*) subunit gene of the SDH, was reported 4 years ago in two siblings with LS and SDH deficiency. We now report a new patient with LS and SDH deficiency. Because two copies of the *Fp* gene are present in the human genome, we first determined the complete structure of these two copies. This allowed us to identify a 1 bp deletion creating a frameshift in the 3q29 copy, confirming that this second copy was a pseudogene. We also sequenced the promoter region of the 5p15 gene and, in addition, screened for mutations in the patient. Sequencing of the *Fp* SDH cDNA in the patient only allowed us to identify a heterozygous C to T transition, changing an alanine to a valine in one allele. This transition was found to be heterozygous in the patient's father but was absent from 150 controls. Transfection of the corresponding mutant cDNA into human Fp-deficient cells failed to restore normal SDH activity, confirming the deleterious effect of this mutation. The second allele, inherited from the mother, carried an A to C substitution changing the methionine translation initiation codon to a leucine. This mutant transcript represented only 10% of total Fp transcript suggesting instability of this transcript. So far, profound deficiencies in complex II activity resulting from mutations in the *Fp* gene of the SDH present only as LS, a striking observation in view of the ubiquitous expression of this typical housekeeping gene in humans.

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Introduction

Complex II deficiency is a rare condition in humans (Rustin et al. 1997), representing 2% of 280 cases of respiratory chain (RC) deficiencies identified in our cohort of patients. Clinically, it may reveal as Leigh syndrome (Bourgeois et al. 1992; Birch-Machin et al. 1996), but myopathic presentation with muscle weakness and exercise intolerance (Reichmann and Angelini 1994), Kearns-Sayre syndrome (Rivner et al. 1989), encephalopathy (Riggs et al. 1984), and isolated cardiomyopathy (Rustin et al. 1993) have also been reported.

Complex II (succinate:ubiquinone oxidoreductase; E.C. 1.3.5.1) is made up of four subunits. It catalyses the oxidation of succinate to fumarate and feeds electrons to the RC ubiquinone pool. It can be resolved into a soluble catalytically competent succinate dehydrogenase (SDH) and a membrane-anchoring fraction. The SDH consists of a 70 kDa flavoprotein subunit (Fp) containing the active site and a covalently bound FAD and a 30 kDa iron-sulfur protein subunit (Ip) carrying three distinct iron-sulfur clusters (Ackrell et al. 1990). The SDH is anchored to the matrix-face of the inner mitochondrial membrane by two smaller subunits (SDHC, 15 kDa, and SDHD, 12 kDa) carrying cytochrome b558 and the ubiquinone-binding sites. All four subunits are nuclearly encoded and considered as typical housekeeping genes with ubiquitous expression.

The corresponding genes or the cDNAs of the four subunits have been previously identified (Kita et al. 1990; Hirawake et al. 1994; Hirawake et al. 1997; Morris et al. 1994; Au et al. 1995). Two copies of the *Fp* subunit gene are present in the human genome, on chromosomes 5p15 and 3q29; the latter is not expressed in hybrid cells (Bourgeron et al. 1995). *Ip* is a single-copy gene mapped to chromosome 1p35–36.1 (Leckschat et al. 1993). The two anchoring subunits, SDHC and D, mapped to 1q21 and 11q23, respectively (Hirawake et al. 1997). Only one active *SDHC* gene has been reported (Elbehti-Green et al. 1998) and one sequence of *SDHD* is available in the databases (Hirawake et al. 1999). Therefore, with the excep-

tion of the Fp subunit, tissue-specific expression of CII genes related to the occurrence of multiple copies can hardly be advocated to account for tissue-specific expression of the disease.

We reported 4 years ago the first mutation in a nuclearly encoded subunit of the RC in two siblings with Leigh syndrome (LS) and a deficiency of the SDH, namely an Arg554Trp substitution in the *Fp* subunit gene (Bourgeron et al. 1995).

Here, we report two compound heterozygous mutations in the *Fp* gene in one patient with LS and CII deficiency. In the course of identification of the underlying mutations, we analyzed the sequences of the four CII genes in the patient. In the meantime, the hitherto unknown nucleotide sequence of the *Fp* exon-intron boundaries and the *Fp* promoter region were determined.

Patient and methods

Patient

The patient, a girl, was the first child of unrelated healthy parents and was born at term after a normal pregnancy and delivery. She developed normally until 9 months of age, when psychomotor delay was noticed. She could not sit unaided before 16 months of age, when truncal ataxia and cerebellar syndrome were noticed. Echocardiogram was normal. There was a mild hyperlactatemia (3.2–4.4 mmol/L; normal: <2.5) with a lactate/pyruvate ratio =31 and 23 (normal: <20). Cerebrospinal fluid lactate was mildly elevated (2.6 mmol/L; normal: <2.3). Cerebrospinal fluid protein, immunoglobulins and α-fetoprotein were normal. Magnetic resonance imaging revealed necrotic lesions in the basal ganglia compatible with the diagnosis of Leigh syndrome.

Enzyme assays

Cytochrome-*c* oxidase (EC 1.9.3.1), succinate phenazine methosulfate (PMS), dichlorophenol indophenol (DCPIP) reductase (EC 1.3.99.1), succinate quinone DCPIP reductase, succinate cytochrome-*c* reductase, decylubiquinone cytochrome-*c* reductase (EC 1.10.2.2) and glycerol-3-phosphate cytochrome-*c* reductase activities were measured spectrophotometrically on lymphoblastoid cell lines as previously described (Rustin et al. 1994).

Fp gene structure and promoter analysis

Cosmid recombinants of a human chromosome 5 PWE15 cosmid library were screened with a [^{32}P]dCTP *Fp* cDNA probe. One positive clone was obtained and subcloned into BlueScript vector after digestion with different restriction enzymes. Recombinant clones were screened by colony hybridization using a *Fp* exon 1 probe. Only one clone containing exon 1 and the 5' part of the *Fp* gene was obtained. The *Fp* promoter sequence was determined from this clone using T3, T7 and new primers designed according to novel sequence information (Table 1).

For sequencing exon-intron boundaries, cosmid as well as genomic DNA of somatic cell hybrids, plus human chromosome 5 (C56 N) or chromosome 3 (C34 U) (Van Cong et al. 1980) were submitted to long range PCR between exonic primers (Table 1).

Sequence analysis

Total DNA extracted from lymphoblastoid cell lines was submitted to PCR amplification using primers specific for the *Fp* and *Ip* promoters and *SDHC*. Total RNA extracted from patient lymphoblastoid cell lines was reverse transcribed using the GeneAmp RNA PCR core kit (Perkin-Elmer) and each cDNA was amplified using specific oligonucleotides (Table 1). After 30 cycles (95°C, 30 s; 50–66°C, depending on the primers used, 30 s; 72°C, 30 s), amplification products were purified on a 1% low melting point agarose gel and recovered by heating for 5 min at 65°C. Direct sequencing was performed using 3.2 pmol of the amplification primers and 8 μl sequencing reaction mixture (Prism Ready Reaction Sequencing Kit; Perkin-Elmer Cetus) on an automatic fluorimetric DNA sequencer (373 A DNA sequencer).

Mutations screening

Table 1 Oligonucleotides used for sequence analyses of complex II subunits. *Fp* promoter region refers to the sequence presented in Fig. 3

Forward primer (nucleotide positions)	Reverse primer (nucleotide positions)	GenBank accession number
Fp promoter region		AF171017
–1950 to –1930	–1557 to –1577	
–1605 to –1585	–1293 to –1313	
–1335 to –1315	–1132 to –1152	
–1181 to –1161	–806 to –826	
–858 to –838	–622 to –642	
–671 to –650	–219 to –239	
–361 to –341		
Fp cDNA		L21936
	80–51	
1–21	131–111	
81–100	320–300	
290–310	465–445	
368–388	572–552	
533–553	767–747	
722–742	842–821	
801–821	1060–1040	
999–1019	1204–1185	
1129–1149	1421–1401	
1372–1392	1570–1550	
1467–1487	1632–1612	
1630–1650	1781–1761	
1731–1751	1901–1881	
1838–1858	2141–2121	
Ip genomic DNA		
124-nt 143	534–514	U17296
488–508	916–897	
863–886	1163–1143	
1117–1137	1484–1464	
1437–1457	2052–2032	
1674–1694	2377–2357	
2325–2344	2585–2566	
Ip cDNA		
2–22	630–611	U17248
570–589	1078–1056	
SDHC intronic primers		
905–924	1002–982	AF039589
453–473	567–547	AF039590
344–363	499–479	AF039591
384–404	499–479	AF039592
227–247	445–425	AF039593
226–246	575–555	AF039594
520–540	1107–1087	AF039594
SDHD cDNA		
5785–5805		AB026906
263–283		NM_003002
515–535	1260–1240	NM_003002

The C to T transition at nt 1595 was screened on genomic DNA with the allele created restriction site (ACRS) technique using the forward 5p15-specific intronic oligonucleotide 5'TTTCTGTTT-AATTTATGTAACTTTT3' and the reverse modified oligonucleotide 5'CGCTTCCCACACGGAACTCG3'. The 160 bp PCR products were then digested using *Taq*I restriction enzyme. Normal and mutant restriction fragments were 160 and 140 bp long, respectively.

The A to C transvertion at nt 25 of the *Fp* gene was detected by *Afl*III restriction analysis of a 416 bp genomic 5p15-specific fragment obtained by using oligonucleotides for nt –361 to nt –341 and nt 80 to nt 51 (Table 1). Mutant and normal restriction fragments were 416 and 361 bp long, respectively.

Transfection of fibroblasts

SDH-deficient skin fibroblasts from a patient harboring an Arg554Trp substitution in the *Fp* subunit gene (Bourgeron et al. 1995) were immortalized by transfection of plasmid pLASwt containing SV40 T antigen (kindly supplied by B. Grospierre). The wild type and C1595 T mutant *Fp* cDNAs were inserted into the mammalian pIRES-neo expression vector (ClonTech). SDH-deficient SV40-immortalized fibroblasts were harvested and resuspended at a density of 5×10^6 cells/ml. The cell suspension (0.4 ml) was electroporated with 10 µg plasmid DNA with settings of 200 V and 2100 µF and infinite resistance (Equibio, Easyject Plus). Cells were immediately diluted with RPMI culture medium and plated at 2×10^6 cells/ 75 cm^2 flask. Transformed cells were selected in culture medium containing 50 µg/ml G418.

Results

Enzyme analyses were performed on patient lymphoblastoid cell lines (Table 2). SDH (SPDR), complex II (SQDR) and complexes II+III (SCCR) activities, which all involve SDH enzyme, were found to be defective, resulting in strongly abnormal ratios to cytochrome-*c* oxidase (COX) ratios. Complex III, glycerol-3-phosphate cytochrome-*c* reductase, citrate synthase and fumarase activities were found to be normal in patient cells (not shown).

The mRNA of three subunits of complex II, namely Fp, Ip and SDHD, from patient lymphoblastoid cell lines was submitted to RT-PCR amplification. The coding sequence of the *SDHC* subunit gene was PCR amplified using genomic DNA. Sequence analysis of the Fp subunit revealed an homozygous C to T transition at nt 1595, changing an alanine to a valine at codon 524 in a moderately conserved domain of the protein. This C to T substitution can be rapidly screened on genomic DNA by the ACRS technique using a modified oligonucleotide surrounding it and could not be found in 150 healthy controls. This transition was tested with chromosome 5p15 *Fp* gene-specific oligonucleotides in the non-consanguineous parents and was found to be heterozygous in DNA from the patient's father but was absent in DNA of the mother (Fig. 1A). However, this C to T transition was heterozygous in genomic DNA of the patient (Fig. 1A). No mutation could be identified either in the Ip subunit, or in the coding sequences of subunits C and D in complex II from the patient.

As two copies of the *Fp* gene are present in the human genome, we next attempted to determine if one may actually represent a pseudogene. Chromosome 5 and 3 *Fp* genes were therefore separately amplified and sequenced using DNA extracted from either C56 N or C34 U somatic hybrids. Alignment of the genomic sequences with previously reported cDNA sequences allowed the identification of the exon-intron boundaries, as well as part of the intronic sequences of both genes. Chromosomes 5p15 and

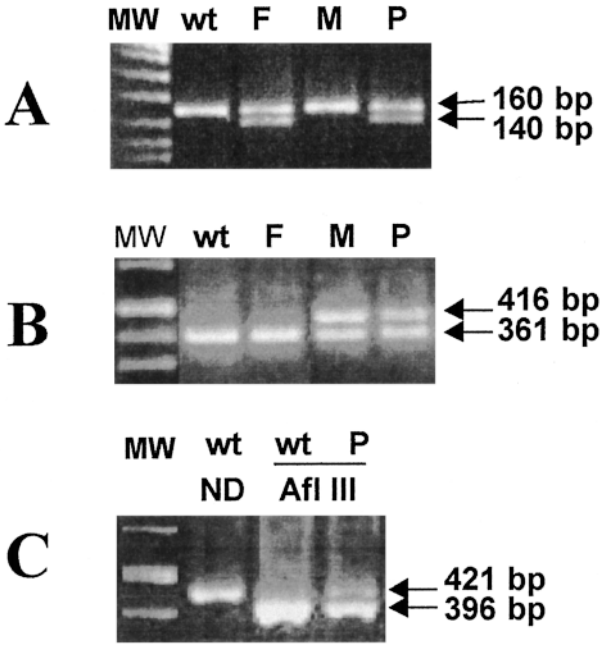

Fig. 1 Segregation of the *Fp* mutations in the patient's family. **A** Segregation of the C1595 T mutation detected by *Taq*I restriction analysis of the 160 bp genomic 5p15 specific fragment. Normal and mutant restriction fragments are 160 and 140 bp long, respectively. **B** Segregation of the A to C mutation at nt 25 of the *Fp* gene detected by *Afl*III restriction analysis of the 416 bp genomic 5p15-specific fragment. Mutant and normal restriction fragments are 416 and 361 bp long, respectively. **C** Relative amount of normal and A to C (nt 25) mutant transcripts detected by *Afl*III restriction analysis. Normal and mutant restriction fragments are 396 and 421 bp long, respectively. (*MW* molecular weight, *wt* wild-type control, *F* father, *M* mother, *P* patient, *ND* non-digested, *AflIII* digested by *Afl*III restriction enzyme)

Table 2 Respiratory chain investigation in patient and control lymphoblastoid cell lines. The absence of normal distribution of absolute control values precludes the use of mean and standard deviation. The ratio values are presented as mean ±1 SD. Abnormal values are in bold. (*COX* cytochrome-*c* oxidase, *SCCR* succinate cytochrome-*c* reductase, *SQDR* succinate quinone DCPIP reductase, *SPDR* succinate PMS DCPIP reductase)

Lympho-blastoid cell lines	Activities (nmol min^{-1} mg^{-1} prot)				Ratios	
	COX	SCCR	SQDR	SPDR	COX/ SCCR	COX/ SQDR
Patient	211	**21.4**	12.9	**10.7**	**9.8**	**16.3**
Control	145–229 (*n*=5)	43–71 (*n*=4)	21–52 (*n*=3)	17–29 (*n*=3)	3.5±0.3 (*n*=3)	6.0±1.3 (*n*=3)

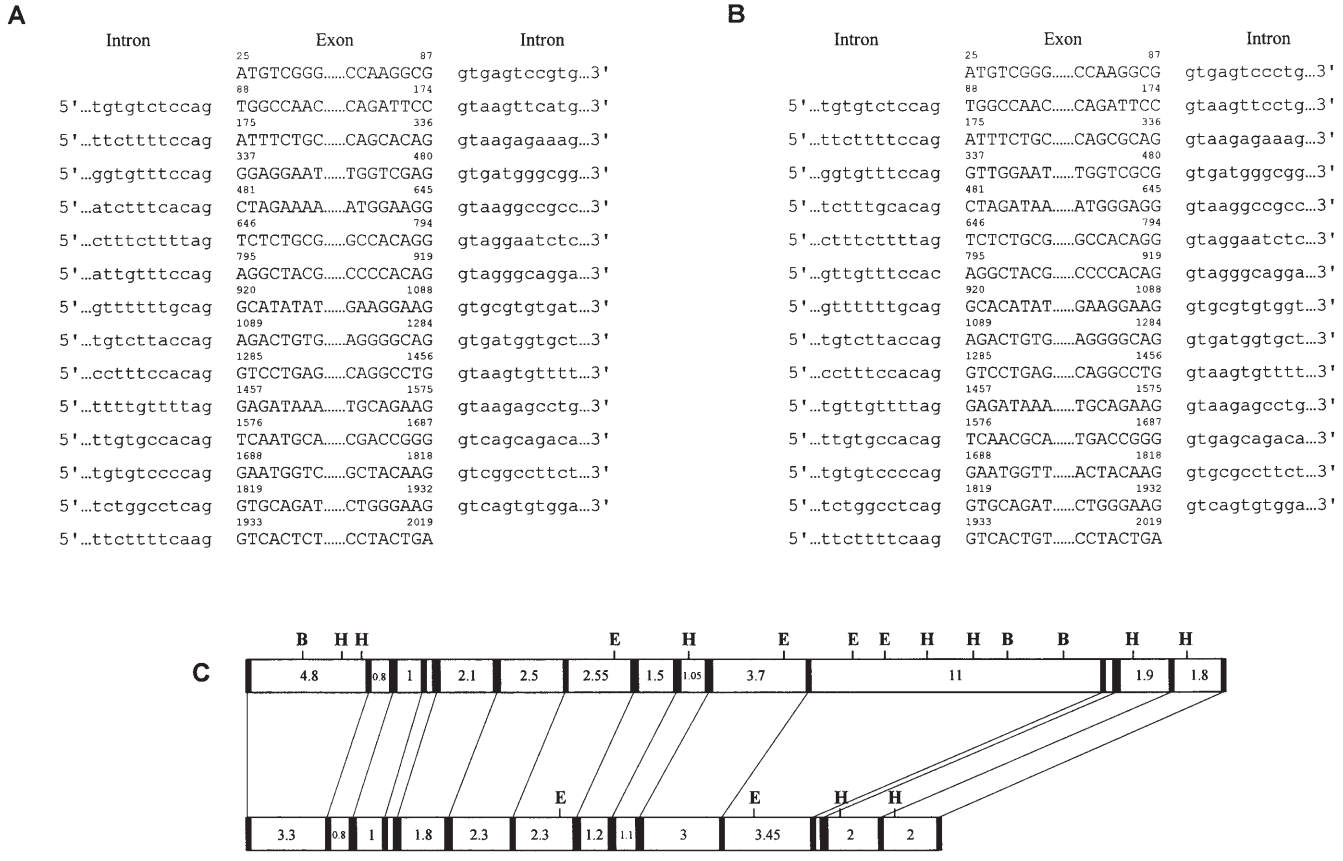

Fig. 2 Sequence of the exon-intron boundaries and structure of the two *SDH* genes. Exon-intron boundaries of chromosome 5p15 (**A**) and chromosome 3q29 (**B**) *Fp* genes. The position of the exon-intron boundaries is indicated with respect to the cDNA sequence (accession number L21931). **C** Physical organization of 5p15 (*top*) and 3q29 (*bottom*) *Fp* genes. The position of exons 1 to 15 are indicated by *black vertical blocks*. The *numbers* indicate the size of introns (kb). Accession numbers: chromosome 5p15 Fp gene AF171017, AF171018, AF171019, AF171020, AF171021, AF171022, AF171023, AF171024, F171025, AF171026, AF171027, AF171028, AF171029, AF171030; chromosome 3q29 Fp pseudogene AF171003, AF171004, AF171005, AF171006, AF171007, AF171008, AF171009, AF171010, AF171011, AF171012, AF171013, AF171014, AF171015, AF171016. (*B Bam*HI, *E Eco*RI, *H Hin*dIII)

3q29 copies presented 15 exons and 14 introns extending over 38 kb and 27 kb, respectively. The sequences of the exon-intron boundaries of chromosomes 5p15 and 3q29 *Fp* genes are summarized in Fig. 2A and B. The approximate size of the introns was determined by PCR amplification using exonic primers. Typical splice acceptor and donor sequences gt-ag were found for all exons, except for exon 7 of the chromosome 3 copy. A comprehensive restriction map of the SDH *Fp* genes and the sizes of the introns are shown in Fig. 2C. Several differences allowed the two *Fp* copies to be distinguished. In particular, the chromosome 3 *Fp* gene presented a 1 bp deletion at nt 139 in exon 2, creating a frameshift leading to a stop codon 17 codons later, and establishing that this gene was actually a pseudogene.

Sequence analysis of the 1950 bp of the 5' untranscribed region of the chromosome 5 *Fp* gene was next performed using a cosmid containing the 5p15 gene and analyzed using the Mat Inspector/Transfac program (1995; 1999). The first 200 bp are extremely GC rich (76% GC) and lack the canonical TATA or CAAT boxes, a feature which is specific of housekeeping genes (Dynan 1986; Dynan 1989). However, six putative Sp1 binding sites were located at nucleotides –24, –319, –358, –384, –720 and –1555 (Fig. 3). In addition, several regions presented homology with protein binding sites involved in coordinated expression of nuclear genes encoding mitochondrial proteins (Scarpulla 1996). Indeed, four 12-nt long motifs sharing a 11/12 nucleotide homology with the consensus nuclear respiratory factor 1 (NRF1) binding site were present at –192, –143, –94 and –45. Moreover, two 13-nt sequences presenting a 10/13 nucleotide homology with the OXBOX transcriptional element were found at –1758 and –315 and three putative mt2, mt3 and mt4 binding sites are present at –1765, –1161 and –667 respectively. Glucocorticoid-responsive elements (GRRE), cAMP-responsive elements (CRE/ATF) and nuclear hormone receptor binding sites are also observed in this 5' upstream region. Finally, tissue specific transcription factor binding sites were present in this region: a putative E-box binding site (nt –1583 and –948), which may act as a muscle-specific enhancer, and several putative binding sites for Nkx-2.5, a homeobox gene with expression restricted to heart tissue (Lints et al. 1993). A detailed com-

CCCCGACTTGAGAGCAGCAAAGCCTTCAGAGGGAACTAAGTTATTAAAACCCACCCCACACAAAATGCTTCTAAACGCCT -1870

TGGCTGACTTAATCCTTGCAGTGTGCTGAGGTTAAGTATGATTATCCATCTCCTGTGAACAGGAAGACTGAGGCTCAGAG -1790
IRE NRRE

CTTAAGTGATTTGCCCACGGTTCACTGCAGGGGATAAAACTCGGCTGTCCCTGGCAGGTCCCAAAGTCACGGCCTTCAGG -1710
Nkx 2.5 mt2 OXBOX

CTCCCACACCATGCTGCCTCCCTCACCGGGGCTCCACACTCTCAGCCTGGAAAACACGAATTTTAAGAAAACCATGACTT -1630

ACGAAAAGCAGCCAATCCCAGCATTGGAACCAACAGGGCATAATTCCACCTGCTTCCATCACCACCATCCGCCCTGGAAT -1550
Myo D sp1

TAGGCCGGATATTCCAATTTGGGGGATCATTTAGGTTATTCATGGAGATATACCTTGAAGTAAAGAATAAAAACGTTAAC -1470

TACTTTTATTCAAGAAATTTTTCTTCAGTTTTAACTATGTGCTTGCTGCACAGCACTGAGGCCCTACGGGATTCAAAGAA -1390
GRE GRE IRE

GAAAAAAACTAGACCAGAGCCGGGCATGGTGGCGGGCACCTGTAGTCCCAGCTACTCGGGAGGCTGAGGCAGGAGATTCG -1310
Alu sequence

CTTGAACCTGGGAGGTGGAGGTTGCAGTGAGTCGAGGTCGCGCCACTGCACTCCAGCCTGGTGACAGAACGAGACTCCGT -1230
Alu sequence

CCCCCCCAAAAAAAAAGAAAAAGAAAAAGGAAAAAAAAAACAAGAACTTAAGATGAAAGCGACTCACAATCTGGTTGGGG -1150
Alu sequence mt3

AAAGTGGAGAAAACAGACATGTCTTAAGCATCTGAAGTGCAGGTAGAATCAGGTAAGTTCTCTGGTACAAATATGTTTAA -1070
Nkx 2.5 NRRE GRE

ATGTATGGCTGCTGCGATGGTTAATTTTGTTTGTCAACTTGACGGCATCATGAGGTGCTCAGATACATGGTTAAGCATTA -990
NRRE CRE/ATF NRRE

TTTCTGGGCGTGTCTGTAAGGGTGTTTTTGGATGAGATTAGCAATTGAACCTTTAAACCAATTCCTCACGTCTCCCTTCT -910
GRE E-Box

ATTTGTTGTGTCTCTGGAGAACCCTCATACAGTTGTATCAAAGATACAGAATCAACTCGATAAAAGTGGGCTCACACCGG -830
GRE

ACACTTTCATATGAGCTAGGGTTTGAAGAGTGAGCAGCATTTTGACAGGCAGAGAAAGAAGGAAAGGGTCTTCTGATCAG -750
GRE

AAGGAAGACTGTGAGGAAGGCAGGGAGGGGTGATGTGGGGAAGGTGCAAACAGTGCTAGAAGCTTCTGAGGCCTCCTCGT -670
sp1 IRE

GGTGGTGATGAATGCAGAGATTACAGAGCTCTCTGCTTCGGGCTCAGCATGCACCCTCCATAGCTAGGCTGGGGGCTAAC -590
mt4

GTTAGTAAGAATTTACTGATTTACTGAGTACCTACACGAAGTTTCACATTTTCACAAAGTAGAGTCACTTTAAGTTTGTT -510

TACATGTGACCTCCACAAACACACACGATTACAAAATAGTTTAGTTAATCAAGGGATGTATAATAAACAAGAGGTC**GGAG** -430
Nkx 2.5

GCCCCGGGCCGCTCCCGCCCACCTCCGCGGACGAGCGCCCCCCTCCGACCCCATTCCCTGCGGTCTGGGCGTTCAGGCCC -350
Fp/Ip homologous sequence sp1 sp1

TTCGGTCTGGGCGATCCCGGAGAACCACCCACGGGGCTTTAAAAATGTTGGTGCCCACCACCTCCCCGGAACAGGGCCCG -270
sp1 OXBOX

CTCTACCTCGGTCGGGGAGCGCGGGACCTCAGCGTTCCCTTAACGCCACCGTCCGCGGGTCCGCTTTGCGCAGGCGCGGC -190
NRF1

GCCCCCACTCAGTACCCGCTCCGGGCGTGGCATGGTGCGCAGGCGCGATGTCCCCCACTGCAGCCCCGCTCGACTCCGGC -110
NRF1

GTGGTGCGCAGGCGCGGTATCCCCCCTCCCCCGCCAGCTCGACCCCGGTGTGGTGCGCAGGCGCAGTCTGCGCAGGGACT -30
NRF1 NRF1

GGCGGGACTGCGCGGCGGCAACAGCAGAC**ATG**
sp1 Translation initiation

Table 3 Expression of wild-type and mutant SDH *Fp* cDNAs in SDH-deficient SV40-immortalized fibroblasts. Abnormal values are in bold. (*SCCR* succinate cytochrome-*c* reductase, *GCCR* glycerol-3-phosphate cytochrome-*c* reductase)

Skin fibroblasts	Activities (nmol min^{-1} mg^{-1} prot)		Ratios SCCR/GCCR
	SCCR	GCCR	
Control fibroblasts (primary culture)	17–58 (*n*=84)	10–21 (*n*=36)	1.80±0.19 (*n*=30)
SV40-immortalized control fibroblasts	26.6	16	1.7
SDH Fp-deficient fibroblasts (primary culture)	**8.3**	13.7	**0.6**
SDH Fp-deficient SV40-immortalized fibroblasts	**7.1**	11.4	**0.6**
SDH Fp-deficient SV40-immortalized fibroblasts transfected with wt-*Fp* cDNA	27.2	14.2	1.9
SDH Fp-deficient SV40-immortalized fibroblasts transfected with C1595 T *Fp* cDNA	**7.5**	13	**0.6**

parison with the previously published *Ip* subunit promoter region indicated the presence of a homologous 22/26 bp region which may represent a target for a co-regulation of these two functionally related genes. Noticeably, as reported for the Ip subunit, two iron-responsive elements (IRE; –1417, –699) were also found in the promoter region of the *Fp* gene.

The complete sequence analysis of the *Fp* gene promoter was next performed on genomic DNA from patient lymphoblastoid cell lines, using oligonucleotides specific for the chromosome 5 copy. This sequence analysis revealed an heterozygous A to C substitution at the 25th nucleotide of the coding sequence, changing the methionine translation initiation codon to a leucine, which was found in both the patient and her mother (Fig. 1B). RT-PCR amplification and restriction enzyme analysis using *Afl*III, a restriction site which is abolished by the A to C substitution at nt 25, allowed us to distinguish between normal and mutated transcripts of the *Fp* gene. Densitometric analysis showed that the mutant transcript represented only 10% of total Fp transcripts, suggesting a high instability of this transcript in the patient (Fig. 1C). Moreover, as the next translation initiation codon is located 339 nt downstream, translation should result in a truncated protein of 551 amino acids (instead of 664) lacking the mitochondrial targeting sequence.

In order to demonstrate the deleterious effect of the A524 V mutation identified in patient lymphoblastoid cell lines, we next transfected both normal and mutant cDNAs into immortalized Fp-deficient skin fibroblasts from a patient previously shown to harbor a deleterious mutation in the *Fp* gene (Bourgeron et al. 1995). We first checked that the immortalization of these latter cells by stable transfection of SV40 T antigen did not modify either the overall respiratory chain enzyme activity or the deficient SDH activity. Stable transfection of pIRES neo/wild-type *Fp* cDNA into these SDH deficient cells resulted in normal SDH activity (Table 3). However, transfection of the A524 V mutant *Fp* cDNA failed to restore normal SDH activity, confirming the deleterious effect of this mutation (Table 3).

◀ **Fig. 3** Sequence of the promoter region of the chromosome 5p15 *Fp* gene. The GenBank accession number is AF171017. The *underlined* elements include (1) transcription factor binding sites specific for mitochondrial genes: NRF1, OXBOX, mt2, mt3, mt4, and (2) other transcription factor binding sites: IRE, NRRE, Nkx2.5, GRE, Myo D, CRE/ATF, Sp1. A 22/26 bp sequence homology with a sequence in the *Ip* promoter is *underlined*, in *bold*

Discussion

Here, we report two compound heterogygous mutations in the *Fp* subunit gene of SDH in a patient presenting with LS. A first heterozygous mutation, inherited from the mother, affected the methionine translation initiation codon and may result in a truncated protein lacking the mitochondrial targeting sequence. A quantitative decrease of the corresponding mRNA was observed. A second heterozygous mutation, inherited from the father, changed an alanine to a valine at codon 524. Pathogenicity of this mutation, occurring in a moderately conserved region of the protein, was proven by expression studies showing that this alanine to valine change led to an altered function of the protein. This functional alteration may result from steric hindrance resulting from the distinctive shape of the alkyl group of the valine side chain as compared to that of alanine.

An examination of the few cases of complex II deficiency for which a molecular basis has been elucidated (Bourgeron et al. 1995; Goto et al. 1998; Birch-Machin et al. 1999) revealed that only mutations in the *Fp* gene have been found in patients with complex II deficiency. Indeed no mutation in the coding regions of either the Ip subunit, or in the SDH C and D subunits has been identified in any patients so far. Similarly, in the six cases of complex II deficiency identified in our group, two of them had an heterozygous mutation in the *Fp* gene, while no mutation in any of the other structural genes encoding complex II subunits could be found (unpublished data). This is strongly reminiscent of the data obtained by systematic sequencing of structural genes encoding respiratory chain complexes III or IV, which in most cases failed to identify mutations in these genes (Parfait et al. 1997; Valnot et al. 1999). This suggests that other genes involved in the biogenesis, assembly and/or maintenance of RC complexes might be involved in a majority of the cases. To date, only one gene involved in complex II assembly is known in yeast, namely TCM62 (Dibrov et al. 1998) but its human counterpart is still unknown.

In the course of this study, we established that the copy of the *Fp* gene present on the chromosome 3 is actually a pseudogene. Similarly, previous results have shown that only one of the three copies of *SDHC* is actually expressed (Elbehti-Green et al. 1998). Thus, this excludes the possibility that the various clinical presentations and the tissue-specific expression of complex II deficiency could be explained by different expression of each of the two *Fp* copies. These variations could rather be due to a differential regulation of complex II gene expression in different tissues or the intervention of additional genes or mechanisms. In keeping with this, the sequence analysis of the promoter region of *Fp*, carried out while searching for potential mutations in our patient, identified several putative regulatory elements that could account for tissue specificity. With respect to co-regulated expression of the genes coding for the four complex II subunits, an NRF1 binding site was the sole site present in the promoter region of all four genes (Au and Scheffler 1998; Elbehti-Green et al. 1998; Hirawake et al. 1999; this work). Finally, a 22/26 bp region common to both the *Ip* and *Fp* promoter regions may represent a favorable target for co-regulation of these two functionally related subunits of complex II.

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