Mutation of a nuclear succinate dehydrogenase gene results in mitochondrial respiratory chain deficiency

Thomas Bourgeron, Pierre Rustin, Dominique Chretien, Mark Birch-Machin, Marie Bourgeois, Evani Viegas-Péquignot[1], Arnold Munnich & Agnès Rötig

We now report a mutation in the nuclear-encoded flavoprotein (Fp) subunit gene of the succinate dehydrogenase (SDH) in two siblings with complex II deficiency presenting as Leigh syndrome. Both patients were homozygous for an Arg554Trp substitution in the Fp subunit. Their parents (first cousins) were heterozygous for the mutation that occurred in a conserved domain of the protein and was absent from 120 controls. The deleterious effect of the Arg to Trp substitution on the catalytic activity of SDH was observed in a *SDH*⁻ yeast strain transformed with mutant Fp cDNA. The Fp subunit gene is duplicated in the human genome (3q29; 5p15), with only the gene on chromosome 5 expressed in human–hamster somatic cell hybrids. This is the first report of a nuclear gene mutation causing a mitochondrial respiratory chain deficiency in humans.

Unité de Recherches sur les Handicaps Génétiques de l'Enfant, INSERM U-393 et Département de Pédiatrie and [1]Unité de Recherches Génétique, Chromosome et Cancer, INSERM U-383, IFREM, Institut Necker, Hôpital des Enfants-Malades, 149, rue de Sèvres, 75743 Paris Cedex 15, France

Correspondence should be addressed to A.R.

Mitochondrial respiratory chain enzyme deficiency is the major cause of congenital lactic acidosis, a frequent condition (1/10,000 live births) associated with variable clinical presentations in humans[1,2]. The respiratory chain catalyzes the oxidation of fuel molecules by oxygen with a concomitant energy transduction into ATP. During the oxidation process, electrons are transferred to oxygen via four multienzyme complexes. Complex I carries reducing equivalents from NADH to coenzyme Q (CoQ). It consists of about 40 different polypeptides, seven of which are encoded by the mitochondrial DNA (mtDNA)[3]. Complex II, specifically involved in the oxidation of succinate, carries electrons from $FADH^2$ to CoQ. It consists of four nuclear-encoded polypeptides, including a flavoprotein (Fp) and an iron-sulphur protein subunit (Ip) which together constitute succinate dehydrogenase (SDH)[4]. The Fp and Ip cDNAs have recently been cloned and sequenced[5,6]. Complex III delivers electrons from the common lipid-soluble CoQ pool to cytochrome *c*. Finally, cytochrome *c* oxidase (complex IV) catalyzes the terminal transfer of electrons from cytochrome *c* to oxygen.

The molecular bases of respiratory chain enzyme deficiencies in humans remain largely unknown, although alterations of the mtDNA (point mutations, rearrangements, depletions) have been described[7]. Despite the overwhelming proportion of components encoded by nuclear genes in the respiratory chain, no mutation in nuclear DNA has hitherto been reported in inborn errors of oxidative phosphorylation. We now report a mutation in the Fp subunit gene of the SDH in two siblings with complex II deficiency presenting with Leigh syndrome[8]. This is the first report of a nuclear gene mutation causing a respiratory chain deficiency in humans.

Enzyme studies

A complex II deficiency was initially identified in the mitochondria isolated from the skeletal muscle of patient 1, based on both the impairment of succinate oxidation [12 nmol/min/mg protein; controls=34–92 (n=80)], and the decrease of succinate-dependent enzyme activities (Table 1). The decrease of SDH activity pointed to a defect of either the iron-sulphur protein subunit (Ip) or the flavoprotein subunit (Fp) of complex II, the two protein moieties which together constitute SDH[4]. The other respiratory chain enzymes were polarographically and spectrophotometrically normal, including the oligomycin-sensitive ATPase and the pyruvate dehydrogenase. Defects of the last two enzymes have occasionally been associated with Leigh syndrome[9]. Similarly, normal activities of complex III [210 nmol/min/mg protein; controls=196–1,110 (n=51)] ruled out a generalized defect of the proteins containing iron-sulphur clusters[10]. The same defect was found in cultured skin fibroblasts and circulating lymphocytes of the patient and her affected sister but not in their healthy consanguineous parents (Table 1).

Further kinetic studies on digitonin-permeabilised fibroblasts revealed normal K_m and K_i for succinate and malonate respectively, as well as normal pH optimum in the patients. However, the enzyme sensitivity to oxaloacetate (OAA; the only known physiological inhibitor of the enzyme) was greatly increased in patients' cells (Fig. 1). The progressive inhibition of SDH by OAA could be partially released by 100 μM ATP in control but not in patient cells (Fig. 1*a*, traces a and b). Unfortunately, the very slow equilibration of the inhibitor with the SDH in normal cells has previously been shown to preclude the determination of the K_i of the enzyme for OAA[11]. Studies

Table 1 Enzyme activities in two patients with complex II deficiency, their parents and controls

	Activities (nmol min^{-1} mg^{-1} prot.)			Ratios	
	COX	SCCR	SPDR	COX/SCCR	COX/SPDR
Patient 1					
Skeletal muscle mitochondria	294	50	12	5.9	24.5
Lymphocytes	92	17	5	5.4	18.4
Fibroblasts	99	16	4.5	6.2	22.0
Patient 2					
Fibroblasts	92	16	5	5.8	18.4
Mother					
Lymphocytes	82	24	13	3.4	6.3
Father					
Lymphocytes	87	25	12	3.5	7.3
Controls					
Skeletal muscle mitochondria (*n*=15)	245–1247	147–402	50–140	3.2 ± 0.3	9.1 ± 1.5
Lymphocytes (*n*=12)	61–216	21–70	12–33	3.1 ± 0.3	7.0 ± 0.6
Fibroblasts (*n*=8)	47–172	17–58	13–31	3.0 ± 0.4	5.2 ± 0.5

of the effect of varying concentrations of OAA (0.05–1 mM) on SDH activity indicated that the enzyme equilibrated very rapidly with the inhibitor even at the lowest OAA concentration in patient as compared to control cells (0.05 mM, Fig. 1*b*). Under these conditions, inhibition at 1 min was about 95% in patient compared to about 40% in control cells (Fig. 1*b*, inset). Apart from the partial loss of activity, this change in the regulatory functions of the mutant enzyme could also account for the severity of the disease.

Analysis of SDH gene and transcript

The cDNAs of wild-type Ip and Fp subunits have been cloned and sequenced[5,6,12]. The SDH Ip RNA from cultured skin fibroblasts of both patients was reverse transcribed and PCR amplified in two overlapping fragments.

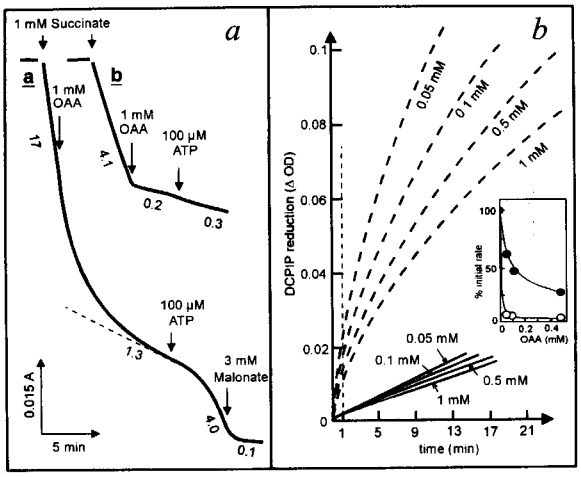

Fig. 1 Changes in the regulatory functions of patient SDH. *a*, Effect of oxaloacetate and ATP on SDH activity of control (trace a) and patient 1 cultured skin fibroblasts (trace b). SDH (complex II) was measured as the succinate quinone dichlorophenol indophenol (DCPIP) reductase activity[25]. Numbers along the traces refer to nmol DCPIP reduced per min/mg protein. *b*, Time-dependent inhibition of control (dotted line) and patient (solid line) SDH by varying OAA concentrations. Inhibitor was added after 30 s incubation with 1 mM succinate; Inset, percent of initial activities of control (dotted line) and patient (solid line) after a 1 min incubation with OAA in the presence of 1 mM succinate plotted as a function of OAA concentration.

Sequence analysis of these fragments showed no differences with six controls (not shown).

The Fp subunit RNA was subsequently reverse transcribed and amplified from skeletal muscle (patient 1), cultured skin fibroblasts (patients 1 and 2) or lymphocytes (parents) in six overlapping fragments using oligonucleotide primers designed from the human heart sequence[5]. Direct sequencing of the fifth fragment (nt 1360–1781) revealed a homozygous C→T transition at nucleotide 1684 in the patients (Fig. 2*a*), causing an Arg544Trp substitution in a highly conserved domain of the protein (Fig. 2*c*). Both parents were heterozygous for the substitution (Fig. 2*a*). This C→T transition abolishes an *Msp*I restriction site, allowing for rapid screening of the mutation (Fig. 2*b*). Northern blot analysis of the patients' and parents' RNAs showed a normal abundance of the Fp transcripts as compared to controls (not shown).

The genomic DNA encompassing the C→T transition was amplified between two primers located 151 bp apart in the cDNA sequence. This produced a 387-bp fragment that contained one intron of the Fp subunit gene (Fig. 3*a*). Heterozygosity of both consanguineous parents for the C→T transition was confirmed by restriction analysis and direct sequencing of the 387-bp genomic fragment (Fig. 3*b*, *c*). This C→T transition was not found in 120 controls. However, to our surprise, restriction enzyme and sequence analyses revealed the presence of both the C→T transition and the normal sequence in the patients' genomic DNA (Fig. 3*b*, *c*). To account for the apparent discrepancy between the cDNA and the genomic DNA sequences in the patients, the copy number of the SDH Fp subunit gene in the human genome was investigated.

Physical mapping of the SDH Fp gene

Southern blot analysis of control genomic DNA digested with *Pvu*II, *Eco*RI, *Rsa*I or *Taq*I and hybridized with the 387-bp genomic fragment of the SDH Fp gene revealed only one restriction fragment (not shown). For this reason, pulsed field gel electrophoresis was performed using control genomic DNA digested with rare-cutter restriction enzymes. Digestion using *Sac*I, *Bss*HII, *Sfi*I, *Kpn*I or *Xho*I and hybridization with the 387-bp genomic fragment revealed at least two restriction fragments (Fig. 4*a*). These results, suggestive of a duplication of the succinate Fp gene, prompted us to carry out fluorescence *in situ*

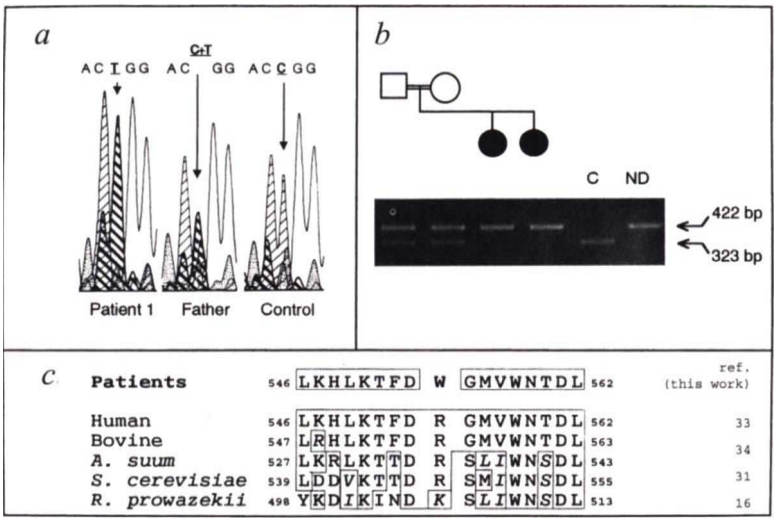

Fig. 2 Molecular analysis of the SDH Fp cDNA. *a*, Sequence analysis of the SDH Fp cDNA from patient 1, her father and a control. *b*, Segregation of the SDH Fp mutation in the family, detected by *MspI* restriction analysis of the cDNA. Mutant and normal restriction fragments were 422 and 323 bp, respectively. *c*, Multiple sequence alignment of the conserved domain of the SDH Fp protein from patients, controls and from different species. Conserved residues are shaded. Residues indicated by italic letters belong to the same amino acid class.

hybridization on metaphase chromosomes (Fig. 4*b*). Two signals were observed, one on the distal long arm of chromosome 3 (3q29; 80% of labelled cells), and the other on the distal short arm of chromosome 5 (5p15, 50% of labelled cells).

Expression of the human SDH Fp gene

To determine which of the two *SDH* genes are expressed, total RNA and genomic DNA were isolated from human-rodent somatic cell hybrids harbouring either human chromosome 3 (C34U) or chromosome 5 (C56N). PCR amplification of the reverse transcribed Fp RNA (fragment 5 or the fragment used for the study of the genomic DNA) only detected an amplification product in somatic cell hybrids harbouring human chromosome 5 (Fig. 4*c*). No specific amplification product was observed in the C34U or the hamster cell line cDNA. Control amplification of the 387-bp genomic fragment was observed in both somatic cell hybrids but not in the hamster cell line. These results indicate that the SDH Fp gene localized on chromosome 5 is the only one expressed in the somatic cell hybrids tested.

Expression of the mutant SDH Fp gene in yeast

To support the deleterious effect of the C→T transition in the SDH Fp gene, expression studies in cultured human cells were originally considered. Unfortunately, the high residual enzyme activity (more than 40%), and the lack of SDH-deficient human strains, hampered functional tests. We therefore used an *SDH⁻ Saccharomyces cerevisiae* mutant strain (sdhA6L) carrying a disrupted SDH Fp gene[13]. We failed to express the human wild-type SDH Fp cDNA in the SDH⁻ yeast strain as previously reported for the SDH Ip subunit[14] (results not shown). As the mutation is located in a conserved domain of the protein, we performed *in vitro* mutagenesis of the yeast SDH Fp cDNA. The deleterious effect of the mutation was eventually confirmed by comparing the SDH activity of the *SDH⁻* yeast strain transformed by the mutant Fp cDNA (*SDHYm*) with that transformed by the wild-type Fp cDNA (*SDHYwt*). Both the complex II and the SCCR activities of the *SDHYm* transformants were decreased to less than 50% of the activity of the *SDHYwt* transformants. The decrease affected both the absolute values and the relative enzyme activities and was largely similar to the activity decrease observed in patients' cells (about 60%).

We subsequently studied the effect of the mutation on the sensitivity of the yeast enzyme to OAA. A markedly increased sensitivity of the wild-type yeast enzyme to OAA as compared to its human counterpart was observed. Similarly, the OAA sensitivity of the *E. coli* enzyme is known to differ greatly from that of the human enzyme, suggesting that the regulation by OAA is a distinctive feature between species[11]. As expected, no significant change in SDH sensitivity to OAA between the *SDHYm* and the *SDHYwt* transformants was observed (not shown).

Discussion

Our study reports the first mutation of the SDH gene (Fp subunit) in two sisters presenting with a Leigh syndrome ascribed to SDH deficiency. The patients were homozygous for a C→T transition in the coding sequence (nt 1684) of the Fp subunit in all tissues tested, and their

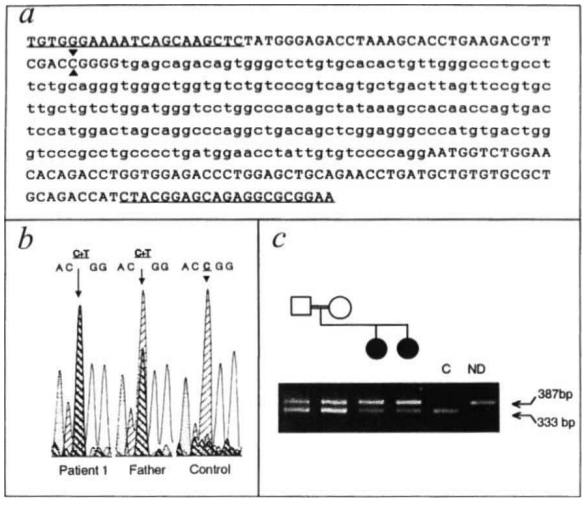

Fig. 3 Molecular analysis of the SDH Fp gene. *a*, Sequence analysis of the 387-bp SDH Fp genomic fragment containing the C→T transition. Bold letters indicate exonic sequences; primer sequences are underlined. The location of the C→T transition is indicated by an arrow. *b*, Sequence analysis from patient 1, her father and a control. *c*, Segregation of the Fp mutation in the family detected by *MspI* analysis of the 387-bp genomic fragment. Mutant and normal restriction fragments are 387 and 333 bp long, respectively.

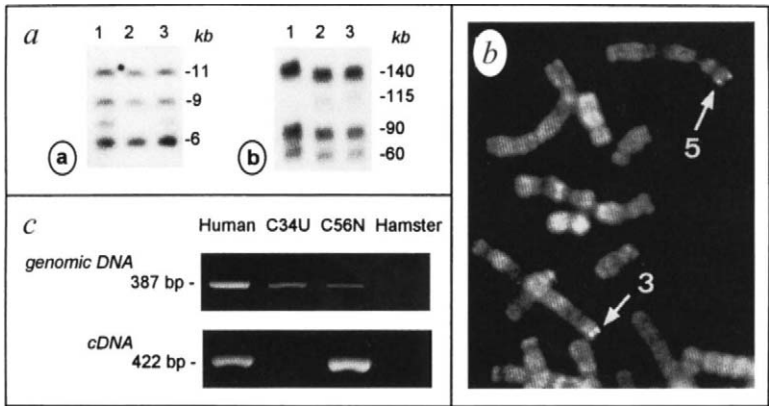

Fig. 4 Physical mapping and expression study of the SDH Fp genes. *a*, Pulsed-field gel electrophoresis analysis of three control DNAs (1–3), digested by *Sac*I (a) or *Bss*HII (b) and hybridized with the 387-bp genomic fragment. *b*, FISH analysis of the SDH Fp cDNA to human metaphase chromosomes using a full-length biotinylated Fp cDNA probe, immunofluorescence detection and propidium iodide counterstaining. SDH gene–specific signals are indicated by arrows. *c*, Expression of the human SDH Fp subunit genes in human–hamster somatic cell hybrids. Amplifications of either the 387-bp genomic fragment or the SDH cDNA (fragment 5) were performed as described under Methods. Similar amplification pattern was obtained on somatic cell hybrid cDNA by using the primer pair used to amplify the 387-bp genomic fragment. Somatic cell hybrids contained all hamster chromosomes plus either human chromosome 3 (C34U) or 5 (C56N).

consanguineous parents were heterozygotes. The C→T transition occurred in a CpG doublet regarded as a hot spot for deamination of the 5-methylcytosine to thymine[15]. This mutation changes a positively charged (Arg) into a neutral cyclic amino acid (Trp) in a conserved domain of the protein. The mutant enzyme has a normal K_m and K_i for succinate and malonate respectively, as well as normal pH optimum. However, there was an increased sensitivity of the mutant enzyme to the inhibitory effect of oxaloacetate (OAA), the only known physiological regulator of the enzyme. This may suggest a distinctive role of the conserved domain encompassing the mutation for the binding of OAA. Alternatively, the mutation could alter either the conformation or the redox state of the protein, both of which might potentially alter enzyme catalysis and response to OAA[11,16]. Moreover, a 50% decrease of the SDH activity was observed in the SDH⁻ yeast strain transformed with the mutagenised yeast SDH Fp cDNA, as compared to control transformants. These functional data, along with the segregation of the mutant allele with the enzyme deficiency, the location of the substitution in a highly conserved domain, and the absence of the mutation in 120 healthy controls, give strong support to the view that this mutation produces the SDH deficiency in our patients. Yet, the effect of the mutation on the sensitivity of SDH to OAA could not be reproduced in the yeast mutant protein, a feature which could be related to the differences between yeast and human SDH.

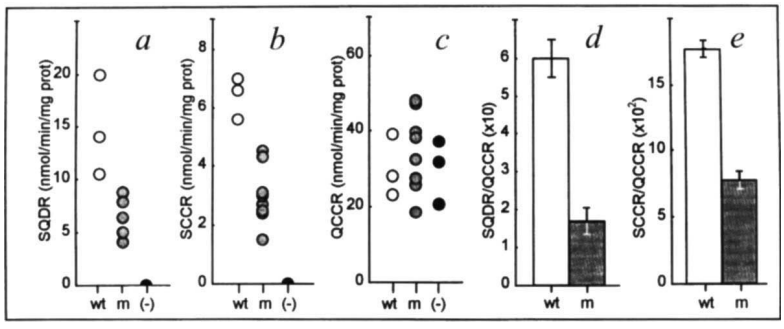

Fig. 5. Expression of wild-type and mutant SDH Fp cDNA in SDH-deficient yeast. Absolute rates of DCPIP (*a*, complex II; SQDR), succinate cytochrome *c* reductase (*b*, complexes II+III; SCCR), quinol cytochrome c reductase (*c*, complex III; QCCR) and relative activities of SQDR to QCCR (*d*) and SCCR to QCCR (*e*) were measured in SDH-deficient yeast strain (sdhA6L) untransformed ((–); dark symbols) or transformed with wild-type (wt; light symbols) or mutant SDH Fp cDNA from yeast (m; shaded symbols). Bars refer to mean values ± 1 SE for each condition.

The discrepancy between the cDNA and the genomic sequence in our patients could be ascribed to a duplication of the SDH Fp genes on chromosomes 3 and 5 respectively. The duplication of nuclear genes encoding mitochondrial proteins has been reported[17,18]. Nothing was known until now regarding the gene organization, the tissue specific expression and the developmental regulation of the two SDH Fp genes. Yet, the amplification of an intronic sequence from either human-rodent somatic cell hybrid speaks against a retrogene and favors the view that the duplication of an ancestral gene has occurred.

The gene localized on chromosome 5p15 is the only one expressed in our somatic cell hybrids. Yet, if both genes are expressed, their tissue-specific and/or developmentally regulated expression could be highly relevant to the clinical heterogeneity of SDH deficiencies in humans[10, 19–24], especially as SDH also includes an iron–protein subunit. Indeed, there is a considerable variation in the clinical presentation of the three reported cases of isolated SDH deficiency[22–24]. The first patient had Kearns-Sayre syndrome (KSS), including the conduction defects[22]. The second had isolated hypertrophic cardiomyopathy[23]. Finally, Reichman and Angelini reported a patient with hypertrophic cardiomyopathy and skeletal muscle myopathy[24]. Based on the above observations, heart involvement appears to be a consistent feature in isolated SDH deficiency. In contrast, it should be noted that heart involvement was not observed in our two patients. Similarly, optic atrophy, cerebellar ataxia, but no heart involvement were observed in two recent cases of isolated SDH deficiency in two sisters (M. B.-M., unpublished results).

In accordance with the variable clinical expression of the disease, its biochemical expression showed a marked tissue-to-tissue variation. In our study, the enzyme defect was detected in all those tissues tested, namely, skeletal muscle, circulating lymphocytes and cultured skin fibroblasts. In contrast, the enzyme defect was found in muscle but not in cultured skin fibroblasts of the KSS patient[22], while it was only found in heart of the patient with isolated hypertrophic cardiomyopathy (normal activity in skeletal muscle, circulating lymphocytes and cultured skin fibroblasts)[23]. Finally, enzyme measurements were only performed in skeletal muscle of the patient with skeletal muscle myopathy and hypertrophic cardiomyopathy[24]. Age at onset also differed among patients: early childhood in our patients and in the patient with isolated cardiomyopathy[23], adulthood (25 years) for the KSS patient[22], and in our recently observed case (55 years, M. B.-M., unpublished data). Altogether, this heterogenous pattern of clinical presentations, organ involvement and biochemical findings is suggestive of the genetic heterogeneity of SDH deficiencies. In conclusion, this study represents the first molecular characterization of a respiratory chain enzyme deficiency of nuclear origin in humans.

Methods

Clinical description. Patients 1 and 2 were born to first cousin Tunisian parents. Their clinical course and presentation have been reported and meet the diagnostic criteria of Leigh syndrome[6].

Tissue samples and cell culture. Skeletal muscle homogenates and mitochondria-enriched fractions were prepared from open microbiopsies of the deltoid (120 mg)[25]. Circulating lymphocytes were isolated from 10 ml of blood on a Ficoll cushion[26]. Somatic cell hybrids, containing all hamster chromosomes plus human chromosome 3 (C34U) or 5 (C56N), have been described[27]. All cells were grown in RPMI 1640 (Life Technologies Inc.) supplemented with 10% undialyzed fetal calf serum, 2 mM glutamine, 2.5 mM sodium pyruvate, 100 μg ml^{-1} streptomycin, 100 U ml^{-1} penicillin and 200 μM uridine at 37 °C, under standard conditions[28].

Enzyme assays. Cytochrome *c* oxidase (EC 1.9.3.1), succinate phenazine methosulphate (PMS) dichlorophenol indophenol (DCPIP) reductase (EC 1.3.99.1), succinate quinone DCPIP reductase, succinate cytochrome *c* reductase and decylubiquinol cytochrome *c* reductase activities were measured spectrophotometrically according to standard procedures[25]. Enzyme measurements on cultured skin fibroblasts were performed on freeze-thawed cells[25]. Polarographic studies were performed in a 250 μl cell using 30 μg protein[25]. Enzyme measurements in yeast were performed on the 1,000*g* supernatant, after sonication (10, 5 and 5 s in 200 μl ice-cooled medium; 20 kiloHertz; VibraCell 72434, BioBlock) of the yeast samples.

Sequence and restriction analyses. Total RNAs were extracted from skeletal muscle, cultured skin fibroblasts or somatic cell hybrids[29] and reverse transcribed using the GeneAmp® RNA PCR kit (Perkin-Elmer). The SDH Ip cDNA[6] was amplified in overlapping fragments using two pairs of primers (fragment 1: primer 5' nt 10–nt 30, primer 3' nt 412–nt 392; fragment 2: primer 5' nt 348–nt 368, primer 3' nt 791–nt 771). The SDH Fp cDNA[5] was amplified in overlapping fragments using six pairs of primers (fragment 1: primer 5' nt 1–nt 21, primer 3' nt 421–nt 401; fragment 2: primer 5' nt 368–nt 388, primer 3' nt 767–nt 747; fragment 3: primer 5' nt 722–nt 742, primer 3' nt 1060–nt 1040; fragment 4: primer 5' nt 999–nt 1019, primer 3' nt 1421–nt 1401; fragment 5: primer 5' nt 1360–nt 1380, primer 3' nt 1781–nt 1761; fragment 6: primer 5' nt 1731–nt 1751, primer 3' nt 2141–nt 2121). A genomic fragment containing one intron was amplified from 200 ng of total DNA using primer 5' nt 1630–nt 1650 and primer 3' nt 1781–nt 1761. After 30 cycles (95 °C, 30 s; 55 °C, 30 s; 72 °C, 2 min), amplification products were purified on a 2% low melting point agarose gel and recovered by heating for 5 min at 65 °C. Direct sequencing was performed on an automatic DNA sequencer (Applied BioSystems 373A) using 3.2 pmol of the amplification primer, 100 ng DNA and 9.5 μl sequencing reaction mixture (Perkin-Elmer). For restriction enzyme analysis, PCR products (500 ng) were digested using 10 U *MspI* enzyme.

Northern blot analysis. Total RNA (20 μg) from each sample were electrophoresed on 1.5% agarose formaldehyde gels and transferred onto Hybond N^+ membranes.

Pulsed field gel electrophoresis. Digested DNA samples were run on a CHEF DRIII electrophoresis system (Biorad), using the running conditions recommended by the manufacturer. Fragments of 50 to 1,200 kb were separated by electrophoresis on 1% agarose Seakem gels, at 200 V for 24 h at 40 °C using a 30 to 70 s ramping pulse time.

Fluorescence *in situ* hybridization. Non-radioactive *in situ* hybridization was performed on metaphase chromosomes obtained from lymphocyte cultures of normal donors. Details of chromosome preparations, banding, probe labelling and hybridization have been described[30]. The SDH cDNA probe of 2.277 kb inserted in pBlueScript SK^- was nick-translated with biotin-11-dUTP (Enzo Laboratories).

***In vitro* mutagenesis and yeast transformation.** *In vitro* mutagenesis of the yeast SDH Fp cDNA was performed with the Sculptor mutagenesis kit (Amersham®). Primer 5'GACGACCGATTGGT-CAATGATCTGG3' (nt 1629–nt 1653) was used to change Arg 547 (AGA) into Trp (CGG). Eight independent mutant constructs were sequenced for the mutation and separately transfected in the deficient yeast strain. The yeast SDH Fp gene cloned in the vector pRS416 has been previously described[31]. The yeast mutant strain (sdhA6L) disrupted for the SDH Fp subunit[13] was transformed by either mutant or wild-type constructs using the lithium acetate method[32]. Transformed yeasts were selected on minimum medium *minus* uracyl and assayed as described above for SDH-dependent activities.

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