The Molecular Basis of Cytochrome Oxidase Deficiency in Childhood

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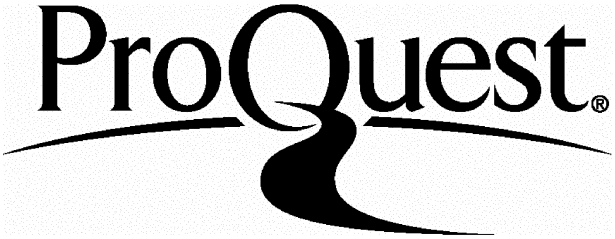

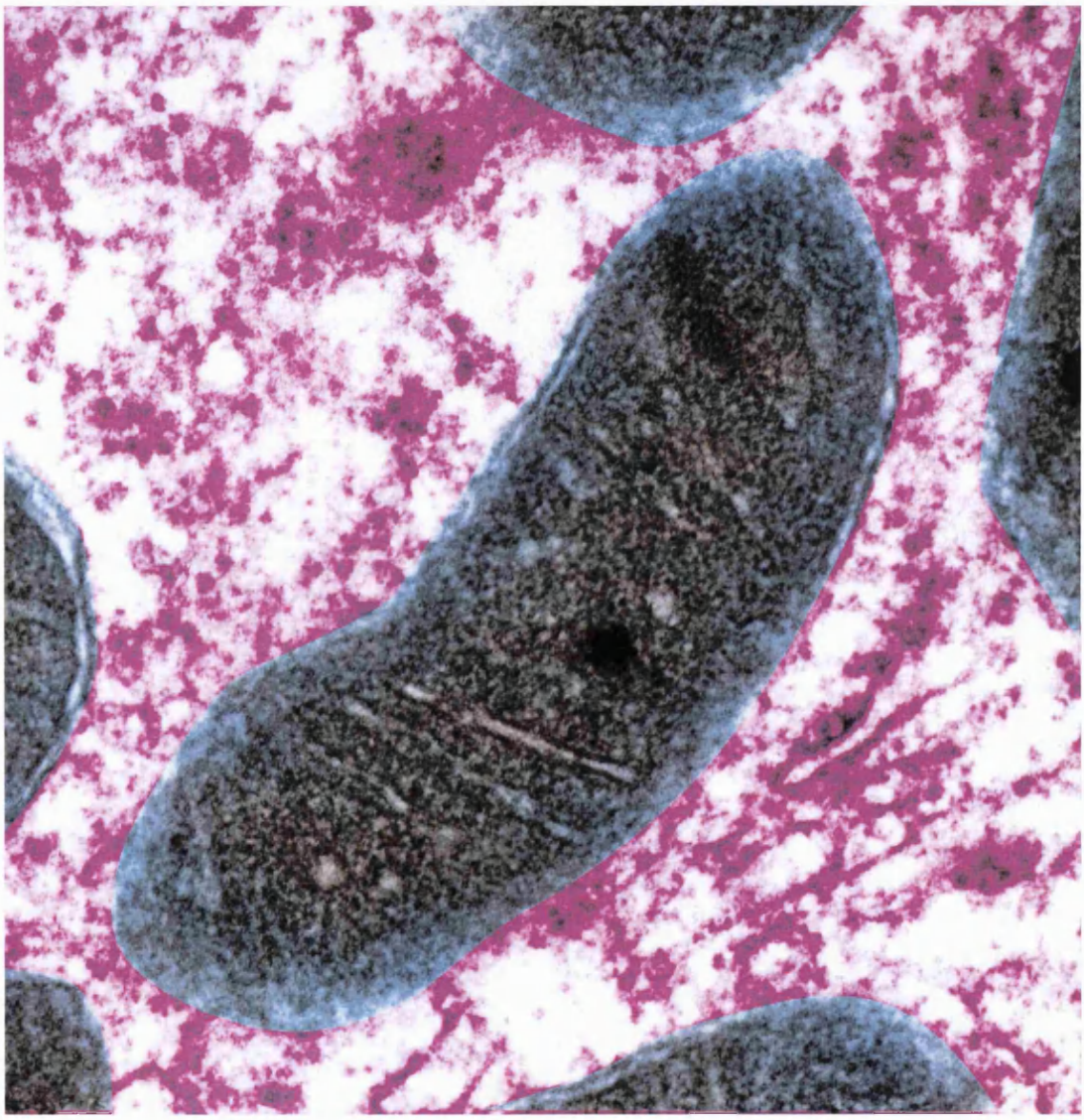

Abstract

The mitochondrial respiratory chain and oxidative phosphorylation system (complexes I-V) produce ATP by aerobic metabolism. Complex IV or cytochrome *c* oxidase (COX) catalyses transfer of electrons from reduced cytochrome *c* to molecular oxygen, coupled with proton pumping across the inner mitochondrial membrane. Human COX has 13 polypeptide subunits. Three subunits (I, II and III) constitute the enzyme's catalytic core and are encoded on the mitochondrial genome. The remaining subunits are nuclear-encoded. COX deficiency, either total or partial, is the most commonly recognised respiratory chain defect in childhood. This may be an isolated defect, or combined with deficiencies of other respiratory chain components. Clinical presentations are heterogeneous but most patients with COX deficiency remain uncharacterised at the molecular level.

COX subunit expression patterns were analysed in 5 patients with known mitochondrial DNA (mtDNA) mutations and 36 uncharacterised patients. A specific pattern of COX subunit loss was identified in COX deficiency secondary to mtDNA mutations. This suggested that immunohistochemistry using monoclonal antibodies may distinguish between mtDNA defects and nuclear defects in COX deficiency. Subsequent sequence analysis, targeted by immunohistochemistry findings, led to identification of a missense mutation of COX subunit II that causes defective assembly and myopathy. Characterisation of this mutation provided information about assembly of the metal centres of COX. Thus identification of naturally occurring COX mutations allows insight into structure-function relationships within the enzyme.

The majority of children with COX deficiency did not have selective loss of mtDNA-encoded subunits, suggesting that nuclear gene defects account for many cases of childhood-onset COX deficiency. One nuclear gene *SURF1* is responsible for COX assembly or maintenance. Four patients had homozygous *SURF1* mutations, associated with reduced expression of both mtDNA- and nuclear-encoded COX subunits. Studying patterns of subunit expression in COX-deficient patients is fundamental to understanding the pathogenesis of respiratory chain enzyme deficiencies.

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Publications Arising from this Work

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'Cytochrome c oxidase deficiency associated with the first stop codon point mutation in mitochondrial DNA'
American Journal of Human Genetics **63**, 29-36

Rahman S and Schapira AHV (1999)
'Mitochondrial Myopathies: Clinical Features, Molecular Genetics, Investigation and Management' in 'Muscle Diseases' eds Schapira AHV and Griggs RC, Butterworth Heinemann

Rahman S, Taanman J-W, Cooper JM, Nelson I, Hargreaves I, Meunier B, Hanna MG, Capaldi RA, Lake BD, Leonard JV and Schapira AHV (1999)
'A Mis-sense Mutation of Cytochrome Oxidase Subunit II Causes Defective Assembly and Myopathy'
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'Cytochrome *c* Oxidase Immunohistochemistry: Clues for Genetic Mechanisms'
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Annals of Neurology **49**, 797-800

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List of Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
AISA	acquired idiopathic sideroblastic anaemia
ALT	alanine transaminase
AST	aspartate transaminase
ATP	adenosine triphosphate
COX	cytochrome *c* oxidase
CRS	Cambridge reference sequence
CSF	cerebrospinal fluid
CT	computed tomography
DAB	diaminobenzidine
ddNTP	dideoxynucleoside triphosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ECG	electrocardiogram
EEG	electroencephalogram
EMG	electromyogram
ERG	electroretinogram
FAD	flavin adenine dinucleotide
HMG	high mobility group
Hsp	heat shock protein
kDa	kiloDaltons
LHON	Leber's hereditary optic neuropathy
MELAS	mitochondrial encephalomyopathy, lactic acidosis, stroke-like episodes
MERRF	myoclonic epilepsy, ragged red fibres
MNGIE	mitochondrial neurogastrointestinal encephalopathy
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA
mtTFA	mitochondrial transcription factor A
NAD	nicotinamide adenine dinucleotide
NARP	neurogenic muscle weakness, ataxia and retinitis pigmentosa
NBT	nitro blue tetrazolium
nDNA	nuclear DNA
np	nucleotide position
NRF	nuclear respiratory factor
PBS	phosphate buffered saline
PC	pyruvate carboxylase
PCR	polymerase chain reaction
PDHC	pyruvate dehydrogenase complex
PEO	progressive external ophthalmoplegia
PICU	paediatric intensive care unit
RNA	ribonucleic acid
RRF	ragged red fibre
rRNA	ribosomal RNA
SDH	succinate dehydrogenase
SNHL	sensorineural hearing loss
SURF1	surfeit 1
tRNA	transfer RNA
VDAC	voltage-dependent anion channel (porin)

Chapter 1

Introduction

Chapter 1 Introduction

1.1 The mitochondrion

The mitochondrion is an intracellular organelle and was first observed microscopically more than a hundred years ago. The name mitochondrion derives from the Greek 'mitos' for thread and 'chondros' for granule and was coined by Benda in 1898, because of the organelle's appearance during spermatogenesis (Ernster and Schatz, 1981). Mitochondria are thought to have evolved up to 2 billion years ago from the endosymbiotic engulfment of a purple photosynthetic bacterium by a primitive eukaryotic cell (Grivell, 1983). Under the electron microscope mitochondria seen in cross-section appear as double-membraned oval structures, approximately 0.5 to 1 μm in diameter and 2 to 5 μm long. The inner membrane is multiply invaginated to form cristae which protrude into the central matrix space (Palade, 1953). However although they are traditionally depicted as sausage or rod-shaped structures, mitochondria may exist as a dynamic network of tubular forms that constitute a single interconnecting mitochondrion (Rizzuto et al. 1998).

It was not until the 1940s that mitochondria were recognised to be the site of the cell's major energy producing pathways (Kennedy and Lehninger, 1949). The structure of the mitochondrion allows different enzymes and reactions to be localised in discrete membrane and aqueous compartments. The two membranes in mitochondria form discrete compartments (inner and outer mitochondrial membranes) and there are also two soluble compartments (the central matrix space and the intermembrane space). The central matrix space is the site of pyruvate oxidation, some fatty acid β-oxidation and the Krebs citric acid (tricarboxylic acid) cycle (Figure 1.1).

A ubiquitous and very important mitochondrial function is synthesis of adenine triphosphate (ATP) from adenine diphosphate (ADP) and inorganic phosphate, by oxidative phosphorylation (OXPHOS). The five enzyme complexes of OXPHOS are embedded in the mitochondrial inner membrane (Figure 1.2) and constitute approximately 50% of the protein content of this membrane in bovine heart mitochondria (Hatefi, 1985).

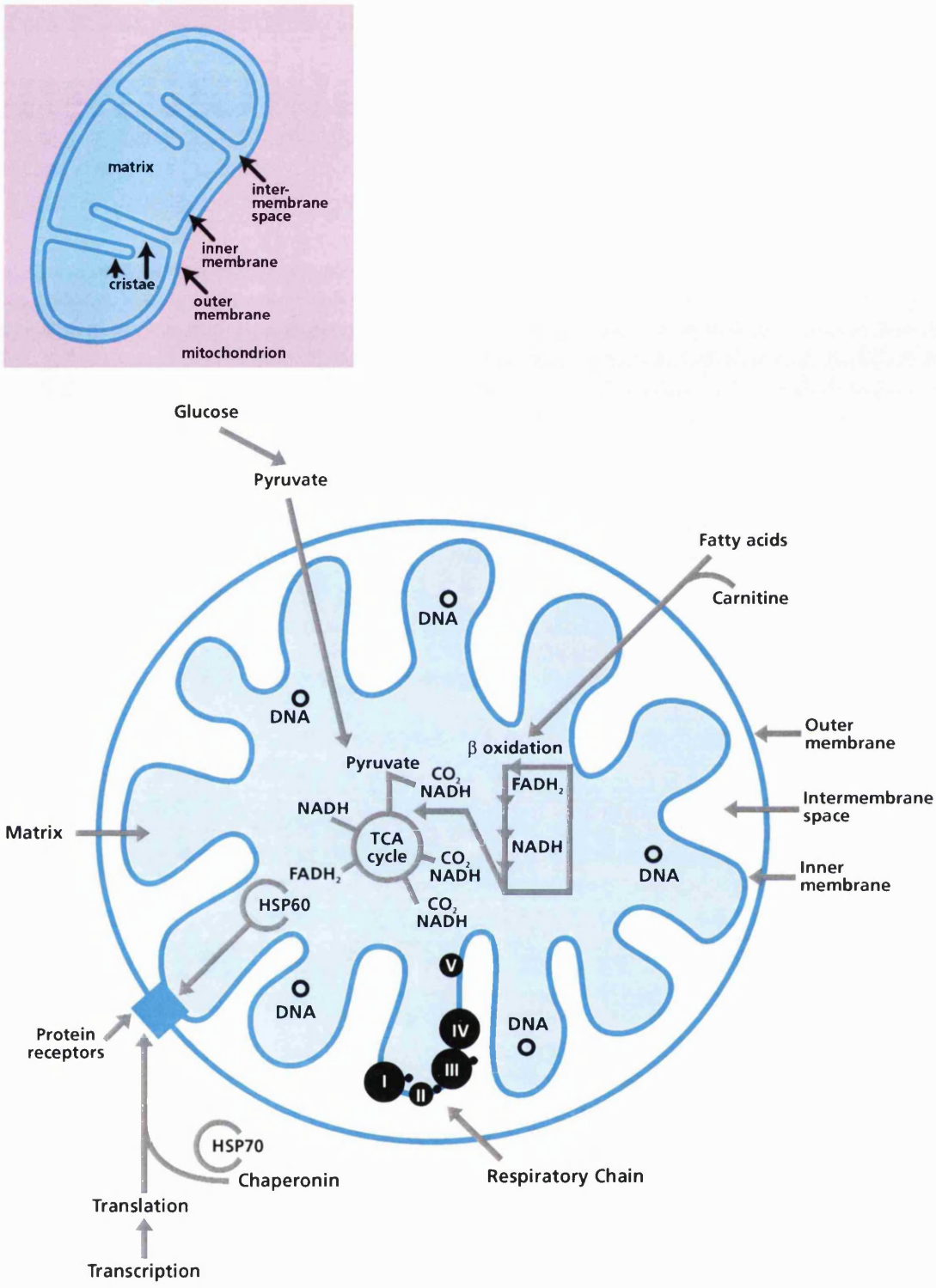


Figure 1.1 The mitochondrion and intermediary metabolism
Schematic illustration of the location of intermediary metabolism in mitochondrial matrix and membrane compartments. $FADH_2$, reduced flavin adenine dinucleotide; HSP, heat shock protein (chaperonin); NADH, reduced nicotinic adenine dinucleotide; TCA, tricarboxylic acid cycle. Adapted from Cooper and Clark 1994.

1.2 The mitochondrial respiratory chain and oxidative phosphorylation system

The mitochondrial respiratory chain (complexes I-IV) and oxidative phosphorylation (OXPHOS) system (complexes I-V) are responsible for ATP production by aerobic metabolism (reviewed by Hatefi, 1985). Human complex I (NADH-ubiquinone oxidoreductase) comprises approximately 43 polypeptide subunits (Loeffen et al. 2000), seven of which are encoded by mitochondrial DNA (mtDNA). Complex II (succinate-ubiquinone oxidoreductase) has only four subunits, all encoded by nuclear genes. Complex II includes succinate dehydrogenase (SDH) which catalyses the oxidation of succinate to fumarate and is a component of the Krebs cycle. Complex III (ubiquinol-cytochrome *c* oxidoreductase) is composed of 11 subunits only one of which (cytochrome b) is encoded by mtDNA. Complex IV (cytochrome *c* oxidase, COX) comprises 13 polypeptides and is discussed in detail below. Mammalian complex V (ATP synthase or F_1F_0 ATPase) has 16 subunits (Houstek et al. 1999), of which two (ATPase 6 and 8) are encoded by mtDNA.

Reducing equivalents generated by the oxidation of pyruvate, fatty acids and the Krebs cycle are transferred to the respiratory chain via NADH (reduced nicotinamide dinucleotide) and $FADH_2$ (reduced flavin adenine dinucleotide). Electrons derived from oxidation of pyruvate and fatty acids are transferred via NADH to complex I, whilst electrons from succinate in the Krebs cycle are transferred to complex II via $FADH_2$. The electron carriers in the respiratory chain are flavins, iron-sulphur (FeS) complexes, quinones and the haem groups of cytochromes. Ubiquinone (Coenzyme Q, CoQ) and cytochrome *c* function as mobile carriers of electrons between OXPHOS complexes. From complexes I and II, electrons are transferred to ubiquinone and then to complex III, and via cytochrome *c* (cyt *c*) to cytochrome oxidase (COX), before finally reducing molecular oxygen to water. The energy released during this sequential electron transfer is used to generate an electrochemical gradient, by translocating protons from the matrix to the intermembrane space. Protons are pumped at three coupling sites (complexes I, III and IV) and the resulting membrane potential (proton motive force, ~150mV) is used by complex V (ATP synthase or F_1F_0 ATPase) to drive ATP synthesis from ADP and inorganic phosphate by free energy transduction (Mitchell, 1961). ATP synthase is

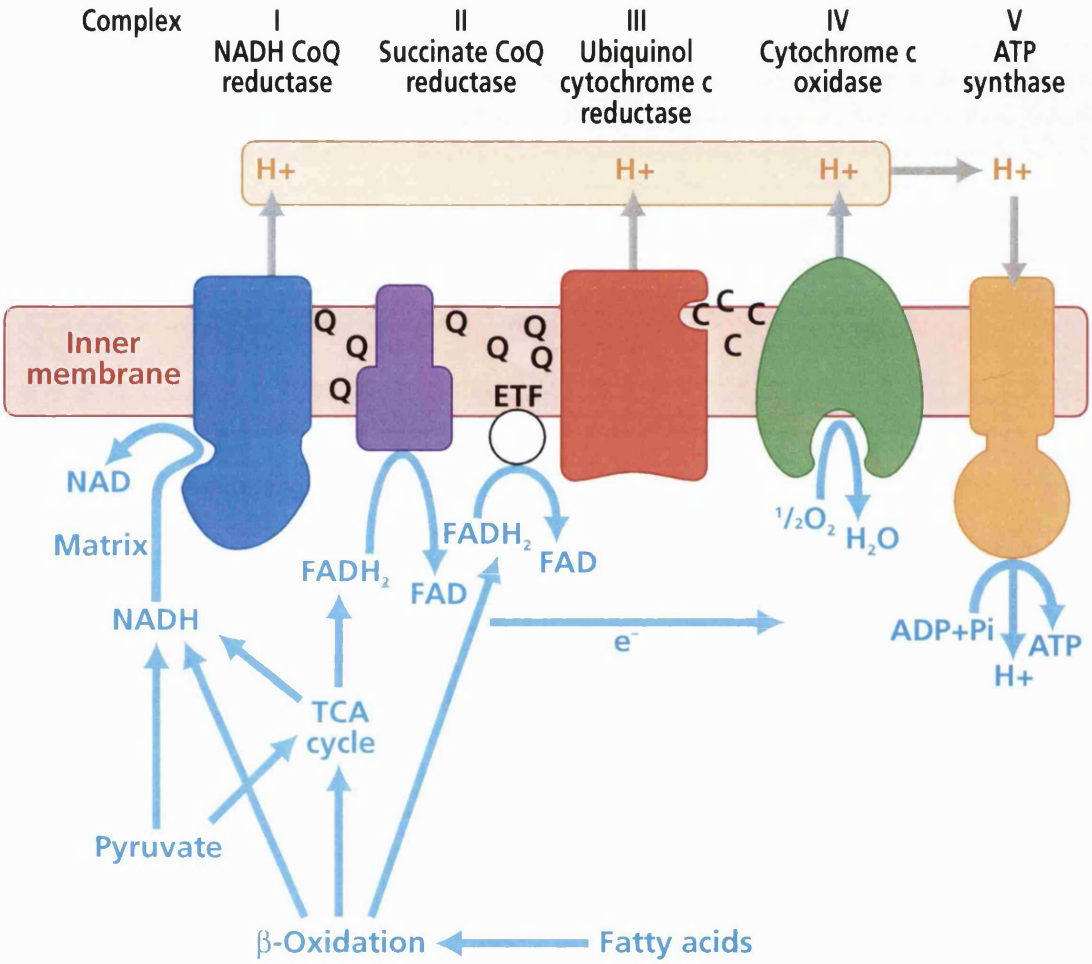


Figure 1.2 Oxidative phosphorylation
Schematic representation of the enzyme complexes of the respiratory chain and oxidative phosphorylation system. C, cytochrome c; ETF, electron transfer flavoprotein; Q, ubiquinone. Adapted from Cooper and Clark 1994.

an enzyme complex that can either hydrolyse or synthesise ATP, according to the energy status of the cell.

The OXPHOS complexes are arranged in the inner mitochondrial membrane, with a stoichiometry of 1: 1-2: 3: 6-7: 3-5 for complexes I-V respectively (Hatefi, 1985; Schagger and Pfeiffer, 2001). Recent studies have suggested that individual respiratory chain enzymes assemble into 'supercomplexes' within the inner membrane of both yeast and mammalian (bovine heart) mitochondria (Schagger and Pfeiffer, 2000; Schagger and Pfeiffer, 2001). These supramolecular assemblies may allow variable stoichiometry of COX relative to the other OXPHOS complexes, according to the energy requirements of the cell. Other potential benefits of these supercomplexes include: 1) substrate channelling of quinol and cytochrome *c* to maximise respiratory chain efficiency at low substrate concentrations; and 2) catalytic enhancement, by reducing the diffusion time required by quinol or cytochrome *c*.

1.3 Structure and organisation of the human mitochondriai genome

Mitochondria are the only mammalian cell organelles to contain their own genetic material. The existence of mitochondrial DNA (mtDNA) was first noted by Nass and Nass in 1963 and the complete sequence of the human mitochondrial genome was elucidated in 1981 (Anderson et al. 1981). The human mitochondrial genome is a 16569 bp circular double-stranded DNA molecule (Figure 1.3). The two mtDNA strands can be separated on denaturing alkaline caesium chloride gradients into heavy (H) and light (L) strands, as the former has a higher G+T content (Kasamatsu and Vinograd, 1974). The human mitochondrial genome encodes 13 structural components of the respiratory chain/OXPHOS system, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) (Anderson et al. 1981). 28 of these 37 genes are encoded by the H-strand (2 rRNAs, 14 tRNAs and 12 polypeptides). The remaining 9 genes (8 tRNAs and a single polypeptide ND6) are encoded on the light strand (Figure 1.3).

One of the most striking features of mtDNA is that it is extremely compact: 37 genes are encoded by only 16.5 kb of DNA. This economy is achieved by a number of

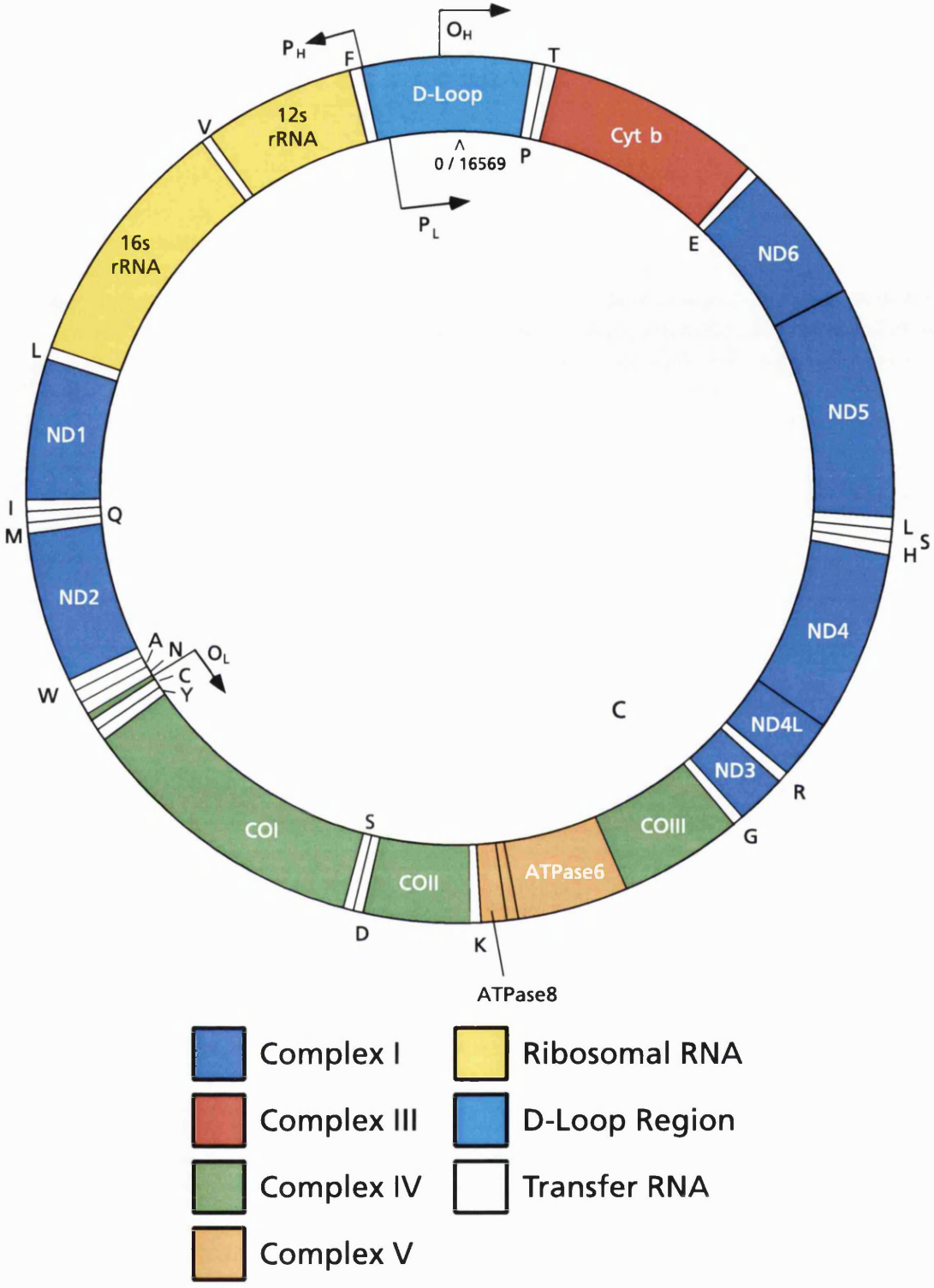


Figure 1.3 The human mitochondrial genome
Map positions are numbered from 1 to 16 569 in an anticlockwise direction, commencing near the origin of heavy strand DNA replication O_H. O_L, origin of light strand DNA replication; P_H, heavy strand promoter; P_L, light strand promoter. The same colours are used to represent individual OXPHOS complexes as in Figure 1.2. Transfer RNA (tRNA) genes are represented by the one letter abbreviation of their respective amino acids. Complex I subunit ND6 and the tRNA genes for glutamine (Q), alanine (A), asparagine (N), cysteine (C), tyrosine (Y), serine UCN (S), glutamic acid (E) and proline (P) are encoded on the light strand. The remaining 28 genes are encoded on the heavy strand.

means (Borst and Grivell, 1981). Virtually the whole sequence of human mtDNA is coding: ~93% compared to only ~3% of the nuclear genome (Strachan and Read, 1996). There are no introns, very little repetitive DNA and the only significant noncoding region is the D-loop. There is even some overlap between the coding sequences of some of the genes, for example the ATPase 6 and 8 genes (Anderson et al. 1981). Finally, the number of tRNAs necessary for mitochondrial protein synthesis is only 22, compared to 32 for protein synthesis on cytosolic ribosomes, because of relaxation of the usual codon-anticodon pairing rules. Thus many mitochondrial tRNA molecules are able to recognise any one of the 4 nucleotides in the third (wobble) position (Barrell et al. 1980). However, protein synthesis using fewer tRNA molecules may be less accurate.

There are a number of differences between the mitochondrial genetic code and the 'universal' genetic code employed by nuclear genes. In humans 4 of the 64 codons have a different meaning to that in the universal genetic code and these are highlighted in red in Table 1.1 (Barrell et al. 1979).

Mitochondrial genetics has other unusual features. Mammalian mtDNA is virtually exclusively maternally inherited (Giles et al. 1980). Mechanisms that are thought to prevent paternal transmission of mtDNA include a low mtDNA copy number in sperm cells (Larsson et al. 1997). Another feature of mtDNA is that it has a ten-fold higher mutation rate than nuclear DNA (Brown et al. 1979). This higher mutation rate can be attributed to a lack of protective histones, comparatively inefficient repair mechanisms for mtDNA, particularly nucleotide-excision repair and mismatch repair (Bogenhagen, 1999), and a higher rate of damage to mtDNA because of exposure to OXPHOS-generated free radicals (Croteau and Bohr, 1997).

Between 2 and 10 copies of the mitochondrial genome are present in each mitochondrion. As there may be hundreds or thousands of mitochondria in a cell, the number of mtDNA molecules in a cell may exceed a hundred thousand, although the precise number may vary between different tissues (Bogenhagen and Clayton, 1974; Robin and Wong, 1988). The presence of multiple copies of the mitochondrial genome leads to the phenomenon of heteroplasmy, in which differing proportions of mutant and wild type mtDNA coexist in the mitochondria of a cell

Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid
AAA AAG	Lys (K)	CAA CAG	Gln (Q)	GAA GAG	Glu (E)	UAA UAG	STOP
AAC AAU	Asn (N)	CAC CAU	His (H)	GAC GAU	Asp (D)	UAC UAU	Tyr (Y)
ACA ACG ACC ACU	Thr (T)	CCA CCG CCC CCU	Pro (P)	GCA GCG GCC GCU	Ala (A)	UCA UCG UCC UCU	Ser (S) (UCN)
AGA AGG	STOP	CGA CGG	Arg (R)	GGA GGG	Gly (G)	UGA UGG	Trp (W)
AGC AGU	Ser (S) (AGU/C)	CGC CGU		GGC GGU		UGC UGU	Cys (C)
AUA AUG	Met (M)	CUA CUG	Leu (L) (CUN)	GUA GUG	Val (V)	UUA UUG	Leu (L) (UUA/G)
AUC AUU	Ile (I)	CUC CUU		GUC GUU		UUC UUU	Phe (F)

Table 1.1 The Human Mitochondrial Genetic Code
Alterations from the universal code are highlighted in red: UGA is used by mtDNA to encode tryptophan rather than as a termination codon, AGA and AGG are read as stop codons rather than as arginine, and AUA encodes methionine rather than isoleucine. In addition AUA and AUU can sometimes serve as initiation codons instead of AUG.

(Lightowlers et al. 1997). This in turn means that mitochondrial genetics bears greater similarities to population genetics than to traditional Mendelian genetics (Schon, 2000). Heteroplasmy has been found to occur in the control region of normal individuals (Jazin et al. 1996), but in other areas of the mitochondrial genome heteroplasmy is usually a feature of pathogenic mutations. Rapid segregation of different mitochondrial genomes to homoplasmy, sometimes even completely switching between homoplasmy for one base to another within a single generation, has been observed both for neutral polymorphisms in Holstein cows (Koehler et al. 1991) and for mutant mtDNA molecules in human disease (Seller et al. 1997; Degoul et al. 1997; Takahashi et al. 1998).

A genetic 'bottleneck' occurring during oogenesis has been proposed to explain this rapid and preferential expansion of one mitochondrial genome over another (reviewed by Brown, 1997). In 1998 Bergstrom and Pritchard suggested that the predominantly uniparental inheritance, apparent lack of recombination and high mutation rates of mitochondrial genomes mean that mitochondria are susceptible to Muller's 'ratchet', in which deleterious mutations accumulate over generations (Bergstrom and Pritchard, 1998). Bergstrom and Pritchard proposed that the development of a mitochondrial genetic bottleneck in the germline would lead to rapid genetic drift and loss of mildly deleterious mutations and thereby counteract the process of Muller's ratchet.

1.4 Replication and maintenance of mitochondriai DNA

The precise mechanism of mtDNA replication is debated and very little is known about the enzymes involved in mammals (Spelbrink et al. 2000). What is known is that the D-loop is the control site for both mtDNA replication and transcription (Anderson et al. 1981). Human mtDNA has two separate origins of replication, the origin of H-strand replication O_H and the origin of light-strand replication O_L (Figure 1.3). O_H is located in the D-loop region, whilst O_L lies in a 30 bp noncoding sequence within a cluster of 5 tRNA (WANCY) genes about two-thirds of the genomic distance around the mtDNA molecule (Clayton, 1982) (Figure 1.3). Replication of mtDNA follows transcription, since transcripts initiated at the light strand promoter (LSP) are used as primers for H-strand mtDNA replication by DNA polymerase γ (POLG) (Shadel and Clayton, 1997). Transcripts are thought to be processed by a mitochondrial RNA processing endonuclease (RNase MRP) and

endonuclease G (ENDOG) in order to produce mature primers with a donor 3' hydroxyl group for H-strand replication (Cote and Ruiz-Carrillo, 1993). Transition from RNA to DNA synthesis occurs over a region of short conserved sequence blocks (CSBs) I-III. Other factors required for replication include single-stranded DNA binding protein (SSBP), DNA helicase and transcription factors (Shadel and Clayton, 1997).

An asymmetric model of mtDNA replication was first proposed almost 20 years ago (Clayton, 1982). In this model two-thirds of the leading H-strand is synthesised before L-strand replication is initiated. Recent studies have, however, suggested that strand-synchronous ('coupled') replication of mammalian mtDNA may occur in some circumstances (Holt et al. 2000). In the Clayton model L-strand replication proceeds in the opposite direction to that of H-strand elongation. The mitochondrial DNA primase required for priming L-strand synthesis has not yet been characterised (Spelbrink et al. 2001). To complete mtDNA replication, the two daughter molecules separate, RNA primers are removed at both origins of replication and any remaining gaps in the molecule are filled and ligated. Superhelical turns are then introduced into the covalently closed circular mtDNA by a putative mitochondrial type II topoisomerase (Taanman, 1999).

Little is known about the regulation of mtDNA copy number in animal cells (Moraes, 2001b). It appears that some mtDNA molecules within a cell may replicate twice before other mtDNA molecules replicate even once (Shadel and Clayton, 1997). This apparent lack of a mechanism to identify each and every molecule for each replication event has led to the term 'relaxed control' to describe the regulation of mtDNA replication. Recent evidence has suggested that mammalian cells maintain a constant mass of mtDNA rather than a constant copy number of mitochondrial genomes (Tang et al. 2000). Maintenance of mitochondrial nucleotide pools is also important for mtDNA replication. Tang et al. suggested that cells could regulate total mitochondrial mass and therefore mtDNA copy number by the size of intramitochondrial nucleoside pools. Support for this hypothesis comes from the recent finding that several human diseases are caused by defects in nucleotide metabolism (see section 1.11.4).

1.5 Transcription of mitochondrial DNA

Transcription commences at promoters in the D-loop region: the heavy strand promoter HSP and the light strand promoter LSP. H-strand transcription commences at an initiation site IT_{H1} at nucleotide position (np) 561 within the HSP, whilst L-strand transcription is initiated at IT_L at np 407 within the LSP (Taanman, 1999). These transcription initiation sites are surrounded by promoter elements containing the consensus sequence 5'-CANACC(G)CC(A)AAAGAYA, which is essential for transcription to proceed (Chang and Clayton, 1984). Transcription of mtDNA requires a mitochondrial RNA polymerase (Tiranti et al. 1997), mitochondrial transcription factor A (h-mtTFA) (Xu and Clayton, 1992) and probably some additional factors that have not yet been characterised in humans (Shadel and Clayton, 1997). These additional factors are likely to include a mtTFB, which has been described in several yeast species and in *Xenopus laevis* (Taanman, 1999). The 25 kDa h-mtTFA protein binds to regions immediately upstream of both HSP and LSP and is thought to activate transcription by binding to DNA, as it contains two high mobility group (HMG) domains. HMG domains have been shown to be important in DNA binding in a large family of proteins known as the HMG-box family (Grosschedl et al. 1994). H-mtTFA can bend and unwind DNA and this is probably how it initiates transcription.

Once transcription has been initiated, it proceeds around the mtDNA molecule, to produce a single polycistronic transcript from each strand (Aloni and Attardi, 1971). These transcripts are subsequently cleaved at specific sites to release tRNA, rRNA and mRNA molecules. Since tRNA genes flank both rRNA genes and virtually all the mitochondrial polypeptide–coding genes, it has been proposed that the secondary structure of these tRNAs may indicate sites for cleavage to the responsible enzymes (mitochondrial RNase P for the 5' end and an uncharacterised endonuclease for the 3' end). This 'tRNA punctuation model' of RNA processing was first proposed by Ojala et al. in 1981. Maturation of mitochondrial tRNAs involves addition of a 3' CCA by an ATP(CTP): tRNA nucleotidyl-transferase (Rossmanith et al. 1995). Unlike their nuclear counterparts, mitochondrial mRNAs are not capped at their 5' termini with 7-methyl guanosine (Grohmann et al. 1978), but they do have a 3' polyadenylated tail that is added by a mitochondrial poly-A polymerase after cleavage and is essential for translation (Rose et al. 1975).

The paucity of noncoding regions in mtDNA means that transcription of mitochondrial genes has to be controlled by different means to those employed in the nuclear genome, where noncoding regions contain signal sequences for the transcribing enzymes. Transcriptional control of mtDNA is mediated at least in part by transcription termination. Transcripts initiated from the HSP are present in different relative amounts: the 12S and 16S rRNAs are thought to be 50 to 100 times more abundant than the mRNAs (Gelfand and Attardi, 1981). The relatively high production of rRNAs can be attributed to early transcription termination, although it may also result from the use of a second initiation site for H-strand transcription, as postulated by Attardi and colleagues (Montoya et al. 1982). A tridecamer sequence located in the tRNAleu(UUR) gene is the binding site for a 34 kDa protein mtTERM (Hess et al. 1991). The resulting protein-DNA complex promotes transcription termination. Differential mitochondrial gene expression may also be mediated at the level of transcription by differences in RNA stability. Nuclear respiratory factors NRF1 and NRF2 are transcriptional regulators of many OXPHOS subunit genes (Scarpulla, 1997). A direct stimulatory effect of thyroid hormone on mitochondrial transcription has also been reported (Enriquez et al. 1999).

1.6 Mitochondrial translation

Modification of mitochondrial gene expression may also occur at the translational level, possibly by differences in translational efficiency of different mRNA species (Cantatore et al. 1987). Translation of mtDNA-encoded polypeptides occurs on mitochondrial ribosomes located in the matrix. Studies in rat hepatocytes suggested there are fewer than 100 mitoribosomes per mitochondrion (Cantatore et al. 1987). The mechanisms of mitochondrial translation have not been fully elucidated (Taanman, 1999). Translation is initiated at N-formylmethionine residues (Montoya et al. 1981). As mitochondrial mRNAs lack a 5' 7-methylguanylate cap, ribosomal recognition of the initiation codon is less efficient than in the nucleus, leading to low translational efficiency of mitochondrial messengers (Cantatore et al. 1987). The initiation codon is likely to be the first AUG from the 5' end of the mRNA, but it is not known how the translation apparatus selects the correct initiation codon (Poyton and McEwen, 1996). Some of the factors involved in initiation and elongation of mitochondrial translation have been characterised but little is known about the termination of mitochondrial protein synthesis (Taanman, 1999). Yeast mRNA-specific translational activator proteins have been identified. For example PET494,

PET54 and PET122 are necessary for translation of COX3 mRNA (Poyton and McEwen, 1996). It is not known whether the same mechanisms are employed for translation of mammalian mitochondrial mRNAs. Most human mitochondrial mRNAs either lack a UAA termination codon to signal the end of translation, or else the stop codon is removed during cleavage of the polycistronic primary transcript. Cleavage usually occurs after a U or UA sequence. Posttranscriptional polyadenylation will provide a stop codon by elongating this terminal U or UA sequence to UAA (Rose et al. 1975).

1.7 Mitochondrial protein import and assembly

Despite the importance of the mitochondrial encoded polypeptides, more than 95% of all mitochondrial proteins are encoded in the nucleus and synthesised in the cytosol. These include the remaining 74 polypeptides of the respiratory chain and oxidative phosphorylation system, all the components of other mitochondrial biochemical pathways (numbering several hundred) and all the proteins required for their import and assembly. These proteins need to be directed to and imported into the mitochondrion. Mitochondria contain two independent protein transport systems: one in the outer membrane and the other in the inner membrane (Pfanner et al. 1996). The former is known as the Tom complex (for transport across the outer membrane) and the latter as the Tim complex (for transport across the inner membrane). Much is known regarding the structure and function of these two protein transport systems (reviewed by Rassow and Pfanner, 2000). Most of this information is derived from studies in the yeast *Saccharomyces cerevisiae* and the mould *Neurospora crassa*. It is likely that similar mechanisms are employed in protein import into mammalian mitochondria.

Mitochondrial proteins synthesised on cytosolic ribosomes have a specific targeting signal. Traditionally these signals were thought to be cleavable N-terminal presequences of approximately 20 amino acids that are predicted to fold into positively charged amphiphilic α-helices in membranes (Roise and Schatz, 1988). It is now thought that only mitochondrial matrix proteins have such presequences and that these sequences are particularly import for interaction with the inner membrane Tim machinery (Rassow and Pfanner, 2000). Proteins destined for other mitochondrial subcompartments, for example most components of the Tom and Tim complexes and the inner membrane ATP/ADP translocase, lack N-terminal

cleavable presequences but instead have internal signals for mitochondrial import. Little is known about these internal signals (Neupert, 1997) but integral membrane proteins may have a hydrophobic 'stop transfer' sequence that leads to interruption of protein translocation across the membrane (Schatz and Dobberstein, 1996). Most mitochondrial proteins are likely to be imported post-translationally, but it is possible that co-translational import occurs for some proteins (Verner, 1993).

Precursor proteins are bound to molecular chaperones in the cytoplasm to prevent misfolding or aggregation prior to mitochondrial import. These chaperones include members of the 70 kDa heat shock protein (Hsp70) family and the ATP-dependent mitochondrial import stimulating factor MSF (Komiya and Mihara, 1996). At the outer mitochondrial membrane the mitochondrial precursor proteins are recognised by the Tom complex, which has receptor components and a general insertion pore GIP (Pfaller et al. 1988). The GIP is a 400 kDa complex that has 5 subunits: Tom40, Tom22, Tom7, Tom6 and Tom5. Preproteins with an N-terminal presequence bind to the Tom20 receptor and then pass through the GIP before inserting into the inner membrane Tim23 import channel to translocate into the matrix (Rassow and Pfanner, 2000). A separate import pathway is employed for inner membrane proteins including the ATP/ADP translocase. These proteins initially bind to Tom70 before passing through the GIP to reach the intermembrane space. Insertion into the inner membrane is achieved by a protein complex containing Tim22 and requires the membrane potential $\Delta\Psi$ (Rassow and Pfanner, 2000).

In most cases targeting signals are removed once the protein reaches its final destination. Presequences of matrix proteins are cleaved by mitochondrial processing peptidase MPP (Neupert, 1997). Once the protein has been imported into the correct mitochondrial subcompartment it needs to be folded. Yeast studies have demonstrated that a number of different chaperones are involved in mitochondrial protein folding (Hartl, 1996). One of these molecules is Hsp60. Deficiency of human Hsp60 has been reported in a single patient with a fatal encephalomyopathy who had deficient activities of multiple mitochondrial enzymes (Agsteribbe et al. 1993).

1.8 Cytochrome *c* oxidase

1.8.1 Cytochrome oxidase structure and function

COX, the terminal enzyme (complex IV) of the respiratory chain, is a member of a superfamily of haem-containing terminal oxidases (Calhoun et al. 1994). COX is an integral protein of the inner mitochondrial membrane and has surfaces exposed to both 'inside' (matrix) and 'outside' (intermembrane space) aqueous compartments. COX catalyses the exergonic transfer of electrons from reduced cytochrome *c* to molecular oxygen ($4H^+ + 4e^- \rightarrow 2H_20$), and couples this reaction to proton pumping across the inner mitochondrial membrane. Four protons are consumed in the reaction and another four are translocated from the matrix to the intermembrane space, so that the overall reaction can be represented as:
4 ferrocytochrome *c* + $8H^+_{inside} + O_2 \rightarrow$ 4 ferricytochrome *c* + $4H^+_{outside} + 2H_2O$
(Wikstrom, 1977). The human enzyme has a molecular weight of approximately 200 kDa and is composed of 13 polypeptide subunits. The three major subunits (I, II and III) constitute the catalytic core of the enzyme and are encoded by mtDNA. The remaining subunits of COX (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII – nomenclature of Kadenbach et al. 1983) are nuclear-encoded. Human subunits VIa and VIIa each exist as two isoforms, encoded by separate genes. The nucleotide sequences of all 15 genes encoding structural subunits of COX are known, and most have been localised to chromosomes (Table 1.2). The enzyme has 6 metal centres – 2 haems, 2 copper (Cu) centres, a magnesium (Mg) centre and a zinc (Zn) centre. The Zn and Mg centres do not act as redox centres and their function is unclear (Capaldi, 1990b).

The crystal structure of bovine heart complex IV was described by Tsukihara et al. in 1996 (Figure 1.4). The availability of three-dimensional structural information at atomic (2.8 Å) resolution from this X-ray crystallographic data has improved our understanding of the functioning of the enzyme. COX I has 12 membrane spanning and no extramembrane domains, whilst COX II is anchored to the membrane with an N-terminal helix hairpin and has a large C-terminal hydrophilic domain which protrudes into the intermembrane space over the surface of COX I. COX III has 7 membrane spanning domains and its N-terminus lies on the matrix side of the membrane. COX II and III both interact with COX I within the membrane, but have no direct contact with each other. The redox centres involved in electron transfer

Human subunit	MW kd	AA	Yeast homologue	Human Gene	Gene localisation of human subunit
I	57	513	I	*MTCO1*	mtDNA H5904-7444
II	25.6	227	II	*MTCO2*	mtDNA H7586-8262
III	30	261	III	*MTCO3*	mtDNA H9207-9990
IV	17.2	147	V	*COX4*	16q24.2
Va	12.5	109	VI	*COX5A*	15q25
Vb	10.6	98	IV	*COX5B*	2cen-q13
VIa-L	9.6	85	VIa	*COX6A1*	12q24.2 or 6p21
VIa-H	9.5	85	VIa	*COX6A2*	16p
VIb	10.1	85	VIb	*COX6B*	19q13.1
VIc	8.6	73	VIIa	*COX6C*	8q22-q23
VIIa-H	6.7	58	VII	*COX7A1*	19q13.1
VIIa-L	6.7	60	VII	*COX7A2*	6 or 14
VIIb	6.4	56	none	*COX7B*	Xp21.1-q21.33
VIIc	5.4	47	VIII	*COX7C*	5q14
VIII	4.9	44	(VIIa)	*COX8*	11q12-q13

H=heart isoform; L=liver isoform

Table 1.2 Genes encoding COX subunits
This table was compiled using information available in the SWISS-PROT (http://www.expasy.ch/sprot/), NCBI LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/), GENATLAS (http://www.citi2.fr/GENATLAS/) and MITOP (http://www.mips.biochem.mpg.de/proj/medgen/mitop/) databases.

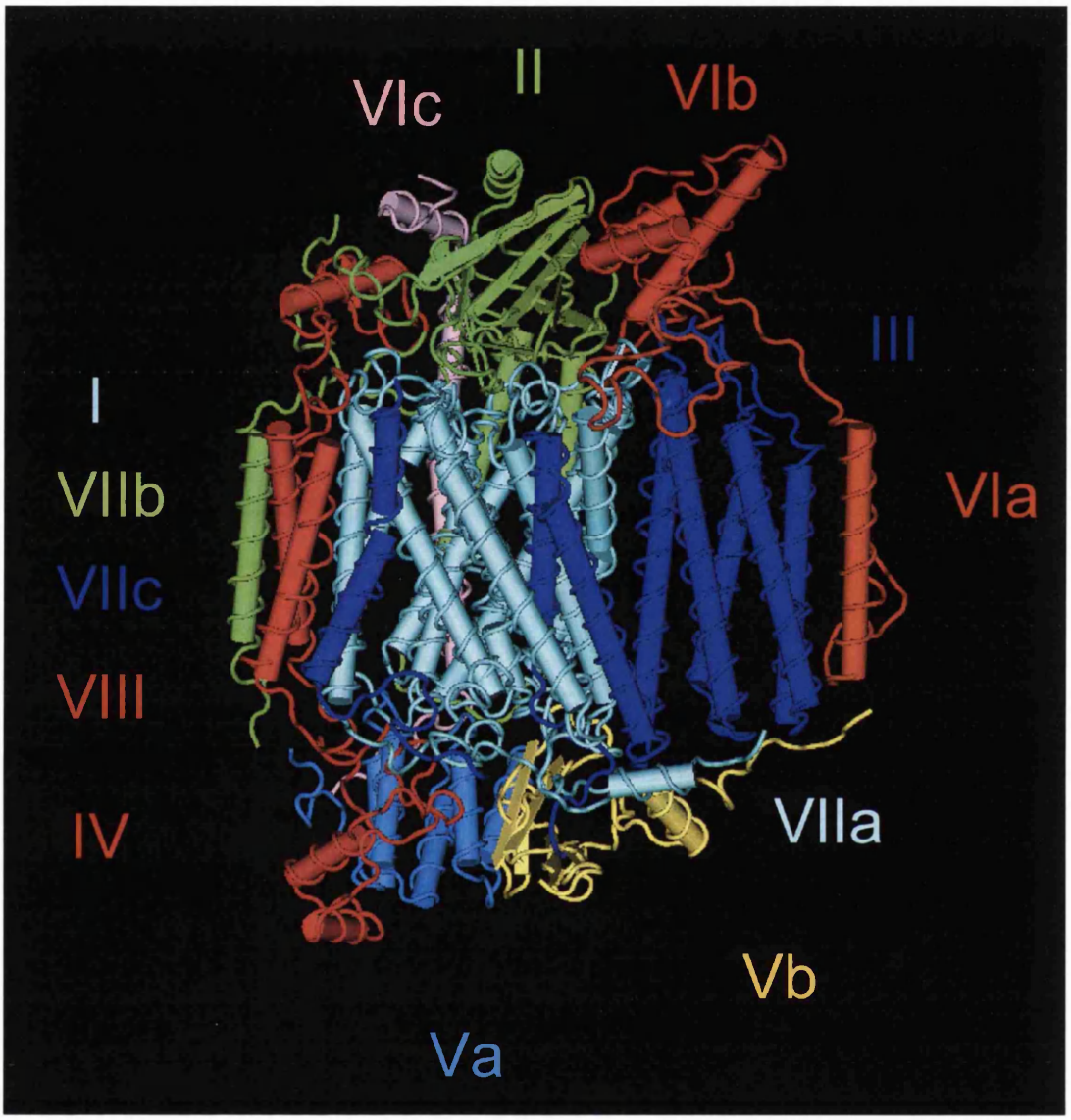


Figure 1.4
Structure of monomer of bovine cytochrome c oxidase
The diagram was constructed with use of the data published by Tsukihara et al 1996.

are the two haem A moieties (*a* and a_3) and the two copper centres (Cu_A and Cu_B). The haem *a* and the haem a_3-Cu_B binuclear centre are associated with COX I, while COX II contains the Cu_A centre and accepts electrons from cytochrome *c*. Electrons are transferred from Cu_A via haem *a* to the haem a_3-Cu_B binuclear centre, where oxygen reduction takes place on the matrix side of the inner membrane (Hill, 1993). COX III does not contain any prosthetic groups and its removal in some systems does not appear to affect COX activity (Capaldi, 1990a). The function of COX III therefore remains unclear. The exact mechanism of proton pumping by COX is still debated (Gennis, 1998; Michel, 1998). The role of the nuclear-encoded subunits remains obscure, despite the availability of sequence and structural information (Grossman and Lomax, 1997). None of the nuclear-encoded subunits has a counterpart in any prokaryotic COX and so it seems unlikely that these subunits play a role in electron transfer or proton pumping (Taanman, 1997). It has been postulated that these subunits may be involved in assembly or stability of the holoenzyme complex, and/or may have regulatory functions by binding ligands that modulate the catalytic function of the enzyme. The identification of tissue-specific isoforms of some nuclear COX subunits is seen as evidence for such a regulatory function. Human subunits VIa and VIIa have both heart (H) and liver (L) specific tissue isoforms (Kadenbach et al. 1982; Yanamura et al. 1988). Only the L isoform is present in liver but both L and H isoforms are found in heart and skeletal muscle. It has been hypothesised that these tissue-specific isoforms may regulate COX activity to the particular needs of the tissue. However different mammalian species have isoforms for different subunits (Linder et al. 1995). This causes difficulties in envisaging how the isoforms might regulate COX activity.

Functional roles for the human nuclear-encoded subunits might be inferred from X-ray crystallography and biophysical studies of the mammalian enzymes and site-directed mutagenesis of the yeast enzyme. X-ray crystallography of bovine COX has demonstrated that none of the 10 nuclear-encoded subunits impinges directly on the metal centres (Tsukihara et al. 1996). In the crystal structure COX exists as a dimer, with subunits VIa and VIb acting as bridging peptides between the monomers. The function of either or both of these subunits may therefore be to stabilise the dimer. X-ray crystallographic data has also demonstrated that subunit Vb provides the 4 cysteine residues that coordinate the Zn atom, and this is the first confirmed role for a nuclear-encoded subunit (Grossman and Lomax, 1997).

Yeast COX is similar to the human enzyme (Table 1.2), and mutagenesis studies in yeast have provided information about the function, assembly and stability of the enzyme complex. For example the yeast homologue of subunit IV appears to modulate the electron transfer rate (Allen et al. 1995). Proteolytic studies have indicated that subunit IV may play a role in proton pumping by mediating access of protons into the transmembrane proton channel (Grossman and Lomax, 1997). This is a potential mechanism by which subunit IV may regulate COX activity. Disruption of the yeast homologue of subunit Va leads to complete loss of cellular respiration (Poyton et al. 1988) whilst disruption of the yeast homologue of subunit Vb appears to prevent COX assembly (Dowhan et al. 1985). Studies of a null mutant for the yeast homologue of subunit VIa have demonstrated that this subunit is not required for full activity of the enzyme. However subunit VIa contains one of two ATP binding sites on COX and may be involved in ATP-mediated modulation of COX activity (Anthony et al. 1993; Taanman and Capaldi, 1993). Yeast VIb is required for assembly of a fully active enzyme (Grossman and Lomax, 1997) and may regulate enzyme activity by acting as an inhibitor of the electron transfer reaction (Taanman, 1997). The yeast equivalent of subunit VIc is also necessary for a fully active enzyme (Taanman, 1997). Subunit VIIa appears to be involved in assembly of the enzyme, possibly by facilitating correct folding or haem incorporation of subunit I (Taanman, 1997). The yeast homologue of subunit VIIc is not necessary for assembly but is required for optimal COX function. The roles of subunits VIIb and VIII are not known.

1.8.2 Cytochrome oxidase assembly

The assembly of COX is still not completely understood. Early studies of COX assembly in rat hepatocytes involved immunoprecipitation of cell lysates labelled with radioactive methionine (Hundt et al. 1980). These studies suggested that COX assembly is a slow sequential process (Kolarov et al. 1981; Wielburski et al. 1982). The precise order of COX assembly could not be deduced from these experiments since it was possible that selective loss of some subunits might have occurred during immunoprecipitation, or that conformational changes in partially assembled complexes might have led to differences in immunoreactivity (Nijtmans, 1998). Other methods were required to study COX assembly in more detail.

Nijtmans developed a two-dimensional blue-native gel electrophoresis system to study cultured human cells treated with specific inhibitors of either mitochondrial or cytosolic protein synthesis (Nijtmans et al. 1998). These studies allowed the identification of 3 intermediates in the assembly of the COX holoenzyme complex: S1, S2 and S3. S1 contains only COX I, S2 consists of COX I and IV and S3 includes all COX subunits except VIa and VIIb. Nijtmans was then able to devise a hypothetical scheme of COX assembly (Figure 1.5), based on his gel electrophoresis data and a review of the literature regarding bacterial and yeast COX assembly mutants (Nijtmans, 1998).

Several factors suggest a vital role of the mitochondrially encoded COX subunits in the assembly of the holoenzyme. They form the catalytic core of the enzyme and contain the prosthetic groups, and a Paracoccus denitrificans mutant containing only subunits I and II had enzymatic activity (Ludwig and Schatz, 1980). It is hypothesised that addition of haem to subunit I is the first step of COX assembly (Nijtmans, 1998) since no COX assembly was observed either in Rhodobacter mutants with impaired haem synthesis or in a yeast Cox 10p (haem A: farnesyl transferase) knockout mutant (Shapleigh et al. 1992; Glerum and Tzagoloff, 1994). Similarly Western blot analysis of fibroblast mitochondrial proteins revealed absence of COX subunit II in a patient with a homozygous missense mutation in *COX10* (Valnot et al. 2000b).

Genetic analysis of yeast respiratory deficient (petite or pet) mutants revealed that there are at least 34 complementation groups for COX deficiency (McEwen et al. 1986). These results imply that at least 34 gene products are required to produce functional COX. This number is obviously greater than the number of genes encoding subunits of COX, and further studies of these yeast mutants have led to the identification of a number of nuclear-encoded factors involved in the assembly of COX (Table 1.3). Some of these are required for the assembly of many mitochondrial proteins, and these include chaperonins such as the heat shock proteins involved in mitochondrial import and sorting pathways (see section 1.7). Other factors are specific for COX assembly and these include COX10 and COX15, which are involved in the synthesis of haem a (Glerum and Tzagoloff, 1994; Barros et al. 2001); SCO1, SCO2 and COX17, which are necessary for copper uptake into mitochondria (Glerum et al. 1996a; Glerum et al. 1996b); and COX11, which is

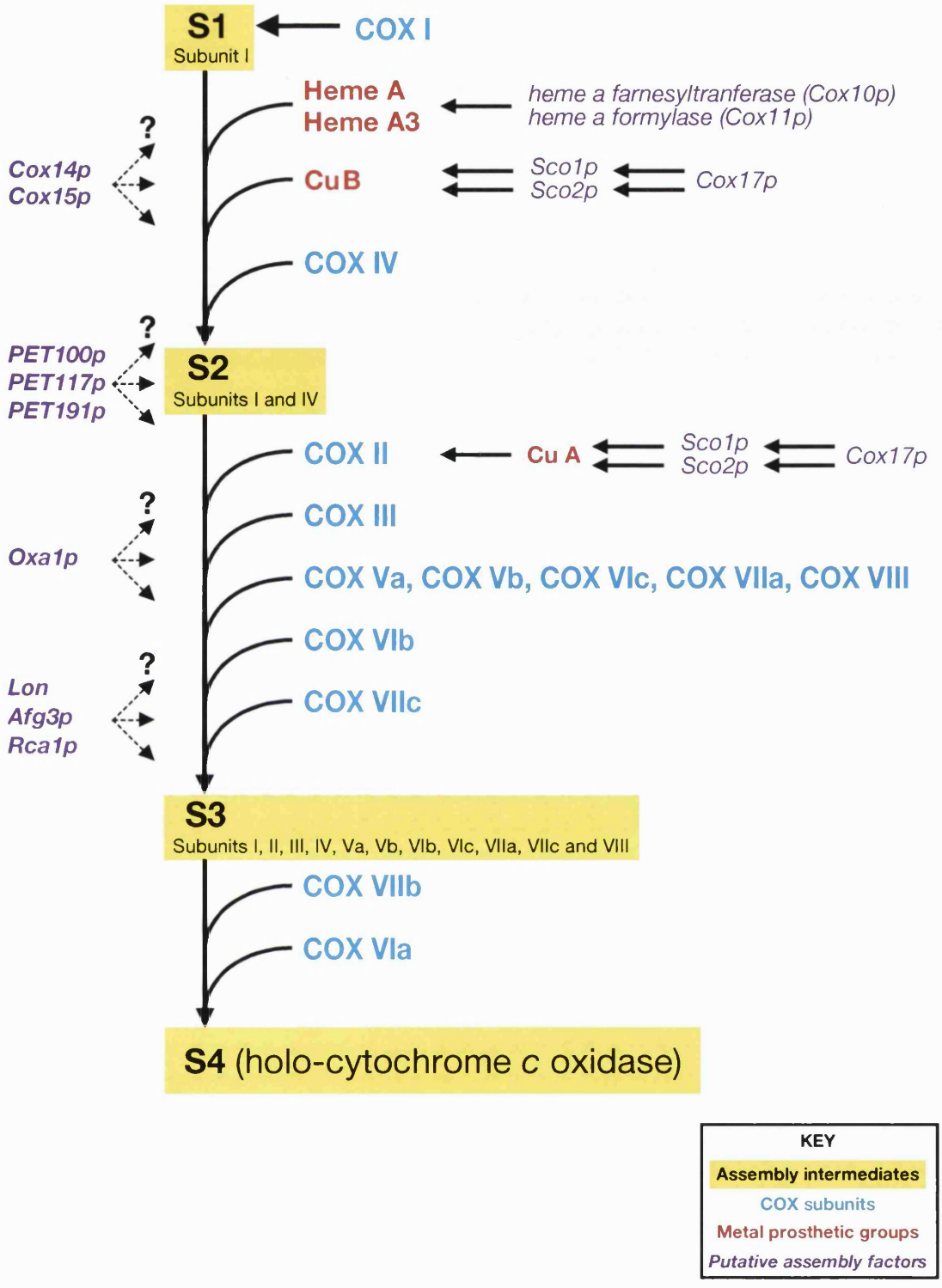


Figure 1.5
Proposed assembly pathway of the COX holoenzyme complex
Schematic representation of the assembly of COX (L Nijtmans, personal communication).

Gene	Product	Location of gene product	Human homologue	Function	Reference
COX10	farnesyl transferase	IMM	*COX10* encodes haem A farnesyl transferase Ch 17p13.1-q11.1	COX assembly protein; required for haem A synthesis	Nobrega et al. 1990; Glerum and Tzagoloff, 1994
COX11		IMM	cDNA isolated	COX assembly protein; involved in synthesis of formyl group of position 8 of haem A	Tzagoloff et al. 1990; Petruzzella et al. 1998; Hiser et al. 2000
COX14				COX assembly protein	Glerum et al. 1995
COX15		IMM	cDNA isolated	biosynthesis of haem A	Glerum et al. 1997; Petruzzella et al. 1998; Barros et al. 2002
COX17		IMM and cytoplasm	cDNA isolated	interacts genetically with SCO1 and SCO2 in COX assembly; involved in copper metabolism	Glerum et al. 1996a; Amaravadi et al. 1997
COX18				required for activity of COX	Hikkel et al. 1997; Souza et al. 2000
COX20		IMM		processing of the COX subunit 2 precursor pCox2p	Hell et al. 2000
MSS1 (PET18, PET53)	GTPase			mitochondrial GTPase involved in expression of Cox1; works in association with small subunit of mitoribosomes; may optimise mitochondrial protein synthesis, possibly by a proofreading mechanism	Decoster et al. 1993
MSS51		not determined		possibly involved in translational activation of Cox1 mRNA	Decoster et al. 1990
NAM1		matrix		Cox1 pre-mRNA splicing	Asher et al. 1989
OXA1 (PET1402 or PET-TS1402)		IMM	*OXA1L* Ch 14q11.2	COX biogenesis protein, required for post-translational step of biogenesis; also necessary for correct assembly of ATP synthase complex; seems to be involved in proteolytic processing of COX II; probably a general membrane insertion protein for mtDNA-encoded polypeptides	Bonnefoy et al. 1994a; Bonnefoy et al. 1994b; Hell et al. 2001
PET54		IMM and matrix		translation of Cox3 mRNA	Costanzo et al. 1986
PET100		not determined		COX assembly protein – not required for synthesis or localisation of COX subunits to mitochondria, but is required at a later step in their assembly into an active holoenzyme	Church et al. 1996; Forsha et al. 2002
PET111		IMM		required for translation of Cox2 mRNA	Poutre and Fox, 1987; Green-Willms et al. 2001
PET112	probable glutamyl-tRNA(gln) amidotransferase subunit b	matrix	cDNA isolated	required for translation of Cox2 mRNA	Mulero et al. 1994; Petruzzella et al. 1998
PET117		IMM		COX assembly factor	McEwen et al. 1993
PET122		IMM		translational activator of Cox3	Kloeckener-Gruissem et al. 1988
PET191		IMM		involved in assembly of COX	McEwen et al. 1993
PET309		spans IMM		required for stability and translation of Cox1 mRNA; interacts with 5'-UTR region of COX1	Manthey et al. 1998
PET494		IMM		translation of Cox3 mRNA	Muller and Fox, 1984
SCO1		IMM	*SCO1* Ch 17p11	involved in stabilisation of COX I and COX II proteins – required for translation and possibly assembly may play a role in mitochondrial copper transport or insertion of copper into the active site of COX	Schulze and Rodel, 1988; Glerum et al. 1996a; Petruzzella et al. 1998
SCO2		IMM	*SCO2* Ch 22q13	strong similarity to SCO1 protein – the two may have overlapping functions	Glerum et al. 1996a; Papadopoulou et al. 1999
SHY1		IMM	*SURF1* Ch 9q34		Mashkevich et al. 1997; Zhu et al. 1998

IMM = inner mitochondrial membrane
Additional information obtained from MITOP database (http://www.mips.biochem.mpg.de/proj/medgen/mitop/)

Table 1.3 Yeast assembly genes required for functional COX

probably responsible for insertion of Cu_B into the haem a_3-Cu_B binuclear centre of COX I (Hiser et al. 2000). Other proteins, termed 'assembly facilitators', are involved late in the biogenesis of COX, after all the subunits have been synthesised and localised to the inner mitochondrial membrane (Poyton and McEwen, 1996; Forsha et al. 2001). These include COX14, COX18, COX20, OXA1, PET100, PET117, PET191, and SHY1 (Glerum et al. 1995; Souza et al. 2000; Hell et al. 2000; Bonnefoy et al. 1994a; Church et al. 1996; McEwen et al. 1993; Mashkevich et al. 1997). The human counterparts of some of these yeast genes have been characterised (Bonnefoy et al. 1994b; Petruzzella et al. 1998; Zhu et al. 1998). Some, for example OXA1, are required for the biogenesis of other respiratory chain enzyme complexes in addition to COX (Altamura et al. 1996). Yeast Oxa1p appears to be a general membrane insertion protein for mtDNA-encoded proteins (Hell et al. 2001). Others, for example SURF1, appear to be specific for COX (Zhu et al. 1998). Yeast SHY1 is the homologue of human SURF1 and SHY1 mutants have decreased levels of COX, increased levels of cyt *c* and inefficient electron transfer from cytochrome *b*-c_1 to COX (Mashkevich et al. 1997). Pet100p appears to be a molecular chaperone that incorporates a subcomplex containing yeast COX subunits VII, VIIa and VIII into the COX holoenzyme complex (Forsha et al. 2001). The complexity of COX assembly probably reflects the central role of COX in the regulation of cellular respiration (Villani and Attardi, 1997; Kunz et al. 2000).

1.8.3 Regulation of cytochrome oxidase gene expression

Regulation of COX activity to meet cellular energy requirements may be via short-term mechanisms such as allosteric regulation by ATP and/or other metabolites, or by long-term mechanisms which involve alteration of COX gene expression (Poyton and McEwen, 1996). The latter may act by increasing or decreasing the number of functionally active COX holoenzyme molecules present in the inner membrane, or by assembling subunit isoforms that alter the catalytic activity of the holoenzyme. In eukaryotes nuclear RNA polymerases require *cis*-acting transcription elements and *trans*-acting transcription factors to initiate transcription. A number of transcription factors that seem to be involved in the regulation of COX gene expression have been identified (reviewed in Lenka et al. 1998). These include Sp1, YY1, NRF1 and NRF2. The genes encoding ubiquitous COX subunits are 'housekeeping' genes, located in CpG islands and rich in GC sequences that act as binding sites for the transcription factor Sp1, which initiates transcription of housekeeping genes (Lenka

et al. 1998). Mutational analysis in rodents has suggested that Sp1 sites are essential for *COX4* and *COX5B* expression (Grossman and Lomax, 1997). Another transcriptional factor YY1 (Yin-Yang-1) appears to be involved in the expression of *COX5B* and *COX7C* (Hahn, 1992; Seelan and Grossman, 1997). NRF1 is a third transcription factor involved in the regulation of COX genes (Evans and Scarpulla, 1990). NRF1 binding sites have been identified in the promoter regions of several genes involved in mitochondrial function, including cytochrome *c*, *COX5B*, *COX7AL*, the mitochondrial transcription factor mtTFA, the mitochondrial processing endoribonuclease mMRP and a gene encoding tyrosine aminotransferase, which catalyses the rate-limiting step of haem biosynthesis (Grossman and Lomax, 1997). Rodent *COX4* seems to require NRF2 rather than NRF1 for its expression (Virbasius and Scarpulla, 1991).

Factors that have been shown to influence transcription of some mammalian COX genes include oxygen concentration, muscle contraction, interferon, oestrogen, vitamin D and cyclic nucleotides (Poyton and McEwen, 1996). Steady-state levels of mRNAs for several human COX subunits have been shown to vary with development, from fetal life to adulthood (Bonne et al. 1993). Developmental switching of VIa isoform expression has also been reported (Ewart et al. 1991) and this may be mediated by thyroid hormone (Meehan and Kennedy, 1997). However not all COX genes appear to have transcriptional regulatory elements, and it is not known how multiple trans-acting factors act in harmony to coordinate COX gene expression. Nor is it known how the mitochondrion sends signals to the nucleus (Poyton and McEwen, 1996).

Even less is known about translational regulation of COX, although some translational activators have been identified for the yeast *Cox1* (MSS51p and PET309p), *Cox2* (PET111p) and *Cox3* (PET54p, PET122p and PET494p) genes (Poyton and McEwen, 1996; Table 1. 3).

1.9 Disorders of the mitochondrial respiratory chain and oxidative phosphorylation

In 1962 Luft reported the first case of a mitochondrial disorder, a Swedish woman with hypermetabolism but without any evidence of thyroid dysfunction (Luft et al.

1962). Further studies demonstrated abnormal mitochondrial structure, and loose coupling of oxidation and phosphorylation. The following year Engel and Cunningham, using a modification of Gomori's trichrome stain, described the morphological hallmark of the mitochondrial myopathies, the ragged red fibre (RRF), in which intense subsarcolemmal red staining represents mitochondrial proliferation (Engel and Cunningham, 1963). Ultrastructural abnormalities of mitochondrial size, number and morphology are also often observed in patients with mitochondrial myopathies.

The first mutations of mtDNA associated with human disease were described in 1988 (Holt et al. 1988). Over the last 13 years many further genetic defects that cause human disease have been recognised in mtDNA, including a large number of rearrangements and approximately 100 different point mutations (Servidei, 2001). Large-scale rearrangements of mtDNA are usually sporadic whilst most point mutations are maternally inherited. Inhibition of mitochondrial protein synthesis by deletions or point mutations involving tRNA genes leads to mitochondrial proliferation, which appears as RRF in biopsied muscle. Mutations are found in the mitochondrial genome in approximately a third of adults but only in 4% of children with respiratory chain disorders (Shoffner, 1996). The mechanism by which mtDNA mutations cause disease is still incompletely understood (Schon et al. 1997).

1.10 Cytochrome oxidase deficiency: clinical features

COX deficiency, either total or partial, is the most commonly recognised respiratory chain defect in childhood (Caruso et al. 1996). In an Italian series of 60 patients with respiratory chain defects 70% had COX deficiency (Caruso et al. 1996). Disease onset was before 3 years of age in virtually all these patients. Activity of COX may be assessed histochemically (Seligman et al. 1968) and measured in tissue homogenates or mitochondrial preparations by spectrophotometric assay (Birch-Machin et al. 1994). COX deficiency may be an isolated defect, or be combined with deficiencies of other components of the respiratory chain.

Clinical presentations of COX deficiency are very heterogeneous (DiMauro et al. 1994). Onset is usually between the neonatal period and 3 years of age but may be later. Cases of isolated COX deficiency with adult onset have been described (Haller et al. 1989). Relatively frequent presentations of COX deficiency in childhood

include fatal infantile myopathy with or without a Fanconi-type renal tubulopathy (DiMauro et al. 1994; Robinson, 2000), Leigh syndrome (Willems et al. 1977; Rahman et al. 1996), cardiomyopathy and myopathy (Zeviani et al. 1986), recurrent myoglobinuria (Saunier et al. 1995; Keightley et al. 1996), and a 'benign' spontaneously reversible COX-deficient myopathy (DiMauro et al. 1983). However the spectrum of clinical features of COX deficiency is enormous and also includes macroglossia (DiMauro et al. 1983; Zeviani et al. 1986), dysmorphic features (Savasta et al. 2001), structural brain abnormalities such as cerebellar hypoplasia (Lincke et al. 1996), infantile spasms (Bakker et al. 1996b) and stroke-like episodes (Morin et al. 1999).

In addition, partial COX deficiency may be seen in certain well-recognised mitochondrial syndromes, such as the Kearns-Sayre, MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes) and MERRF (myoclonus epilepsy and ragged red fibres) syndromes (Johnson et al. 1983; Hammans et al. 1995; Taanman, 1997). These syndromes are associated with mtDNA mutations and the COX deficiency is patchy with some fibres deficient and others apparently normal, reflecting the heteroplasmy of the mutations. A critical mutant load is required to produce a biochemical defect and fibres with mutant loads below this threshold appear biochemically normal (Schon et al. 1997).

The following is a clinical classification of COX deficiency according to the tissue predominantly involved.

1.10.1 Fatal infantile myopathy

COX deficiency associated with a fatal infantile myopathy was first reported by Van Biervliet et al. in 1977 and was subsequently noted to be one of the most frequent presentations of COX deficiency (Bresolin et al. 1994). Presentation is usually early in the neonatal period. Initial features include failure to thrive, weakness (involving facial and pharyngeal as well as limb muscles), hypotonia and severe lactic acidosis. This is rapidly followed by ventilatory insufficiency. In some cases other organ systems may be involved, for example Fanconi-type renal tubulopathy as in the original case of Van Biervliet, or cardiomyopathy (Zeviani et al. 1986; DiMauro et al. 1990). Most of these infants die from respiratory failure and/or aspiration pneumonia at less than a year of age.

Residual COX activity is typically less than 10% in skeletal muscle in patients with isolated myopathy (DiMauro et al. 1990). Patients with tubulopathy have residual COX activity of 40% in kidney (DiMauro et al. 1990). Some of these patients were reported to have RRF (Zeviani et al. 1985; DiMauro et al. 1994; Bresolin et al. 1994). Immunohistochemical analysis of these patients revealed variable findings. In the first study reported there was a general decrease of immunostaining of all COX subunits in skeletal muscle (Bresolin et al. 1985) but later studies suggested a specific deficiency of subunit VIIa/b (Tritschler et al. 1991; Possekel et al. 1995). Expression of subunit VIa isoforms has not been analysed in these patients.

The genetic basis of this condition remains unknown, but pedigree studies in a few informative families suggested autosomal recessive inheritance and thus a nuclear gene (DiMauro et al. 1990). Selective involvement of the VIaH or VIIaH isoform would in theory explain severe skeletal muscle disease and normal cardiac function, but no mutation has ever been reported in the genes encoding either of these subunits nor, in fact, in any nuclear COX subunit gene. A nuclear regulatory gene may be responsible but this has yet to be identified.

1.10.2 Benign infantile myopathy

Eight children have been described in whom a severe COX-deficient myopathy resolved spontaneously by the age of 3 years (DiMauro et al. 1983; Zeviani et al. 1987; Suzuki et al. 1989; Tritschler et al. 1991; Salo et al. 1992; Wada et al. 1996). These children presented between birth and 2 months with profound hypotonia and weakness leading to feeding and breathing difficulties, and associated severe lactic acidosis. Four required artificial ventilation for periods up to 19 months (Zeviani et al. 1987; Suzuki et al. 1989; Salo et al. 1992; Wada et al. 1996). Extraocular muscles were spared (DiMauro et al. 1983). Mild hepatomegaly was noted in 3 cases (Zeviani et al. 1987; Salo et al. 1992; Wada et al. 1996) and macroglossia in one (DiMauro et al. 1983). None of these children had renal tubulopathy.

Muscle histology revealed the presence of RRF, ultrastructural mitochondrial abnormalities and fewer than 5% COX-positive fibres (DiMauro et al. 1983; Suzuki et al. 1989). Biochemical analysis confirmed severe COX deficiency, with residual COX activity between 8 and 25% in skeletal muscle (DiMauro et al. 1983; Zeviani et al. 1987; Suzuki et al. 1989; Salo et al. 1992; Wada et al. 1996). COX activity was

normal in cultured skin fibroblasts (Zeviani et al. 1987). A ninth case had combined deficiencies of complex I and COX (Roodhooft et al. 1986). In all cases clinical improvement began between 6 and 9 months of age and was accompanied by gradual return of COX activity, demonstrated both histochemically and biochemically (DiMauro et al. 1983).

Benign and fatal infantile myopathies are indistinguishable early in the clinical course, until the spontaneous improvement in the former becomes apparent at approximately 6 months (Bresolin et al. 1985). It has been suggested that fatal and benign myopathies may be differentiated by COX subunit immunohistochemistry. In one study 4 patients with fatal myopathy had selective loss of immunostaining of subunit VII whereas 4 patients with the benign myopathy had loss of staining of subunit II and subunit VII (Tritschler et al. 1991). The benign reversible infantile myopathy was proposed to be caused by a defect in a developmentally regulated COX subunit, with resolution of the COX deficiency being secondary to isoform switching (Tritschler et al. 1991), but this has never been proved.

1.10.3 Late-onset myopathy

Exercise intolerance may be the sole clinical feature of COX deficiency. Weakness in the mitochondrial myopathies is mainly proximal, mild and most easily detected in the upper limbs (Petty et al. 1986). Fatigue characteristically occurs after sustained or repeated contraction. Weakness and exercise intolerance may be accompanied by recurrent exertional myoglobinuria. The clinical course is usually relatively benign. Defects of mtDNA associated with isolated skeletal myopathy and COX deficiency include multiple deletions (Ohno et al. 1991), point mutations in tRNA genes (Table 1.4) and mutations in COX subunit genes (Table 1.5) and. Mutations in nuclear genes have not yet been associated with this phenotype.

1.10.4 Leigh syndrome

Isolated COX deficiency is also a common biochemical defect in Leigh syndrome (Rahman et al. 1996) and Leigh syndrome is probably the most frequent presentation of COX deficiency (Robinson, 2000). Leigh syndrome or subacute necrotising encephalomyelopathy is a progressive neurodegenerative disorder with characteristic neuropathological features. In 1951 Denis Leigh reported the

neuropathology of a seven month old infant who died following a progressive neurological illness of six weeks' duration, with somnolence, blindness, deafness and generalised limb spasticity (Leigh, 1951). Leigh's findings were focal bilaterally symmetrical subacute necrotic lesions in the thalamus, extending to the pons, and in the inferior olives and spinal cord. Histological characteristics of these lesions were intense capillary proliferation, gliosis, demyelination and vacuolation with relative preservation of neurons.

This neuropathology was subsequently reported in a large number of patients with clinical features of psychomotor retardation, hypotonia, failure to thrive, breathing abnormalities, oculomotor disturbances, optic atrophy, seizures and lactic acidosis (van Erven et al. 1987a). In clinical practice neuropathology is rarely available so a presumptive diagnosis of Leigh syndrome is often made on the basis of a characteristic clinical course, lactic acidosis in blood and/or CSF and radiological findings (Rahman et al. 1996). Bilateral symmetrical basal ganglia and brainstem lesions are typical neuroradiological findings. These appear as hypodensities on computerised tomography (CT) and as areas of high signal intensity on T2-weighted magnetic resonance imaging (MRI) (Schwartz et al. 1981; Koch et al. 1986).

A number of biochemical defects have been associated with Leigh syndrome, including COX deficiency (Willems et al. 1977), complex I deficiency (Van Erven et al. 1987b), pyruvate dehydrogenase deficiency (Kretzschmar et al. 1987) and biotinidase deficiency (Baumgartner et al. 1989). Mutations have also been reported in the mitochondrial ATPase 6 gene in a number of patients with maternally inherited Leigh syndrome (Rahman et al. 1996; Wilson et al. 2000; Servidei, 2001). This has led to the hypothesis that the neuropathology seen in this condition is the end result of a failure of cerebral mitochondrial energy production (DiMauro and De Vivo, 1996).

Pedigree analysis suggested autosomal recessive inheritance of COX-deficient Leigh syndrome (Rahman et al. 1996) and this was confirmed by transmitochondrial cybrid analysis (Tiranti et al. 1995b). More extensive cell fusion studies suggested that most patients belong to a single complementation group (Brown and Brown, 1996; Munaro et al. 1997). Sequence analysis failed to identify pathogenic mutations in either mitochondrial (Adams et al. 1997) or nuclear (Lee et al. 1998)

COX subunit genes, but more recently mutations were identified in the *SURF1* gene in a proportion of patients with COX-deficient Leigh syndrome (Zhu et al. 1998; Tiranti et al. 1998b, see section 1.11.3.1). All patients with SURF1 mutations reported to date had severe isolated COX deficiency in all tissues studied (Pequignot et al. 2001b). Residual COX activities ranged from 5 to 46% in skeletal muscle, 2 to 20% in cultured skin fibroblasts and 13 to 31% in lymphoblastoid cell lines (Tiranti et al. 1999c; Sue et al. 2000; Pequignot et al. 2001a).

A variant of Leigh syndrome seen in the remote Saguenay Lac St Jean region of Quebec province in Canada has a characteristic phenotype (Morin et al. 1993). Affected patients have facial dysmorphism, acidotic crises and microvesicular fatty infiltration of the liver, in addition to more typical features of Leigh syndrome (hypotonia, psychomotor retardation and ataxia). These patients also have isolated COX deficiency but the biochemistry differs from the biochemical profile of SURF1 deficient patients. There is severe COX deficiency in brain and liver, but approximately 50% residual enzyme activity in skeletal muscle, cultured skin fibroblasts and amniocytes, and approaching normal levels in kidney and cardiac muscle (Merante et al. 1993). Immunoblot analysis demonstrated a generalised decrease of COX subunits, proportional to the decrease of COX activity and suggesting an assembly defect as for the 'classical' form of COX-deficient Leigh syndrome (Merante et al. 1993). Recently a genomewide linkage disequilibrium scan demonstrated a locus on chromosome 2p16 for this French Canadian variant of Leigh syndrome, but the responsible gene has not yet been identified (Lee et al. 2001).

1.10.5 Cardiomyopathy

Cardiac involvement was noted in 9% of a French series of patients with COX deficiency (von Kleist-Retzow et al. 1998) and in some cases of COX deficiency cardiomyopathy may be the only clinical feature (Rimoldi et al. 1982). Cardiomyopathy associated with OXPHOS defects is usually hypertrophic but may occasionally be dilated (Antozzi and Zeviani, 1997). A number of mtDNA point mutations, mainly clustered in the tRNA leucine and isoleucine genes, are associated with maternally inherited cardiomyopathy syndromes (Servidei, 2001). In addition hypertrophic cardiomyopathy has been reported in pedigrees bearing the MELAS A3243G mutation (Hammans et al. 1995) and the MERRF A8344G

mutation (Shoffner et al. 1990) and in patients with multiple mtDNA deletions (Takei et al. 1995). Dilated cardiomyopathy has also been described in patients with multiple mtDNA deletions (Suomalainen et al. 1992b). Cardiac conduction defects can also occur in patients with COX deficiency, and are particularly well recognised in the Kearns-Sayre syndrome (Berenberg et al. 1977). Both heart block and Wolf Parkinson White syndrome have been reported in patients with the A3243G MELAS mutation (Moraes et al. 1993; Hammans et al. 1995).

In other patients with cardiomyopathy and COX deficiency, with or without complex I deficiency, mtDNA mutations have not been identified and nuclear gene defects may be responsible. Overall, the frequency of mtDNA mutations in cardiomyopathy appears low (Turner et al. 1998). Severe cardiomyopathy also occurs in an autosomal recessively inherited syndrome with PEO and multiple mtDNA deletions (Bohlega et al. 1996), but the causative gene has not yet been identified. Early-onset cardiomyopathy was the presenting feature in a group of children with mutations in the *SCO2* gene, whose protein product is involved in copper delivery to the COX holoenzyme (Papadopoulou et al. 1999). These children also had an encephalopathy (see section 1.11.3.2).

1.10.6 Liver disease

Liver disease is a frequent manifestation of COX deficiency, particularly complicating the Pearson and mtDNA depletion syndromes, and is often the cause of death in these patients (Rotig et al. 1995; Morris et al. 1998). In a French series 10% of children found to have OXPHOS defects had been referred because of liver dysfunction (Cormier-Daire et al. 1997). Clinical features of these patients included hypoglycaemia, jaundice, hepatomegaly and liver failure. Liver histology was performed in 15 of 22 cases and revealed steatosis in 7, cirrhosis (usually micronodular) in 7, and both steatosis and micronodular cirrhosis in one case. COX deficiency was a frequent finding in these patients: 8 patients had isolated COX deficiency in liver; 4 had combined deficiencies of complex I and COX; and 4 had generalised OXPHOS defects including COX deficiency. In another French series 20% of 44 patients with isolated COX deficiency had hepatic failure (von Kleist-Retzow et al. 1998). Mutations of *SCO1*, a nuclear gene encoding another protein involved in copper delivery to COX, have been reported in one family with liver disease and isolated COX deficiency (Valnot et al. 2000a, see section 1.11.3.3).

1.10.7 Renal involvement

Proximal tubular defects of the Fanconi type are the most common renal manifestation of COX deficiency (Niaudet and Rotig, 1996). Renal disease was noted in 9% of 44 COX deficient patients in a French series (von Kleist-Retzow et al. 1998). In a literature review of tubulopathy associated with respiratory chain defects, 16 of 31 cases had COX deficiency (Niaudet and Rotig, 1996). Extra-renal symptoms were present in all these patients. Tubulopathy has been reported in patients with COX-deficient fatal infantile myopathy (see section 1.10.1) and can be a feature of the Pearson, Kearns-Sayre, Leigh and mtDNA depletion syndromes. Proximal renal tubulopathy was a prominent feature in a family with isolated COX deficiency, in which mutations of the *COX10* gene encoding haem A: farnesyltransferase were subsequently shown to be causative (Ogier et al. 1988; Valnot et al. 2000b, see section 1.11.3.4).

1.10.8 Multisystem disorders

1.10.8.1 Mitochondrial DNA depletion syndrome

A quantitative defect of mtDNA, with severe tissue-specific reduction in mtDNA copy number, was first described in 1991 in 4 patients with variable clinical features including progressive generalised hypotonia, PEO, severe lactic acidosis, liver failure and Fanconi-type renal tubulopathy (Moraes et al. 1991). At least 50 patients with mtDNA depletion have now been reported (Taanman et al. 1997; Vu et al. 1998; Campos et al. 1998), making this a relatively common cause of primary lactic acidosis in infancy (Macmillan and Shoubridge, 1996). As with other mitochondrial disorders, clinical features are heterogeneous but typically neonatal or infantile onset fatal lactic acidosis is associated with severe hypotonia and progressive liver failure (Morris et al. 1998). Variable features include seizures (Moraes et al. 1991; Telerman Toppet et al. 1992), PEO, cataracts (Morris et al. 1998), Fanconi syndrome and congestive heart failure (Figarella-Branger et al. 1992). This multisystem disorder is often fatal in infancy, but cases with less severe depletion of mtDNA associated with later-onset myopathy and slower progression have been reported (Vu et al. 1998).

Muscle histology typically shows abnormal mitochondrial proliferation and patchy reduction of COX staining (Moraes et al. 1991; Morris et al. 1998). Muscle

immunohistochemistry revealed reduced staining of mtDNA-encoded COX subunits but normal or increased staining of nuclear-encoded COX subunits (Moraes et al. 1991; Tritschler et al. 1992). MtDNA is depleted by up to 99% in affected tissues compared to age-matched control subjects (Moraes et al. 1991). All mtDNA-encoded polypeptides are affected, so that COX deficiency in this syndrome is usually accompanied by defects of all other respiratory chain enzymes, with the exception of complex II (Hargreaves et al. 2002). The underlying aetiology is likely to be heterogeneous: so far mutations have been reported in the deoxyguanosine kinase gene *DGUOK* in patients with hepatocerebral disease (Mandel et al. 2001) and in the thymidine kinase 2 gene *TK2* in patients with muscle-specific mtDNA depletion (Saada et al. 2001; see section 1.11.4).

1.10.8.2 Alpers syndrome

Alpers syndrome or progressive neuronal degeneration of childhood with liver disease is a rare familial disorder of unknown aetiology (Alpers, 1931; Huttenlocher et al. 1976; Harding, 1990). Onset of symptoms in Alpers syndrome typically follows normal delivery and early development. Infants most often present with intractable generalised seizures associated with developmental delay, marked hypotonia, episodes of vomiting and failure to thrive (Harding, 1990). There may be signs of liver disease at presentation, although these may not occur until later (Huttenlocher et al. 1976). Investigations reveal occipital and posterior temporal hypodensities and atrophy on CT scan, characteristic EEG features (very slow activity of very high amplitude interspersed by lower amplitude polyspikes; Boyd et al. 1986), absent visual evoked responses and abnormal liver histology with fatty change, hepatocyte loss, bile duct proliferation, fibrosis or frank cirrhosis (Harding, 1990). The clinical course is of progressive deterioration and death usually occurs before the age of 3 years, although presentation in adulthood has been reported (Harding et al. 1995). Neuropathology reveals patchy cortical thinning and discoloration, particularly of the striate cortex, with spongiosis, neuronal loss and astrocytosis on histological examination (Harding et al. 1986).

COX deficiency has been described in several patients with Alpers syndrome (Prick et al. 1983; Chabrol et al. 1994; Morris et al. 1996) and one patient had a few RRF as well as COX-negative fibres (Rasmussen et al. 2000). Recently a patient with Alpers was reported to have mtDNA depletion associated with deficiency of mtDNA

polymerase gamma POLG activity (Naviaux et al. 1999). However so far *POLG* mutations have not been reported in Alpers syndrome. Like Leigh syndrome, Alpers syndrome probably encompasses a heterogeneous group of genetic disorders, and the responsible genes have not yet been identified.

1.10.8.3 Mitochondrial neurogastrointestinal encephalopathy (MNGIE)

This autosomal recessive syndrome is characterised by gastrointestinal dysmotility associated with peripheral neuropathy, ptosis, PEO and histological features of mitochondrial myopathy (RRF, increased SDH staining or ultrastructurally abnormal mitochondria) (Hirano et al. 1994). Presentation is usually between the second and fifth decades but onset in infancy has been reported (Nishino et al. 2000). Initial symptoms are usually gastrointestinal, but ptosis and ophthalmoplegia are also frequent early symptoms. The most common gastrointestinal symptoms in these patients are recurrent nausea, vomiting and diarrhoea, but pseudo-obstruction may occur. Neuroimaging may demonstrate leukodystrophy and neurophysiological studies a sensorimotor polyneuropathy. COX-negative fibres appear to be a universal feature in the skeletal muscle of MNGIE patients and in one series 50% of patients had reduced COX activity, either as an isolated defect or in combination with deficiencies of other respiratory chain enzymes (Nishino et al. 2000). Southern blot of muscle DNA reveals multiple mtDNA deletions (Hirano et al. 1994) and/or partial mtDNA depletion (Papadimitriou et al. 1998). A disease locus was mapped to chromosome 22q13.32-qter (Hirano et al. 1998) and subsequently mutations were found in the thymidine phosphorylase gene in more than 20 families with MNGIE (Nishino et al. 1999).

1.11 Cytochrome oxidase deficiency: molecular genetics

1.11.1 Mutations of Mitochondrial DNA

Reduction of COX activity has been reported in patients with large scale rearrangements of mtDNA and in patients with point mutations. The latter may be in tRNA or rRNA genes or in polypeptide-coding genes.

Table 1.4 rRNA and tRNA point mutations associated with COX deficiency

Gene	Mutation	%COX-negative fibres	RRF	RC enzyme activities (%control)	Clinical features	Reference
12S rRNA	A1555G	none	none	CI 38%, **COX 50%**	restrictive cardiomyopathy	Santorelli et al. 1999
tRNA phenylalanine	A606G	68%	1-2%	**COX 50%**, other complexes normal	acute rhabdomyolysis	Chinnery et al. 1997b
tRNA valine	A1606G	5%	<3%	all complexes normal	mitochondrial encephalomyopathy	Tiranti et al. 1998a
	G1644T	11-13%	none	NS	Leigh syndrome	Chalmers et al. 1997
tRNA leucine UUR	A3243G	<5%	12-20%	reduced CI+III, II+III, COX (COX normal in only 2/16 patients)	MELAS	Clafaloni et al. 1992; Moraes et al. 1993; Hammans et al. 1995
	A3243G	3-6%	12-16%	NS	PEO	Hammans et al. 1995
	A3243G	NS	NS	CI 50%, COX 90%	diabetes mellitus and deafness	van den Ouweland et al. 1992
	A3243T	NS	NS	CI 8%, CII normal, **COX 29%**, CIII 65%	mitochondrial encephalomyopathy	Shaag et al. 1997
	G3249A	numerous	numerous (COX-)	CI 50%, CII, CIII and COX normal	KSS	Seneca et al. 2001
	T3250C	±	+	CI ↓↓, **COX ↓**	myopathy	Goto et al. 1992
	T3250C	none	none	CI 6%, **COX 47%***	myopathy	Ogle et al. 1997
	A3251G	NS	numerous	**COX 67%**, other complexes normal	myopathy and sudden death	Sweeney et al. 1993
	A3252G	5%	10-15%	CI 52%, CII+III and COX normal	MELAS	Morten et al. 1993
	C3254G	many	profusion	NS	myopathy	Kawarai et al. 1997
	C3256G	NS	NS	**COX >3SD below control**, CI and CIII >2SD below control	PEO / multisystem disease	Moraes et al. 1993
	A3260G	+	+	reduced activities of CI and COX	MIMyCa	Zeviani et al. 1991
	A3260G	+	+	NS	MELAS	Nishino et al. 1996a
	A3302G	NS	NS	CI <5%, CIII 63%, **COX 30%**	myopathy	Bindoff et al. 1993
	C3303T	NS	+	CI 23%, **COX 16%**	MIMyCa	Silvestri et al. 1994
tRNA isoleucine	A4269G	+ (focal)	+	**COX 30%**, others NS	multisystem disease with cardiomyopathy	Taniike et al. 1992
	T4274C	51%	+	CI 30%, **COX 20%**	PEO	Chinnery et al. 1997a
	T4285C	abundant	abundant (COX-)	**COX 38%**	PEO	Silvestri et al. 1996
	A4295G	NS	NS	**COX 20% in heart, 33% in liver**, normal in muscle; CI+III also low in heart and liver	HCM	Merante et al. 1996
	G4298A	20%	10%	CI 60%, **COX 40%**	PEO and multiple sclerosis	Taylor et al. 1998
	G4309A	+	+	CI 94%, CII+III 71%, **COX 64%**	PEO	Franceschina et al. 1998
	A4317G	NS	NS	severe defects of CI and COX (heart)	fatal infantile cardiomyopathy	Tanaka et al. 1990
tRNA glutamine	G4332A	+	3%	normal	atypical MELAS	Bataillard et al. 2001
	4370insA	89%	+	NS	Myopathy	Dey et al. 2000
tRNA methionine	U4409C	~50%	+	NS	myopathy	Vissing et al. 1998
tRNA tryptophan	G5521A	many	many	CI 85%, CI+III 69%, CII+III 55%, **COX 15%**	late-onset myopathy	Silvestri et al. 1998
	5537insT	few	+	CI 47%, CI+III 43%, **COX 11%**	mitochondrial encephalomyopathy	Santorelli et al. 1997c
	G5540A	numerous	numerous (COX-)	**COX 16%**, other complexes normal	mitochondrial encephalomyopathy	Silvestri et al. 2000
	G5549A	+	32%	complex I defect (polarography)	dementia, chorea, cerebellar ataxia, deafness, peripheral neuropathy	Nelson et al. 1995
tRNA asparagine	A5692G	NS	few	slight reduction of CI and COX	PEO	Seibel et al. 1994
	G5703A	NS	many	**COX >3SD below control**, CI and CIII >2SD below control	PEO	Moraes et al. 1993
tRNA cysteine	A5814G	+	abundant	NA	mitochondrial encephalomyopathy	Santorelli et al. 1997a
	A5814G	NS	numerous (COX+)	CI 22%, CI+III 31%, CII+III 70%, **COX 24%**	MIMyCa	Karadimas et al. 2001
tRNA tyrosine	A5874G	24-73%	24-73% (COX-)	?combined deficiencies of CIII and COX (polarography)	exercise intolerance	Pulkes et al. 2000
tRNA serine UCN	7472Cins	48%	none	CI 47%, **COX 36%**	HAM / MERRF	Tiranti et al. 1995a
	T7511C	+	none	**COX 44%**, other complexes normal	isolated SNHL	Sue et al. 1999
	T7512C	many	many (COX-)	NS	MERRF / MELAS	Nakamura et al. 1995
tRNA aspartic acid	G7543A	-	-	**COX 74%**	myoclonic epilepsy and psychomotor regression	Shtilbans et al. 1999
tRNA lysine	G8313A	+	numerous	CI and **COX >3SD below control**	gastrointestinal symptoms and encephalopathy	Verma et al. 1997
	G8328A	10%	frequent	COX 94%	mitochondrial encephalomyopathy	Houshmand et al. 1999
	G8342A	diffuse severely decreased staining	none	CI 105%, CII 67%, CIII 43%, **COX 8%**, CV 29%	PEO and myoclonus	Tiranti et al. 1999a
	A8344G	NS	+	CI 4%, CIII 44%, **COX 2%; residual COX 16-66% in 13/16 cases**	MERRF	Shoffner et al. 1990; Silvestri et al. 1993
	A8344G	35-40%	+	NS	familial multiple symmetric lipomatosis	Gamez et al. 1998
	T8356C	numerous	+ (COX-)	NS	MERRF/ MELAS overlap syndrome	Zeviani et al. 1993
	G8363A	+	+	**COX 25%***	multisystem cardiomyopathy	Santorelli et al. 1996a
	G8363A	30-80%	0.5%	CI-III 7-43%, CII-III normal, **COX 20-88%**	MERRF	Ozawa et al. 1997
	G8363A	50%	+	reduced activities of all complexes	cerebellar ataxia and lipomas	Casali et al. 1999
tRNA glycine	T9997C	NA	NA	reduced activities of CI, CII and COX	HCM	Merante et al. 1994
	A10044G	NS	NS	CI 47%, **COX 43%**	sudden unexpected death, multisystem disorder	Santorelli et al. 1996b
tRNA leucine CUN	T12311C	1%	0.1%	normal	PEO	Hattori et al. 1994
	G12315A	90%	90% (COX-)	NS	PEO, ptosis, limb weakness, SNHL, pigmentary retinopathy	Fu et al. 1996
	A12320G	68%	18%	CI 37%, CII 101%, CIII 82%, **COX 30%**	myopathy	Weber et al. 1997
tRNA glutamic acid	T14709C	few	many	CI 52%, **COX 54%**	congenital myopathy, mental retardation, cerebellar ataxia, diabetes mellitus	Hanna et al. 1995a
tRNA threonine	G15915A	+ (focal)	few	NS	mitochondrial encephalomyopathy	Nishino et al. 1996b
	A15923G	NS	NS	CI NA, CIII N, **COX <6%**,	fatal infantile multisystem disorder	Yoon et al. 1991a
	A15924G	NS	NS	CI NA, CIII **<10%, COX <10%**,	fatal infantile multisystem disorder	Yoon et al. 1991a
	15940del T	+	+	NS	axonal sensorimotor peripheral neuropathy	Seneca et al. 1998
tRNA proline	G15990A	NS	many	**COX >3SD below control**, CI+III and CII+III >2SD below control	myopathy	Moraes et al. 1993

Notes:
RC enzymes assayed in skeletal muscle and refer to % activity compared to lowest control, unless otherwise stated (* = % of control mean)

Abbreviations:

HAM	**hearing loss, ataxia, myoclonus**
HCM	**hypertrophic cardiomyopathy**
KSS	**Kearns-Sayre syndrome**
MELAS	**mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes**
MERRF	**myoclonic epilepsy with ragged red fibres**
MIMyCa	**maternally inherited myopathy and cardiomyopathy**
NA	**not analysed**
NS	**not stated**
PEO	**progressive external ophthalmoplegia**
SNHL	**sensorineural hearing loss**

1.11.1.1 Deletions and Duplications

Focal COX deficiency has been observed in muscle biopsies from many patients with large-scale mtDNA deletions (Holt et al. 1989a). The clinical phenotypes most commonly associated with large deletions of mtDNA are PEO and the Kearns-Sayre and Pearson syndromes (Moraes et al. 1989; Holt et al. 1989a; Rotig et al. 1989). Duplications are seen in the Kearns-Sayre and Pearson syndromes and in patients with diabetes and deafness (Poulton et al. 1989; Superti-Furga et al. 1993; Dunbar et al. 1993).

1.11.1.2 tRNA and rRNA point mutations

Focal COX deficiency has also been reported in association with point mutations in 18 of the 22 mitochondrial tRNA genes, but does not always involve sufficient fibres to produce a demonstrable defect on enzyme assay (Table 1.4). The presence of COX deficiency appears to be related to the mutant load and to which tRNA gene is involved, since some amino acids are relatively over-represented in COX subunits (Silvestri et al. 1998). Reduced COX activity has also been reported in a patient with cardiomyopathy and a point mutation in the 12S rRNA gene (Santorelli et al. 1999).

1.11.1.3 Mutations in COX genes

Several mutations have been reported in the mitochondrial COX subunit genes, in association with a number of different phenotypes (Table 1.5). A 15 bp in-frame microdeletion in COX III was associated with severe isolated COX deficiency in a girl with recurrent myoglobinuria and muscle cramps (Keightley et al. 1996). Three other mutations in COX III have been described, all point mutations, and associated with different phenotypes. These are: T9957C in a patient with MELAS (Manfredi et al. 1995a); a stop mutation G9952A leading to the production of a truncated protein in a 36 year old woman with recurrent episodes of encephalopathy (Hanna et al. 1998b); and a single base pair insertion 9537insC in an 11 year old girl who had progressive spastic paraparesis, ophthalmoplegia, mental retardation, severe lactic acidosis and putaminal lesions on MRI of the brain (Tiranti et al. 2000).

Three mutations in COX I have been reported in association with neuromuscular disease. A man with a motor neuron disease-like syndrome, with progressive spastic quadriparesis and speech and swallowing difficulties, had a heteroplasmic 5

Gene	Mutation	Amino acid change	Inheritance	Age at Onset	% Residual Muscle COX Activity**	Phenotype	Reference
COI	G5920A	W6X (truncated protein Δ6-513)	sporadic	childhood	32%	exercise intolerance, myoglobinuria	Karadimas et al. 2000
	6015del5 5 bp microdeletion	truncated protein Δ43-513 (frameshift)	sporadic	29 years	38%	atypical motor neuron disease	Comi et al. 1998
	G6264	G121X	somatic*	adult	NS	colorectal tumour	Polyak et al. 1998
	T6721	M273T	somatic	adult		acquired idiopathic sideroblastic anaemia (AISA)	Gattermann et al. 1997
	T6742C	I280T	somatic	adult		AISA	Gattermann et al. 1997
	G6930A	G343X (truncated protein Δ343-513)	sporadic	3 years	8%	cataract, SNHL, myoclonic epilepsy, ataxia, visual loss, lactic acidosis	Bruno et al. 1999
	A7339G	K479X	somatic	adult		AISA	Gattermann, 2000
	G7444A	X514K,Q,K,X	maternal*	adult	NS	Leber's hereditary optic neuropathy (LHON)	Brown et al. 1992b
COII	T7587C	M1T	maternal	5 years index case ~50 years mother	38%	gait ataxia, cognitive impairment, optic atrophy, pigmentary retinopathy	Clark et al. 1999
	G7896A	W104X (truncated protein Δ104-227)	sporadic	3 months	13%	hypotonia, psychomotor retardation, failure to thrive, hypertrophic cardiomyopathy, pigmentary retinopathy	Campos et al. 2001
	G8009A	V142M	somatic*	adult	NS	colorectal tumour	Polyak et al. 1998
	8042delAT	truncated protein Δ153-227 (frameshift)	?sporadic	in utero	55%	severe lactic acidosis with apnoea and bradycardia	Wong et al. 2001
COIII	T9438C	G78S	maternal*	adult	NS	LHON	Johns and Neufeld, 1993
	9537insC	truncated protein Δ111-260 (frameshift)	sporadic	4 years	11%	'Leigh-like' syndrome (progressive spastic paraparesis, ophthalmoplegia, moderate mental retardation, severe lactic acidosis, putaminal lesions)	Tiranti et al. 2000
	G9738T	A178T	maternal*	adult	NS	LHON	Johns et al. 1994
	G9804A	A200T	maternal*	adult	NS	LHON	Johns and Neufeld, 1993
	9480del15 15 bp microdeletion	5 amino acid deletion (ΔF94-F98)	sporadic	15 years	14%	cramps and recurrent myoglobinuria	Keightley et al. 1996
	G9949A	V248I	somatic*	adult	NS	colorectal tumour	Polyak et al. 1998
	G9952A	W249X (truncated protein Δ249-261)	sporadic	17 years	20%	encephalopathy, lactic acidosis, exercise intolerance and proximal myopathy	Hanna et al. 1998b
	T9957C	F251L	maternal	18 months	79%	MELAS	Manfredi et al. 1995a

***homoplasmic mutation**
****% mean control activity in skeletal muscle**
NS not stated

Table 1.5 Mutations reported in COX subunit genes

bp out of frame deletion in COX I (Comi et al. 1998). A 21 year old Italian woman with childhood onset of cataracts and progressive sensorineural hearing loss (SNHL), myoclonic epilepsy, cerebellar ataxia, muscle weakness and progressive visual loss with optic atrophy had a nonsense mutation G6930A (Bruno et al. 1999). The third patient had a missense mutation G5920A associated with recurrent myoglobinuria of childhood onset (Karadimas et al. 2000).

Mutations reported in COX II are also associated with a wide range of phenotypes. Maternal inheritance of a T7857C transition in the initiation codon was reported in a man with progressive gait ataxia, severe cognitive impairment and visual disturbance with optic atrophy and pigmentary retinopathy (Clark et al. 1999). His mother also had gait ataxia and fatigue. Recently a 3 year old girl with psychomotor delay, hypotonia and failure to thrive noted from 3 months of age was reported to have a nonsense mutation G7896A in COX II (Campos et al. 2001). She also had mild hypertrophic cardiomyopathy and pigmentary retinopathy.

Phenotypes associated with mutations in mitochondrially encoded COX subunit genes are thus extremely heterogeneous. Genotype-phenotype correlation is not possible, since all are private mutations and there is extreme clinical heterogeneity even for different mutations in the same COX subunit. Furthermore, mutations in mtDNA-encoded COX subunits have also been reported in patients with sideroblastic anaemia (Gattermann et al. 1997; Gattermann, 2000), in colorectal carcinomas (Polyak et al. 1998) and in Leber's hereditary optic neuropathy (LHON) pedigrees (Brown et al. 1992b; Johns and Neufeld, 1993). The mutations in sideroblastic anaemia and colorectal tumours are thought to be somatic. The significance of the mutations associated with LHON is not clear, but they are unlikely to be primary disease-causing mutations as they have also been found in healthy controls (Howell, 1997). However they may act synergistically with the three primary LHON mutations G11778A, G3460A and T14484C.

1.11.1.4 Other mtDNA mutations

In the few point mutations described which involve other mitochondrially-encoded polypeptides (subunits of complexes I, III and V), COX activity is usually normal. Scattered COX-negative fibres were reported in a patient with MELAS syndrome caused by a point mutation G13513A in the ND5 gene encoding a subunit of

complex I (Santorelli et al. 1997b). However overall COX enzyme activity was normal in this patient, and 4 other MELAS patients subsequently described to have the same G13513A point mutation did not appear to have COX-negative muscle fibres (Pulkes et al. 1999). COX-negative fibres have also been reported in three patients with mutations in the ATPase 6 gene of complex V. Two had the T8993G mutation associated with maternally inherited Leigh syndrome (Santorelli et al. 1997d; Uziel et al. 1997), and one of these had reduced COX enzyme activity in skeletal muscle (67% of lowest control, Uziel et al. 1997). The third had transient lactic acidosis associated with a microdeletion 9204-5delAT (Seneca et al. 1996). All muscle fibres showed a reduced histochemical reaction for COX and fibroblast COX activity was 43% of the mean control value in Seneca's patient. The 9204-5 microdeletion removes part of the translation stop codon of the ATPase 6 gene and disrupts the cleavage site of *COIII*. Seneca et al. therefore hypothesised that the reduced COX activity in their patient was secondary to abnormal processing of *COIII* mRNA, and not related to the position of the mutation in the ATPase 6 gene.

1.11.2 Pathogenic mechanisms of mtDNA mutations

There is still much unknown about the pathogenesis of mtDNA mutations, but cell culture systems employing transmitochondrial cybrids have been particularly helpful in beginning to unravel the molecular mechanisms involved. In these model systems cells lacking mtDNA (ρ^0 cells) are fused with enucleated cells containing the mutation of interest to produce transmitochondrial cybrids in which the mtDNA mutation may be studied in the context of a different nuclear background (King and Attardi, 1989). However, despite many detailed studies it is still difficult to explain the lack of correlation between genotype and phenotype for many mtDNA mutations. Although some variation in phenotype may be explained by differing tissue distributions of heteroplasmic mutations other factors are likely to be involved. These include other modifying mutations or polymorphisms, either mitochondrial (Lertrit et al. 1994) or nuclear (Holt et al. 1997), immunological mechanisms (Harding et al. 1992b) and environmental factors (Prezant et al. 1993).

1.11.2.1 Deletions and duplications

Large-scale rearrangements of the mitochondrial genome are always heteroplasmic and may involve single or multiple deletions or partial duplications. There is no

obvious relationship between the clinical phenotype and size, location or percentage of the mtDNA deletion(s) in muscle. However the proportion of deleted mtDNA molecules varies widely between tissues, and tissue distribution and segregation is likely to be important in determining the phenotype. Evolution of a phenotype, for example in patients with Pearson syndrome who later develop Kearns-Sayre syndrome, may be explained by selection against deleted mtDNA molecules in rapidly dividing tissues such as bone marrow, paralleled by accumulation of deleted molecules in tissues with slow cell turnover, such as muscle and the central nervous system (Larsson et al. 1990).

Although many different mtDNA deletion molecules have now been described, ranging in size from 1 to 10 kb, 30-40 % of all deletions are identical, spanning 4977 bp from ATPase 8 to ND5, and flanked by a perfect 13 bp direct repeat (Holt et al. 1989b). This has led to speculation that the deletion may be produced by homologous recombination or slippage during replication (Schon et al. 1989; Mita et al. 1989). The mechanism of production is not clear in other cases where the deletions are not flanked by repeats, but may involve breakage and ligation of replication intermediates. Single deletions are usually sporadic, although they may occasionally be maternally inherited (Bernes et al. 1993). They are present at low levels in human oocytes (Chen et al. 1995) but transmission is thought to be prevented in most cases by the bottleneck effect (Brown, 1997). Multiple deletions, however, are inherited as Mendelian traits, either recessive or dominant (see section 1.11.4).

The mechanism of pathogenesis of deletions is thought to be mediated by impaired mitochondrial translation and this has been supported by cybrid studies. Impaired mitochondrial translation has been observed in transmitochondrial cybrids containing 60% or more deleted mtDNA (Hayashi et al. 1991). Single fibre analysis of muscle from patients with mtDNA deletions, demonstrating a higher percentage of deleted mtDNA in COX-negative fibres compared to COX-positive fibres, also provides evidence for impaired mitochondrial translation secondary to the deletion (Mita et al. 1989). Impairment of mitochondrial translation most probably is a consequence of insufficient tRNAs due to deletion of tRNA genes.

It has been proposed that duplications may be an intermediate in forming all

deletions (Brockington et al. 1993; Poulton et al. 1993). Maternal inheritance of duplications has been reported (Ballinger et al. 1992; Brockington et al. 1993; Rotig et al. 1992). The pathogenic mechanism of duplications is uncertain but those involving the D-loop may interfere with the binding and function of trans-acting nuclear factors. However there is much controversy regarding whether duplications are intrinsically pathogenic (Manfredi et al. 1997).

1.11.2.2 tRNA point mutations

Point mutations in mitochondrial tRNA genes are usually heteroplasmic and often display marked phenotypic heterogeneity. Pathogenic mechanisms are most well characterised for the point mutations A8344G and A3243G associated with the MERRF and MELAS syndromes respectively. A8344G is found in 80 to 90% of cases of MERRF (Shoffner et al. 1990) and two other mutations in the tRNA lysine gene, T8356C and G8363A, have also been reported in families with this phenotype (Silvestri et al. 1992; Ozawa et al. 1997). Studies of cultured myotubes bearing the A8344G mutation revealed a threshold of 85% mutant load for pathogenicity (Boulet et al. 1992). Cybrids containing the A8344G and T8356C point mutations had decreased mitochondrial protein synthesis, with concomitant reduction in the activities of complexes I and IV (Chomyn et al. 1991; Masucci et al. 1995). Further cybrid studies demonstrated defective aminoacylation of the mutant tRNA lysine in cell lines containing the A8344G mutation, leading to premature termination of translation at lysine codons (Enriquez et al. 1995).

Pathogenic mechanisms are less clear for MELAS mutations. Reductions in both protein synthesis and complex I activity were observed in cybrids containing >95% A3243G and T3271C mutations (King et al. 1992; Koga et al. 1995). In addition, a partially processed polycistronic RNA, RNA 19, has been observed in A3243G mutant cell lines, but its pathogenic significance is unclear as it is also found at low levels in normal tissues (King et al. 1992; Kaufmann et al. 1996). The large number of tRNA leucine^{UUR} mutations documented may be due to the critical role of this gene in controlling mtDNA transcription rates. Transcription of mitochondrial ribosomal RNA genes is modulated by a termination factor mTERF that binds to a 13 bp sequence within the tRNA leucine^{UUR} gene to promote transcription termination. In vitro studies have demonstrated impaired mTERF binding and decreased efficiency of transcription termination in cell lines containing the A3243G

mutation (Hess et al. 1991), but there is no evidence for this occurring in vivo (Hammans et al. 1992; Moraes et al. 1992a).

1.11.2.3 Mutations in COX genes

Several COX mutations were not present in cultured myoblasts or skin fibroblasts and so detailed studies of their pathogenic effects was not possible (Keightley et al. 1996; Hanna et al. 1998b; Karadimas et al. 2000). However analysis of in vitro protein synthesis in fibroblasts from a patient with a T7587C point mutation in the initiation codon of the *COII* gene suggested that translation of mutant COX II mRNA did not occur (Clark et al. 1999). Single muscle fibre PCR analysis in this patient demonstrated that a threshold of 55-65% mutant mtDNA was necessary to produce a COX-deficient phenotype, and similar results were obtained for a nonsense mutation in *COIII* (Hanna et al. 1998b). COX mutations thus appear to have a much lower threshold for phenotypic expression than tRNA mutations (see section 1.11.2.2). The reason for this is not known, but it has been suggested that there is tight post-transcriptional or translational regulation of respiratory chain polypeptides compared to excess production of mitochondrial tRNAs (Bruno et al. 1999). Another possibility is that, since functional COX exists as a dimer, mutant COX polypeptides may exert a dominant negative effect if only COX enzyme complexes containing two wild type COX subunits are functional (Bruno et al. 1999).

Two studies have analysed the effects of COX mutations in transmitochondrial cybrid systems. Transmitochondrial cybrids containing a nonsense mutation G6930A in *COIII* had a biochemical defect directly proportional to the mutant load (Bruno et al. 1999). In the second study transmitochondrial cell lines harbouring a 15 bp deletion in the *COIII* gene (9480del15) were generated by fusing human ρ^0 cells with platelet and lymphocyte fractions isolated from a patient who had this mutation (Hoffbuhr et al. 2000). Using pulse chase experiments, this study demonstrated normal translation but reduced stability of the COX III polypeptide. Furthermore, there was no demonstrable incorporation of COX I, II and III into a multisubunit complex in cells homoplasmic for the 9480del15 mutation, suggesting that stable COX III polypeptide is necessary for assembly of COX holoenzyme (Hoffbuhr et al. 2000).

Gene	Chromosomal location	Clinical Phenotype	Reference
SURF1	9q34	Leigh syndrome	Zhu et al. 1998; Tiranti et al. 1998b
SCO2	22q13	fatal infantile cardioencephalo-myopathy	Papadopoulou et al. 1999
SCO1	17p11	hepatic failure and encephalopathy	Valnot et al. 2000a
COX10	17p13.1-q11.1	tubulopathy and leukodystrophy	Valnot et al. 2000b
?	2p16	Saguenay Lac St Jean variant of Leigh syndrome	Lee et al. 2001

Table 1.6 Nuclear gene defects associated with COX deficiency

1.11.3 Mutations of Nuclear DNA

As most subunits of COX are nuclear-encoded and many nuclear-encoded factors are essential for assembly of the holoenzyme complex, it is thought that most cases of COX deficiency are caused by nuclear gene mutations. However, in contrast to the exponentially expanding number of reported mtDNA mutations, very few nuclear gene defects have been described in OXPHOS proteins. Although mutations have been reported in nuclear-encoded subunits of complexes I and II (Bourgeron et al. 1995; Loeffen et al. 1998; van den Heuvel et al. 1998; Triepels et al. 1999; Schuelke et al. 1999; Parfait et al. 2000; Baysal et al. 2000; Budde et al. 2000; Petruzzella et al. 2001; Benit et al. 2001; Loeffen et al. 2001), mutations have yet to be described in the nuclear COX subunit genes, despite extensive sequence analysis (Adams et al. 1997; Lee et al. 1998; Jaksch et al. 1998).

The nuclear-encoded factors required for correct assembly of COX are candidate genes for autosomally inherited COX deficiency syndromes. So far only a few of the human genes encoding these factors have been characterised (Table 1.3) and only 4 of these have been demonstrated to be mutated in human COX deficiency (Table 1.6).

1.11.3.1 SURF1

The first nuclear gene mutations reported to cause COX deficiency were in the *SURF1* gene (Zhu et al. 1998; Tiranti et al. 1998b). The *SURF1* gene is part of the surfeit locus, an extremely tight cluster of housekeeping genes that maps to chromosome 9q34 in humans. There are 6 genes in the surfeit locus, but these do not share any sequence or amino acid homology (Duhig et al. 1998). The structure of the gene cluster is thought to be functionally significant, since it has been conserved during 250 million years of divergent evolution (Colombo et al. 1992). *SURF3* is the only gene in the surfeit locus whose function is known. It encodes the L7a ribosomal protein. The SURF4 product appears to be an integral membrane protein associated with the endoplasmic reticulum whilst *SURF5* encodes 2 cytoplasmic proteins (as a result of differential splicing). The SURF6 protein has been localised to the nucleolus.

SURF1 encodes a 300 amino acid protein, human surfeit locus protein 1, whose precise function is unknown. It is a homologue of yeast Shy1p which has been localised to the inner mitochondrial membrane (Mashkevich et al. 1997). SURF1 has an N-terminal mitochondrial targeting signal and has also been shown to be an integral inner mitochondrial membrane protein (Yao and Shoubridge, 1999; Tiranti et al. 1999b). It is thought to have an essential role in the assembly or maintenance of the functional COX holoenzyme.

Mutations in the *SURF1* gene have now been reported in a significant proportion of patients with COX-deficient Leigh syndrome (Zhu et al. 1998; Tiranti et al. 1998b; Tiranti et al. 1999c). This proportion varies between centres, from 26% (Sue et al. 2000) to 75% (Tiranti et al. 1999c). The clinical phenotype associated with *SURF1* mutations appears to be remarkably homogeneous, which is particularly surprising when one considers the enormous clinical variability associated with respiratory chain defects. It has been suggested that *SURF1* mutations are exclusively associated with COX-deficient Leigh syndrome of infantile onset (Tiranti et al. 1999c), but the reason for this is not clear. It is possible that it may reflect ascertainment bias since mutation analysis of a large number of patients with other COX-deficient phenotypes has not yet been described.

Most *SURF1* mutations appear to be small-scale rearrangements, leading to a frameshift and predicting a truncated protein product (Table 1.7). In addition several nonsense and splice-junction mutations, which also result in a truncated protein product, have been reported. Relatively few missense mutations have been identified, but these are also associated with typical Leigh syndrome (Teraoka et al. 1999; Poyau et al. 2000). Several mutations have occurred more than once in different ethnic groups and are thus likely to represent mutational 'hotspots'. In particular the 312del10/insAT mutation in exon 4 appears to be the most common *SURF1* mutation in Caucasian patients, and has been estimated to account for 35-50% of all disease-causing *SURF1* alleles (Robinson, 2000).

Northern blot analysis demonstrated ubiquitous expression of *SURF1* (Yao and Shoubridge, 1999) although expression in brain appeared relatively low compared to heart, skeletal muscle and kidney. This is surprising since all patients with *SURF1* mutations reported to date had Leigh syndrome, and had almost exclusively

Site of mutation	Mutation	Predicted Protein Sequence	Clinical Phenotype	Reference*
Exon 1	37_38ins17	Frameshift	LS	Tiranti et al. 1998b
Exon 1-2	delCCCCGCA	Frameshift (?ss)	LS	Hanson et al. 2001
Exon 2	74G>A	W25X	LS	Tiranti et al. 1999c
Exon 3-4	240+1G>T	Frameshift	LS	Tiranti et al. 1999c
Exon 4	244C>T	Q82X	LS+periph neuropathy	Santoro et al. 2000
Exon 4	312_321del10, 311_312insAT	Frameshift (ss)	LS	Tiranti et al. 1998b
Exon 4	323+2T>C	Frameshift (ss)	LS	Zhu et al. 1998
Exon 5	371G>A	G124E	LS	Poyau et al. 2000
Exon 5-6	515+2T>G	Frameshift (ss)	LS	Tiranti et al. 1998b
Exon 5-6	516-516_1delAG	Frameshift (ss)	LS	Pequignot et al. 2001a
Exon 6	550_551delAG	Frameshift	LS	Tiranti et al. 1998b
Exon 6	552delG	Frameshift	LS	Tiranti et al. 1999c
Exon 6	539G>A	G180E	**	von Kleist-Retzow et al. 2001
Exon 6	1 base deletion	V187X	LS	von Kleist-Retzow et al. 1999
Exon 6	574_575insCTGC	Frameshift	LS	Tiranti et al. 1999c
Exon 6	587_588insCAGG	Frameshift	LS	Sue et al. 2000
Exon 6-7	588+1delG	Frameshift (ss)	LS	Pequignot et al. 2001a
Exon 6-7	589-1G>C	Deletion I197 + E198 (ss)	**	von Kleist-Retzow et al. 2001
Exon 7	688C>T	R230X	LS	Coenen et al. 1999
Exon 7	737T>C	I246T	LS	Poyau et al. 2000
Exon 7	751C>T	Q251X	LS	Tiranti et al. 1998b
Exon 7-8	751+6T>G	Frameshift (ss)	LS	Pequignot et al. 2001b
Exon 7-8	752-3C>G	Frameshift (ss)	LS	Poyau et al. 2000
Exon 8	758_759delCA	Frameshift	LS	Tiranti et al. 1999c
Exon 8	771_774delACCC	Frameshift	LS	Hanson et al. 2001
Exon 8	772_773delCC	Frameshift	LS	Tiranti et al. 1998b
Exon 8	790_1delAG	Frameshift	LS	Teraoka et al. 1999
Exon 8	808G>T	E270X	LS	Tiranti et al. 1999c
Exon 8	814_815delCT	Frameshift	LS	Sue et al. 2000
Exon 8	820T>G	Y274D	LS	Teraoka et al. 1999
Exon 8-9	821_835+3del18	Frameshift (ss)	LS	Williams et al. 2001
Exon 9	841_842delCT	Frameshift	LS	Zhu et al. 1998
Exon 9	845_846delCT	Frameshift	LS	Tiranti et al. 1998b
Exon 9	868_869insT	Frameshift	LS	Tiranti et al. 1998b

Mutation numbering is from the first ATG (start) codon.
LS = Leigh syndrome
ss = splice-site mutation
***Refers to first report of mutation.**
****This patient had hypertrichosis, partial villous atrophy and neurological dysfunction.**

Table 1.7
SURF1 mutations

neurological symptoms. Imunohistochemical studies (Sue et al. 2000) and immunoblot analysis (Yao and Shoubridge, 1999; Poyau et al. 2000) of patients with *SURF1* mutations demonstrated reduced immunoreactivity to both nDNA and mtDNA-encoded subunits of COX. Two-dimensional blue-native gel electrophoresis of fibroblasts from 4 SURF1 deficient patients revealed reduced levels of both holo-COX (S4) and the final assembly intermediate S3, proportional to the decrease in COX activity, but relatively increased levels of the early assembly intermediates S1 and S2 (Coenen et al. 1999). Taken together, these results indicate that SURF1 is not necessary for the biosynthesis of COX subunits but is required at a later stage in COX assembly or maintenance.

1.11.3.2 SCO2

The gene encoding SCO2, a mitochondrially-targeted protein thought to be required for insertion of copper into COX I and COX II (see section 1.8.2), has been found to be mutated in patients with a fatal infantile form of COX deficiency characterised by hypertrophic cardiomyopathy and encephalopathy (Papadopoulou et al. 1999). COX deficiency in these patients was expressed most severely in cardiac and skeletal muscle (0 to 18% residual COX activity), with relatively high residual COX activity in cultured skin fibroblasts (12 to 50%) compared to patients with *SURF1* mutations (Sue et al. 2000; Jaksch et al. 2000). Immunohistochemical studies suggested that the enzyme deficiency is due to loss of mtDNA-encoded COX subunits (Papadopoulou et al. 1999).

All patients reported to have *SCO2* mutations share a common missense mutation E140K near the putative CxxxC copper binding domain of SCO2. This might be an ancient founder allele or a mutational hotspot. Most patients are compound heterozygotes (Papadopoulou et al. 1999; Sue et al. 2000; Jaksch et al. 2000) but recently three patients were reported who were homozygous for this mutation (Jaksch et al. 2001). These patients had delayed onset of hypertrophic obstructive cardiomyopathy. The phenotype thus appears to be milder in E140K homozygotes and it has been postulated that two severe *SCO2* alleles might lead to embryonic lethality (Shoubridge, 2001).

1.11.3.3 SCO1

Mutations in the *SCO1* gene, whose product is also thought to be involved in copper delivery to COX, have been demonstrated in a French family with isolated COX deficiency associated with neonatal-onset hepatic failure and encephalopathy (Valnot et al. 2000a). Residual COX activity was 0.5% in skeletal muscle and 20% in liver of the index case. Affected infants were compound heterozygotes, with a 2 bp frameshift deletion in exon 2 resulting in a premature stop codon on the paternal allele, and a missense mutation P174L in exon 3 on the maternal allele. This missense mutation alters a highly conserved proline residue adjacent to the copper-binding domain of SCO1 and is thus analogous to the common E140K missense mutation in *SCO2*.

The patients with *SCO1* mutations did not have the cardiac symptoms observed in patients with *SCO2* mutations. The observation of different clinical phenotypes associated with *SCO1* and *SCO2* mutations, with predominantly liver involvement in the former and cardiac involvement in the latter but neurological symptoms in both, has led to the suggestion that there might be tissue-specific pathways for mitochondrial copper delivery in humans (Shoubridge, 2001).

A Canadian study that sequenced *SCO1* and *COX17* in 30 COX-deficient patients with heterogeneous clinical presentations suggested that mutations in *SCO1* are not a common cause of isolated COX deficiency (Horvath et al. 2000). Furthermore no mutations were identified in *COX17*, whose product is also thought to be involved in the delivery of copper to the COX holoenzyme.

1.11.3.4 COX10

A genetic linkage study of an African consanguineous family with an isolated COX defect allowed identification of a homozygous missense mutation in the *COX10* gene (Valnot et al. 2000b). The *COX10* gene encodes haem A: farnesyltransferase, which catalyses the first step in the conversion of protohaem to the haem A prosthetic group of COX subunit I. The clinical phenotype in this family was lactic acidosis and proximal renal tubulopathy associated with neurological features including hypotonia, myopathy, ataxia and seizures. Residual COX activities were 2%, 17% and 51% in skeletal muscle, kidney and liver respectively (Ogier et al.

1988). Immunoblot analysis of COX subunits in fibroblasts revealed severe reduction of steady state levels of subunit II, moderate reduction of subunits III and VIc and only slight reduction of all other COX subunits studied including I and IV (Valnot et al. 2000b).

1.11.4 Defects of intergenomic communication

Mutations in the nuclear genes responsible for regulating mtDNA transcription, replication and repair may also produce human disease. Such mutations were postulated to be responsible for the autosomally inherited multiple mtDNA deletion syndromes, in which there is a qualitative defect of mtDNA (Zeviani et al. 1989), and the mtDNA depletion syndrome, where there is a quantitative defect of mtDNA (Bodnar et al. 1993). Linkage studies were used to identify loci for these disorders of intergenomic communication (Suomalainen et al. 1995; Kaukonen et al. 1996; Kaukonen et al. 1999) and more recently several responsible genes have been identified.

Mutations of four genes, encoding thymidine phosphorylase TP, the heart and skeletal muscle-specific adenine nucleotide translocator ANT1, DNA polymerase gamma POLG and the novel putative helicase Twinkle, have been reported in pedigrees with autosomally inherited multiple mtDNA deletions. TP mutations occur in the recessive MNGIE syndrome (Nishino et al. 1999), whilst mutations in ANT1, POLG and Twinkle are all associated with autosomal dominant PEO (Kaukonen et al. 2000; Van Goethem et al. 2001; Spelbrink et al. 2001). POLG is the only known mitochondrial DNA polymerase for mtDNA replication and so it is easy to understand how mutations in the POLG gene may lead to the accumulation of deleted mtDNA molecules as a result of inefficient mtDNA replication (Van Goethem et al. 2001). Both TP and ANT1 however are thought to cause mtDNA deletions by a more indirect mechanism. TP and ANT1 mutations are believed to deplete intramitochondrial dNTP pools (dTTP and dATP respectively), leading to aberrant mtDNA synthesis (possibly by increasing the error rate of POLG) and the accumulation of deleted mtDNA molecules (Suomalainen and Kaukonen, 2001). TP catalyses the phosphorolysis of thymidine to thymine, which is then recycled for dTTP synthesis in the thymidine salvage pathway (Brown and Bicknell, 1998). TP deficiency is associated with a 20-fold increase in plasma thymidine levels, and this thought to lead to secondary depletion of intramitochondrial dTTP pools (Nishino et

al. 2001). ANT1 shuttles ATP and ADP across the inner mitochondrial membrane of cardiac and skeletal muscle fibres. The precise pathogenic mechanism of ANT1 mutations is not known, but it is possible that ANT1 regulates intramitochondrial dATP concentrations (Kaukonen et al. 2000). The mechanism by which defects of the Twinkle helicase cause multiple mtDNA deletions is also not known (Spelbrink et al. 2001) but it is possible that they also alter the intramitochondrial dNTP pool, perhaps as a result of increased nucleotide hydrolysis (Moraes, 2001a).

Family studies have suggested autosomal recessive inheritance of the mtDNA depletion syndrome in some cases (Bakker et al. 1996a; Morris et al. 1998). There is no evidence of maternal inheritance and cell fusion studies demonstrated complementation of the defect by a donor nucleus (Bodnar et al. 1993). It was proposed that mtDNA depletion may be due to the absence of mtTFA (Larsson et al. 1994; Poulton et al. 1994), but subsequent studies suggested that reduction in mtTFA protein levels are likely to be a secondary effect (Taanman et al. 1997). Mouse embryos in which both copies of the gene encoding mtTFA have been disrupted exhibit severe mtDNA depletion, but die in utero (Larsson et al. 1998). Haplotype analysis of candidate genes excluded *SSBP1*, *ENDOG*, *POLG*, *TFAM* and *NRF1* as causative genes in one consanguineous family with hepatic mtDNA depletion (Spelbrink et al. 1998). Recently homozygosity mapping studies have led to the identification of mutations in the deoxyguanosine kinase *DGUOK* gene and the mitochondrial thymidine kinase gene *TK2* in patients with the hepatocerebral and myopathic forms of mtDNA depletion syndrome respectively (Mandel et al. 2001; Saada et al. 2001). Both genes are involved in the maintenance of intramitochondrial nucleotide pools by deoxynucleoside salvage. Human deoxyguanosine kinase phosphorylates deoxyguanosine, deoxyadenosine and deoxyinosine, whilst TK2 phosphorylates deoxythymidine and deoxycytidine (Mandel et al. 2001). The reason for tissue specificity of deficiencies of these enzymes is not known, but it is possible that tissue-specific pathways of deoxynucleoside salvage exist.

Thus disturbance of nucleoside pools, the building blocks of mtDNA, appears to be a common feature of disorders of intergenomic communication (Suomalainen and Kaukonen, 2001). This was not predicted and leads to the hypothesis that many disorders of the mitochondrial respiratory chain, including COX deficiency, may in

future be found to be caused by a variety of housekeeping genes whose role in maintaining mitochondrial integrity is not currently suspected.

1.12 Aims of this thesis

Deficiency of cytochrome oxidase is relatively common amongst patients with respiratory chain disorders seen at Great Ormond Street Hospital. Prior to this study the underlying molecular defect had been identified in very few patients.

The aims of this thesis were to determine whether immunohistochemistry using monoclonal antibodies directed against individual COX subunits could be used as a screening method to differentiate patients with mtDNA defects from those with nuclear defects, and to define the responsible mutations in the former group. To achieve these aims, COX subunit expression patterns were analysed in biopsied skeletal muscle from a large number of patients with COX deficiency. Sequence analysis of mtDNA and of a candidate nuclear gene *SURF1* was then performed in selected patients, to determine whether the immunohistochemical screening had increased the likelihood of finding the underlying molecular defect.

Chapter 2

Patients and Methods

Chapter 2 Patients and Methods

2.1 Patients

Patients were selected both retrospectively and prospectively for this study. All muscle biopsies were obtained for diagnostic purposes from children with suspected respiratory chain disorders, after informed parental consent. For retrospective patients (patients presenting to Great Ormond Street Hospital for Children between 1985 and 1995), consultants in paediatric metabolic medicine and neurology were asked to recall cases of COX deficiency which they had managed. Retrospective cases were also identified from the records of the professor of histochemistry at Great Ormond Street Hospital. This led to the identification of a total of 40 retrospective cases. Of these, 22 retrospective samples with decreased or absent COX activity, as determined by histochemistry, were included in the present study. Patients were excluded from the study if frozen muscle was not available for analysis: three patients were excluded because of sample decay during storage; and insufficient tissue remained for study in a further 6 cases. Nine additional cases were excluded because routine COX staining performed as part of the original investigations was normal.

Patients selected prospectively were all those having a muscle biopsy at Great Ormond Street Hospital from 1996 onwards in whom COX-deficient fibres were identified on routine histochemistry. These numbered 14.

Three control patient groups were also included in the study. Biopsies were studied from 5 patients with known mtDNA mutations, 3 'disease' controls and 3 normal controls. The mtDNA mutations were: 3 point mutations in tRNA genes [7472insC (Hanna et al. 1998a), T14709C (Hanna et al. 1995a) and A8344G (Sweeney et al. 1994)]; a point mutation G9952A in COX subunit III (Hanna et al. 1998b); and a 4.9 kb deletion (McShane et al. 1991). The 3 'disease' controls were patients with congenital lactic acidosis who had normal spectrophotometric assay of COX activity. These patients had isolated deficiencies of pyruvate carboxylase (PC), the pyruvate dehydrogenase complex (PDHC) and complex I of the respiratory chain. The 3 'normal' individuals had orthopaedic surgery for conditions not associated with

neuromuscular disease, and routine histology of the muscle biopsies from these patients was entirely normal.

In summary, a total of 36 cases (22 historical and 14 prospective) and 11 controls (5 with mtDNA mutations, 3 with other energy defects and 3 normal biopsies) were included in this study. The underlying molecular defect was not known in any of the 36 cases prior to this study.

2.2 Storing and sectioning muscle biopsies

Open or needle muscle biopsies were performed under general anaesthesia and snap frozen immediately in hexane maintained in an acetone/CO_2 bath at -80^{0}C. All muscle biopsies were from quadriceps, except for one patient who had a paraspinal muscle biopsy during spinal surgery. After sectioning for diagnostic purposes, they were stored at -50^{0}C until the time of this study. I cut and stained new muscle sections for all the studies described in Chapter 3.

2.3 Histochemical staining of muscle biopsies

A number of techniques allow visualisation of both normal and pathological mitochondria on frozen tissue sections. These include the modified Gomori trichrome and haematoxylin and eosin stains, and also histochemical methods to demonstrate oxidative enzyme activity, such as the succinate dehydrogenase (SDH) and cytochrome oxidase (COX) stains. The cytochemical method for the microscopic demonstration of SDH activity in frozen tissue sections uses a tetrazolium salt (nitro blue tetrazolium, NBT) as electron acceptor with phenazine methasulphate as intermediate electron donor to NBT. Addition of sodium malonate, an SDH inhibitor, to the incubation medium can be used to test the specificity of the method. SDH staining of normal muscle sections produces a checkerboard pattern, with light blue type II fibres and darker blue type I fibres. Type I muscle fibres are highly oxidative and contain more mitochondria than type II fibres, which rely on glycolysis. Ragged red fibres (RRF), in which there is mitochondrial proliferation, stain intensely blue with SDH. In the COX cytochemical stain, 3,3'-diaminobenzidine (DAB) is used as the electron donor for cytochrome *c*. Oxidation of DAB produces a brown reaction product in the distribution of mitochondria within the section. Addition of 10mM potassium cyanide, a COX inhibitor, can be used to test the specificity of

the method. As for SDH, COX staining of normal muscle produces a checkerboard pattern, with dark type I fibres and less intensely stained type II fibres.

In the present study, for histochemical analysis cryostat sections cut at 10 μm were stained to demonstrate the activities of SDH and COX (Stoward and Pearse, 1991; Filipe and Lake, 1990). COX activity was assayed using a medium containing 4 mM 3,3'-diaminobenzidine tetrahydrochloride and 100 μm cytochrome *c* in 0.1 mM phosphate buffer pH 7.0 at 25^0C. In addition 5 μm cryostat sections were stained with the modified Gomori trichrome, as described by Engel and Cunningham in 1963, to demonstrate the presence of RRF.

2.4 Immunohistochemical staining of muscle biopsies

Sections were incubated at optimal concentration, i.e. the lowest concentration of antibody that gave a clear particulate immunostain corresponding to the localisation of mitochondria in normal muscle fibres. Unfixed frozen sections were used. In normal muscle a checkerboard pattern was observed as for the histochemical stains SDH and COX, with type I fibres appearing brighter because of their higher mitochondrial density. The immunological probes used were highly specific monoclonal antibodies directed against mitochondrial and nuclear-encoded subunits of COX. For muscle biopsies the targets of these immunoprobes were visualised using an enzyme-linked method, horse radish peroxidase.

Serial 8 μm cryostat sections were cut from patient and control biopsies, mounted adjacently on polysine slides (BDH) and air-dried for one hour.

A battery of subunit-specific monoclonal antibodies was used to identify COX subunits immunohistochemically (Taanman et al. 1996). The primary mouse monoclonal antibodies (Molecular Probes) used were anti-COX I (clone 1D6-E1-A8), anti-COX II (clone 12C4-F12), anti-COX IV (clone 10G8-D12-C12), anti-COX Va (clone 6E9-B12-D5) and anti-COX VIc (clone 3G5-F7-G3). Subunits I and II are mitochondrially encoded; the other subunits are nuclear-encoded. The anti-COX Va monoclonal antibody was a hybridoma cell culture supernatant; the other antibodies were purified monoclonals. A mouse monoclonal antibody directed against fast myosin (Novocastra) was used to identify type I and type II muscle fibres.

Mouse monoclonal antibodies directed against COX subunits and fast myosin were optimally diluted in phosphate-buffered saline (PBS) pH 7.4, and 200 μl of each was applied to serial sections for each patient and control. Optimal dilutions were as follows: anti-COX I (1 in 250), anti-COX II (1 in 1000), anti-COX IV (1 in 500), anti-COX Va (1 in 500), anti-COX VIc (1 in 500) and anti-fast myosin (1 in 200). Sections were incubated with primary antibody overnight in a humidified chamber to prevent dehydration. One set of sections was incubated in PBS alone, without primary antibody, as a control. All sections were washed in PBS and then incubated for 45 minutes in biotinylated rabbit anti-mouse IgG secondary antibody diluted 1 in 250 (Dako). The immunoreaction was visualised after a 45 minute incubation in streptavidin-biotinylated horseradish peroxidase complex (ABC complex, Dako) by developing the peroxidase activity in a solution containing 0.05% 3,3'-diaminobenzidine hydrochloride (DAB) and 0.03% hydrogen peroxide in PBS for ten minutes. Sections were washed with PBS between each step of the procedure, and finally in tap water for 10 minutes after incubating in DAB. Carazzi's haematoxylin was used as a nuclear counterstain. Sections were dehydrated, cleared and mounted in DPX. All incubations were at room temperature.

2.5 Cell culture

2.5.1 Normal growth medium

Myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and 4 mM L-glutamine and supplemented with 20% fetal calf serum, 2% detoxified chick embryo extract, 1 mM sodium pyruvate, 2 mM uridine, 50 U/ml penicillin and 50 mg/ml streptomycin ('normal growth medium'). Cells were cultured at 37^0C in a humidified atmosphere of 8% CO_2 in air.

2.5.2 Primary myoblast culture

Primary myoblast cultures were established from skeletal muscle biopsies using a method in which dormant myoblasts known as satellite cells were stimulated to re-enter the cell cycle (Yasin et al. 1977). Fresh skeletal muscle from diagnostic muscle biopsies was placed in a sterile 100 ml beaker containing 10 ml dissociation solution [bovine serum albumin (essentially fatty acid free) 0.1% w/v, collagenase (Sigma type II) 0.15% w/v and trypsin (pH 7.3 Difco 1:250) 0.15% w/v, sterile filtered through a 0.2 μm Acrodisc] and made up to a final volume of 40 ml using sterile

DMEM. Muscle fibres were teased apart using sterile needles and then incubated at 37^{0}C (shaking) for 15 minutes. 5 ml normal growth medium (see section 2.5.1) was added to neutralise the dissociation enzymes, and the solution was gently pipetted in and out of a 10 ml pipette to further dissociate the muscle fibres. The muscle fragments were allowed to settle, before filtering the supernatant (containing the dissociated satellite cells) through two layers of nylon mesh (upper layer 50 μm, lower layer 20 μm) supported by a 40 mm plastic funnel. The mesh and funnel had previously been sterilised by washing with 70% alcohol, and then rinsed several times with sterile DMEM. The filtered supernatant was collected into a sterile universal container and centrifuged at 1000 rpm for 10 minutes to pellet the cells. The supernatant was then aspirated off and the cells were resuspended in a small volume of normal growth medium and placed in a 37^{0}C incubator until required. This cycle was repeated 3 times and the contents of the 4 universal containers (final volume <1.5 ml) were combined and plated on a 35 mm tissue culture plate. The plate was incubated overnight at 37^{0}C in 8% CO_2. The following day the medium was aspirated off and the plate washed twice with sterile DMEM to remove red blood cells. Fresh growth medium was added and the plate returned to the 37^{0}C 8% CO_2 incubator. The medium was then changed every 3 to 4 days.

Cells were cultured in a humidified 37^{0}C incubator containing 8% CO_2 in air. Cells were harvested by trypsinisation, using 0.1% trypsin solution in Versene, when confluent and passaged by 1:2 or 1:4 dilution.

Clonal myoblast cultures were obtained by plating a mass myoblast culture at low density followed by ring cloning.

2.5.3 Fibroblast culture

Primary fibroblast cultures were established from diagnostic skin biopsies by the explant method (Martin, 1973). Skin biopsies were either full thickness 3 or 4 mm punch (Stiefel) biopsies, or were small full thickness skin ellipses taken from the incision site at the time of open muscle biopsy. To obtain fibroblasts, the skin biopsies were divided extremely finely under sterile conditions and plated on to 60 mm culture plates with a drop of growth medium (see section 2.5.1) over each piece of skin. After 3 to 4 days fibroblasts could be observed, using inverted light microscopy, migrating from the edges of the biopsy. Cultured skin fibroblasts were

grown in the same medium as myoblasts but lacking chick embryo extract and with 10% rather than 20% fetal calf serum.

Cultured myoblasts and fibroblasts were stained for COX activity and immunostained for COX subunits I, IV and VIc.

2.6 COX staining of cultured cells on coverslips

Myoblasts and fibroblasts were seeded on glass coverslips. Cells growing at moderate density on coverslips were airdried to permeabilise and then incubated in a 10 ml solution containing 5 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB), 1 ml catalase (20 μg/ml), 10 mg cytochrome *c* and 9 ml 0.1 M phosphate buffer pH 7.4 for 2 hr at 37^{0}C. The cells were washed twice with phosphate-buffered saline (PBS), incubated in Carazzi's haematoxylin for 2 minutes to counterstain the nuclei, washed in tap water to blue, dehydrated in absolute alcohol, cleared in xynol and mounted in DPX on a microscope slide.

2.7 Immunostaining of cultured cells on coverslips

For studies on cultured cells, fluorochromes were used to visualise antigen-antibody binding sites directly. Myoblasts and fibroblasts seeded on glass coverslips were cultured for 45 minutes in medium containing 2 μM MitoTracker^{TM} CMTMRos (Molecular Probes), followed by a period of 30 minutes culture in normal growth medium. MitoTracker^{TM} is a mitochondria-specific dye. Cells on coverslips were then washed in PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilised in methanol at –20^{0}C for 20 minutes, and washed again in PBS. Protein binding sites were saturated by incubating the cells with 10% normal goat serum in PBS at 37^{0}C in a humidified chamber for 30 minutes, to block non-specific binding. The cells were then incubated with primary monoclonal antibodies directed against COX subunits (Molecular Probes) and 10 μg bis-benzimide per ml PBS at 37^{0}C (humidified) for 45 minutes. The primary monoclonal antibodies used were anti-COX I (1D6-E1-A8), anti-COX IV (10G8-D12-C12) and anti-COX VIc (3G5-F7-G3). The cells were then washed in PBS and incubated with fluorescein-conjugated secondary antibody (goat anti-mouse IgG-FITC) in PBS at 37^{0}C (humidified) for 45 minutes, before a further wash in PBS and mounting onto glass slides in Citifluor-glycerol-PBS solution. Fluorescence was observed using a Zeiss Axiophot

photomicroscope with a 40x Plan-Neofluar lens, and cells were photographed using Kodak Ektachrome™ P1600 film.

2.8 Isolation of DNA

DNA was extracted from frozen muscle biopsy samples and cultured cells using proteinase K digestion, followed by phenol/chloroform extraction and ethanol precipitation. Extractions were performed as described by Sambrook et al. (1989), with modifications for small samples. All extractions were performed in Class I fume cabinets. Frozen (-70^{0}C) muscle samples were transferred on dry ice to the Class I cabinet. For each patient and control an extremely small (≤10 mg) piece of muscle was divided extremely finely in a tissue culture dish using two scalpels and then transferred to a 1.5 ml eppendorf tube containing 600 µl DNA extraction buffer (0.1 M NaEDTA, 10 mM Tris-HCl, pH 8.0), 40 µl 10% SDS and 100 µl proteinase K (20 mg/ml). The scalpel blades were changed between samples in order to avoid cross contamination of nucleic acids between samples. The samples were incubated at 56^{0}C with mixing until the muscle fragments had been completely digested (2 to 6 hours, depending on sample size). A further 100 µl proteinase K solution was added after 3 hours, if longer incubation was necessary. Samples could then be stored at -20^{0}C before proceeding to the phenol-chloroform extraction. For phenol-chloroform extraction, samples were thawed and incubated with 600 µl phenol (liquefied, washed in Tris buffer, stored at 4^{0}C - PhiφBio) for 15 minutes at room temperature (rotating). The samples were microcentrifuged at 13000 rpm for 15 minutes and the supernatants transferred to new eppendorfs. Extreme care was taken to transfer only the organic phase. 300 µl each of phenol and 24:1 chloroform: isoamyl alcohol were added to each sample before mixing gently by rotation for 15 minutes at room temperature. The samples were then centrifuged at 13000 rpm for 15 minutes and the supernatants were carefully transferred to new eppendorf tubes. Three further phenol: chloroform: isoamyl alcohol extractions were performed, until there was no visible 'interphase' separating the organic and aqueous phases. A final extraction was performed using 600 µl 24:1 chloroform: isoamyl alcohol. After rotating for 15 minutes at room temperature and centrifuging at 13000 rpm for 15 minutes, the aqueous (top) phase of each sample was divided between two fresh eppendorf tubes and placed on ice. One tenth volume of sodium acetate and two volumes 100% ethanol were added to

each sample and mixed. DNA was observed to swirl out in some samples, whereas in others it formed a white precipitate. The DNA was pelleted by centrifuging at 13000 rpm at 4^0C for 10 minutes, washed with 70% ethanol and dried before dissolving in TE buffer (20-40 μl, according to sample size) on a 37^0C heating block.

DNA was extracted from cell pellets by incubating the cell pellets in 190 μl TNE buffer (10 mM Tris, 0.4 M NaCl, 2 mM EDTA), 2 μl proteinase K (0.2 mg/ml) and 10 μl 0.5% SDS at 60^0C for 90 minutes, followed by phenol extraction, chloroform extraction and ethanol precipitation as above. One phenol and one chloroform extraction was found to be sufficient for cell pellets.

DNA was extracted from blood samples using the salting out lysis method, in which red cells were lysed with ice-cold water and cell membranes were degraded using ice-cold 0.1% Igepal. Nuclei were then lysed using a buffer containing 10 mM Tris, 400 mM NaCl, 2 mM NaEDTA, before digesting cellular proteins with SDS and proteinase K solution at 60^0C for 90 minutes. Proteins were then precipitated with saturated ammonium acetate. DNA was then precipitated with absolute ethanol, as for the muscle biopsy and cultured cell samples above.

For *SURF1* sequencing studies genomic DNA was extracted from cultured skin fibroblasts using the Nucleon BACC2 kit (Nucleon) according to the manufacturer's instructions.

2.8.1 Estimation of DNA yield and quality

DNA yield was estimated by diluting the DNA solution in distilled water and measuring its absorbance at 260 nm wavelength, using a Hitachi split beam spectrophotometer in single beam mode. The DNA concentration was calculated by assuming that DNA contains approximately equal amounts of purine and pyrimidine bases (Sambrook et al. 1989) and therefore that one absorbance unit at 260 nm is equivalent to 50 ng/μl of double stranded DNA. The quality of the extracted DNA was assessed by electrophoresing 0.5 μg undigested DNA through a 0.6% agarose gel. The observation of single sharp DNA bands was interpreted as evidence of lack of degradation of the DNA. Even loading of all sample lanes implied that the optical density measurements were reliable.

2.9 Isolation of RNA

Fibroblasts harvested by trypsinisation were pelleted, washed in PBS, repelleted and resuspended in 200 ml PBS. RNA was isolated from these fibroblasts using the High Pure RNA Isolation Kit (Boehringer Mannheim), according to the manufacturer's instructions. Initially the cells were lysed by incubating in lysis/binding buffer (which inactivates RNases) containing 4.5 M guanidine hydrochloride, 50 mM Tris-HCl and 30% Triton X-100 (w/v), pH 6.6, at room temperature. Nucleic acids were then bound to a glass fibre fleece in the upper chamber of a filtration tube. Nucleic acids bind specifically to the surface of glass fibres, in the presence of a chaotropic salt. The binding process is specific for nucleic acids, and binding conditions used in this kit have been optimised for RNA. Contaminating DNA was digested using RNase free DNase I applied directly to the glass fibre fleece. The bound RNA was purified by a series of washing steps to remove salts, proteins and other cellular impurities, and finally eluted in 50 μl nuclease-free water.

2.10 Isolation of protein

A mitochondrial cell fraction was prepared from frozen (-70^{0}C) skeletal muscle by a modification of the method of Darley-Usmar et al. 1983, scaled down for small sample size. Approximately 20 mg of human control and patient skeletal muscle was divided extremely finely in a 35 mm tissue culture dish, using a clean scalpel blade for each sample, and placed in 200 μl buffer containing 0.2 M sucrose, 0.13 M NaCl and 1 mM Tris-HCl, pH 7.4 and 280 μg collagenase (Sigma type VII) in a 1.5 ml eppendorf tube. The muscle mixtures were incubated on ice for 30 minutes, mixing intermittently, and a cocktail of protease inhibitors was added before transferring the samples to precooled small glass homogenisers. The stocks of protease inhibitors used were pepstatin A (Sigma) 1 mg/ml in methanol, leupeptin (Sigma) 1 mg/ml in water, and phenylmethylsulphonyl fluoride (PMSF, Sigma) 1M in acetone, all stored at -20^{0}C. Final concentrations of protease inhibitors used were 1 mM pepstatin A, 1 mM leupeptin and 1 mM PMSF. Samples were homogenised manually for 4 minutes and then transferred to new eppendorf tubes. The homogenisers were rinsed three times with buffer containing protease inhibitors, to give a final volume of 1 ml per sample. Samples were centrifuged at 500 g (2300 rpm) for 10 minutes at 4^{0}C. The precipitate was discarded and the supernatant was

spun at 8000 g (9000 rpm) for 15 minutes at 4^{0}C. The resulting crude mitochondrial pellets were stored at –70^{0}C until required for immunoblot analysis.

For cultured cells, cellular proteins including mitochondrial membrane proteins were extracted from 1 confluent plate (approximately 10^6 cells) of myoblasts or fibroblasts harvested by trypsinisation. All steps were carried out on ice to prevent proteolytic degradation of the cellular proteins. Cell pellets were washed in PBS, repelleted and then resuspended in 1 ml cold (4^{0}C) PBS containing 1.5% lauryl (*n*-dodecyl-β-D-) maltoside and a cocktail of protease inhibitors (extraction buffer) (Capaldi et al. 1995). Laurylmaltoside is a mild detergent that solubilises all mitochondrial inner membrane enzyme complexes but does not solubilise nuclear proteins. Final concentrations of protease inhibitors used were 1 mM pepstatin A, 1 mM leupeptin and 1 mM PMSF, as above. The resuspended cells were transferred to a 1.5 ml eppendorf tube and cooled on ice. The cells were then centrifuged at 6500 rpm for 3 minutes in a microcentrifuge. The supernatant was discarded and the cell pellet placed on ice. The cells were resuspended in a further 200 μl extraction buffer and incubated for 30 minutes on ice, mixing occasionally. The cell suspension was centrifuged at 13000 rpm at 4^{0}C for 20 minutes. The supernatant containing the cellular extracts was transferred to a precooled tube and stored at -70^{0}C until required for immunoblot analysis.

2.11 Immunoblot analysis of mitochondrial proteins

Cellular extracts, prepared as in section 2.10 and stored at -70^{0}C, were placed on ice. The cellular extracts were incubated at 37^{0}C with 50 μl of 5x dissociation buffer (6 ml glycerol, 2 g SDS, 5 mg bromphenol blue and 1 ml 1 M Tris-HCl, pH 6.5, made up to 10 ml in water), liquefied by placing in a beaker of hot water, and mixed thoroughly. Once the extracts had thawed 5 μl β-mercaptoethanol was added (in a fume hood), mixed and incubated at 37^{0}C for 30 minutes to reduce the formation of disulphide bonds. Proteins were separated on SDS-polyacrylamide (37.5:1 acrylamide:bisacrylamide, Bio-Rad) minigels containing 5.5 M urea. The percentage of polyacrylamide was 10 to 20%, according to the molecular weight of the COX subunits of interest. For COX I a 12.5% gel was used and for COX IV a 15% gel. Urea was included in the gels to improve resolution of hydrophobic membrane proteins. 5-20 μl aliquots of the dissociated mixtures were loaded, together with

2.5 μl prestained low molecular weight marker (Gibco BRL) per lane, for orientation of the gels. Gel electrophoresis was performed using the Mini-PROTEAN II system (Bio-Rad) with electrophoresis buffer containing 14.4 g glycine, 3 g Tris base and 1 g SDS per litre. Gels were stained for 2 hours in Coomassie blue to check even loading of samples and destained in water overnight.

Proteins in the gel were electroblotted onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore), using precooled Towbin's blotting buffer. After blotting membranes were air dried overnight. After wetting with methanol, the membranes were blocked with 10% milk solution and washed with PBS containing 0.3% Tween-20 (PBS-Tween) prior to the addition of primary antibodies. Blots were developed with monoclonal antibodies directed against COX subunits (as for immunohistochemistry, but with slightly different dilutions of primary antibodies: 1 in 1000 for anti-COX I and 1 in 2000 for anti-COX IV), the flavoprotein subunit of SDH and porin (31HL; Calbiochem-Novabiochem). Porin (or voltage dependent anion channel, VDAC) is an integral mitochondrial membrane protein and is used to demonstrate equal loading of samples (Taanman et al. 1997). Incubations with primary antibodies were overnight at room temperature (shaking). After a further wash in PBS-Tween, blots were incubated with a 1:3000 dilution of HRP-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad) for 2 hours at room temperature (shaking). The blots were washed again in PBS-Tween and then in plain PBS. Immunoreactive material was visualised using an enhanced chemiluminescence kit (DuPont).

2.12 Southern blot analysis of mitochondrial DNA

Southern blot analysis was used for the detection of both mtDNA rearrangements and mtDNA depletion. For Southern blots 2-4 μg total genomic DNA was digested with *Pvu* II to linearise mtDNA and size-fractionated by electrophoresis through a 0.8% agarose gel with a *Hind* III-digested I marker. After electrophoresis DNA within the gel was depurinated by incubating the gel in 0.2 M HCl for 10 minutes whilst shaking and then denatured by incubating in 0.5 M NaOH, 1.5 M NaCl for 30 minutes (shaking), before neutralising the gel in 1 M Tris-HCl (pH 7.4), 1.5 M NaCl for 40 minutes (shaking). DNA from the gel was subsequently blotted overnight onto a Hybond™-N membrane (Amersham) as recommended by the supplier. DNA within the blot was cross-linked by exposure to ultraviolet light for 2.5 minutes. The blot

was hybridised at 65^{0}C overnight simultaneously with two probes: one a cloned 5.8 kb *Eco*RI fragment of the gene encoding 18S rRNA, and the other the entire mtDNA sequence obtained by long range PCR. Probes were labelled with [α-^{32}P]dCTP using the Rediprime random primer labelling system (Amersham). The blot was prehybridised, hybridised and washed as recommended by the membrane supplier, and exposed to Kodak Biomax MS film. Signals were quantified using the Molecular Dynamics phosphorimaging system with correction for local background noise and the mtDNA signal was expressed relative to the nDNA (18S rRNA) signal.

2.13 Long range PCR

Recent developments in polymerase chain reaction (PCR) technology have allowed amplification of the entire mitochondrial genome (16.6 kb) in a single step, making PCR a potentially useful technique for the detection of mtDNA rearrangements (Cheng et al. 1994). Approximately 1 μg total genomic DNA was subjected to PCR amplification using the primers: forward 5'(L15149)-TGA GGC CAA ATA TCA TTC TGA GGG GC-(15174)3' and reverse 5'(H14841)-TTT CAT CAT GCG GAG ATG TTG GAT GG-(14813)3' and the GeneAmp XL PCR kit (Applied Biosystems). Cycling conditions were: 94^{0}C for 90 seconds (sec); 80^{0}C 'hot start' addition of *rTth* DNA polymerase; 94^{0}C for 35 sec then 69^{0}C for 14 minutes (min) for 35 cycles; 72^{0}C for 10 min; then hold at 4^{0}C.

2.14 DNA sequencing

2.14.1 Polymerase chain reaction amplification of DNA fragments

DNA samples were diluted to 50 ng/μl for use in PCR reactions, and 25-100 ng (0.5-2 μl) DNA template was used in a 50 μl volume PCR reaction. Oligonucleotide primers with M13 tails at the 5' end were designed to amplify all 22 mitochondrial tRNA genes and all three mitochondrially-encoded COX subunit genes, and are detailed in Table 2.1. For all reactions cycling was performed in a Hybaid omnigene thermal cycler using the following conditions: initial denaturation step at 94 ^{0}C for 4 min followed by 'hot start' addition of 0.5 μl *Taq* polymerase (Boehringer Mannheim); 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, 72 ^{0}C for 30 sec, for 30 cycles; then a final extension at 72 ^{0}C for 10 min.

Table 2.1 Primers for mtDNA Amplification

Gene	Nucleotide Position	Forward Primer	Reverse Primer
COX genes			
COX I	5904-7444	L5866	H6485
		L6426	H6968
		L6799	H7196
		L7115	H7650
COX II	7586-8262	L7588	H7992
		L7865	H8445
COX III	9207-9990	L9151	H9746
		L9638	H10107
tRNA genes			
P phenylalanine	577-647	L336	H706
V valine (+12S rRNA)	1602-1670	L1412	H2022
L leucine (UUR)	3230-3304	L3056	H3406
I isoleucine	4263-4331	L4215	H4643
Q glutamine	4329-4400		
M methionine	4402-4469		
W tryptophan	5512-5576	L5464	H5988
A alanine	5587-5655		
N aspartame	5657-5729		
C cysteine	5761-5826		
Y tyrosine	5826-5891		
K lysine	8295-8364	L7865	H8445
S serine	7445-7516	L7115	H7650
D aspartic	7518-7585		
G glycine	9991-10058	L9638	H10107
R arginine	10405-10469	L10362	H10724
H histidine	12138-12206	L12069	H12395
S serine (AGY)	12207-12265		
L leucine (CUN)	12266-12336		
E glutamic acid	14674-14742	L14595	H15038
T threonine	15888-15953	L15788	H16084
P proline	15955-16023		

All primers are 20 base oligonucleotides and are named according to the nucleotide position of the first base of their sequence, according to the Cambridge reference sequence CRS (Anderson et al. 1981). Forward primers are identical to the L strand sequence, whilst reverse primers are identical to the H strand sequence.

PCR products were separated in 3.2 % agarose gels run at 50 V with 1x TBE electrophoresis buffer. DNA in agarose gels was visualised by staining the gels with ethidium bromide and viewing under ultraviolet light. Comparison with a 100 bp DNA ladder (Gibco BRL) was used to confirm amplification of DNA fragments of the expected size.

2.14.2 Purification of PCR products

PCR products were purified prior to sequencing using the Microcon 30 microconcentrator containing a low-binding, anisotropic, hydrophilic YM membrane (Amersham). The column was assembled by placing the sample reservoir in the vial provided. 40 μl PCR product was placed in the sample reservoir, together with 400 μl sterile water. The column was spun at 10000 rpm at 4^0C for 10 minutes. The eluent was discarded and the column was inverted into a second vial and spun again at 2000 rpm at 4^0C for 2 minutes to collect the purified sample. The sample was diluted with sterile water to a total volume of 80 μl and was stored at -20^0C prior to sequencing.

2.14.3 Automated cycle sequencing of DNA

Automated DNA sequencing was performed using the ABI PRISM™ Dye Primer Cycle Sequencing Ready Reaction Kit and the ABI 373 DNA sequencer (Applied Biosystems). The chemistry used in the dye primer cycle sequencing method is derived from the Sanger dideoxy chain termination method (Sanger et al. 1977). The method relies on the ability of DNA polymerase to synthesise a fluorescently-labelled complementary copy of single-stranded (ss) DNA, using a short complementary ss DNA fragment as primer, and to incorporate randomly a dideoxynucleotide (ddNTP) instead of a deoxynucleotide (dNTP). Since dideoxynucleotides lack the 3'-hydroxyl group necessary for the formation of the next phosphodiester bond during chain elongation, chain elongation is terminated whenever a dideoxynucleotide is incorporated into the strand. If both dideoxynucleotide (for example, ddATP) and deoxynucleotide (dATP) are present, chain elongation will terminate randomly according to the site at which the ddATP is incorporated. For sequence analysis, four separate reactions are performed simultaneously, each with a differently coloured fluorescently labelled primer.

In the ABI PRISM™ Dye Primer Ready Reaction Kit, reagents are premixed into A, C, G and T cocktails. The sequencing enzyme in this kit is AmpliTaq DNA Polymerase FS. This is a variant of *Taq* DNA polymerase with a point mutation in the active site, leading to less discrimination against dideoxynucleotides and a more even peak intensity. Each reaction cocktail contains AmpliTaq DNA polymerase FS, all four deoxynucleotides and only one of the four dideoxynucleotides, together with

an M13 primer (either forward or reverse) fluorescently labelled with a differently coloured dye for each reaction cocktail. To use the kit, the reaction cocktails were aliquoted, DNA template added, the reactions cycled and the products precipitated in ethanol, pelleted and dried according to the manufacturer's instructions. The DNA templates were PCR products containing M13 sequences, to allow cycle sequencing using either the –21M13 (forward) or M13rev (reverse) sequencing dye primers. The reactions were overlaid with mineral oil prior to cycling on a Perkin Elmer 480 thermal cycler. Cycling conditions were: 95^{0}C for 30 sec, 55^{0}C for 30 sec, 70^{0}C for 1 min, for 15 cycles; then 95^{0}C for 30 sec, 70^{0}C for 1 min, for 15 cycles; then hold at 4^{0}C. The extension products were precipitated in 100% ethanol, pelleted and dried. If sequence analysis was not performed on the same day the dried pellets were stored at –20 ^{0}C for up to 3 weeks prior to use. The pelleted sequencing products were resuspended in 3 μl of a 5:1 mixture of deionised formamide and EDTA 25 mM with Blue dextran (50 mg/ml). The samples were then heated to 94^{0}C to denature before loading on a sequencing gel in an ABI 373 sequencer.

In the ABI 373 automated sequence analyser an argon laser causes excitation of the fluorochrome resulting in the release of photon quanta corresponding to specific wavelengths. The raw data was analysed using ABI Sequence Analysis software run on a Power Macintosh computer. Sequence Navigator software was used to compare sequences for mutation analysis. Previous reports of polymorphisms were checked in Mitomap, the Mitochondrial Human Genome Database at Emory University in Atlanta (http://www.gen.emory.edu/mitomap.html). Comparative mitochondrial genome sequence data was obtained from the Organelle Genome Database GOBASE (http://megasun.bch.umontreal.ca) for polypeptide-coding genes and from Sprinzl et al. 1998 for tRNA genes (http://www.uni-bayreuth.de/departments/biochemie/sprinzl/trna/).

Problems with the cycle sequencing method include uneven peak intensities, which can affect base calling late into the run, reducing the accuracy within that region. Uneven peak intensity can also lead to difficulties for the basecalling software in calling heterozygous or heteroplasmic positions.

2.15 Restriction fragment length polymorphism (RFLP) detection of T7671A point mutation

A mismatch PCR method was designed to detect the mutant mtDNA molecule by creating a restriction site for the enzyme *Tru*9 I (Promega). The sequences of the PCR primers used were: forward 5'(L7651)-CTT TCA TGA TCA CGC CCT TA-(7670)3' (mismatch nucleotide in red) and reverse 5'(H7865)-AGG GAT CGT TGA CCT CGT CT-(7846)3'. The numbers correspond to nucleotide positions (np) in the standard mitochondrial genome sequence (Anderson et al. 1981). The PCR cycling conditions used to amplify the target sequence were: 4 min denaturation at 94^{0}C followed by hot start addition of *Taq* polymerase, then 30 cycles of 30 sec denaturation at 94^{0}C, 30 sec annealing at 55^{0}C and 30 sec extension at 72^{0}C. A final extension step of 10 min at 72^{0}C was performed after the last cycle. Ten μl PCR product was incubated with 1 μl *Tru*9 I (10 u/μl) and buffer F (Promega) in a total reaction volume of 20 μl at 65^{0}C for 1 hour. After digestion with *Tru*9 I the 215 bp PCR product from the mutant allele yields two fragments of 197 and 18 bp, whilst the wild type allele remains uncut.

2.16 Quantitation of mtDNA mutant ioad using Genescan analysis

The relative proportions of wild type and mutant mtDNA were analysed by adding 0.5 μl 100μM fluorescent dUTP (R6G, Applied Biosystems) just before the final (20th) cycle of the PCR, followed by *Tru*9 I restriction digestion of the PCR product and separation on a 5% nondenaturing polyacrylamide gel. The fluorescent products were analysed using Genescan software (Applied Biosystems). The addition of fluorescence in the last cycle avoids the detection of heteroduplexes that can skew the RFLP result.

2.17 Single fibre PCR analysis

Percentage mutant mtDNA in individual fibres was correlated with COX activity by a single fibre PCR assay (Sciacco and Bonilla, 1996). 30 μm cryostat muscle sections on polysine slides were stained for COX activity as described above (see section 2.3) and fixed by immersion in a 100 mm^2 cell culture dish containing 50 ml of 50%

ethanol (w/v). Individual muscle fibres were selected according to COX staining (positive or negative) and microdissected using a borosilicate microcapillary tube under an inverted light microscope. The desired fibre was isolated by pulling away adjacent fibres, and then picked up using the microcapillary tube. Suction was not necessary to pick up the fibres. Fibres were removed under direct microscopic observation, to ensure that only the desired fibre was removed on each occasion. Fibres were also isolated from a control section on the same microscope slide. Fibres were isolated with as little liquid (50% ethanol) as possible. The removed fibre segments were added to tubes containing 10 μl water at room temperature. Once all required fibres had been removed, the tubes were centrifuged for 10 minutes at 13000 rpm at room temperature and then lysed at 65^{0}C for 1 hour in a solution containing 900 μl 220 mM potassium hydroxide (final concentration 200 mM) and 100 μl 500 mM dithiothreitol (DTT, final concentration 50 mM), to release DNA. The samples were then neutralised in 5 μl of a solution containing 900 mM Tris-HCl pH 8.3 and 200 mM HCl, before being subjected to fluorescent mismatch PCR amplification and restriction digestion, as described above, to analyse the proportion of mutant mtDNA in single muscle fibres. Successful PCR amplification was checked on an agarose gel before performing the restriction digestion and quantitation with the Genescan software (see sections 2.15 and 2.16 above).

2.18 RFLP detection of other base changes

2.18.1 Detection of A10044G in the tRNA glycine gene

For RFLP analysis of the A10044G point mutation in the tRNA glycine gene two PCR assays were used: one in which the mutation created a restriction site and another in which the mutation resulted in loss of a restriction site present in the wild type sequence. For the former the primers used were: forward 5'(L9911)-CGA AGC CGC CGC CTG ATA CT-(9930)3' and reverse 5'(H10107)-GTA GTA AGG CTA GGA GGG TG-(10088)3', and the cycling conditions were: 4 min denaturation at 94^{0}C followed by hot start addition of *Taq* polymerase, then 30 cycles of 30 sec denaturation at 94^{0}C, 30 sec annealing at 60^{0}C and 30 sec extension at 72^{0}C. A final extension step of 10 min at 72^{0}C was performed after the last cycle. Ten μl PCR product was incubated with 1 μl *Mae* II (2 u/μl, Boehringer Mannheim) and specialised incubation buffer (100 mM Tris-HCl, 440 mM NaCl, 12 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 200 μg/ml bovine serum albumin, pH 8.8 at 50^{0}C, Boehringer Mannheim) in a total

reaction volume of 20 µl at 50^{0}C for 2 hours (incomplete digestion occurred during incubation for 1 hour). After digestion with *Mae* II the 197 bp PCR product from the mutant allele yields two fragments of 132 and 65 bp, whilst the wild type allele remains uncut.

Quantitation of mtDNA mutant load was performed by using Genescan analysis to analyse the relative proportions of wild type and mutant mtDNA, as described in section 2.16 above.

In the second RFLP assay a mismatch PCR method was designed to detect the wild type mtDNA molecule by creating a restriction site for the enzyme *Hsp*92 II (Promega). The sequences of the PCR primers used were: forward 5'(L9911)-CGA AGC CGC CGC CTG ATA CT-(9930)3' and reverse 5'(H10064)-GTT TAT TAC TCT TTT TTG CA-(10045)3' (mismatch nucleotide in red). Cycling conditions were as for the first method except that an annealing temperature of 55^{0}C was used. Ten µl PCR product was incubated with 1 µl *Hsp*92 II (10 u/µl, Promega) and buffer K (Promega) in a total reaction volume of 20 µl at 37^{0}C for 1 hour. After digestion with *Hsp*92 II the 154 bp product from the wild type molecule yields two fragments of 136 and 18bp whilst the mutant molecule remains uncut.

2.18.2 Detection of T5773C in the tRNA cysteine gene

A mismatch PCR method was designed to detect the T5773C mutant mtDNA molecule by creating a restriction site for the enzyme *Mbo* I (Promega). The sequences of the PCR primers used were: forward 5'(L5651)-CCC TTA CTA GAC CAA TGG GA-(5670)3' and reverse 5'(H5793)-TTC GAA GAA GCA GCT TCA GA-(5774)3'(mismatch nucleotide in red). The PCR cycling conditions used to amplify the target sequence were: 4 min denaturation at 94^{0}C followed by hot start addition of *Taq* polymerase, then 30 cycles of 30 sec denaturation at 94^{0}C, 30 sec annealing at 55^{0}C and 30 sec extension at 72^{0}C. A final extension step of 10 min at 72^{0}C was performed after the last cycle. Ten µl PCR product was incubated with 1 µl *Mbo* I (8 u/µl) and buffer C (Promega) in a total reaction volume of 20 µl at 37^{0}C for 1 hour. After digestion with *Mbo* I the 143 bp PCR product from the mutant allele yields two fragments of 123 and 20 bp, whilst the wild type allele remains uncut.

2.19 cDNA synthesis using the reverse transcriptase-polymerase chain reaction (RT-PCR)

The Omniscript RT kit (Qiagen) was used to synthesise first strand cDNA from mRNA isolated from cultured skin fibroblasts (as described in section 2.9). 2 μl of the mRNA template was mixed with 2 μl oligo DT primer and 11 μl nuclease-free water. The reaction was set up on ice to avoid premature cDNA synthesis and to minimise the risk of RNA degradation. The mixture was then incubated at 65^{0}C for 5 minutes, to denature the RNA, and then placed immediately on ice. 1 μl reverse transcriptase (4U/μl), 2 μl Buffer RT, 1 μl RNase inhibitor (10 U/μl) and 2 μl dNTP mix (5 mM) were added (total reaction volume 20 μl) and the mixture was incubated at 37^{0}C for one hour. The resultant cDNA was then used to amplify *SURF1* in the PCR described below.

2.20 *SURF1* PCR and sequence analysis

SURF1 cDNA was amplified using oligonucleotide primers:
forward 5'-AGGAGCGTCCTCAGGGTC-3' and
reverse 5'-CATGATCCAGCATAAAGGCA-3'.
The amplified fragment covers all of the *SURF1* coding region apart from exon 1 and the first part of exon 2. Amplification was performed in a reaction volume of 25 μl and the following cycling conditions were used on a Hybaid PCR Sprint thermal cycler: 2 min denaturation at 94^{0}C followed by hot start addition of *Taq* polymerase, then 34 cycles of 1 min denaturation at 94^{0}C, 1 min annealing at 61^{0}C and 1 min extension at 72^{0}C. In the final (35th) cycle the extension step was increased to 4 minutes.

The PCR products were purified using the QIAquick purification kit (Qiagen) prior to performing sequencing reactions using the above PCR primers and the Big Dye^{TM} Terminator Cycle Sequencing kit (Applied Biosystems). For each reaction 3 μl of purified cDNA was used in a total reaction volume of 10 μl. Cycling conditions were: 1 min at 96^{0}C and then 25 cycles of 30 sec at 96^{0}C, 15 sec at 50^{0}C and 1 min at 60^{0}C (on a Hybaid PCR sprint thermal cycler). Once cycling was complete, sequencing products were precipitated by adding 2 μl 3M sodium acetate (pH 4.6) and 50 μl 95% ethanol to each sequencing reaction, vortexing and then placing on

ice for 10 minutes. The samples were pelleted by microcentifuging for 20 to 30 minutes at maximum speed. The supernatants were discarded and the pellets were washed with 200 µl 70% ethanol and then repelleted and dried. Sequence analysis was performed in an automated ABI 377 sequencer (Applied Biosystems).

2.21 RFLP detection of the 790-1delAG mutation in *SURF1* exon 8

A DNA fragment encompassing exons 6-9 was amplified using oligonucleotide primers:
forward 5'-TGCCTGAGTGACCATGAGTG-3' and
reverse 5'-TGGGAAAGTTCTTTGGACTGA-3'.
PCR cycling conditions were as for amplification of *SURF1* cDNA (see section 2.20). The mutation in exon 8 was confirmed by restriction endonuclease digestion of the exon 6-9 genomic fragment with *Bsr* I (New England Biolabs) as the mutation creates an additional restriction site for this enzyme. The normal fragment of 337 bp which includes the mutation site is digested into fragments of 262 and 75 bp in the presence of the mutation.

2.22 RFLP detection of the 312del10,insAT mutation in *SURF1* exon 4

A DNA fragment encompassing exons 3 and 4 was amplified using oligonucleotide primers:
forward 5'-CTGTGTGTGCCTTGTTCTGG-3' and
reverse 5'-CATATGTCAGGAGGCGGTCT-3'.
PCR cycling conditions were: 2 min denaturation at 94^{0}C followed by hot start addition of *Taq* polymerase, then 35 cycles of 30 sec denaturation at 94^{0}C, 1 min annealing at 60^{0}C and 2 min extension at 72^{0}C (on a Hybaid PCR sprint thermal cycler). The insertion-deletion mutation at np 312 is associated with two polymorphisms: a T→C at np 280 and a C→G at np 573. The 573 polymorphism results in loss of a restriction site for the enzyme *Msp* I, so that digestion of the mutant PCR product with *Msp* I yields fragments of 534 and 333 bp, compared to 399, 333 and 135 bp fragments from the wild type product.

Chapter 3

Immunohistochemistry

Chapter 3 Immunohistochemistry

3.1 Introduction

Most patients with COX deficiency remain uncharacterised at the molecular level. In the absence of an identified mtDNA mutation or a strong maternal family history of neuromuscular disease it is difficult to be certain whether the genetic defect is mitochondrial or nuclear in individual cases. In this chapter I sought to determine whether COX subunit expression patterns in muscle, detected immunohistochemically using highly specific monoclonal antibodies, could provide clues to the genetic origin of COX deficiency. I initially characterised patients with known mtDNA mutations and then analysed all patients presenting to Great Ormond Street Hospital in whom COX deficiency had been diagnosed histochemically over a 12 year period. The results of the immunohistochemical studies described in this chapter were used to direct the subsequent molecular genetic investigations described in Chapters 4, 5 and 6.

3.2 Clinical features

The clinical features of patients from whom muscle biopsies were available for immunohistochemical studies are summarised in Table 3.1 (P1 to P36). Patient identification numbers used in Table 3.1 are used throughout this thesis to refer to the same patients. Patients were aged between 5 days and 15 years 10 months (median 1 year 5 months) at the time of biopsy, with the majority being infants. There was a male preponderance of 1.8:1. Nine patients were from consanguineous pedigrees and there was a positive family history in 18 cases. Most patients (86%) had features of a myopathy and 64% had encephalopathy. Cardiomyopathy was noted in only 6 patients (17%), whilst 12 (33%) had liver disease, and a renal tubular defect was documented in 7 cases (19%). Three patients were dysmorphic. Seven had peripheral neuropathy, including 3 with Leigh syndrome. Eye abnormalities included cataracts in 3 cases and corneal clouding in two others.

Patient	Sex	Age at onset	Age at biopsy	Family history	Myo-pathy	Encephalo-myopathy	Cardio-myopathy	Liver disease	Renal tubular defect	Hypo-glycaemia	Lactate Blood mmol/L (<1.8)	Lactate CSF mmol/L (<2.0)	Lactate: pyruvate ratio (<20)	Other features
Known mtDNA mutations														
C1	F	11y	16y	-	+	-	-	-	-	-	ND	ND	ND	
C2	F	45y	49y	+	+	-	-	-	-	-	1.9	ND	ND	diabetes
C3	M	birth	7y10m	-	+	+	-	+	+	-	2.2-3.0	5.9	26	
C4	F	17y	29y	-	+	+	-	-	-	-	3.0	ND	ND	
C5	M	7y	18y	+	-	+	-	-	-	-	4.1	2.9	ND	Leigh syndrome (LS), peripheral neuropathy
Other patients														
P1	M	3y	10y3m	+	+	-	-	-	-	-	3.2	2.1	ND	peripheral neuropathy
P2	M	birth	2y6m	- C	+	+	-	-	-	-	4.4-8.5	6.4	32-35	
P3	M	9m	8y8m	+	-	+	-	-	-	-	0.9-2.0	1.9	14	
P4	M	birth	2.5m	+ C	+	+	-	-	-	-	2.8-11.8	4.3-6.5	18	LS
P5	M	1.5m	4m	- C	+	-	-	+	+	+	3.0-14.2	ND	66	
P6	M	birth	2m	-	-	+	-	+	-	-	6.8-38.1	ND	62	LS
P7	F	birth	5d	- C	+	-	+	-	-	+	18.3-27.3	ND	ND	
P8	M	1.5m	3.5m	-	+	+	+	-	-	-	10.1-16.8	15.8	55	
P9	M	6m	2y1m	-	+	+	-	+	-	+	0.7-9.0	3.3-4.1	22-30	LS
P10	F	6m	1y8m	-	+	+	-	-	-	-	4.3	2.3	ND	LS
P11	M	birth	1y2m	-	+	+	-	+	+	+	2.3-8.1	1.9-3.8	ND	
P12	F	birth	9m	+	+	+	-	+	+	+	3.9-14.6	ND	ND	
P13	M	5m	2y2m	+	+	+	-	-	-	-	8.2	3.4	ND	peripheral neuropathy
P14	F	8m	3y10m	-	+	+	-	-	-	-	2.3-8.9	8.2	38-42	
P15	M	birth	15y7m	+	+	+	-	-	-	-	2.0	ND	ND	peripheral neuropathy
P16	M	1y	15y10m	-	+	-	-	+	-	-	1.3-4.0	1.6	9-13	
P17	M	birth	16d	+	+	+	-	-	-	-	2.9-20.3	5.4-6.1	28-51	dysmorphic
P18	F	9m	3y1m	-	+	+	-	-	-	-	2.1-9.7	4.1-11.4	ND	LS
P19	M	birth	11.5m	-	+	-	-	-	-	+	0.3	ND	ND	dysmorphic
P20	M	birth	6m	+ C	+	+	+	+	-	+	5.7-11.1	9.3	43	dysmorphic
P21	M	1y1m	1y9m	+	+	+	-	-	-	-	3.7-4.5	5.2	ND	LS, peripheral neuropathy
P22	M	3m	1y1m	+ C	+	-	+	+	-	-	ND	ND	ND	
P23	F	2m	5m	+	+	+	-	+	-	-	5.8-8.5	ND	25	
P24	M	3m	1y6m	- C	+	+	-	-	-	-	1.7	2.2-3.5	ND	
P25	M	birth	6d	-	+	+	+	-	+	-	5.9-12.2	5.2	71	clouded cornea
P26	M	8y	11y	-	+	-	-	-	-	-	4.1	ND	ND	
P27	F	9m	2y3m	- C	+	+	-	-	-	-	2.3-.38	4.0	24-39	LS, clouded cornea, peripheral neuropathy
P28	F	9m	11y9m	+	+	+	-	-	-	-	1.1-4.4	ND	14-21	LS, peripheral neuropathy, hypertrichosis
P29	F	15d	4.5m	+	+	-	-	+	+	-	4.2-8.6	ND	59	cataracts
P30	M	3m	9.5m	+ C	+	+	-	-	-	-	8.6	4.2	ND	LS
P31	F	birth	17d	+	-	-	+	+	+	+	5.3-27.0	21.6	40-86	
P32	F	1y	1y7m	-	-	+	-	-	-	-	3.55	7.0	24	
P33	F	1y5m	7y2m	+	+	-	-	-	-	-	0.8-4.1	1.9	ND	cataracts
P34	F	3y	10y6m	+	+	-	-	-	-	-	2.3-5.6	2.0	36	cataracts
P35	M	birth	2.5m	-	-	-	-	+	-	-	10.9-20.0	ND	ND	
P36	M	1m	1y4m	+	+	-	-	-	+	-	2.1-4.1	3.7-4.0	ND	peripheral neuropathy

Table 3.1 Clinical and Biochemical Features of Patients Studied
F=female; M=male; y=year(s); m=month(s); C=consanguineous; ND=not determined
The lactate range refers to measurements from the same patient on different occasions.
Reference ranges for blood and CSF lactate and lactate:pyruvate ratios are given in brackets.
Each patient has been allocated a unique P number. This is used throughout the thesis whenever the patient is discussed.

Patient	Complex I	Complex II	Complex III	Complex II+III	Complex IV (COX)	COX (% mean control)	COX (% lowest control)
Control[a]	0.104-0.268	0.052-0.258	0.008-0.028	0.04-0.204	0.014-0.034 (0.024±0.005*)	-	
Control[b1]	185±31*	204±30*	820±78*	-	0.52±0.15*	-	
Control[b2]	0.107-0.275 (0.166±0.047*)	0.122-0.466 (0.208±0.070*)	-	-	0.932-2.747x10^3 (1.803±0.553x10^3*)		
Control[c]	1.72±0.23	583±127	-	1.98±0.38	42±4.3	-	
P3[a]	0.181	-	-	0.074	0.021	**87**	150
P4[a]	0.163	-	-	0.068	**0.007**	**29**	50
P5[a]	0.122	-	-	0.076	**0.013**	**54**	93
P6[c]	1.97	854		**1.59**	**3**	**7**	-
P8[a]	**0.014**	-	-	**0.003**	**0.001**	**4**	7
P9[a]	**0.097**	0.069	**<0.001**	**<0.001**	**0.006**	**25**	43
P10[a]	**0.094**	-	-	**0.012**	**0.010**	**42**	71
P11[b2]	**0.063**	**0.121**	-	-	**0.697x10^3**	**39**	75
P13[a]	0.106	-	-	**0.021**	**0.009**	**37**	64
P14[c]	**0**	-	-	**1.47**	**28**	**67**	-
P15[a]	0.155	-	-	0.106	0.014	**58**	100
P16[a]	0.142	-	-	0.113	0.016	**67**	114
P18[b1]	**24.9**	228	-	-	**0.342**	**66**	-
P19[a]	0.226	0.095	**0.0031**	**0.011**	0.015	**62**	107
P24[a]	**0.068**	-	-	**0.010**	**0.010**	**42**	71
P26[a]	**0.095**	-	-	0.05	**0.001**	**4**	7
P29[a]	**0.058**	0.109	**0.005**	**0.014**	**0.004**	**17**	28
P30[b1]	**21**	217	**375**	-	**0.08**	**15**	-
P31	**Low**	-	-	-	**Low**		
P32[a]	0.162	-	-	**0.030**	**0.009**	**37**	64
P34[c]	**0**	923	-	**1.44**	**16**	**38**	-
P35[c]	**0.722**	-	-	**1.218**	**16**	**38**	-

Table 3.2 Spectrophotometric Assay of Respiratory Chain Enzyme Activities in Muscle

[a] Laboratory a: all values given as ratio to citrate synthase;

[b] Laboratory b: b1 units for all complexes = nmol/min/mg protein; b2 all values given as ratio to citrate synthase

[c] Laboratory c: complex I = mmol NADH/min/g, complex II = nmol/min/g, complex II+III = mmol cyt c/min/g, complex IV = rate constant K/min/g; *mean–1 SD.

Hypoglycaemia was observed in 8 patients. Four of these had liver disease. Lactate levels were raised in blood and/or CSF in 34 of 35 patients. Lactate: pyruvate ratios were determined in 19 patients and were elevated in 16 of these. Nine patients had Leigh syndrome, defined by clinical features, lactic acidosis and neuroimaging, and confirmed neuropathologically in two cases (P6 and P9).

3.3 Histochemistry

COX staining was normal in all fibres in the 3 normal and 3 'disease' (proven pyruvate carboxylase (PC) deficiency, pyruvate dehydrogenase (PDHC) deficiency and isolated complex I deficiency) control biopsies (Figure 3.1A). COX histochemical staining was reduced in the majority of fibres in the biopsies from patients with known mtDNA mutations (C1 to C5, Figure 3.1C). All patients (P1 to P36) also had reduced COX activity on histochemical staining. In some cases there was complete absence of COX staining (Figure 3.1B), whilst in others there was a more patchy loss (Figure 3.1D and E). In 22 patients the decreased COX staining activity shown histochemically was associated with reduced COX enzyme activity on biochemical assay performed in diagnostic laboratories (Table 3.2). Residual COX enzyme activity ranged from 4 to 87% (median 38%) of the mean control value. In 17 patients there was reduction of one or more respiratory chain complexes in addition to COX. There was insufficient material to perform enzyme assays retrospectively in the other patients.

All but two patients (P14 and P20) had increased neutral lipid droplets within their muscle fibres. Only three cases (P1, P9 and P15) had classical RRF on Gomori trichrome staining but 20 had evidence of mitochondrial proliferation suggested by increased SDH staining (Figure 3.2).

3.4 Immunohistochemistry

Clear particulate immunoreactivity was observed in muscle fibres from the normal control biopsies with all antibodies directed against COX subunits (Figure 3.3). Permeabilisation, as used by Johnson et al. 1988, had no effect on the staining intensity in control samples and was not used in this study. In control muscle immunoreactivity was more prominent in type I fibres, which have a higher mitochondrial content. No immunostaining was observed in sections incubated with

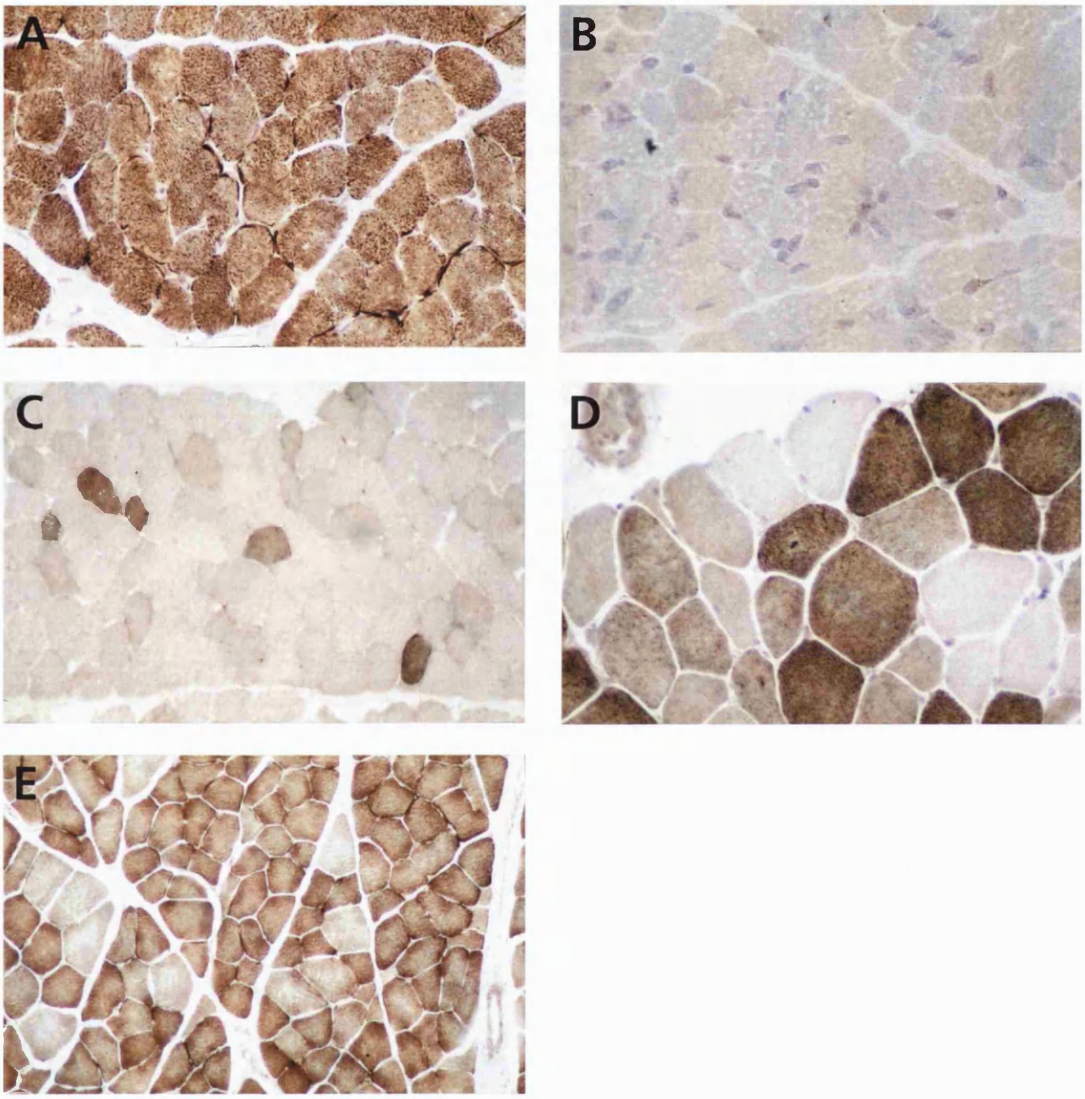


Figure 3.1 Cytochrome oxidase histochemistry

A Intense punctate staining of all muscle fibres in a normal control

B Absent staining in P8

C Mosaic appearance, with many COX-negative fibres and a few COX-positive fibres in C1, a positive control with a known mtDNA mutation 7472insC in tRNA serine^{UCN}

D Mosaic appearance, with approximately equal numbers of COX-positive and COX-negative fibres, in P1

E Mosaic appearance, with many COX-positive fibres and only a few COX-negative fibres in P15

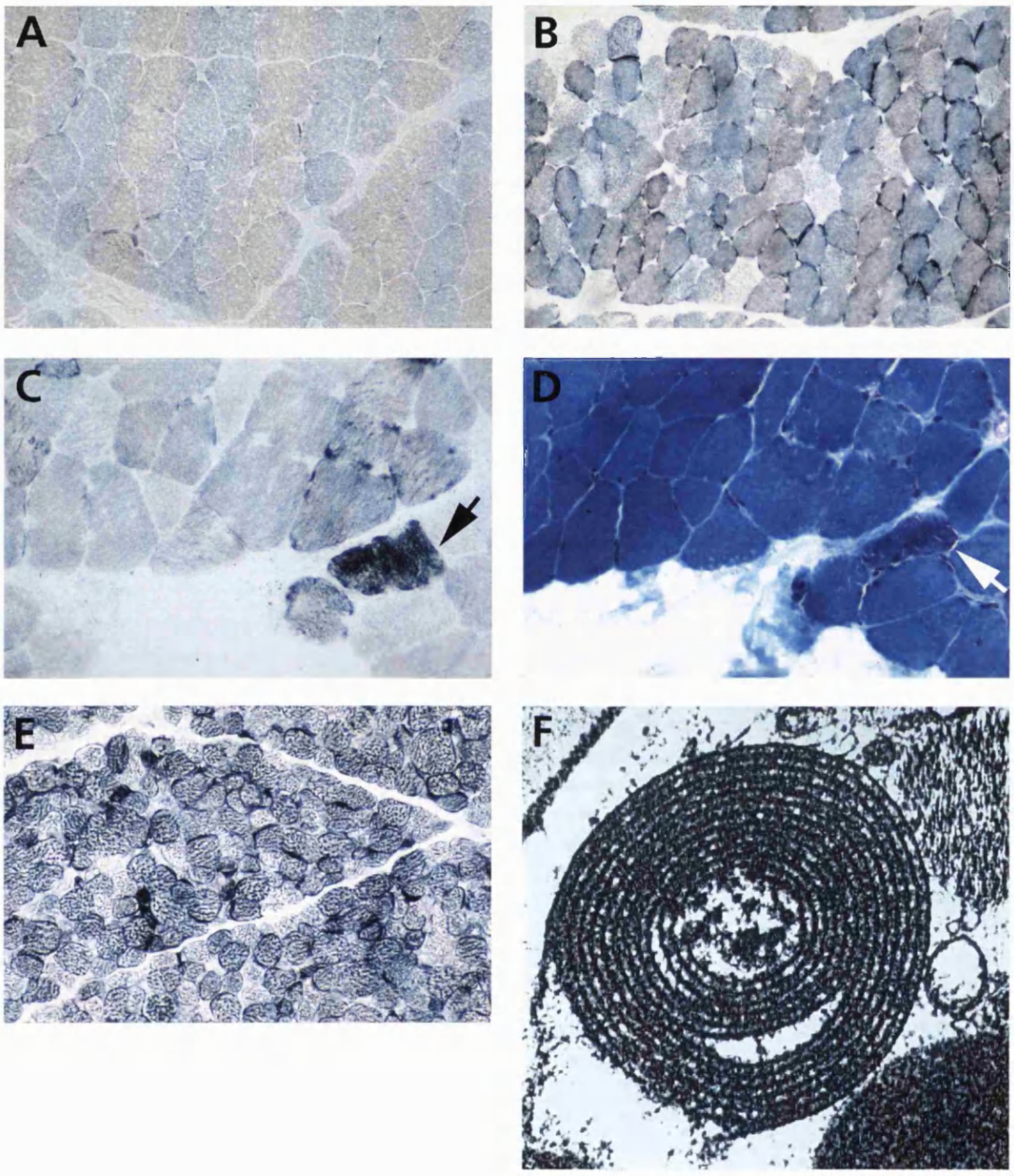


Figure 3.2 Mitochondrial proliferation

A Succinate dehydrogenase (SDH) staining of normal control muscle

B Enhanced SDH staining of muscle fibres in C1, a positive control with a known mtDNA mutation 7472insC in tRNA serine^{UCN}

C Increased SDH staining of muscle fibres in P1; the arrow marks a strongly SDH-positive fibre

D Modified Gomori trichrome stain of muscle fibres in P1; the strongly SDH-positive fibre can be seen to be a ragged red fibre (arrow)

E Markedly increased SDH staining in virtually all muscle fibres of P8

F Electron micrograph of a muscle mitochondrion from P8; there is proliferation of cristae, which appear as a series of concentric rings in this mitochondrion

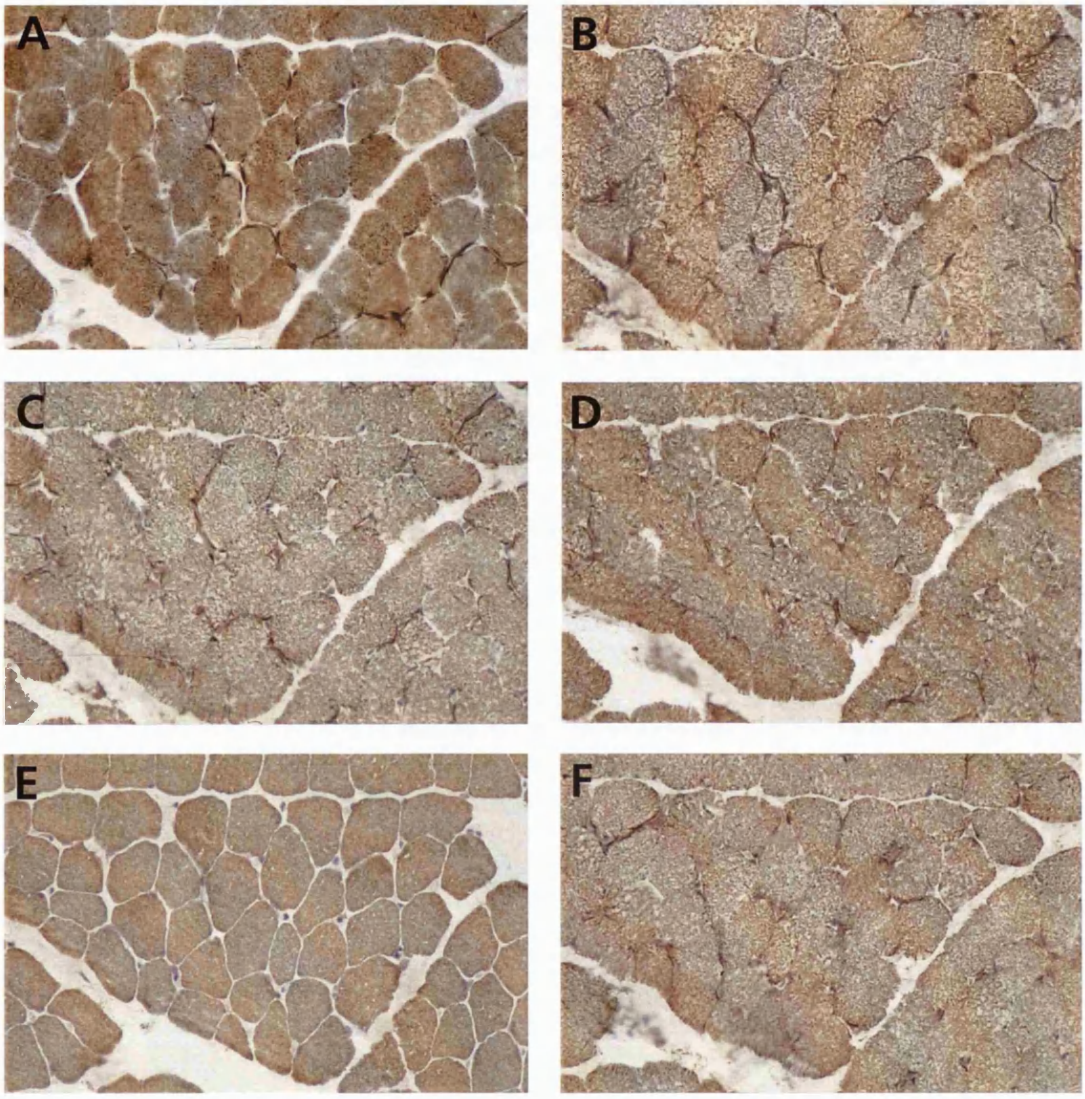


Figure 3.3 Immunohistochemistry of cytochrome oxidase (COX) subunits in normal control muscle
Serial sections of muscle are shown.
A COX histochemistry
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining

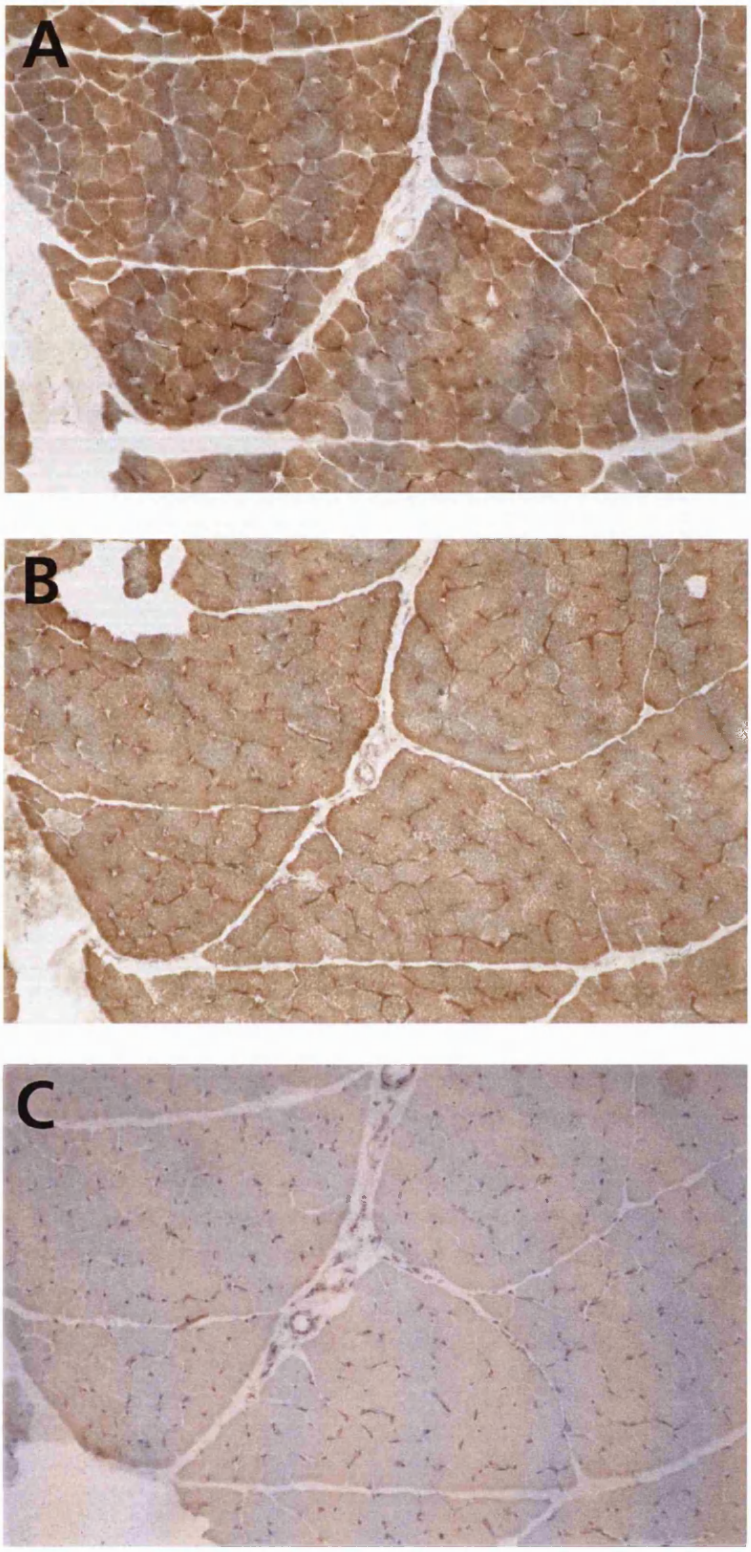


Figure 3.4 Lack of COX immunostaining in absence of primary antibody
Serial sections of control muscle are shown.
A COX histochemistry
B COX immunostaining using COX I primary antibody
C Lack of COX immunostaining in the absence of primary antibody

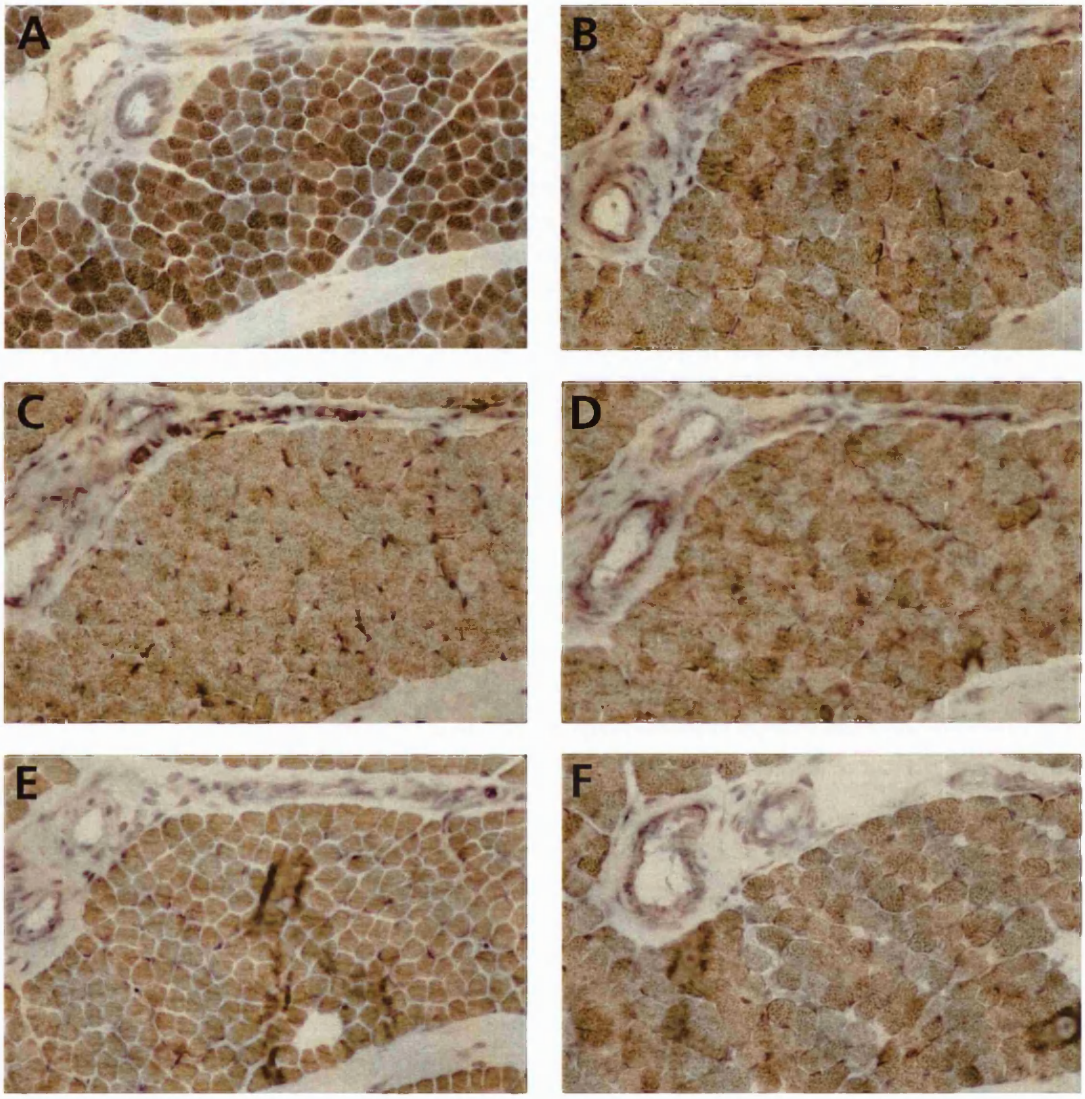


Figure 3.5 Immunohistochemistry of COX subunits in disease control muscle from a patient with pyruvate carboxylase deficiency
Serial sections of muscle are shown.
A COX histochemistry
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining

PBS pH 7.4 in place of primary antibody, as a negative control (Figure 3.4). Patients with proven PC, PDHC and isolated complex I deficiency also had normal immunostaining of all COX subunits as well as normal COX activity (Figure 3.5).

3.4.1 Patients with known mtDNA mutations

All 5 patients with known mtDNA mutations (C1 to C5) had a very similar pattern of COX subunit immunostaining, with selective loss of staining of mtDNA-encoded subunits I and II, together with reduced staining of subunit VIc (Figures 3.6 and 3.7). This reduction was limited to COX-deficient fibres. All subunits stained normally in COX-positive fibres.

The other 36 patients fell into two groups as defined by their immunostaining: those with the same pattern of immunostaining as patients with known mtDNA mutations (Group 1, Table 3.3), and those with other patterns of immunostaining (Group 2A/B, Table 3.3). The majority of patients did not have the same pattern of immunostaining as those with known mitochondrial mutations: 13 patients had reduced staining of all subunits (Group 2A), whilst 10 had normal staining of all subunits despite reduced enzyme activity (Group 2B). No consistent pattern of immunostaining was observed in the 3 patients with mtDNA depletion.

3.4.2 Group 1: Reduced immunostaining of mtDNA-encoded COX subunits

Ten of the COX deficient patients with unknown aetiology had a pattern of COX immunostaining similar or identical to the 5 patients with known mtDNA mutations (see Figures 3.6 and 3.7). Three examples (patients P1, P8 and P15) are illustrated in Figures 3.8 to 3.10. One of these 10 patients (P26) had reduction of subunit II only, with normal staining of subunit I (Figure 3.11). Subunit VIc was also reduced in 6 of these 10 patients, including the patient with isolated reduced staining of subunit II.

This group of patients was clinically heterogeneous, including patients with Leigh syndrome or other encephalopathy in infancy, multisystem disease, cardiomyopathy and isolated myopathy. Screening for mtDNA deletions and point mutations at nucleotide positions (np) 3243, 8344 and 8993 was negative in these 10 patients.

Patient	COX staining	Immunohistochemistry to COX subunits				
		I	II	IV	Va	VIc
Normal control muscle						
Disease control muscle (PC /PDHC/ Complex I deficiency)						
Known mtDNA mutations						
C1 7472insC						
C2 T14709C						
C3 4.9 kb deletion						
C4 G9952A						
C5 A8344G						
mtDNA depletion						
P9						
P23						
P29						
Group 1 Selective reduction of mtDNA-encoded subunits +/- VIc						
P1						
P6						
P8						
P10						
P14						
P15						
P25						
P26						
P31						
P35						
Group 2A Reduction of all subunits						
P2						
P3						
P7						
P13						
P16						
P18						
P20						
P24						
P27						
P28						
P30						
P33						
P34						
Group 2B All subunits stain normally						
P4						
P5						
P11						
P12						
P17						
P19						
P21						
P22						
P32						
P36*						

Absent staining

Severely reduced staining

Reduced staining

Mildly reduced staining

Normal staining

Table 3.3 Results of COX Immunohistochemistry
*sample dehydrated
shading refers to COX negative fibres only

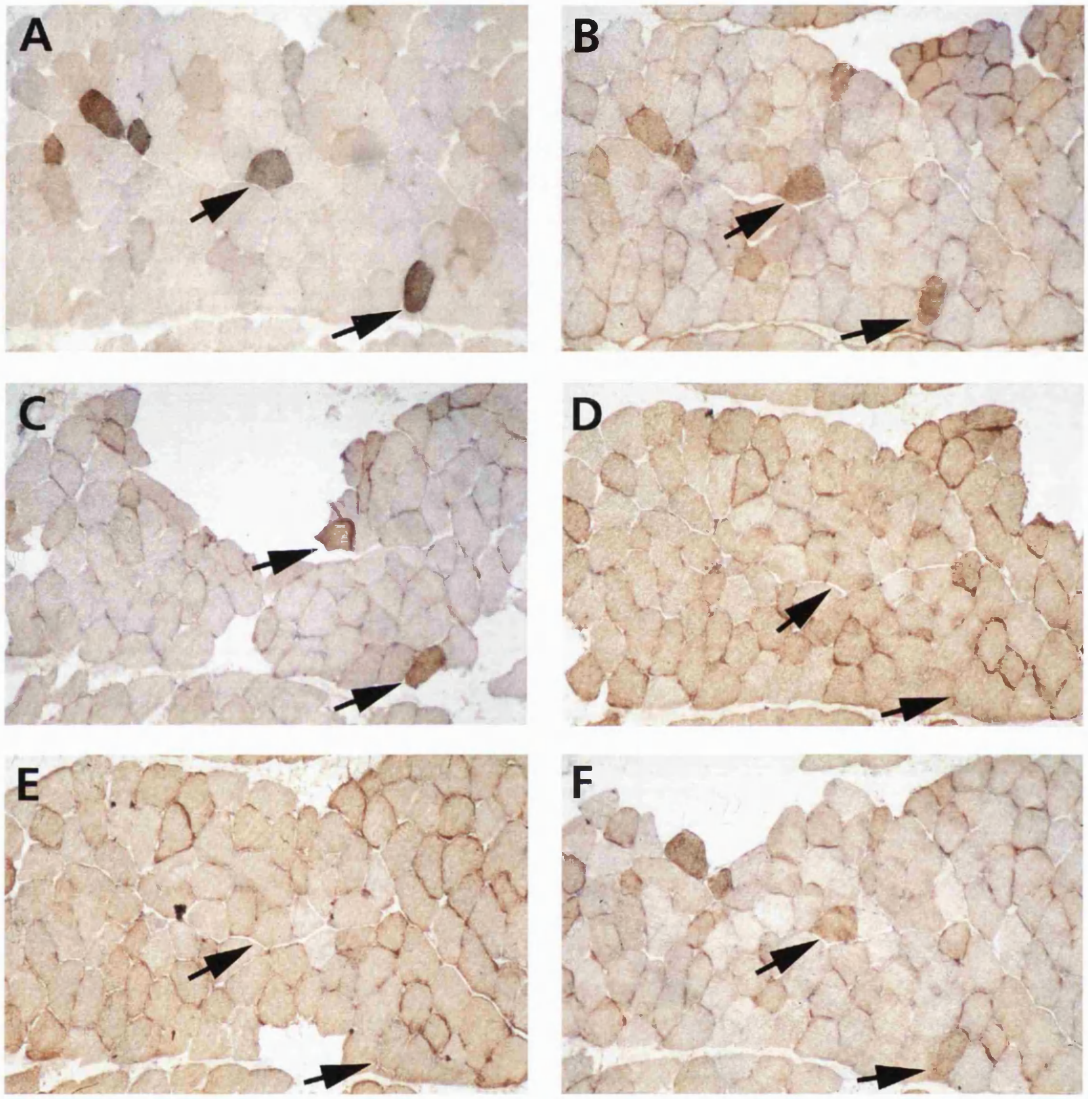


Figure 3.6 Immunohistochemistry of COX subunits in C1, a patient with mtDNA mutation 7472insC in tRNA serine^{UCN}

Serial sections of muscle are shown.

A COX histochemistry: arrows indicate two positive fibres
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining

Arrows indicate the same (COX-positive) fibres in each section.

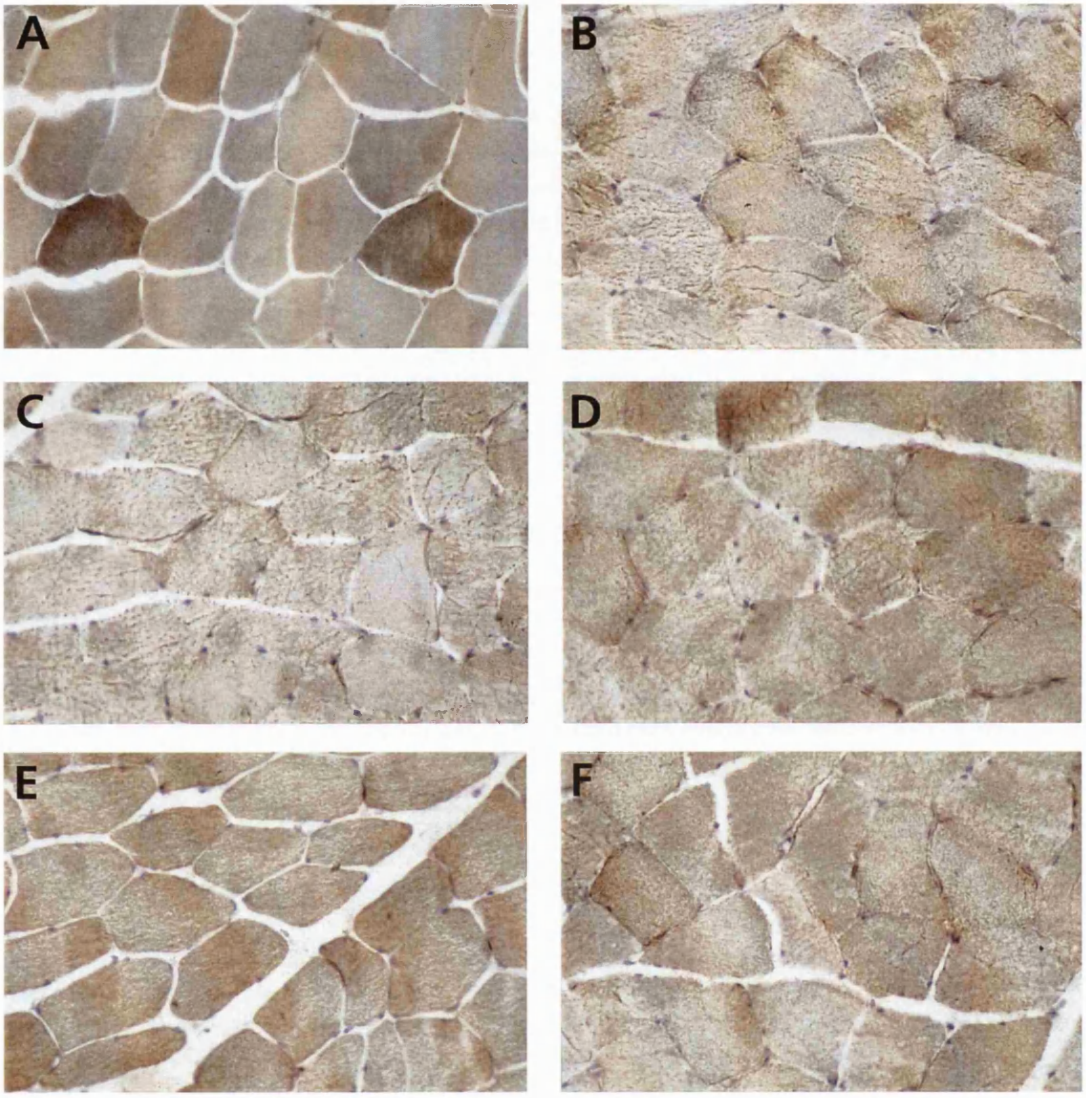


Figure 3.7 Immunohistochemistry of COX subunits in C5, a patient with mtDNA mutation G9952A in COX subunit I

Serial sections of muscle are shown.

A COX histochemistry
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining

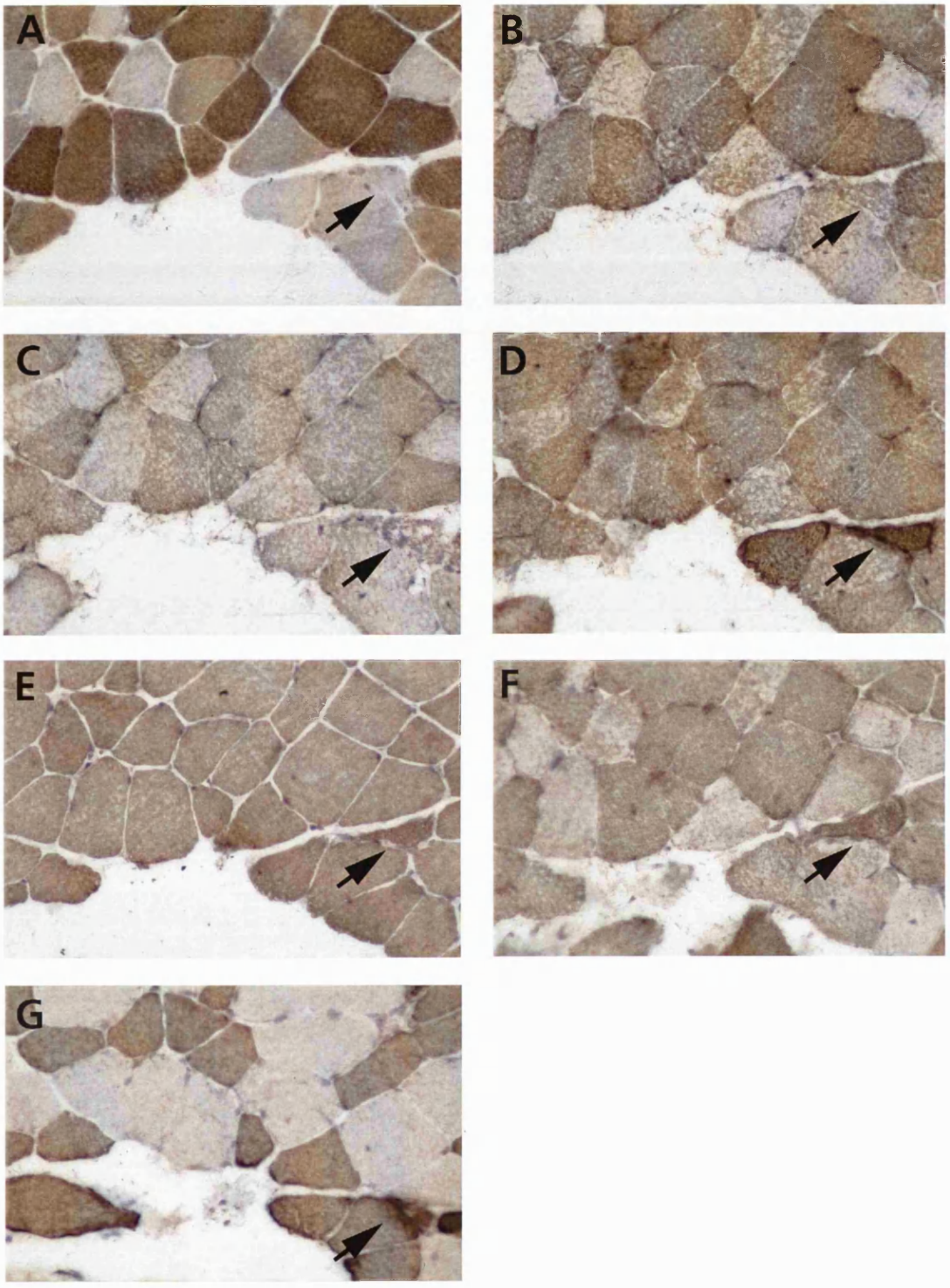


Figure 3.8 Immunohistochemistry of COX subunits in P1
Serial sections of muscle are shown.
A COX histochemistry
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining
Arrow indicates the same COX-negative ragged red fibre in each section.

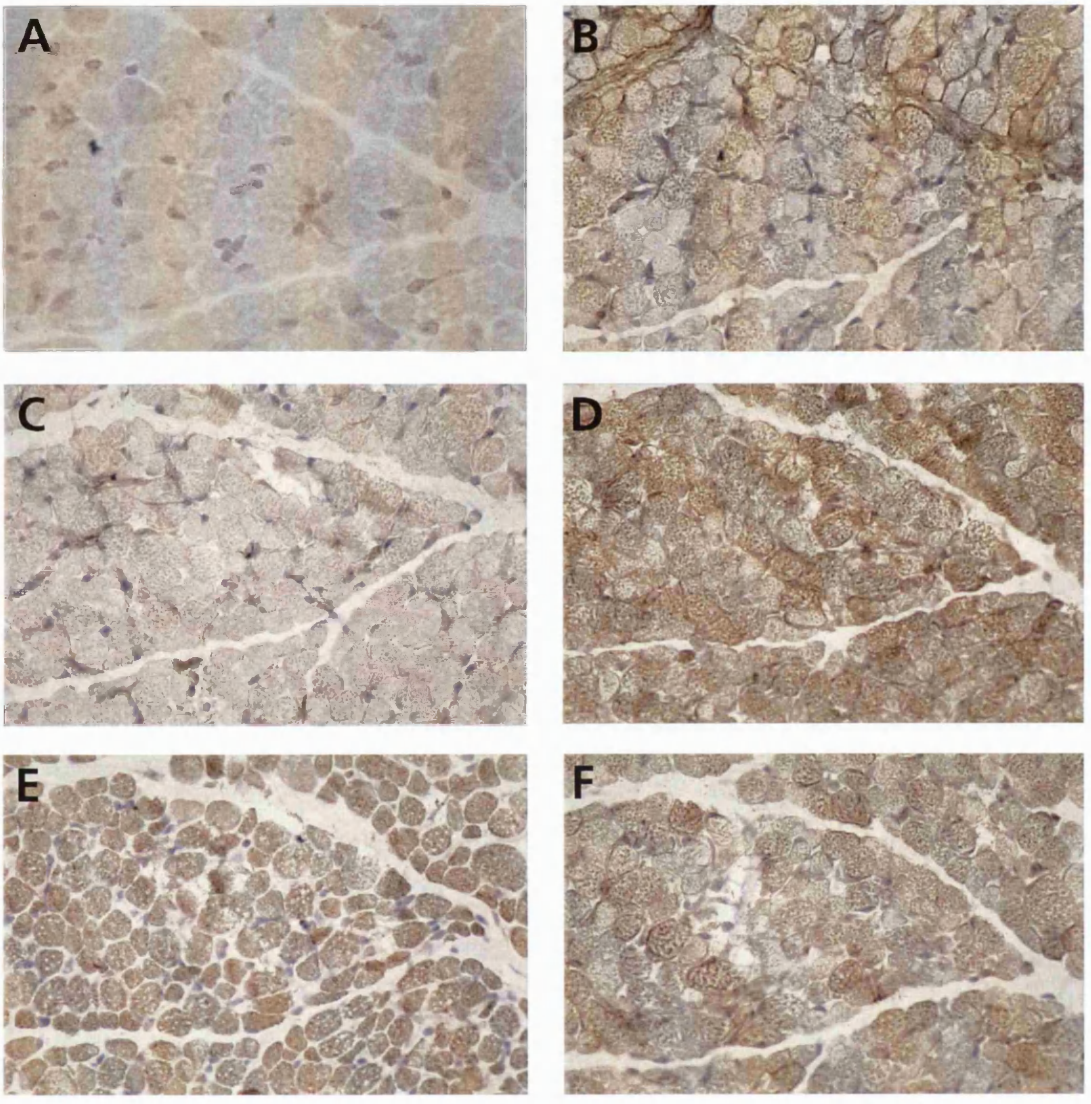


Figure 3.9 Immunohistochemistry of COX subunits in P8
Serial sections of muscle are shown.
A COX histochemistry
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining

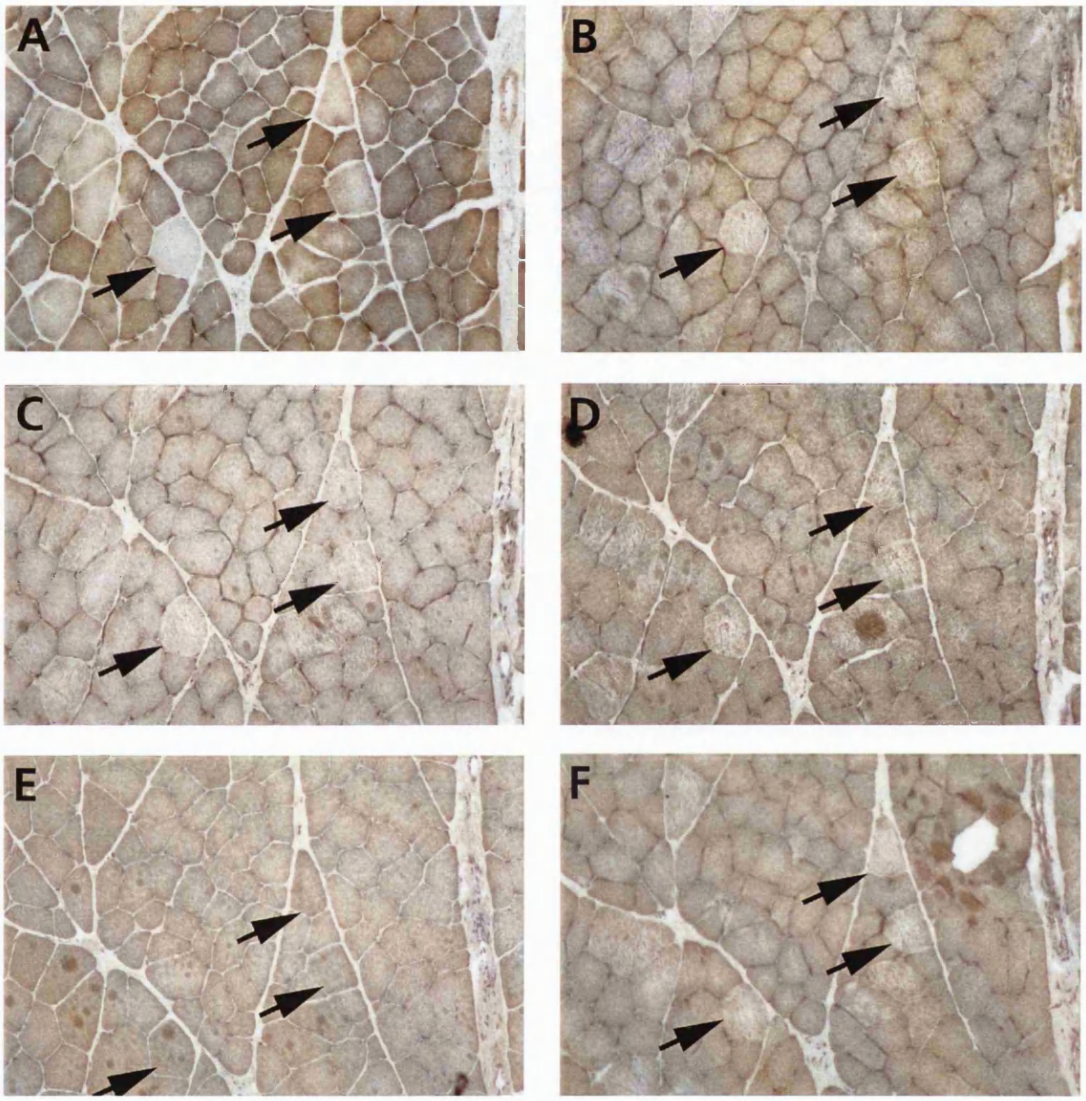


Figure 3.10 Immunohistochemistry of COX subunits in P15
Serial sections of muscle are shown.
A COX histochemistry: arrows indicate 3 COX-negative fibres
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining
Arrows indicate the same (COX-negative) fibres in each section.

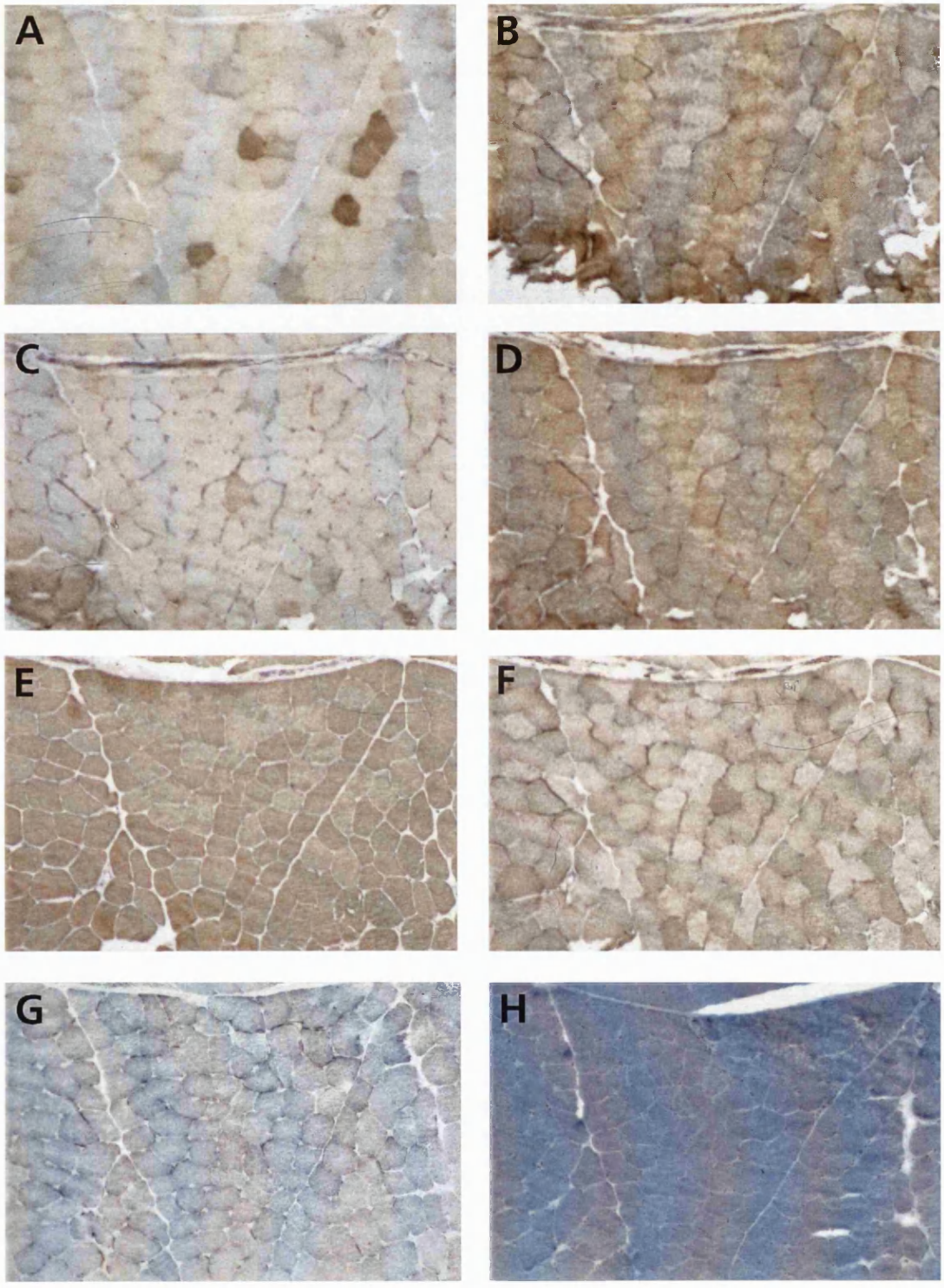


Figure 3.11 Immunohistochemistry of COX subunits in P26
Serial sections of muscle are shown.
A COX histochemistry
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining
G SDH stain
H Modified Gomori trichrome stain

Two other patients, with mtDNA depletion on Southern blot analysis of muscle DNA, also had selective loss of mtDNA-encoded subunits (Table 3.3, P9 and P23). A third patient with mtDNA depletion had patchy loss of staining of all subunits (P29).

3.4.3 Group 2: Other patterns of COX subunit expression

The majority of patients did not have the same pattern of immunostaining as those with known mtDNA mutations. Thirteen patients had reduced staining of all subunits (Group 2A in Table 3.3), whilst 10 had normal staining of all subunits despite reduced COX activity on histochemical staining (Group 2B in Table 3.2). These patterns of immunostaining are illustrated in Figures 3.12 (P28) and 3.13 (P12). Muscle from P36 had been stored for 9 years and there was some desiccation artefact in the sections. This made quantitative assessment of immunostaining difficult. However as immunostaining was present to all subunits P36 was assigned to Group 2B.

3.5 Discussion

A number of different types of genetic defect could lead to partial or total COX deficiency. These include mtDNA deletions, point mutations in mitochondrial COX and tRNA genes, mtDNA depletion and mutations in nuclear genes coding for subunits and assembly factors of COX or coding for factors involved in transcriptional or post-transcriptional regulation of COX genes. Neither clinical, biochemical nor histochemical features enable patients with mtDNA mutations and others with presumed nuclear defects to be differentiated reliably. For example, although mitochondrial proliferation commonly occurs in association with primary mtDNA defects, it has been reported in patients with fatal infantile COX-deficient myopathy, which is thought to be an autosomal recessive condition (DiMauro et al. 1994). This study sought to determine whether COX subunit expression patterns detected immunohistochemically could distinguish between mtDNA defects and nuclear defects in COX deficiency.

The monoclonal antibodies used in the present study have been shown to be specific for individual COX subunits (Taanman et al. 1996) and should therefore allow identification of patterns of subunit loss which could prove to be informative.

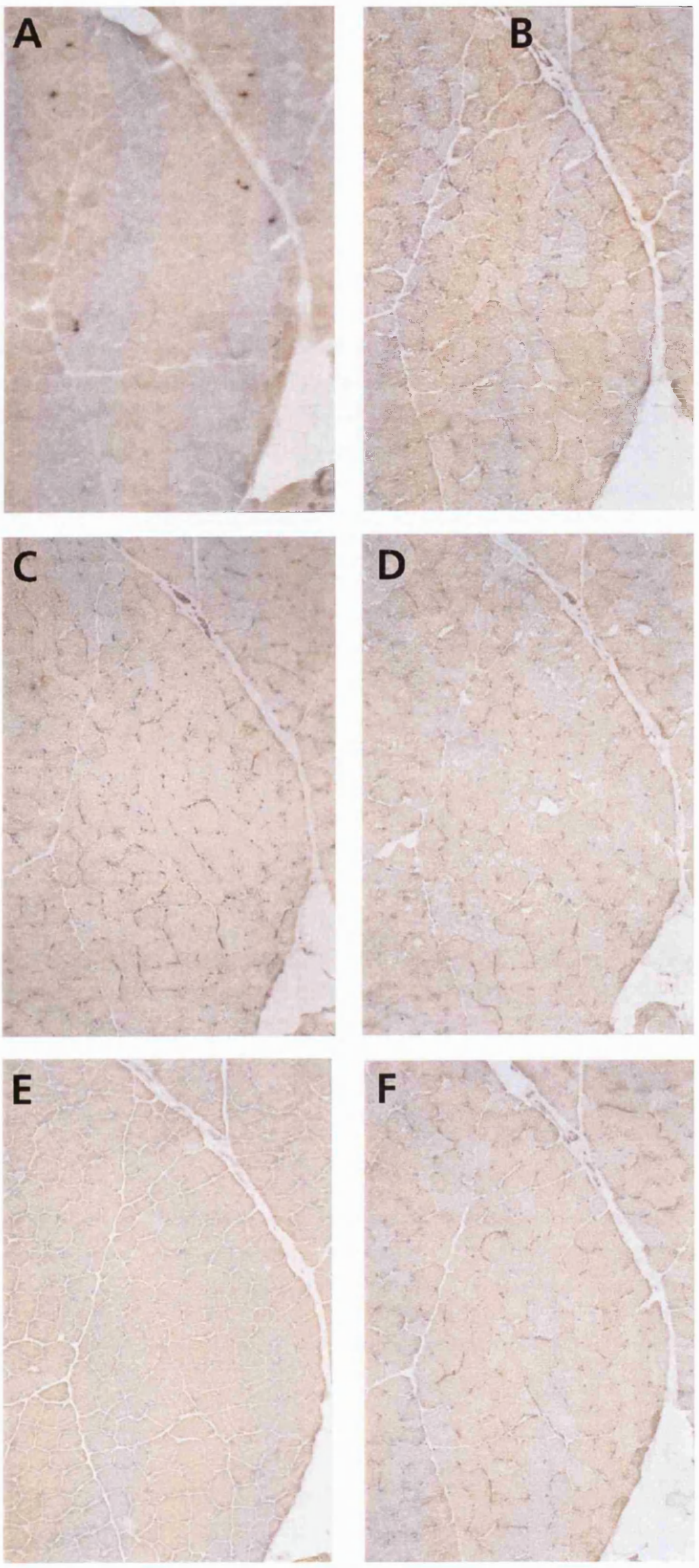


Figure 3.12 Immunohistochemistry of COX subunits in P28: decreased staining of all subunits
Serial sections of muscle are shown.
A COX histochemistry
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining

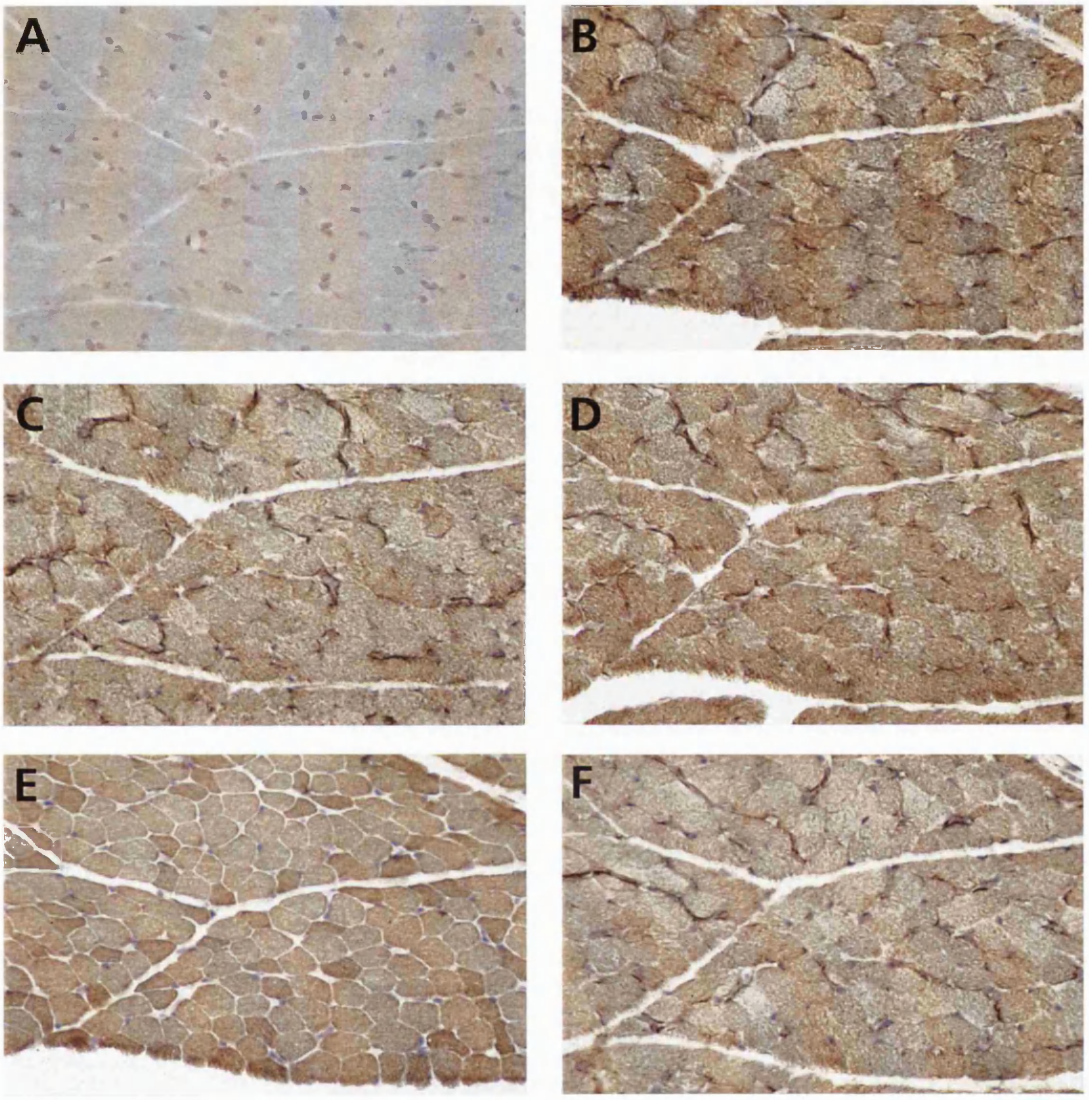


Figure 3.13 Immunohistochemistry of COX subunits in P12: normal staining of all subunits
Serial sections of muscle are shown.
A COX histochemistry
B COX I immunostaining
C COX II immunostaining
D COX IV immunostaining
E COX Va immunostaining
F COX VIc immunostaining

The clear particulate immunoreactivity observed in normal control muscle with all COX subunit antibodies, with differential staining intensities between type I and II fibres, implies that the observed staining is mitochondrial.

3.5.1 COX subunit expression pattern in patients with mtDNA mutations

The steady-state expression pattern of COX subunits was investigated using immunohistochemistry in patients with a range of known mtDNA mutations, including a large-scale deletion, point mutations in tRNA genes and a point mutation in the COX III protein-coding gene. Selective and marked reduction of mtDNA-encoded COX subunits I and II was observed in all these patients. This reduction was restricted to COX-deficient fibres, with all subunits appearing normal in COX-positive fibres. In addition, less marked reduction of the nuclear-encoded COX subunit VIc was also observed in COX-deficient fibres in these patients. Immunostaining with antibodies directed against nuclear-encoded subunits COX IV and Va, however, was normal in all muscle fibres from all five patients with known mtDNA mutations.

Selective loss of mtDNA-encoded COX subunits has previously been observed in patients with mtDNA deletions (Mita et al. 1989; Moraes et al. 1992b; Taanman et al. 1996), a point mutation in the mitochondrial $tRNA^{leu(CUN)}$ gene (Fu et al. 1996) and a 15 bp microdeletion in the COX III gene (Keightley et al. 1996). Levels of COX subunit IV were preserved in the patients studied by Mita et al. 1989, Moraes et al. 1992b, and Fu et al. 1996. However no antibodies against other nuclear-encoded subunits were used in these studies and so the fate of other nuclear-encoded COX subunits was not determined in these patients. Taanman et al. (1996) examined immunostaining of COX Va, Vb and VIc as well as IV in a CPEO patient harbouring a mtDNA deletion and found greatly reduced levels of COX I, II, Va, Vb and VIc in COX-negative fibres, with relatively preserved levels of COX IV. The patient with the COX III microdeletion had little or no reactivity with antibodies to COX I and VIc but intact immunostaining of subunit IV (Keightley et al. 1996). Immunoblot analyses have also demonstrated relative preservation of COX IV and Va and reduction of COX VIc in ρ^0 cells lacking mtDNA-encoded subunits (Taanman et al. 1996; Marusich et al. 1997).

Differential loss of immunostaining of nuclear-encoded COX subunits in patients with known mtDNA mutations may be related to the quaternary structure of the holoenzyme, which has been determined for the bovine enzyme (Tsukihara et al. 1996). Subunit VIc forms a dumb-bell, with a transmembrane helix in contact with helix I of subunit II (see Figure 1.4 in Chapter 1). It is possible that the stability of subunit VIc may be impaired when there are reduced levels of subunit II, since the two subunits are intimately related. Subunits IV and Va, on the other hand, have interactions with several nuclear subunits. These may form stable partial complexes in the absence of mitochondrial subunits and thus be more resistant to proteolytic degradation (Hanson et al. 2001). Two other mechanisms may explain the differential expression of nuclear subunits in these patients. Incomplete inhibition of mitochondrial gene expression, with some mitochondrial subunits remaining, may enable COX sub-assemblies to form and may lead to relative protection of some nuclear subunits from rapid degradation. A third but less likely possibility is that lack of mtDNA-encoded subunits as a result of impaired mitochondrial translation may alter nuclear COX gene expression, down-regulating transcription of nuclear-encoded COX subunits. This might not affect all nuclear subunits equally, leading to differential protein expression.

3.5.2 Reduced immunostaining of mtDNA-encoded COX subunits

In the present study the COX-deficient patients who had lost subunits I and II were not typical clinically of the group of patients usually associated with mtDNA defects. Some of the patients presented in the neonatal period with lactic acidosis, variably associated with encephalomyopathy, cardiomyopathy, liver disease and renal tubulopathy. Two had Leigh syndrome confirmed neuropathologically (P6 and P9). In the past these patients may have been assumed to have nuclear defects. Three patients did have an isolated myopathy presenting late in childhood (P1, P15 and P26), a common phenotype associated with mtDNA defects. Two of these had classical RRF (P1 and P15). The third had selective loss of subunit II with normal immunostaining of subunit I (P26). Loss of COX II immunostaining in this patient was associated with reduced immunostaining of subunit VIc. This may be seen as further evidence that subunit II is required for stability of subunit VIc. None of the 12 patients with selective loss of mtDNA-encoded COX subunits in this study have the common mtDNA point mutations A3243G, A8344G or T8993G/C. Southern blot analysis has revealed mtDNA depletion in two of these patients (P9 and P23),

including one with pathologically proven Leigh syndrome (P9). Direct sequencing studies of mtDNA from 8 of the remaining 10 patients are described in Chapter 4. A point mutation has been identified in the COX subunit II gene in P26, who had selective loss of immunostaining to COX II (Rahman et al. 1999), and will be described in detail in Chapter 5.

Recently selective loss of immunoreactivity to mtDNA-encoded COX subunits has been described in a small group of patients who did not have mtDNA mutations. Two reports described this pattern of immunostaining in patients with mutations in nuclear genes involved in COX assembly (Papadopoulou et al. 1999; Valnot et al. 2000b). The first of these studies described immunohistochemical findings in two patients with isolated COX deficiency caused by mutations in the *SCO2* gene. Both patients had marked reduction of immunostaining to COX subunits I and II in skeletal muscle and more mild reduction of immunostaining to COX subunits IV and Va (Papadopoulou et al. 1999). The SCO2 gene product is thought to be involved in delivery of copper to COX I and II. Mutations have also been described in a single family with mutations in another gene involved in copper delivery to COX, *SCO1*, but neither immunohistochemistry nor Western blot analysis was performed on samples from affected members of this family (Valnot et al. 2000a).

The second report described Western blot analysis of fibroblast mitochondrial proteins in a patient with a homozygous mutation in the *COX10* gene. COX II was barely detectable (<3% residual immunoreactivity), whilst there was moderate reduction of COX III and VIc (approximately 55%) and only slight reduction of all other COX subunits studied (70 – 82%) including COX I and IV (Valnot et al. 2000b). Immunohistochemistry was not done on this patient's muscle. The *COX10* gene encodes haem A: farnesyl transferase which catalyses the first step in the conversion of protohaem to haem A, the prosthetic group in COX I. The reason why steady state levels of COX II were more affected than COX I in this patient is not clear, but it was suggested that haem groups are not necessary to stabilise COX I (Valnot et al. 2000b). It seems that COX assembly intermediates S1 and S2 were able to form in this patient, since relatively normal levels of COX I and IV were observed (see Figure 1.5 in Chapter 1 for detailed COX assembly pathway). However association of COX II with COX I appears to have been impaired by the lack of haem groups, leading to increased proteolytic degradation of COX II by

mitochondrial proteases (Rep and Grivell, 1996) and low steady state levels of COX II.

Taken together, these studies suggest the hypothesis that two main mechanisms may lead to selective reduction of immunoreactivity to mtDNA-encoded COX subunits in patients with COX deficiency: 1) mtDNA mutations and 2) mutations in genes involved in delivery of prosthetic groups (copper and haem) to COX I and COX II.

3.5.3 COX subunit expression patterns in the mtDNA depletion syndrome

COX subunit expression has previously been studied in 10 patients with the mtDNA depletion syndrome (Moraes et al. 1991; Tritschler et al. 1992; Macmillan and Shoubridge, 1996; Taanman et al. 1997; Marusich et al. 1997). Eight had selective loss of mtDNA-encoded subunits (I and/or II), with variable loss of nuclear-encoded subunits. Two patients, however, had homogeneous reduction of all COX subunits compared to controls. In the present study two patients with mtDNA depletion (P9 and P23) had loss of mtDNA-encoded subunits whilst a third (P29) had loss of all subunits. These results therefore confirm that different patterns of COX subunit expression may be seen in the mtDNA depletion syndrome. This may reflect genetic heterogeneity. Recently three genes causing mtDNA depletion have been reported. These genes encode thymidine phosphorylase, deoxyguanosine kinase and thymidine kinase (Nishino et al. 2000; Mandel et al. 2001; Saada et al. 2001). Immunohistochemistry of patients with mutations in these genes has not been described, so it is not yet known whether these patients have uniform or heterogeneous COX subunit expression patterns.

An alternative explanation for the heterogeneous COX subunit expression patterns seen in the mtDNA depletion syndrome is that they may be related to the stage of the disease. It is known that there is progressive loss of mtDNA in this condition and hence progressive loss of mitochondrially-encoded respiratory chain subunits (Taanman et al. 1997). A study of human leukaemic cells (Molt- cells) which lack mitochondrial protein synthesis suggested that all COX subunits are lost if the mitochondrial translation defect is sufficiently severe (Nijtmans et al. 1995b).

Studies of ρ^0 cells, which lack mtDNA, have yielded similar results (Taanman et al. 1996; Marusich et al. 1997).

3.5.4 Other patterns of COX subunit expression

The remaining patients in the present study had COX subunit patterns falling into one of two broad groups: intact staining of all subunits tested, or reduced staining of all subunits. These patterns of immunostaining have not been reported in association with mtDNA mutations causing COX deficiency described in the literature. It is postulated that patients with these immunostaining patterns are likely to have nuclear DNA defects. To date no mutations have been described in nuclear-encoded COX subunits; therefore patients with known mutations cannot be used for comparison. However, pedigree analysis indicates autosomal recessive inheritance in some distinct clinical entities associated with COX deficiency, such as fatal infantile myopathy and Leigh syndrome (Rahman et al. 1996). The present study suggests that Leigh syndrome associated with COX deficiency is aetiologically heterogeneous. Two patients had loss of immunostaining of mitochondrial subunits (including one with the mtDNA depletion syndrome) whilst others had loss of staining of all subunits or normal staining of all subunits.

A recent study demonstrated a uniform pattern of COX subunit immunohistochemistry in 3 patients with COX-deficient Leigh syndrome caused by mutations in the *SURF1* gene, another COX assembly gene. These patients had reduced immunostaining for both mtDNA-encoded (COX II) and nuclear-encoded (COX IV and VIIa) subunits in frozen muscle sections (Sue et al. 2000). Immunoblot analysis of fibroblast mitochondrial proteins from other SURF1 deficient patients revealed similar results, with reduced steady state levels of COX subunits I, II, IV and Vb (Yao and Shoubridge, 1999; Poyau et al. 2000). Another immunoblot study found marked reduction of COX I, II, III, VIa, VIb and VIc, with moderate reduction of COX IV and relatively normal levels of COX Va and Vb in a single SURF1 deficient patient (von Kleist-Retzow et al. 1999). Two-dimensional polyacrylamide gel electrophoresis followed by Western blotting with monoclonal antibody directed against COX I has been used to study COX assembly in fibroblasts of patients with *SURF1* mutations (Tiranti et al. 1999b; Coenen et al. 1999). This demonstrated reduction of the COX holoenzyme (S4) to 20-30% of control levels and also reduced

levels of assembly intermediate S3 but relatively increased intermediates S1 and S2 (Coenen et al. 1999).

Sequence analysis of the *SURF1* gene in four patients with 'nuclear' patterns of COX subunit immunostaining (P20, P27, P28 and P36) is described in Chapter 6. Causative *SURF1* mutations were identified in P27, P28 and P36 but not in P20.

Patients with intact immunostaining of all subunits despite reduced or absent COX staining appear to have reduced function despite apparently normal assembly of the holoenzyme. These patients may have a kinetic defect. A single patient with COX deficiency has been described who had an altered K_m for reduced cytochrome c (Nijtmans et al. 1995a). Studies in cultured fibroblasts from this patient revealed decreased COX activity but normal synthesis, assembly and stability of both mitochondrial and nuclear-encoded COX subunits. This patient was thought to have a defect in one of the nuclear subunits of the enzyme, as mtDNA analysis failed to identify a mutation and the parents were consanguineous. A yeast mutant for COX IV (equivalent of human subunit Vb) had altered COX enzyme kinetics (Lightowlers et al. 1991), suggesting that human COX Vb mutations might result in kinetic defects. However no such mutations have been reported to date. Recently two further patients with apparently normal COX subunit steady state levels but reduced enzyme activity were identified by an approach combining immunoblot analysis with sucrose gradient centrifugation (Hanson et al. 2001). The underlying genetic defect in these patients has not been determined.

Although it is more likely that this group of patients has nuclear subunit mutations, it is theoretically possible that a mtDNA defect involving a COX gene could alter the kinetic or binding properties of the enzyme but allow normal assembly and therefore normal immunostaining of COX subunits.

3.5.5 Conclusions

There appears to be a relatively specific pattern of COX subunit loss in COX deficiency secondary to mtDNA mutations. This means that COX subunit immunohistochemistry can be used to identify patients likely to have mtDNA defects. After excluding mtDNA depletion by Southern blot analysis, mtDNA should be selectively sequenced in these patients. However mutations in genes involved in

delivery of prosthetic groups to mtDNA-encoded COX subunits may mimic this pattern of immunostaining. The *COX10*, *SCO1* and *SCO2* genes should therefore be sequenced in any patient with selective loss of immunostaining to mtDNA-encoded COX subunits who does not appear to have a primary mtDNA mutation.

The majority of children with COX deficiency do not have selective loss of mtDNA-encoded COX subunits. It therefore seems likely that nuclear gene defects account for a large proportion of childhood-onset COX deficiency.

Chapter 4

Analysis of Mitochondrial DNA

Chapter 4 Analysis of Mitochondrial DNA

4.1 Introduction

In Chapter 3 patients were identified who had a characteristic 'mitochondrial' pattern of COX subunit immunostaining in skeletal muscle. These patients had reduced immunostaining of one or more mtDNA-encoded COX subunits (see section 3.4.2). Chapter 4 describes further analysis of this subgroup of patients. These patients were investigated using the techniques of Southern blotting, long range polymerase chain reaction (PCR) and mtDNA sequence analysis, to determine whether a precise mtDNA defect could be identified in each case.

4.2 Case histories

In Chapter 3 patients P1, P8, P14, P15, P25, P26, P31 and P35 were found to have a similar pattern of COX subunit immunostaining to control patients with known mtDNA mutations (C1 to C5). Patient identification numbers are those used in Table 3.1 in Chapter 3. These 8 patients were therefore selected for further analysis of mtDNA. However two patients (P6 and P10) with this pattern of staining were not included in the mtDNA analysis described below. The reason for this is that immunostaining of these patients was performed after the mtDNA sequencing studies had already been commenced for the other patients and insufficient time remained to add P6 and P10 to the analysis.

4.2.1 P1 Epilepsy and progressive myopathy

P1 was first seen by paediatric neurologists at the age of 10 years. The elder child of healthy nonconsanguineous parents, he was born at term by normal delivery and had normal early development. His 5 year old sister was well but another sibling, born prematurely at 8 months gestation, had intrauterine growth retardation and dysmorphic features and died less than 6 hours after birth.

At the age of 3 years P1 developed brief episodes of sudden collapse, which were thought to be seizures. These were eventually well controlled with sodium valproate. At the age of 6 years he developed weakness, affecting upper limbs more than lower limbs and distal muscles more than proximal, with decreased deep tendon

reflexes. He also developed bilateral ptosis and difficulty with eye movements. Creatine kinase was elevated at 429 IU/L (normal <150 IU/L). Plasma and CSF lactates were elevated at 3.2 and 2.1 mmol/L respectively (reference range <1.8 mmol/L for blood and <2.0 mmol/L for CSF). Neurophysiological studies revealed myopathy on electromyogram (EMG) and sensory neuropathy on nerve conduction studies. Visual evoked potentials were normal, but a small amplitude electroretinogram (ERG) was suggestive of dysfunction of the outer retinal receptors. Magnetic resonance imaging (MRI) of the brain and electrocardiogram (ECG) were normal. Muscle biopsy revealed numerous RRF and COX-negative fibres. Routine screening for mtDNA deletions and the 'common' mtDNA point mutations at nucleotide positions (np) 3243, 8344 and 8993 was negative.

4.2.2 P8 Fatal infantile lactic acidosis with multisystem disease

The younger child of healthy unrelated parents, this boy was born at term weighing 4 kg, with good Apgar scores. He was initially well, although his mother felt that he was always a slow feeder and an 'unhappy' baby. At his 6 week check he was not fixing, following or smiling and a hearing test revealed bilateral sensorineural hearing loss (SNHL). At 9 weeks his feeding problems worsened, with episodes of choking, and at 10 weeks he developed a right-sided divergent squint. Computed tomography (CT) of the brain was normal at this time. During admission for investigation of his neurodevelopmental delay at 16 weeks he developed respiratory syncytial virus bronchiolitis, leading to cardiorespiratory failure requiring ventilatory support. He was transferred to the regional paediatric intensive care unit (PICU) where he was noted to be hypotonic with absent reflexes, and to have lactic acidosis (plasma lactate 16.8 mmol/L, CSF lactate 15.8 mmol/L, CSF pyruvate 0.29 mmol/L, lactate/pyruvate ratio 55). Echocardiogram revealed biventricular hypertrophy. The liver appeared enlarged and diffusely echogenic on ultrasound examination, hepatic transaminases were elevated (aspartate transaminase AST 134 U/L, reference range 20 to 60; alanine transaminase ALT 305 U/L, reference range 5 to 45) and he had a coagulopathy. There were encephalopathic changes on the electroencephalogram (EEG), with diffuse slowing that was more marked on the right. Ophthalmological examination raised the suspicion of a faint salt and pepper pigmentary retinopathy.

Muscle biopsy demonstrated a gross excess of lipid droplets and virtually undetectable COX staining in muscle fibres (but normal COX staining of smooth muscle in vessel walls), and specific enzyme assays revealed a reduction in the activities of all respiratory chain components (see Table 3.2 in Chapter 3). In addition, there was evidence of mitochondrial proliferation, with an elevated citrate synthase activity of 347.7 nmol/min/mg protein (control range 22.3-168.0). This was confirmed by electron microscopy, which demonstrated the presence of very abnormal mitochondria, with proliferation of concentrically arranged cristae (Figure 3.2F in Chapter 3). Routine screening for mtDNA deletions and mtDNA point mutations at np 3243, 8344 and 8993 was negative.

He continued to deteriorate, with a worsening metabolic acidosis and increasing requirement for bicarbonate and inotropes, and died 6 days after admission to PICU, at the age of 4 months. Post mortem examination was not performed.

4.2.3 P14 Leigh syndrome

The only child of healthy unrelated parents, this girl was born by forceps delivery at term, with a birth weight of 2.78 kg. Gross motor delay was noted by the age of 8 months. She also had ataxia and problems with coordination and expressive language. Family history was significant in that maternal grandmother had diabetes and a maternal aunt was slow to walk.

Initial investigations revealed lactic acidosis (lactate 8.9 mmol/L in plasma with pyruvate 214 μmol/L and lactate/pyruvate ratio 42) and a mild reduction in PDHC activity in cultured skin fibroblasts (0.61 nmol/mg protein/min, with a normal range of 0.7-1.1) assayed by the diagnostic laboratory in Oxford. However the significance of a slightly low PDHC activity in girls is not easy to assess. Subsequent studies demonstrated normal activity of the lipoamide dehydrogenase (E3) component of the PDHC and so far no mutations have been identified in the E1α subunit of the complex. She commenced a daily dose of 100 mg thiamine at the age of 3.5 years, with initial apparent improvement in her ataxia. However thiamine was subsequently withdrawn without any apparent change in her clinical course. CSF lactate was also elevated (8.1 mmol/L) and CT brain revealed low attenuation lesions in the caudate head and the lentiform nuclei corresponding to high signal abnormality on subsequent T2 weighted MRI. These findings are consistent with a diagnosis of

Leigh syndrome. EMG demonstrated evidence of myopathy. A muscle biopsy showed COX staining of all fibres, but this appeared less than in a control stained alongside. Respiratory chain enzyme assays revealed reduced activities of complexes I, II/III and COX (see Table 3.2 in Chapter 3). Routine screening for mtDNA point mutations at np 3243, 3271 and 8993 was negative.

Her clinical course has been one of gradual deterioration. Between the ages of 10 and 11 years she developed a right hemiplegia. She has also had episodes of irregular breathing. Currently at 15 years she is unable to walk independently, and has a windswept posture and four limb dystonia with generalised weakness and brisk reflexes. She has jerky nystagmus and pale optic discs but a full range of eye movements. She has mild to moderate learning difficulties and attends a special needs school.

4.2.4 P15 Multisystem disease with dysmorphic features

This 15 year old boy was admitted to the intensive care unit following spinal fusion to correct a thoracic scoliosis. The elder son of related parents (grandparents were half siblings), he was born at term weighing 1.87 kg. Initially he fed poorly and required admission to the special care baby unit for 4 weeks. His hips were noted to be stiff during an admission for bilateral inguinal herniae repair at 3 months. He subsequently developed Perthes disease affecting both hips. He also had SNHL, severe developmental delay, absence seizures, a generalised sensorimotor polyneuropathy and short stature treated with growth hormone. He had facial dysmorphism which was not typical of any described syndrome. Plasma lactate was normal (2.0 mmol/L) as were creatine kinase, liver function, CT brain and echocardiogram. Raised AST and lymphopaenia were noted on occasion. Following spinal fusion he had a prolonged postoperative course on PICU because of difficulties in extubation due to poor respiratory reserve. He was eventually discharged home ten weeks postoperatively, with a tracheostomy in situ. A younger brother, aged 13 years at the time of P15's admission, has similar problems, including SNHL, speech delay, orthopaedic abnormalities and short stature treated with growth hormone.

Paraspinal muscle biopsy taken at the time of spinal fusion revealed type I fibre predominance and a small number of RRF. These RRF were COX-negative and

had enhanced SDH activity. Overall COX activity was at the bottom end of the normal range (see Table 3.2 in Chapter 3). Abnormally structured mitochondria were noted on electron microscopy. Routine screening for mtDNA deletions and point mutations at np 3243, 8344 and 8993 was negative.

4.2.5 P25 Fatal infantile lactic acidosis with multisystem disease

P25 was born at term with a birth weight of 3 kg. His Apgar scores were normal but by 12 hours of age he was dusky and floppy. He was admitted to the neonatal intensive care unit, where he was noted to have bilateral cloudy corneas, excess skin at the nape of the neck and bilateral pedal oedema. Initial investigations revealed metabolic acidosis with a pH of 7.08 and base deficit 14 mmol/L. He developed seizures on day 2, required ventilatory support from 48 hours of age, and at 4 days he was transferred to the regional PICU. Further investigations revealed hypertrophic cardiomyopathy, renal tubular Fanconi syndrome, bilateral basal ganglia (putaminal) calcification on CT brain, and profound lactic acidosis in blood and CSF (6.3 and 5.2 mmol/L respectively). The EEG was of diffuse low amplitude. Muscle biopsy revealed a mild excess of lipid droplets in some fibres, but with neither RRF nor COX-negative fibres, although the overall staining of COX appeared lower than a control biopsy stained at the same time. The biopsy size was insufficient for respiratory chain enzyme assays. Routine screening for mtDNA mutations was negative.

He was transferred back to his local hospital where intensive support was withdrawn, with informed parental consent, and he subsequently died.

Parents were healthy and unrelated. Mother has previously suffered a single first trimester miscarriage. P25's older brother is well at the age of 5 years and a sister, born since his death, is also well. A maternal aunt has hypothyroidism, whilst another maternal aunt has hyperthyroidism and infertility. There is no maternal history of neuromuscular disease, diabetes, deafness or cardiac problems.

4.2.6 P26 Isolated skeletal myopathy

This boy initially presented at the age of 11 years with a two year history of gradual onset of exertional muscle weakness and fatigue. He had previously been a very

active child, playing rugby, soccer and tennis, but now noted that after prolonged exercise his legs would become painful and weak and that he would usually vomit. He had never noticed dark urine during these episodes of muscle pain and fatigue. By 14 years of age he was only able to walk 10 yards uphill or 20 to 30 yards on the flat before developing tired legs and cramps. He was able to walk through the pain to a maximum of about one mile. Upper limbs were much less severely affected than lower limbs, although he occasionally had weakness and fatigue of his arms after lifting heavy objects. There was no family history of neuromuscular disease, parents were healthy and unrelated, and four younger siblings were all asymptomatic.

On examination at 14 years P26 was generally thin but there was no focal muscle wasting. His weight was between the 10th and 25th percentile for age whilst height was greatly above the 97th percentile. There was mild weakness of shoulder abduction and adduction and hip extension and flexion. Tendon reflexes were normal. There was no evidence of ophthalmoplegia or retinopathy. Resting blood lactate was elevated (4 mmol/L) at the age of 11 years but was normal (0.97 mmol/L) at 14 years. Mild elevation of CSF lactate (2.37 mmol/L) was noted at 14 years. MRI of the brain, echocardiography, ECG, blood creatine kinase and investigations of renal tubular function were all normal. Histochemical staining of biopsied skeletal muscle at the age of 11 years had revealed reduced COX activity, but biochemical assays of other respiratory chain complexes were not performed. A further quadriceps muscle biopsy was obtained at the age of 14 years for detailed biochemical studies, after informed patient and parental consent. These confirmed isolated deficiency of COX (see Table 5.1 in Chapter 5).

4.2.7 P31 Cardiomyopathy, lactic acidosis and liver disease

The second child of healthy unrelated parents, P31 was born by normal delivery after a normal pregnancy including antenatal ultrasound. She was floppy at birth and required resuscitation with oxygen via bag and mask. She had a good Apgar score by 5 minutes. At 12 hours she was noted to be feeding poorly and was tachypnoeic, hypoglycaemic and acidotic. On day 3 of life an echocardiogram revealed dilated cardiomyopathy with biventricular hypertrophy. She had no dysmorphic features, cataracts or hepatosplenomegaly. She was jaundiced and had severe coagulopathy secondary to liver disease. She had lactic acidosis, with

plasma lactate of 15.7 mmol/L, CSF lactate of 21.6 mmol/L and lactate/pyruvate ratio of 58. CT brain was normal, as was plasma creatine kinase. A previous sibling died on day 2 of life with hydrops, cardiomyopathy and hypoglycaemia.
P31 had an open quadriceps muscle biopsy on day 18. This revealed normal muscle architecture but very low COX activity in muscle fibres with much stronger staining activity in extrafusal fibres. She died on day 21 after developing cardiac failure, fever, hypernatraemia and anuric renal failure. Post mortem examination revealed cardiomyopathy and bronchitis with normal neuropathology. Further investigation showed that she did not have any of the mtDNA mutations at np 3243, 3260, 4269, 8344 and 8993, which have all been associated with maternally inherited cardiomyopathy (Turner et al. 1998).

4.2.8 P35 Lactic acidosis, liver disease and renal tubulopathy

The first child of healthy unrelated Nigerian parents, P35 was delivered at term by Caesarean section following fetal distress. Birth weight was 3.1 kg. He had poor weight gain from birth and at 3 weeks was readmitted to hospital because of persistent vomiting and weight loss. Physical examination revealed wasting and mild dehydration. He had metabolic acidosis with base deficit of 14 mmol/L and increased urinary lactate and pyruvate excretion. Plasma lactate was 15.1 mmol/L. He subsequently became jaundiced and developed severe coagulopathy with elevated hepatic transaminase levels (AST 390 U/L, ALT 119 U/L). Ophthalmological examination and CT brain were normal. Metabolic investigations excluded a diagnosis of tyrosinaemia type I. He continued to deteriorate, with worsening lactic acidosis, and died at the age of 12 weeks.

Post mortem examination revealed extensive hepatic necrosis and microvesicular steatosis, with mild intrahepatic cholestasis. Scattered muscle fibres, distal renal tubules and all cardiac muscle cells contained a small amount of neutral fat as fine droplets. There were no RRF but histochemical staining for COX was markedly reduced in skeletal muscle. Respiratory chain enzyme assays demonstrated reduced activities of complexes I, II/III and COX in skeletal muscle (see Table 3.2 in Chapter 3), liver and heart (data not shown). Brain histology appeared normal.

4.3 Results of mtDNA analysis

4.3.1 DNA yield

For several patients only a very small sample of frozen muscle (<10 mg) remained and in some cases this was embedded in OCT fixative. Genomic DNA was extracted from frozen muscle samples of all patients, even if only a small amount of tissue remained, since only a small amount of DNA is required for sequencing studies. Total DNA yield ranged from 4 to 78 μg (median 26 μg).

4.3.2 Southern blot analysis

All patients studied (P8, P14, P15, P31 and P35) had a single strong band with the mtDNA probe, with no evidence of mtDNA rearrangements. Comparison of mtDNA:nDNA signal ratios from patients P8, P14, P15, P31 and P35 with those from a large number of age-matched controls revealed normal levels of mtDNA in all patients with no evidence of depletion (Figure 4.1). Southern blots were not performed for P1, P25 and P26 because of low DNA yield (4 to 6 μg).

4.3.3 Long range PCR

Long range PCR requires less DNA template than Southern blot analysis and was used as an alternative means of detecting mtDNA deletions, particularly for those patients with insufficient DNA available for Southern analysis. The whole mitochondrial genome can be amplified as a single PCR fragment and any abnormal band would be expected to represent a mtDNA rearrangement. Long range PCR of the 8 patients described above (P1, P8, P14, P15, P25, P26, P31 and P35) did not reveal any additional bands consistent with large-scale rearrangements of the mitochondrial genome. Figure 4.2 illustrates the results for P8, P14, P15, P31 and a control.

4.3.4 Mitochondrial DNA sequencing studies

Sequence analysis was performed on muscle DNA in all cases. The mtDNA-encoded genes for COX subunits I, II and III, all 22 mitochondrial tRNA genes and flanking regions were sequenced in 8 patients (P1, P8, P14, P15, P25, P26, P31 and P35) and a control. These mtDNA regions were chosen because mutations of COX subunit and tRNA genes are much more likely to lead to COX deficiency,

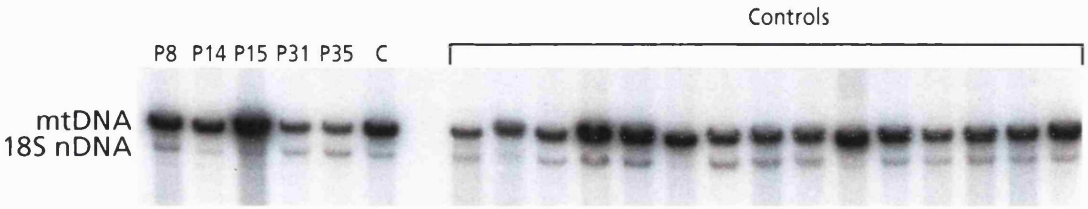


Figure 4.1 Southern blot
Levels of mtDNA in patient skeletal muscle samples (P8, P14, P15, P31 and P35) compared to age-matched controls (C) analysed by Southern blot hybridisation. The Southern blot with *Pvu* II digested total genomic DNA was hybridised simultaneously with two radiolabelled probes: one a cloned fragment of the nuclear gene encoding 18S rRNA, and the other the entire mtDNA sequence. Mitochondrial (mtDNA) and nuclear (nDNA) signals are indicated.

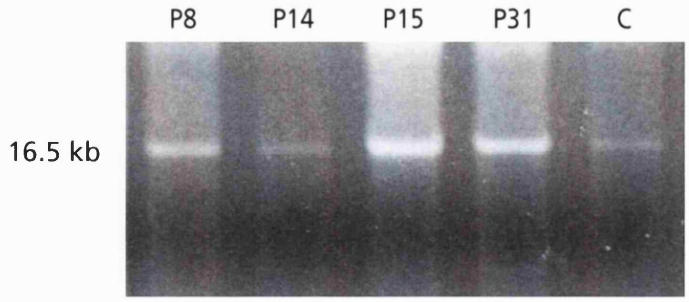


Figure 4.2 Long range PCR of entire mitochondrial genome
A single PCR product of approximately 16.5 kb was amplified from muscle DNA of all patients (P8, P14, P15 and P31 are shown here) and a control (C).

either partial or total, than mutations affecting other mtDNA regions (see section 1.11.1.4 in Chapter 1).

Sequencing studies identified a number of mtDNA base variants from the standard Cambridge reference sequence CRS (Anderson et al. 1981) in each patient. These are listed below and summarised in Tables 4.10 and 4.11. Two base variations have been omitted from these tables because they have recently been identified as errors in the original CRS (Andrews et al. 1999). These errors are: 1) the correct nucleotide at np 9559 (in the COX subunit III gene) is cytosine rather than guanosine; and 2) there is only a single cytosine at np 3106-7 (in the 16S rRNA gene).

4.3.4.1 Base changes observed in P1

Base	Change	Gene	Amino acid change	Comments
7028	C→T	*CO I*	alanine → alanine	known polymorphism
7961	T→C	*CO II*	leucine → leucine	silent polymorphism
8396	A→G	ATPase 8	threonine → alanine	not previously reported
9540	T→C	*CO III*	leucine → leucine	known polymorphism
9758	T→C	*CO III*	serine → serine	silent polymorphism

Table 4.1 mtDNA base changes in P1

Five sequence variations were observed in patient P1 (Table 4.1). Comparison with sequence data available from the Mitomap database (http://www.gen.emory.edu/mitomap.html) revealed that two of these (C7028T and T9540C) have been reported previously as polymorphisms. Two other transitions (T7961C and T9758C) did not alter the encoded amino acid and were thus silent polymorphisms. A fifth base change A8396G, which appeared to be homoplasmic in the sequence chromatogram (Figure 4.3), has also not been reported previously. This transition leads to an encoded alanine instead of threonine at amino acid 11 (T11A) in ATP synthase subunit A6L. Comparative amino acid sequence analysis suggested that this is not a conserved residue (Figure 4.4).

4.3.4.2 Base changes observed in P8

All 6 base changes identified in patient P8 (Table 4.2) are either previously reported or silent polymorphisms.

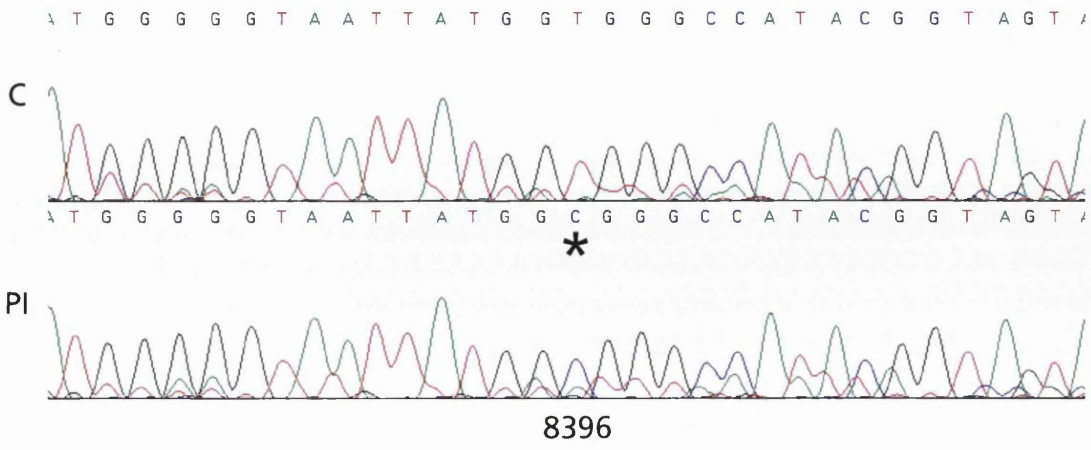


Figure 4.3 Point mutation in the ATPase 8 gene
Electropherogram of automated sequence analysis of a reverse complement region of the ATPase 8 gene encompassing the A8396G mutation (asterisk) in muscle mtDNA from a control (C, upper panel) and P1 (lower panel). Different colours indicate peaks corresponding to each nucleotide. The mutation appears to be homoplasmic.

										11															
Patient	M	P	Q	L	N	T	T	V	W	P	**A**	M	I	T	P	M	L	L	T	L	F	L	I	T	Q	L	K	M
Human	M	P	Q	L	N	T	T	V	W	P	**T**	M	I	T	P	M	L	L	T	L	F	L	I	T	Q	L	K	M
Bovine	M	P	Q	L	D	T	S	T	W	L	**T**	M	I	L	S	M	F	L	T	L	F	I	I	F	Q	L	K	V
Murine	M	P	Q	L	D	T	S	T	W	F	**I**	T	I	I	S	S	M	I	T	L	F	I	L	F	Q	L	K	V
Xenopus	M	P	Q	L	N	P	G	P	W	F	**L**	I	L	I	F	S	W	L	V	L	L	T	F	I	P	P	K	V
Chicken	M	P	Q	L	N	P	N	P	W	F	**S**	I	M	L	L	T	W	F	T	F	S	L	L	I	Q	P	K	L
Honeybee	M	P	Q	M	M	P	M	K	W	F	**L**	I	Y	F	I	Y	L	L	I	F	Y	L	F	I	**M**	L	I	N
Yeast	M	P	Q	L	V	P	F	Y	F	M	**N**	Q	L	T	Y	G	F	L	L	M	I	T	L	L	I	L	F	S

Figure 4.4 Evolutionary comparison of ATP synthase subunit A6L
Amino acid sequence alignment of ATPase subunit A6L from different species. The A8396G mutation in the ATPase 8 gene leads to substitution of alanine for threonine at amino acid residue 11 (shown in red). Mitochondrial genome sequence data obtained from the Organelle Genome Database GOBASE (http://megasun.bch.umontreal.ca/gobase/gobase.html).

Base	Change	Gene	Amino acid change	Comments
1438	A→G	12S rRNA		known polymorphism
4493	C→T	ND2	valine → valine	silent polymorphism
7028	C→T	*CO I*	alanine → alanine	known polymorphism
9899	T→C	*CO III*	histidine → histidine	known polymorphism
10463	A→G	tRNA arginine		known polymorphism
15928	G→A	tRNA threonine		known polymorphism

Table 4.2 mtDNA base changes in P8

4.3.4.3 Base changes observed in P14

Base	Change	Gene	Amino acid change	Comments
1438	A→G	12S rRNA		known polymorphism
7028	C→T	*CO I*	alanine → alanine	known polymorphism
9157	G→T	ATPase 6	alanine → serine	not previously reported
9180	A→G	ATPase 6	valine → valine	known polymorphism
9966	G→A	*CO III*	valine → isoleucine	known polymorphism
10463	A→G	tRNA arginine		known polymorphism
14905		cytochrome b	methionine → methionine	known polymorphism
15928	G→A	tRNA threonine		known polymorphism

Table 4.3 mtDNA base changes in P14

Seven known polymorphisms and a single novel base change G9157T (Figure 4.5) were identified in patient P14 (Table 4.3). The G9157T transversion in the ATPase 6 gene results in the substitution of serine for alanine at amino acid 211 (A211S) in ATP synthase subunit 6. Comparative amino acid sequence analysis of ATP synthase subunit 6 (known as subunit a in yeast) demonstrated that this is a highly conserved residue from yeast to humans, although the honeybee does have a serine at this position (Figure 4.6).

4.3.4.4 Base changes observed in P15

Sequence analysis of mtDNA from P15 revealed 6 known polymorphisms and two new but silent polymorphisms (Table 4.4). In addition there was a 260 bp duplication in the noncoding D-loop region (Figure 4.7). This mutation has been reported previously and is a direct repeat which duplicates both H-strand (nucleotide

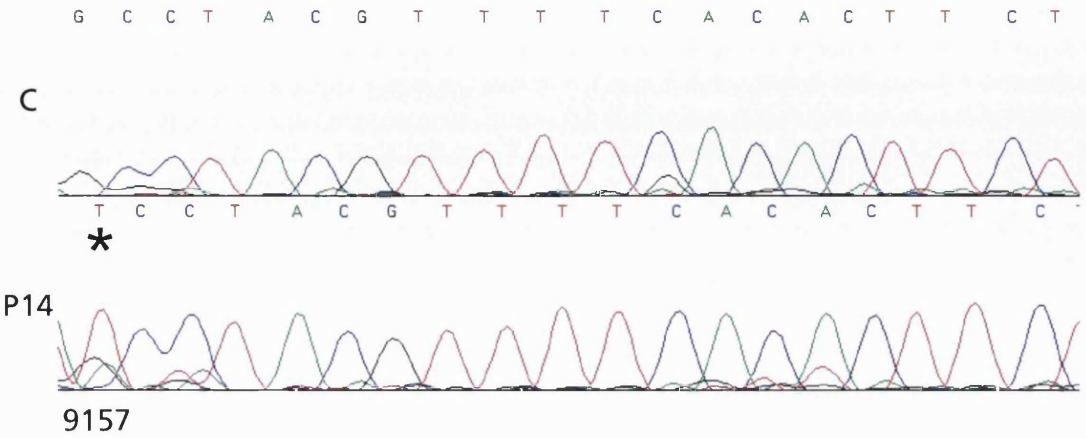


Figure 4.5 Point mutation in the ATPase 6 gene
Electropherogram of automated sequence analysis of a region of the ATPase 6 gene encompassing the G9157T mutation (asterisk) in muscle mtDNA from a control (C, upper panel) and P14 (lower panel). The mutation appears to be heteroplasmic.

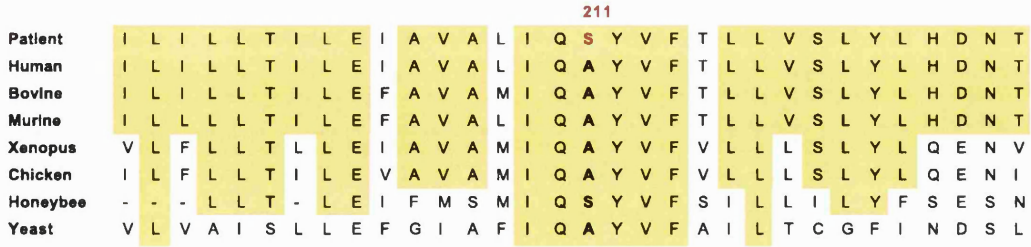


Figure 4.6 Evolutionary comparison of ATP synthase subunit 6
Amino acid sequence alignment of ATPase subunit 6 (yeast subunit a) from different species. The yellow boxes indicate conserved amino acid sequences. The G9157T mutation in the ATPase 6 gene leads to substitution of serine for alanine at amino acid residue 211 (red). Mitochondrial genome sequence data obtained from the Organelle Genome Database GOBASE (http://megasun.bch.umontreal.ca/gobase/gobase.html).

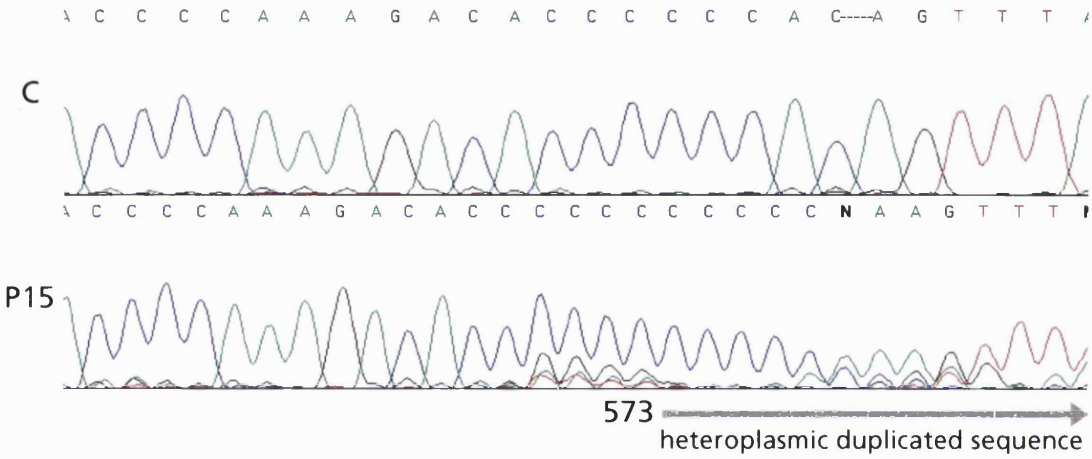


Figure 4.7 D-loop duplication
Electropherogram of automated sequence analysis of part of the D-loop region in muscle mtDNA from a control (C, upper panel) and P15 (lower panel). The region from np 573 onwards is duplicated in P15. The duplication is heteroplasmic, leading to difficulty in reading the sequence in the lower panel since duplicated and wild type sequences are superimposed.

positions np 545-567) and L-strand (np 392-445) promoters, together with the two intervening mitochondrial transcription factor binding sites at np 418-445 and 523-550, the CSBIII (np 346-363), part of the CSBII (np 299-315) and the putative replication primer processing site at np 317-321 (Brockington et al. 1993; Torroni et al. 1994).

Base	Change	Gene	Amino acid change	Comments
573	260bp duplication	D-loop noncoding region		?pathogenic mutation ?polymorphism
6671	T→C	*CO I*	histidine → histidine	known polymorphism
7028	C→T	*CO I*	alanine → alanine	known polymorphism
7476	C→T	tRNA serine UCN		known polymorphism
8386	G→A	ATPase 8	threonine → threonine	silent polymorphism
9181	A→T	ATPase 6	serine → glycine	known polymorphism
10398	A→G	ND3	threonine → alanine	known polymorphism
10499	A→G	ND4	leucine → leucine	known polymorphism
14656	A→G	ND6	lysine → lysine	silent polymorphism

Table 4.4 mtDNA base changes in P15

4.3.4.5 Base changes observed in P25

Base	Change	Gene	Amino acid change	Comments
521-2	del AC	D-loop CA repeat		known polymorphism
1438	A→G	12S rRNA		known polymorphism
10044	A→G	tRNA glycine		?pathogenic mutation
14766	T→C	cytochrome b	isoleucine → threonine	known polymorphism

Table 4.5 mtDNA base changes in P25

Four base changes were observed in patient P25 (Table 4.5). All four have been reported previously. Three are listed as polymorphisms in Mitomap. The fourth base change observed in this patient, A10044G in the tRNA glycine gene (Figure 4.8), has been reported to be pathogenic in a single family previously (Santorelli et al. 1996b). To investigate the aetiological role of the A10044G transition further, two RFLP assays were designed to detect this mutation: one in which the mutation created a restriction site and a second (mismatch PCR) method in which the mutation resulted in loss of a restriction site present in the wild type sequence. In

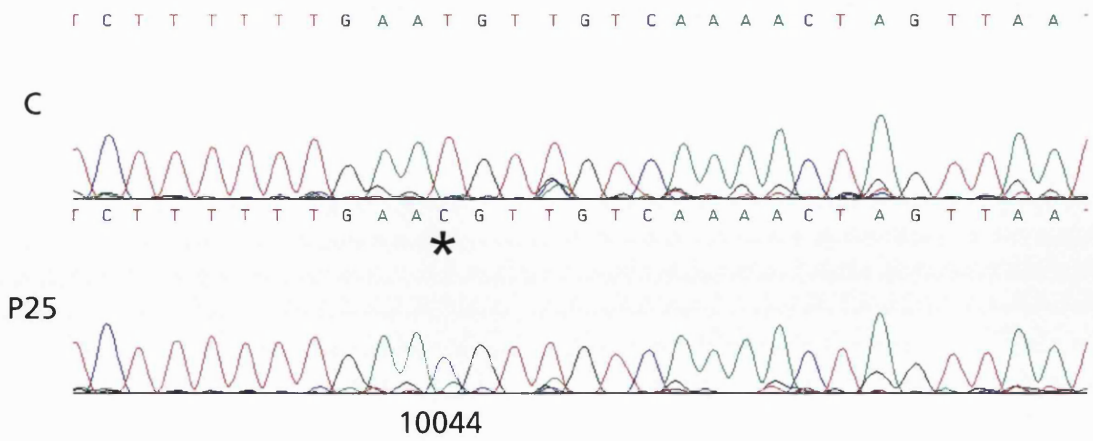


Figure 4.8 Point mutation in mitochondrial tRNA glycine
Electropherogram of automated sequence analysis of a reverse complement region of the tRNA glycine gene encompassing the A10044G mutation (asterisk) in muscle mtDNA from a control (C, upper panel) and P25 (lower panel).

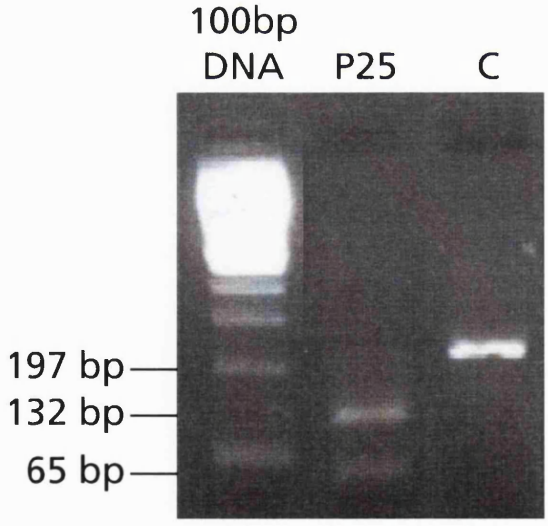


Figure 4.9 Restriction fragment length polymorphism (RFLP) analysis of the A10044G transition in tRNA glycine
Ethidium bromide stained agarose gel showing restriction analysis with Mae II of a 197 bp PCR product obtained from muscle DNA. After digestion with Mae II the PCR product from P25 is completely digested to two fragments of 132 and 65 bp, indicating that P25 is homoplasmic for the A10044G mutation. The PCR product from control muscle (C) does not contain a recognition site for Mae II and therefore remains uncut.

both cases no wild type sequence was detected, even using ABI genescan software, in either the patient s skeletal muscle (Figure 4.9) or cultured skin fibroblasts, or in blood from his asymptomatic mother and brother. This demonstrated that the mutation was homoplasmic in these three individuals. In contrast, only wild type sequence was detected in the father. Figure 4.10 illustrates the position of the A10044G transition in the TψC loop of the predicted secondary structure of the tRNA glycine molecule (upper panel) and evolutionary alignment of mitochondrial tRNA glycine sequences (lower panel). An adenine is conserved at this position in the TψC (pseudouridine) loop in all species except the bovine sequence (Sprinzl et al. 1998).

4.3.4.6 Base changes observed in P26

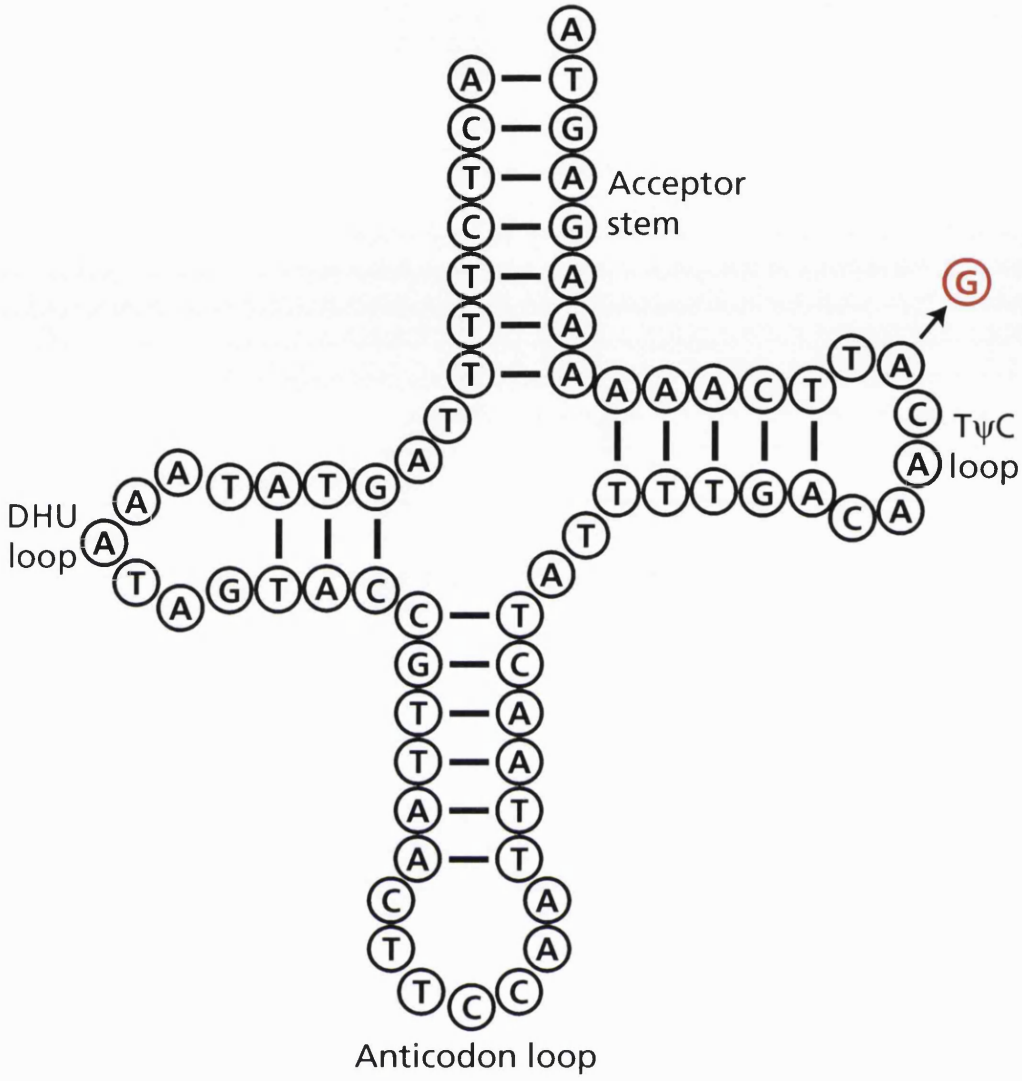
Base	Change	Gene	Amino acid change	Comments
462	C→T	D-loop		known polymorphism
499	T→C	D-loop		known polymorphism
1438	A→G	12S rRNA		known polymorphism
7028	C→T	*CO I*	alanine → alanine	known polymorphism
7671	T→A	*CO II*	methionine → lysine	not previously reported
9755	G→A	*CO III*	glutamic acid → glutamic acid	silent polymorphism
10398	A→G	ND3	threonine → alanine	known polymorphism
14798	T→C	cytochrome b	phenylalanine → leucine	known polymorphism

Table 4.6 mtDNA base changes in P25

P26 had 6 polymorphisms that have previously been reported in Mitomap, and also a previously unreported silent polymorphism G9755A in COX subunit III (Table 4.6). He had one other novel base change, a T to A transversion at np 7671 in COX subunit II (Figure 4.11). Further analysis of the T7671A mutation is described in detail in Chapter 5.

4.3.4.7 Base changes observed in P31

One new but silent transition (G8386A) and 6 known polymorphisms were identified in P31 (Table 4.7).



	TψC stem					TψC loop						TψC stem												
Base position	49	50	51	52	53	54	55	56	57	58	59													
Patient	T	T	T	G	A	C	A	A	C	G		T	T	C	A	A	A	A	A	A	G	A	G	T	A
Human	T	T	T	G	A	C	A	A	C	A		T	T	C	A	A	A	A	A	A	G	A	G	T	A
Bovine	T	T	C	G	G	T	C	T	A	G		T	C	C	G	A	A	A	A	A	G	A	A	T	A
Mouse	T	C	T	G	A	A	T	A	A	A		C	C	C	A	G	A	A	G	A	G	A	G	T	A
Rat	T	C	T	G	A	A	A	A	A	A		C	T	C	A	G	A	A	G	A	G	A	G	T	A
Xenopus	C	T	T	A	G	T	A	G	A	A		T	C	T	A	A	G	A	G	A	A	A	G	T	A
Strongyloc purp	C	T	T	G	G	T	G	A	A	A	A	T	T	T	A	A	G	A	T	A	A	A	G	C	A
Paracentrot liv	C	T	T	A	G	G	G	A	A	A	A	T	C	T	A	A	G	A	T	A	G	A	G	T	A
Drosophila mel	C	T	A	T	T	A	A	T	T				A	A	T	A	G	T	A	T	A	G	A	T	A
Lupinus luteus	G	A	G	G	G	T	T	C	A	A	G	T	C	C	C	T	C	C	T	T	C	C	G	C	T
Saccharomy cer	G	C	G	A	G	T	T	C	G	A	T	T	C	T	C	G	C	C	T	A	T	C	T	A	T	A
Aspergillus nid	G	T	C	G	A	T	T	C	G	A	A	T	T	C	G	A	C	T	A	G	T	C	A	T	A

Figure 4.10 tRNA glycine

Diagrammatic representation of tRNA glycine (upper panel)

Theoretical two dimensional structure of the human mitochondrial tRNA glycine molecule indicating the position of the A10044G transition (red) in the pseudouridine (TψC) loop. DHU, dihydrouridine loop.

Evolutionary comparison of the mitochondrial tRNA glycine gene (lower panel)

Primary sequence alignment of human mitochondrial tRNA glycine with those from various species showing the conservation of an adenine residue at a position analogous to the 10044 position, except in the bovine sequence (comparative sequence data from Sprinzl et al 1998).

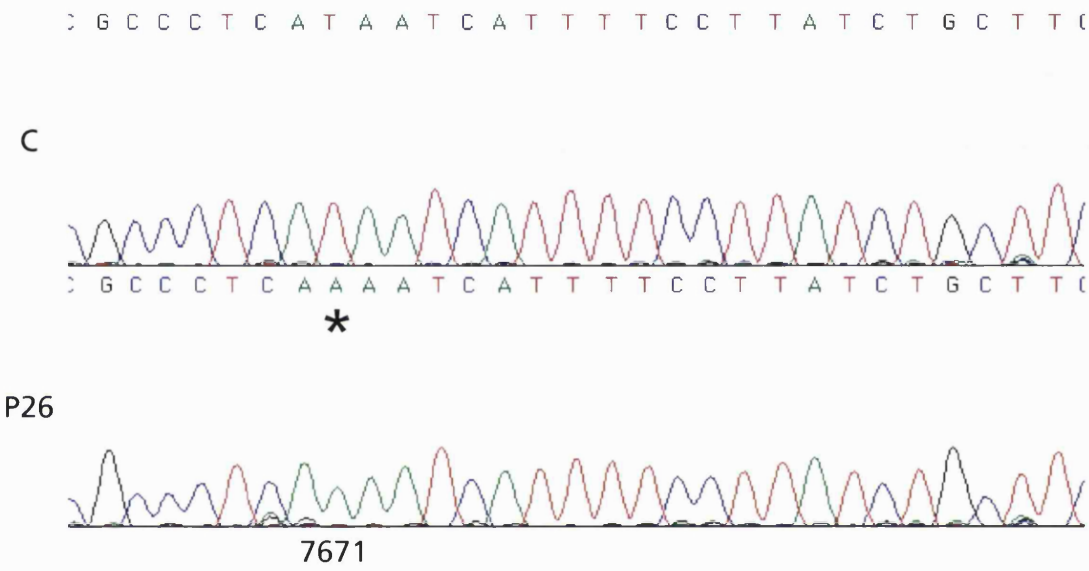


Figure 4.11 Point mutation in COX II
Electropherogram of automated sequence analysis of the region encompassing the T7671A mutation (asterisk) in muscle mtDNA from a control (C, upper panel) and P26 (lower panel).

Base	Change	Gene	Amino acid change	Comments
499	T→C	D-loop		known polymorphism
6671	T→C	*CO I*	histidine → histidine	known polymorphism
7028	C→T	*CO I*	alanine → alanine	known polymorphism
7476	C→T	tRNA serine UCN		known polymorphism
8386	G→A	ATPase 8	threonine → threonine	silent polymorphism
10398	A→G	ND3	threonine → alanine	known polymorphism
10499	A→G	ND4	leucine → leucine	known polymorphism

Table 4.7 mtDNA base changes in P31

4.3.4.8 Base changes observed in P35

Base	Change	Gene	Amino acid change	Comments
521-2	del AC	D-loop CA repeat		known polymorphism
1438	A→G	12S rRNA		known polymorphism
5773	C→T	tRNA cysteine		not previously reported
6221	T→C	*CO I*	proline → proline	known polymorphism
7028	C→T	*CO I*	alanine → alanine	known polymorphism
7396	G→A	*CO I*	cysteine → tyrosine	not previously reported
9449	C→T	*CO III*	tyrosine → tyrosine	silent polymorphism
9540	T→C	*CO III*	leucine → leucine	known polymorphism
10086	T→C	ND3	asparagine → aspartic acid	not previously reported
10398	A→G	ND3	threonine → alanine	known polymorphism
10640	T→C	ND4L	asparagine → asparagine	silent polymorphism
15824	A→G	cytochrome b	threonine → alanine	not previously reported
15940-4	del T	tRNA threonine		?pathogenic mutation ?polymorphism

Table 4.8 mtDNA base changes in P35

Six known polymorphisms, two silent polymorphisms (C9449T and T10640C), 4 novel base changes and a previously reported putative pathogenic mutation 15940-4delT were noted in the sequence analysis of P35 (Table 4.8).

The C5773T transition appeared to be homoplasmic in the sequence chromatogram of P35 s muscle DNA (Figure 4.12). A mismatch PCR method was designed to detect the C5773T mutation in tRNA cysteine by RFLP analysis. This demonstrated

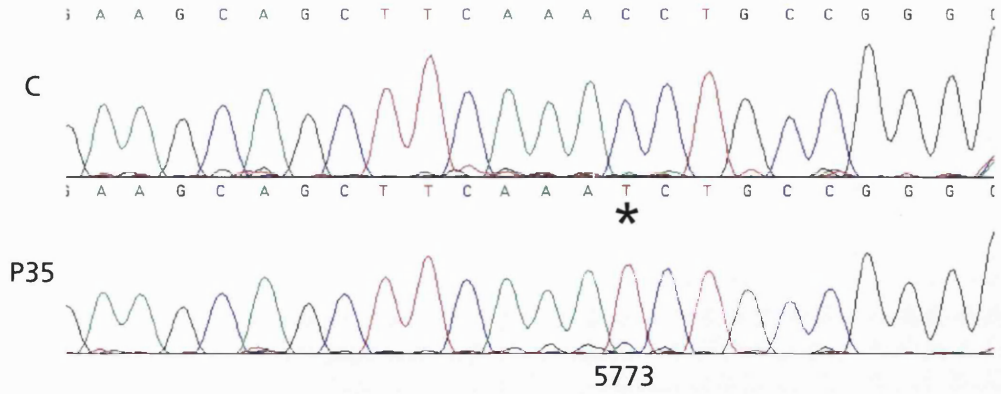


Figure 4.12 Point mutation in mitochondrial tRNA cysteine
Electropherogram of automated sequence analysis of the region encompassing the C5773T mutation (asterisk) in muscle mtDNA from a control (C, upper panel) and P35 (lower panel).

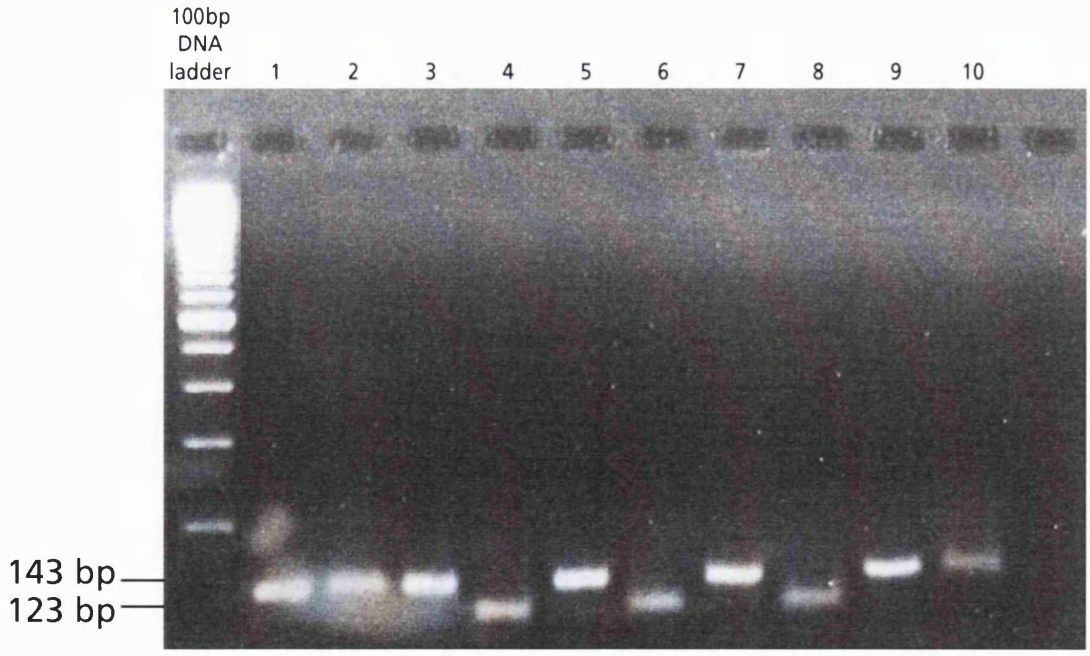
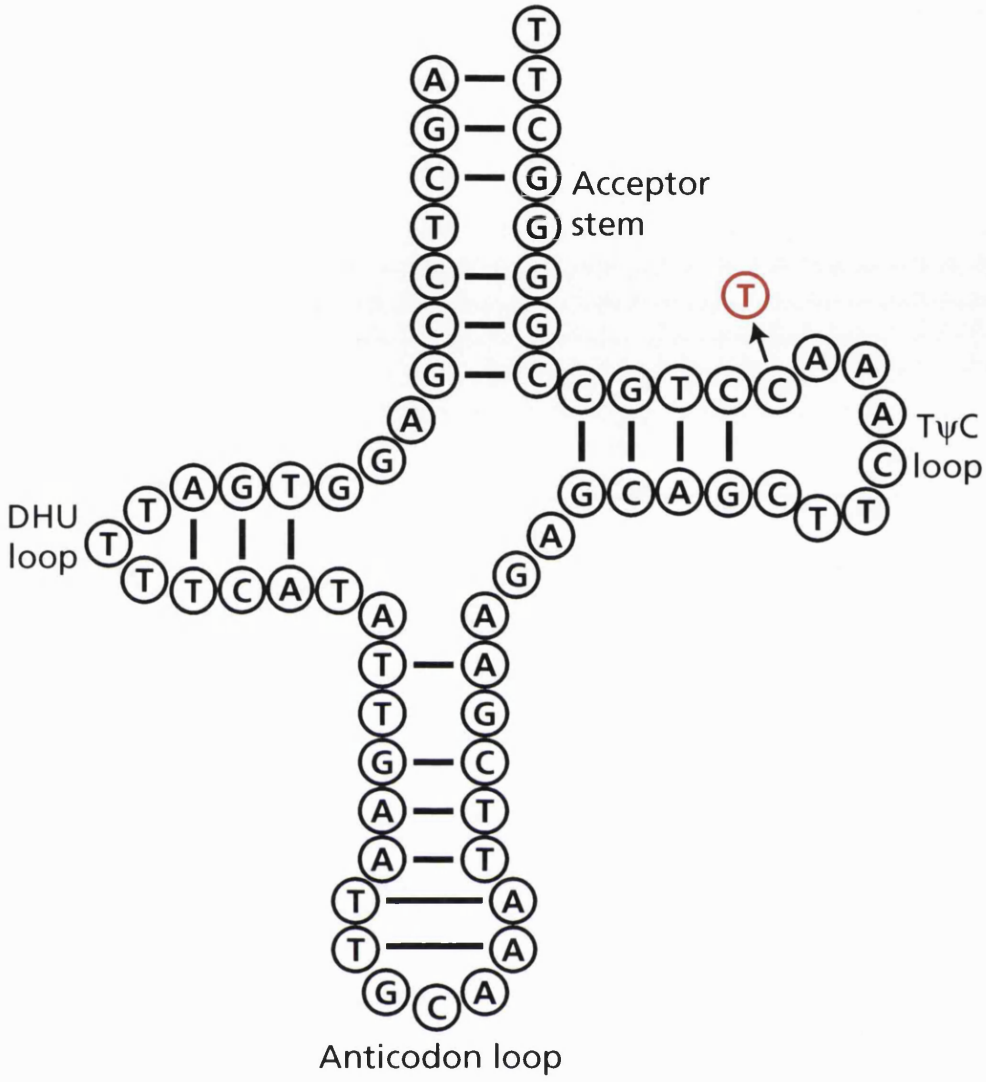


Figure 4.13 RFLP analysis of the C5773T transition in tRNA cysteine
After digestion with *Mbo* I the 143 bp PCR product from the mutant allele yields two fragments of 123 and 20 bp, whilst the wild type allele remains uncut. The 20 bp fragment is not seen in this photograph. ***Lanes 1 and 9:*** PCR products from control muscle DNA; ***lanes 2 and 10:*** control PCR products are not digested by *Mbo* I; ***lane 3:*** PCR product from P35's muscle DNA; ***lane 4:*** P35 muscle PCR product is completely digested by *Mbo* I to yield a 123 bp fragment; ***lane 5:*** PCR product from P35's liver DNA; ***lane 6:*** P35 liver PCR product digested with *Mbo* I; ***lane 7:*** PCR product from P35's kidney DNA; ***lane 8:*** P35 kidney PCR product digested with *Mbo* I. The C5773T mutation appears to be homoplasmic in all tissues studied from P35.



	TψC stem					TψC loop							TψC stem					Aminoacyl stem							
Base position	49	50	51	52	53	54	55	56	57	58	59	60	61	62											
Patient	G	C	A	G	C	T	T	C	A	A		A	T	C	T	G	C	C	G	G	G	G	C	T	T
Human	G	C	A	G	C	T	T	C	A	A		A	C	C	T	G	C	C	G	G	G	G	C	T	T
Bovine	G	C	A	G	C	T	T	C	A	A		T	T	C	T	G	C	C	G	G	G	G	C	T	T
Mouse	G	T	A	G	A	G	A	A	A	T		C	T	C	T	A	C	T	A	A	G	A	C	T	T
Rat	G	T	A	G	A	G	A	A	T	C	T	CC	T	C	T	A	C	T	A	A	G	G	C	T	T
Xenopus	G	C	A	A	A	C	G	A	A	G		G	T	T	T	G	C	C	G	G	G	C	T	T	C
Strongyloc purp	G	C	A	A	A	T	A	G	G	A	C		G	T	T	G	C	C	A	A	A	G	C	T	T
Paracentrot liv	G	C	A	A	T	T	A	G	T	T	C		A	T	T	G	C	C	A	A	A	G	C	T	T
Drosophila mel	G	T	A	A	A	T						T	T	T	T	A	C	T	A	A	G	A	C	T	T
Saccharomy cer	A	A	G	A	G	T	T	C	G	A	T	T	C	T	C	T	T	C	A	T	C	A	T	C	T
Neurospora cra	T	A	G	G		T	T	C	G	A	T	T		C	C	T	A	C	G	T	A	A	T	C	C
Aspergillus nid	A	G	G	G	A	T	T	C	G	A	T	TT	C	C	C	C	G	G	A	C	T	C	C	T	C

Figure 4.14 tRNA cysteine

Diagrammatic representation of tRNA cysteine (upper panel)

Theoretical two dimensional structure of the human mitochondrial tRNA cysteine molecule indicating the position of the C5773T transition (red) in the TψC stem.

Evolutionary comparison of the mitochondrial tRNA cysteine gene (lower panel)

Primary sequence alignment of human mitochondrial tRNA cysteine showing moderate conservation of thymine at residue 61 in the TψC stem of various species. Comparative sequence data from Sprinzl et al 1998.

NCBI Accession no.	Ethnic origin	Base at nucleotide position				
		5773*	7396	10086	15824	15940-4
CRS		C	G	T	A	TTTTT
P35		T	A	C	G	TTTT
AF346980	Ewondo	C	T	C	A	TTCTT
AF346985	Hausa	A	C	C	A	TTTTT
AF346986	Ibo	A	C	C	A	TTTTT
AF346987	Ibo	A	C	C	A	TTTT
AF346992	Kikuyu	C	A	T	A	TTTTT
AF346994	Lisongo	A	C	A	A	TTTTT
AF346995	Mandenka	A	C	A	A	TTTTT
AF346996	Mbenzele	A	C	A	A	TTTTT
AF346997	Mbenzele	A	C	C	A	TTTTT
AF346998	Mbuti	C	T	C	A	TTTTT
AF346999	Mbuti	T	A	T	A	TTTTT
AF347000	Mkamba	C	G	A	A	TTTTT
AF347008	San	A	C	A	A	TTTTT
AF347009	San	A	C	C	A	TTTTT
AF347014	Yoruba	A	C	C	A	TTTTT
AF347015	Yoruba	C	T	C	A	TTTTT
AF346977	Effik	C	G	A	A	TTTTT
AF346976	Effik	C	G	A	A	TTTTT
AF346969	Biaka	A	C	C	A	TTTTT
AF346968	Biaka	A	C	G	A	TTTTT
AF346967	Bamileke	A	C	C	A	TTTTT

Blue refers to Cambridge reference sequence
Red refers to sequence in P35
***denotes light strand sequence**

Table 4.9 African mtDNA sequences

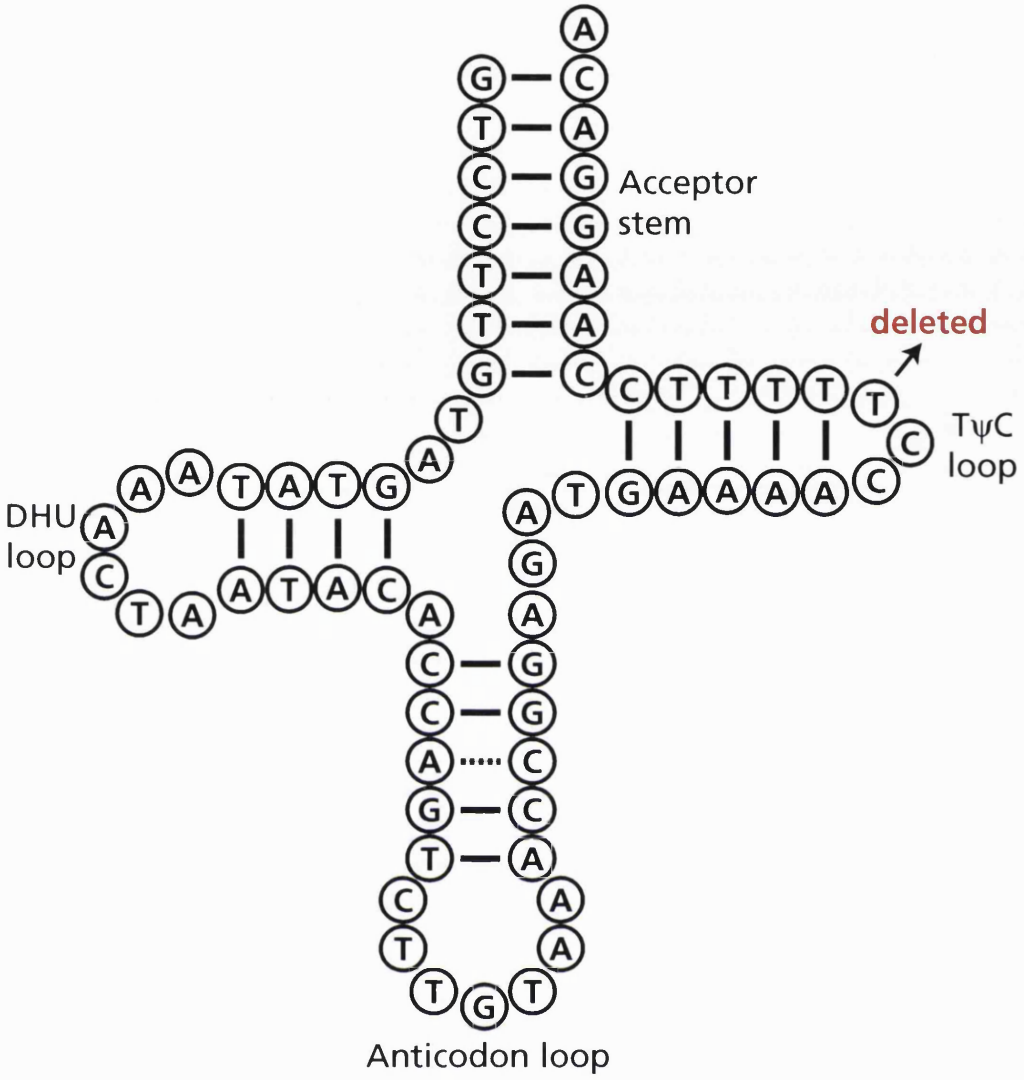
that the mutation was homoplasmic in all tissues available for study from P35 (skeletal muscle, liver, kidney and cultured skin fibroblasts, Figure 4.13). The mutation alters position 61 in the TψC stem of tRNA cysteine (Figure 4.14 upper panel). This nucleotide is not involved in Watson-Crick base-pairing and comparative sequence data suggest that evolutionary conservation of this site is low (Figure 4.14 lower panel). The patient sequence at this site is the same as several other mammals (Sprinzl et al. 1998). Furthermore review of 21 mtDNA sequences from control African individuals in the GenBank database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) revealed the C5773T base change in a Mbuti individual (Table 4.9).

The G7396A transition substitutes an encoded tyrosine for cysteine at amino acid 498 near the C-terminus of COX subunit I. An adenine residue is present at np 7396 in two (a Kikuyu and a Mbuti) of 21 control African sequences in the GenBank database (Table 4.9). Comparative sequence data show that amino acid 498 is conserved from chicken to humans, but not in lower eukaryotes such as yeast (Figure 4.15).

The T10086C transition results in replacement of asparagine by aspartic acid in the ND3 subunit of complex I, whilst the A15824G transition leads to substitution of alanine for threonine in the cytochrome b subunit of complex III. T10086C appears to be a common African polymorphism but the A15824G transition was not present in 21 African control sequences (Table 4.9).

The 15940-4delT microdeletion involves one of a series of 5 thymines located in the TψC stem and loop of the tRNA threonine gene (Figure 4.16 upper panel). Only 4 thymines were present in a control Ibo African sequence (Table 4.9) and comparative sequence data demonstrate low evolutionary conservation of this series of thymine residues (Figure 4.16 lower panel).

Figure 4.15 Evolutionary comparison of COX subunit I
Mitochondrial genome sequence data obtained from the Organelle Genome Database GOBASE (http://megasun.bch.umontreal.ca/gobase/gobase.html).



	TψC stem				TψC loop							TψC stem					Aminoacyl stem							
Base position	49	50	51	52	53	54	55	56	57	58	59	60	61											
Patient	G	A	A	A	A	C	C					-	T	T	T	T	C	C	A	A	G	G	A	C	A
Human	G	A	A	A	A	C	C					T	T	T	T	T	C	C	A	A	G	G	A	C	A
Bovine	G	G	A	G	A	A	C	A	A	C	T	AA	C	C	T	C	C	C	T	A	A	G	A	C	T
Mouse	G	A	A	G	A	T	C	T	T			C	T	C	T	T	C	T	C	A	A	G	A	C	A
Rat	G	A	A	G	A	G	T	C	A			G	T	C	T	T	C	T	C	A	G	G	A	C	A
Xenopus	T	G	A	G	G	C	T	A	A	A	A	C	C	C	T	C	C	T	C	A	A	G	A	C	T
Strongyloc purp	G	A	G	G	G	T	A	A	A	C	T		C	C	C	T	C	T	C	A	A	G	G	C	T
Paracentrot liv	G	A	G	G	G	T	T	A	A	A	G	T	C	C	C	T	C	T	C	A	A	G	G	C	T
Drosophila mel	A	A	G	A	T	T	A					T	T	T	C	T	T	T	A	A	A	A	A	C	T
Saccharomy cer	T	G	G	G	G	T	T	C	A	A	A	T	C	C	C	T	A	A	T	A	T	A	A	C	A
Aspergillus nid	A	C	A	A	G	T	G	C	G	A	T	A	C	T	T	G	T	A	C	T	G	G	G	C	T

Figure 4.16 tRNA threonine

Diagrammatic representation of tRNA threonine (upper panel)

Theoretical two dimensional structure of the human mitochondrial tRNA threonine molecule indicating the position of the 15940 deletion (red) in the TψC loop. The mutation is predicted to decrease the size of the TψC loop compared to the wild type molecule.

Evolutionary comparison of the mitochondrial tRNA threonine gene (lower panel)

Primary sequence alignment of human mitochondrial tRNA threonine with those from various species showing that the run of 5 thymine residues in the human sequence is not conserved across species (bold box). Comparative sequence data from Sprinzl et al 1998.

Base	Change	Gene	Amino acid change	Comments	No. Cases
462	C→T	D-loop		known polymorphism	1
499	T→C	D-loop		known polymorphism	2
521-2	del AC	D-loop CA repeat		known polymorphism	2
1438	A→G	12S rRNA		known polymorphism	5
4493	C→T	ND2	valine → valine	silent polymorphism	1
6221	T→C	*CO I*	proline → proline	known polymorphism	1
6671	T→C	*CO I*	histidine → histidine	known polymorphism	2
7028	C→T	*CO I*	alanine → alanine	known polymorphism	7
7476	C→T	tRNA serine UCN		known polymorphism	2
7961	T→C	*CO II*	leucine → leucine	silent polymorphism	1
8386	G→A	ATPase 8	threonine → threonine	silent polymorphism	3
9180	A→G	ATPase 6	valine → valine	known polymorphism	1
9181	A→T	ATPase 6	serine → glycine	known polymorphism	1
9449	C→T	*CO III*	tyrosine → tyrosine	silent polymorphism	1
9540	T→C	*CO III*	leucine → leucine	known polymorphism	2
9755	G→A	*CO III*	glutamic acid → glutamic acid	silent polymorphism	1
9758	T→C	*CO III*	serine → serine	silent polymorphism	1
9899	T→C	*CO III*	histidine → histidine	known polymorphism	1
9966	G→A	*CO III*	valine → isoleucine	known polymorphism	1
10398	A→G	ND3	threonine → alanine	known polymorphism	4
10463	A→G	tRNA arginine		known polymorphism	2
10499	A→G	ND4	leucine → leucine	known polymorphism	2
10640	T→C	ND4L	asparagine → asparagine	silent polymorphism	1
14656	A→G	ND6	lysine → lysine	silent polymorphism	1
14766	T→C	cytochrome b	isoleucine → threonine	known polymorphism	1
14798	T→C	cytochrome b	phenylalanine → leucine	known polymorphism	1
14905	G→A	cytochrome b	methionine → methionine	known polymorphism	1
15928	G→A	tRNA threonine		known polymorphism	2

Table 4.10 New Silent and Known Polymorphisms

Base	Change	Gene	Amino acid change	Comments	No. Cases
573	260bp duplication	D-loop		?pathogenic mutation ?polymorphism	1
5773	C→T	tRNA cysteine		not previously described	1
7396	G→A	*CO I*	cysteine → tyrosine	not previously described	2
7671	T→A	*CO II*	methionine → lysine	not previously described	1
8396	A→G	ATPase 8	threonine → alanine	not previously described	1
9157	G→T	ATPase 6	alanine → serine	not previously described	2
10044	A→G	tRNA glycine		?pathogenic mutation	1
10086	T→C	ND3	asparagine → aspartic acid	not previously described	1
15824	A→G	cytochrom e b	threonine → alanine	not previously described	1
15940-4	del T	tRNA threonine		?pathogenic mutation	1

Table 4.11 Novel or Known (Putative) Pathogenic Base Changes

4.4 Discussion

4.4.1 Mitochondrial DNA sequencing studies

Fluorescence-based direct sequence analysis was selected for further analysis of mtDNA in these patients because it is considered to be the 'gold standard' for detecting mtDNA variations (Rieder et al. 1998). Other methods that have been used to identify mtDNA base variations include RFLP analysis (Whittam et al. 1986), hybridisation with sequence-specific oligonucleotide probes (Stoneking et al. 1991), denaturing gradient gel electrophoresis DGGE (Yoon et al. 1991b), single strand conformation polymorphism SSCP analysis (Suomalainen et al. 1992a), high-density oligonucleotide arrays (Chee et al. 1996) and denaturing high performance liquid chromatography DHPLC (van Den Bosch et al. 2000). The advantages of direct sequence analysis are that it provides complete information in a single step and is amenable to automation. A potential drawback is that minor degrees of heteroplasmy may be missed on automated sequence analysis. However the clinical significance of low levels of heteroplasmy is not at all clear.

4.4.2 Difficulties in assigning pathogenicity to mtDNA base changes

The highly polymorphic nature of mtDNA leads to considerable difficulties in assigning pathogenicity to novel base changes. Individuals have been reported with as many as 54 base differences from the standard Anderson sequence (Nishino et al. 1996b). Criteria for pathogenicity have been suggested (DiMauro and Schon, 2001) but none are absolute.

Absence of the identified base change in a large number of unaffected individuals from different ethnic groups is generally considered to be an essential criterion to establish pathogenicity of a mtDNA mutation. Population genetics considerations are also helpful as mutations that have arisen more than once in the human population, in association with the same disease phenotype and on different background mtDNA haplotypes, are much more likely to be pathogenic. This is true for the 'common' tRNA point mutations associated with MELAS and MERRF syndromes.

Comparative sequence analysis may also be helpful, as a base change in an evolutionarily conserved region (that is therefore thought to be important for

function) would appear likely to be pathogenic. However interspecies comparisons of mtDNA are not absolute and may be misleading.

For mutations in protein-coding genes a base change that results in a premature stop codon or frameshift would almost certainly be pathogenic. Slightly less strong evidence for pathogenicity would be an amino acid substitution that could be hypothesised to alter the structure and/or function of the gene product. For tRNA base changes the situation is more complicated in that the mutation needs to be thought likely to introduce a functionally relevant change in the tRNA cloverleaf structure. Reported ways of assessing pathogenicity of tRNA mutations include computer modelling and stability calculations according to the Zuker algorithm (Seneca et al. 1998). However since there is no information available about the three-dimensional structure of mitochondrial tRNAs (Sprinzl et al. 1998), the results of such calculations should be interpreted with extreme caution.

Heteroplasmy is a common feature of pathological mtDNA mutations. Heteroplasmy is a transitional state and suggests either that an observed base change has arisen recently (for benign polymorphisms) or that it is pathogenic and is lethal when homoplasmic (Schon et al. 1997). However homoplasmic base changes may be pathogenic (Wallace et al. 1988), although this can prove extremely difficult to verify. Heteroplasmy of an identified base change is useful as it can be used to demonstrate genotype-phenotype correlation of the mutation, either between individuals, or between tissues or even between individual cells. Degree of heteroplasmy correlating with disease phenotype, either between tissues within an affected individual, or between individuals in a maternal lineage, can be used as an argument for pathogenicity of the identified mutation.

The most compelling argument for pathogenicity is demonstration of a functional effect of the mutation. Single muscle fibre mutation analysis is increasingly being used to achieve this purpose, but is limited to patients with a histochemically definable abnormality such as COX-negative fibres or RRF. Cybrid studies are also a useful (but laborious) means of demonstrating a functional effect of mtDNA mutations. Pathogenicity is inferred if respiration deficiency is observed in cybrids obtained by repopulating mtDNA-less ρ^0 cells with the mutant mitochondrial genome.

A potential danger in the identification of pathogenic mtDNA mutations is inadvertent amplification of ancient mtDNA variants that have been preserved as pseudogenes in the nuclear genome (Wallace et al. 1997). Such sequences include nuclear *CO1* and *CO2* pseudogenes which appear to have diverged from modern human mtDNAs approximately 770 000 years ago (Wallace et al. 1997). One study mistakenly assigned pathogenic mtDNA mutations in mtDNA *CO1* and *CO2* genes in patients with Alzheimer's disease (Davis et al. 1997). However the samples had to be boiled in order to amplify these nuclear pseudogenes and only very low levels of apparent heteroplasmy were detected. In this thesis DNA for sequencing studies was extracted by standard techniques (direct SDS-proteinase K digestion with organic extractions) and the mutations discussed below were all present at high heteroplasmic or homoplasmic loads and are therefore unlikely to represent pseudogene sequences. Furthermore the sequence was otherwise identical to modern human mtDNA (nuclear pseudogenes usually have a number of base changes on the same strand) and so it seems unlikely that amplification of nuclear pseudogenes occurred in this study.

4.4.3 Significance of base changes observed in P1

The C7028T polymorphism is characteristic of European haplogroup H, which represents 40.5% of all European mtDNAs (Wallace et al. 1999). The two silent base changes T7961C and T9758C identified in P1 should be added to the list of known human mtDNA polymorphisms, but linked to disease unless they are subsequently observed in healthy control individuals. The only novel and potentially pathogenic base change noted in P8 is the A8396G transition in the ATPase 8 gene.

4.4.3.1 The A8396G transition in ATPase 8

The A8396G transition in the ATPase 8 gene results in the substitution of alanine for threonine in ATP synthase subunit A6L. Several features suggest that A8396G may not be the pathogenic mutation in P1. Firstly it appeared to be homoplasmic on the sequence chromatogram (Figure 4.3). Secondly threonine and alanine are of a similar size and neither is charged (the former is an uncharged polar amino acid whilst the latter is nonpolar), so that substitution of alanine for threonine may not significantly alter protein function. Since the amino acid change appears to be

conservative in terms of chemical properties, it might be selectively neutral and occur rapidly. Indeed interchange between threonine and alanine is one of the most frequently observed amino acid changes in humans (Horai et al. 1995). Thirdly comparative sequence analysis showed lack of evolutionary conservation of this residue (Figure 4.4). Finally it is difficult to explain why a mutation in the ATPase gene might lead to COX deficiency. The function of subunit A6L of ATP synthase is not known (Taanman and Williams, 2002), so it is not possible to postulate a pathogenetic mechanism. Mutations reported in the mtDNA ATPase 6 gene, including T8993G, T8993C and T9176C, are usually associated with normal activities of respiratory chain complexes I to IV in biopsied skeletal muscle (De Meirleir et al. 1995; Vazquez-Memije et al. 1996; Santorelli et al. 1996), although there are occasional reports of COX-negative fibres in these patients (Santorelli et al. 1997d; Uziel et al. 1997; Seneca et al. 1996).

4.4.4 Significance of base changes observed in P8

A pathogenic base change was not identified in the tRNA or COX genes of P8 in this study. The biochemical defect observed in this patient was severe reduction of all respiratory chain enzyme complexes (Table 3.2 in Chapter 3). Such a global reduction of respiratory chain enzyme activity is most often seen in patients with tRNA mutations, large-scale mtDNA rearrangements or mtDNA depletion. The former has been excluded by sequence analysis and no mtDNA rearrangements were observed on long range PCR analysis. However insufficient DNA was extracted from this patient's muscle to allow Southern blot analysis, so mtDNA depletion has not been excluded in this patient.

Other possibilities are that he has a mutation elsewhere in the mitochondrial genome, for example in the D-loop region or involving one of the rRNA genes, or that he may have a nuclear gene defect that affects global mtDNA expression, i.e. a defect of intergenomic communication.

The presence of the A10463G and G15928A tRNA substitutions in P8 means that he belongs to mtDNA haplogroup T. Interestingly, significantly reduced activities of complexes I and IV have been observed in spermatozoa of individuals from haplogroup T, compared to members of haplogroup H. This has led to the

suggestion that haplogroup T may be particularly prone to developing respiratory chain defects (Ruiz-Pesini et al. 2001).

4.4.5 Significance of base changes observed in P14

P14 also appeared to belong to haplogroup T. The only novel and potentially pathogenic base change noted in P14 is the G9157T transversion in the ATPase 6 gene.

4.4.5.1 The G9157T transversion in ATPase 6

The G9157T transversion in the ATPase 6 gene results in the substitution of serine for a highly conserved alanine in ATP synthase subunit 6 (Figure 4.6). Serine and alanine are very similar in size and differ only by a hydroxyl group, which makes serine an uncharged polar amino acid, whereas alanine is nonpolar. Although the former is relatively hydrophilic and the latter relatively hydrophobic, the similarity in size between these two amino acids suggests that the difference in polarity might not be that significant. Substitution of serine for alanine may thus not be a significant change.

The pathogenic mutation in P14 therefore remains obscure. However as she had a generalised respiratory chain defect (see Table 3.2 in Chapter 3), it is possible that the responsible mutation may lie in a part of the mitochondrial genome not sequenced in the present study, for example in a mtDNA-encoded subunit of complex I.

4.4.6 Significance of base changes observed in P15

The presence of the A10398G polymorphism means that P15 belongs to one of three European mtDNA haplogroups: I, J or K (Wallace et al. 1999). The most significant sequence variation observed in P15 was a 260 bp duplication in the noncoding D-loop region.

4.4.6.1 The 260 bp D-loop duplication

There has been much debate in the literature about the pathogenic role of the 260 bp D-loop duplication. It was initially reported by Brockington, in 18 of a series of 58 patients with large-scale mtDNA deletions (Brockington et al. in 1993). The

duplication was also present in 5 of their mothers (all those in whom biopsied muscle was available for study), none of whom had deletions. It was not found in 62 normal controls and it was suggested that the duplication might somehow predispose to the formation of the large-scale deletions. In all cases the duplication was heteroplasmic and present at low abundance (≤ 5%) in muscle. However Torroni reported that it appeared to be an ethnic polymorphism associated with the Caucasian mtDNA haplogroup I (Torroni et al. 1994). In individuals from this ethnic group the duplication was present at low levels, <2% of total mtDNA. Subsequently Manfredi demonstrated high levels of the duplication (32% in muscle) in a patient with a slowly progressive mitochondrial myopathy with RRF and partial COX deficiency (Manfredi et al. 1995b). Manfredi postulated that the duplication might be pathogenic per se, if present at sufficiently high levels. However transmitochondrial cybrid studies (Hao et al. 1997) demonstrated that cybrids homoplasmic for the duplication have normal OXPHOS function and normal mtDNA copy number and transcription, suggesting that the duplication is unlikely to be primarily pathogenic. It may represent an ethnic polymorphism, as suggested by Torroni et al. in 1994, but it remains possible that it may act synergistically with another more pathogenic mutation to produce a disease phenotype. The report by Li , in which a patient with the 'common' A3243G MELAS mutation also had the 260 bp duplication, would seem to support such a synergistic role for this duplication (Li et al. 1996). In Li's patient the duplication was heteroplasmic, 12.5% in muscle and 1.6% in blood, and was also present in maternal relatives.

It is not clear whether the duplication is contributing to the disease phenotype in P15. No primary mutations have been identified in tRNA or COX subunit genes in P15, but a complete mitochondrial genome sequence is necessary to exclude another primary mutation.

4.4.7 Significance of base changes observed in P25

The most significant base change observed in P25 is the A10044G transition in the tRNA glycine gene.

4.4.7.1 The A10044G transition in tRNA glycine

The A10044G mutation has previously been reported to be pathogenic in a single family whose members had severe encephalopathy and sudden unexpected death in association with partial defects of complexes I and IV (Santorelli et al. 1996b). Three generations of this family, including both asymptomatic and affected individuals, had very high mutant loads in blood, ranging from 85% to 99%. In contrast the A10044G mutation was homoplasmic in both the patient (P25) and his asymptomatic mother and older brother in the present study. Santorelli et al. reported absence of this mutation in 90 controls (including whites, African-Americans and Asians). More recently an unpublished report of this mutation has appeared in Mitomap. The mutation was homoplasmic in pancreatic tumour and normal duodenum of a 43 year old Caucasian American male (data submitted to Mitomap in August 2000 by Jessa B Jones from Johns Hopkins University, http://www.gen.emory.edu/cgi-bin/mitomapmutant#20000825012).

Severe COX deficiency is compatible with a tRNA glycine mutation, since all 3 mitochondrially encoded COX subunits have relatively abundant glycine residues (Merante et al. 1994). The A10044G mutation alters position 58 in the TψC loop of the tRNA glycine secondary structure (Figure 4.10 upper panel). Comparative sequence data (Sprinzl et al. 1998; http://www.uni-bayreuth.de/departments/biochemie/sprinzl/trna/) suggest that evolutionary conservation of this position is fairly high, although the normal bovine sequence is the same as P25 (Figure 4.10 lower panel).

Taken together, the lack of complete evolutionary conservation of np 10044 and the observation of homoplasmy of the mutation in two asymptomatic individuals and a normal tissue from a third subject, suggest that evidence for a primary disease-causing role of the A10044G mutation is weak. Another base change involving the TψC loop of tRNA glycine, at np 10042, has been reported in a patient with PEO (Sternberg et al. 1998). This was also homoplasmic and was not considered to be pathogenic. The TψC loop of tRNA glycine may thus be a relative hotspot for well-tolerated base changes.

In contrast the only one other pathogenic mutation reported in the tRNA glycine gene, a T→C transition at nucleotide 9997, involved the acceptor stem of the tRNA.

This heteroplasmic mutation caused maternally inherited hypertrophic cardiomyopathy associated with a RRF myopathy (Merante et al. 1994). A subsequent study confirmed pathogenicity of this mutation by demonstrating that transfer of mitochondria from a patient with hypertrophic cardiomyopathy carrying the mtDNA 9997 mutation to ρ^0 cells, which lack mtDNA, resulted in respiratory chain dysfunction of the resulting transmitochondrial cybrids. Cybrid clones exhibited COX and complex I deficiency, and had an elevated lactate/pyruvate ratio (Raha et al. 1999).

In conclusion the A10044G base change seems more likely to represent a benign, although rare, polymorphism than a pathogenic mutation.

4.4.8 Significance of base changes observed in P26

The silent polymorphism G9755A in COX subunit III should be added to the list of known polymorphisms, but linked to disease unless it is subsequently reported in a healthy control.

4.4.8.1 The T7671A mutation in COX subunit II

Further analysis of the T7671A transversion has demonstrated that this mutation is pathogenic in P26 and is described in detail in Chapter 5.

4.4.9 Significance of base changes observed in P31

A pathogenic base change was not identified in the tRNA or COX genes of P31 in this study. However as she had a combined defect of complexes I and IV in her skeletal muscle, it is possible that she has an ND or rRNA mutation. The G8386A mutation in ATPase 8 should be added to the list of known human mtDNA polymorphisms, but linked to human disease unless it is identified in a healthy control individual.

4.4.10 Significance of base changes observed in P35

A large number of base changes were identified in P35, including four that have not been reported previously (C5773T, G7396A, T10086C and A15824G). This baby was born to African parents and some of the observed base changes might

represent polymorphisms specific to an African mtDNA haplotype. It is known that African and European mtDNA sequences may vary by as many as 93 nucleotides (Horai et al. 1995). The A10398G polymorphism is present in 96% of Africans, compared to 25% of Europeans (Wallace et al. 1999). The two novel silent polymorphisms C9449T and T10640C should be added to the list of known polymorphisms, but linked to disease unless they are subsequently found in healthy control individuals.

4.4.10.1 The C5773T transition in tRNA cysteine

Several features suggested that the C5773T transition is likely to be a benign polymorphism. It was homoplasmic in all tissues of P35, affected a nonconserved residue that is unlikely to be of functional significance (Figure 4.14) and occurred in a control African sequence in the GenBank database (Table 4.9).

4.4.10.2 The G7396A transition in COX subunit I

Pathogenicity of the G7936A transition in *CO I* also seems unlikely, for similar reasons. It leads to substitution of tyrosine for cysteine in a nonconserved region of COX subunit I (Figure 4.15). Both cysteine and tyrosine are uncharged polar amino acids. The former has a sulfhydryl group whilst the latter has a hydroxyl group, but replacement may not cause a large functional effect in the polypeptide. Furthermore structural considerations suggest that this residue is not in contact with any other subunit in the COX holoenzyme and is thus unlikely to have a significant functional role. This base change occurs in 2 of 21 control African sequences (Table 4.9) and I have also observed it in a 9 year old Caucasian boy with the Hutchinson-Gilford progeria syndrome, a completely different phenotype from P35. The sequence chromatogram of both patients was apparently homoplasmic for G7396A. Taken together, these observations suggest that G7396A probably represents a benign polymorphism, although it is possible that it may be contributing to the disease phenotype in P35.

4.4.10.3 The T10086C transition in ND3

The T10086C transition results in replacement of asparagine by aspartic acid in ND3. Asparagine and aspartic acid are of similar size and differ only by an amide group, which makes the former an uncharged polar amino acid whilst the latter is a

charged polar amino acid. Both are hydrophilic and substitution between the two is unlikely to be significant. Control African sequence data demonstrated that T10086C appears to be a common polymorphism (Table 4.9).

4.4.10.4 The A15824G transition in cytochrome b

The A15824G transition in cytochrome b leads to substitution of alanine for threonine. As discussed for P1, threonine and alanine are of similar size and substitution is unlikely to be significant (see section 4.4.3.1 above). The A15824G transition was not noted in sequence analysis of the cytochrome b gene of 32 Caucasians (Andreu et al. 1999a) nor in 21 control African sequences (Table 4.9). It may therefore represent either a rare polymorphism or a private polymorphism or possibly be contributing to the disease phenotype in P35.

4.4.10.5 The 15940-4 microdeletion in tRNA threonine

The 15940-4 delT point deletion has been described previously in two apparently unrelated families, one Turkish and the other Italian (Seneca et al. 1998). The clinical phenotypes of affected family members were heterogeneous, but all were homoplasmic for the mutation. In the Turkish family 3 of 4 children were affected. The first suffered 'severe neurological distress' at 6 hours of age and had hyperammonaemia. This child died on day 4 of life. A second child had dysmorphic features, truncus arteriosus and severe hypotonia, whilst the third had severe hypertrophic cardiomyopathy with large mitochondria in a myocardial biopsy. The proband of the Italian family was a 38 year old man with painful cramps and sensory neuropathy. His muscle biopsy revealed subsarcolemmal mitochondrial aggregates and patchy loss of COX staining. His brother's muscle was also abnormal and their mother had severe retinopathy, chronic renal failure and sensorimotor peripheral neuropathy. These clinical phenotypes are obviously very diverse and differ from that observed in P35. Furthermore although Seneca et al. did not identify the 15940-4 delT mutation in more than 70 disease and healthy controls, this base change has since been observed in association with another phenotype – colorectal tumour (reported to Mitomap by Kornelia Polyak at the Johns Hopkins Oncology Center in March 1999, http://www.gen.emory.edu/cgi-bin/mitomapmutant#19990316054).

Using a computer algorithm, Seneca et al. predicted that the 15940-4 delT mutation would destabilise the TΨC loop of the mutant tRNA threonine. They also argued that the absence of any other identifiable tRNA mutation in either family supported a pathogenic role for this mutation. However it is possible that the mutation could lie in another part of the mitochondrial genome, such as the rRNA genes or the polypeptide-coding genes, or that the causative mutation might be in a nuclear gene. Furthermore the 15940-4 point deletion was homoplasmic and present in asymptomatic family members in their study. In conclusion, a primary pathogenic role for this mutation seems doubtful.

Two other mutations have been reported in the tRNA threonine gene, both associated with lethal infantile phenotypes. Yoon et al. reported two cases of lethal infantile mitochondrial myopathy (LIMM), with lactic acidosis and severe respiratory chain enzyme deficiency, who had mutations in the mtDNA tRNA threonine gene, at nucleotides 15923 and 15924 (Yoon et al. 1991a; Yoon et al. 1993). Both mutations occurred within the tRNA anticodon stem-loop structure. However in a subsequent study Brown found the np A15924G mutation in approximately 11% (11/103) of Caucasian controls (Brown et al. 1992a). This therefore appears to be a relatively frequent ethnic polymorphism and is unlikely to be the cause of the LIMM phenotype in Yoon's patient. The np 15923 mutation was not detected in 163 controls (91 Caucasians, 35 Africans, and 57 Asians) but its aetiological role in LIMM is not yet proven.

Thus no proven pathogenic mutations have been reported in the tRNA threonine gene, and evidence for a pathogenic role of the 15940-4 del T mutation is weak. In summary, none of the base changes noted in P35 appear to be conclusively pathogenic.

4.4.11 Conclusions

This chapter has described the results of mtDNA analysis in 8 patients, confirmed the extremely polymorphic nature of mtDNA and highlighted the difficulties inherent in trying to establish the pathogenic status of novel base changes. Only one mutation observed in this study (T7671A in COX subunit II) incontrovertibly fulfils criteria for pathogenicity. The evidence for this is described in detail in Chapter 5. In other cases (A10044G in tRNA glycine, 15940-4 delT in tRNA threonine and the

260 bp D-loop duplication) it was not possible to conclusively assign pathogenicity to the observed base changes, even though they have previously been reported to be pathogenic. Haplogroup classification of patient mtDNAs and search for the putative disease mutation in phylogenetically closely related control mtDNAs is vitally important in determining pathogenicity (Rocha et al. 1999). Finally, in some of the patients described in this chapter a potentially pathogenic mtDNA mutation has not been identified. There are various possible explanations for this. A pathogenic mtDNA mutation may lie outside the regions sequenced in this study; a heteroplasmic mutation present at relatively low load might have been overlooked on the automated sequence analysis; or the primary mutation may lie in the nuclear genome. Furthermore some of the apparently homoplasmic base changes observed here may play a synergistic role in disease pathogenesis, either in combination with other mtDNA base changes or with nuclear gene mutations – the so-called 'polygenic model' of mtDNA disease (Rocha et al. 1999).

Chapter 5

The T7671A Mutation in mtDNA

Chapter 5 The T7671A Mutation in mtDNA

5.1 Introduction

In the immunohistochemical studies described in Chapter 3 P26 was shown to have a 'mitochondrial' pattern of subunit staining, with specific loss of immunostaining to COX subunit II (see Table 3.3 and Figure 3.11 in Chapter 3). In subsequent work described in Chapter 4 a specific mutation of mtDNA, T7671A in the gene encoding COX II, was identified in P26 (see Figure 4.11). Chapter 5 describes investigations to determine the detailed consequences of this mutation on enzyme structure and function using immunoblot analysis, spectrophotometric studies, quantitative single fibre PCR analysis and computerised modelling.

5.2 Clinical history

The detailed clinical history of P26 is presented in Chapter 4 (section 4.2.6).

5.3 Morphological and biochemical studies

Histology of biopsied skeletal muscle revealed severe COX deficiency in 97% of fibres (Figure 3.11 in Chapter 3), with increased SDH staining but no frank RRF with the modified Gomori trichrome stain (Figure 3.11G and H). Muscle morphology and histochemistry remained unchanged in two biopsies taken three years apart, at the ages of 11 and 14 years respectively. A defect localised to COX was demonstrated by polarographic and spectrophotometric enzyme assays performed on the second biopsy (Table 5.1 - data from Dr Iain Hargreaves).

5.4 Immunological studies

Immunostaining of frozen muscle sections demonstrated almost complete absence of COX II, with preserved immunostaining of subunits I, IV and Va but reduced staining of subunit Vlc (Figure 3.11). Immunoblot analysis of a mitochondrial protein fraction prepared from skeletal muscle confirmed the immunohistochemical finding of a severe reduction of COX II compared to controls (Figure 5.1). Further immunoblot analysis with antibodies directed against other COX subunits demonstrated reduced levels of

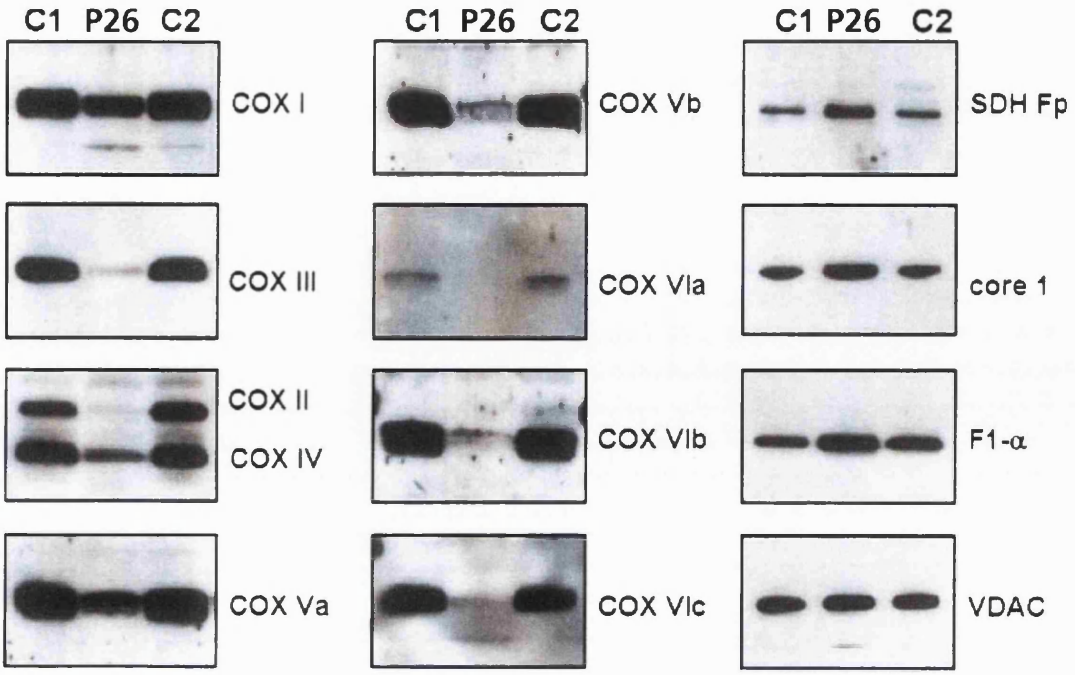


Figure 5.1 Western blot of muscle from P26
Immunoblot analysis of skeletal muscle mitochondrial fractions, from P26 (P) and two controls (C). Blots were developed with subunit-specific monoclonal antibodies to COX subunits, the flavoprotein subunit of SDH (SDH F_p), core protein 1 of complex III (core 1), the a subunit of F_1-ATP synthase (F_1-α), and VDAC.

Polarography - Mitochondrial Oxygen Uptake Rates		
Substrate	**n atoms O/min/mg**	**Control range[1]**
glutamate/malate	**26.5**	53.3-312.0
succinate	**34.5**	68.6-376.1
TMPD-ascorbate[a]	**65.4**	124.7-464.7
Respiratory Chain Enzyme Assays		
Enzyme complex	**nmol/min/mg**	**Control range[2]**
Complex I	167.1	27.8-190.0
Complex II/III	87.3	18.3-234.3
Complex IV[b]	**2.4**	5.4-46.0
Citrate synthase	1745.92	289.7-1916.5

Table 5.1
[1] Controls for polarographic studies were 25 muscle biopsies obtained with from patients receiving hip transplants (age range 25 – 70 years).
[2] Controls for spectrophotometric studies were initially 6 muscle biopsies from paediatric patients having orthopaedic surgery who had no neuromuscular disease. These data were later extended to include 120 'disease controls', aged 2 months - 8 years, assigned retrospectively after no evidence of mitochondrial respiratory chain defects were detectable in their skeletal muscle biopsies.
All controls were taken with informed patient/parent consent and local ethics committee approval.
[a] TMPD = N,N,N',N'-tetramethyl-p-phenylenediamine.
[b] Units measured were k/min/mg.

all subunits investigated. There was mild reduction of subunits I, IV and Va, with more marked reduction of subunits III, Vb, VIa (heart/muscle isoform), VIb and VIc (Figure 5.1). In addition immunoblot analysis revealed increased steady-state levels of other respiratory chain and oxidative phosphorylation subunits: flavin protein of SDH, core 1 subunit of complex III and the alpha subunit of F_1F_0-ATP synthase (Figure 5.1). Immunoblotting for the voltage-dependent anion channel (VDAC or porin) was used to demonstrate equal loading of samples (Figure 5.1).

5.5 Spectrophotometric studies

These studies were performed by Dr Brigitte Meunier in the Department of Biology at University College London. In order to measure the COX I-associated haem a_3 levels in the muscle biopsies from controls and the COX deficient patient, mitochondria were prepared from 10-20 mg of skeletal muscle. The samples were reduced by dithionite, treated with carbon monoxide (CO), and CO flash-photolysis and recombination signals were monitored (Meunier and Rich, 1998a). Figure 5.2 shows the optical signals obtained with mitochondria from a control and P26. The traces were biphasic. The fast component (k_{obs} of 500-600 s^{-1}) was due to CO recombination by contaminating haemoglobin (or myoglobin). The slow component (k_{obs} of 60-65 s^{-1}) arose from CO recombination to haem a_3. The major component (approximately 95%) of the CO recombination signal obtained with the COX deficient mitochondria was due to haemoglobin. In the control trace, the main signal (approximately 65%) arose from CO reaction with haem a_3 (k_{obs} of around 60 s^{-1}). The concentration of haem a_3 estimated from the slow component photolysis signal using an extinction coefficient of 113 $mM^{-1}.cm^{-1}$ at 430-445 nm was around 0.7 nM in the sample from P26 and around 11 nM in the control sample. Haem a_3 content was determined in a second control sample and gave 6 nM (data not shown). It appeared therefore that CO-reactive haem a_3 content was significantly decreased in the mitochondria from P26.

5.6 Mitochondrial DNA studies

Long range PCR analysis excluded the presence of large-scale rearrangements of mtDNA in total genomic DNA extracted from the patient's muscle. Routine screening for the common mtDNA point mutations at np 3243, 8344 and 8993 was also negative. In view of the immunohistochemical and immunoblot findings further

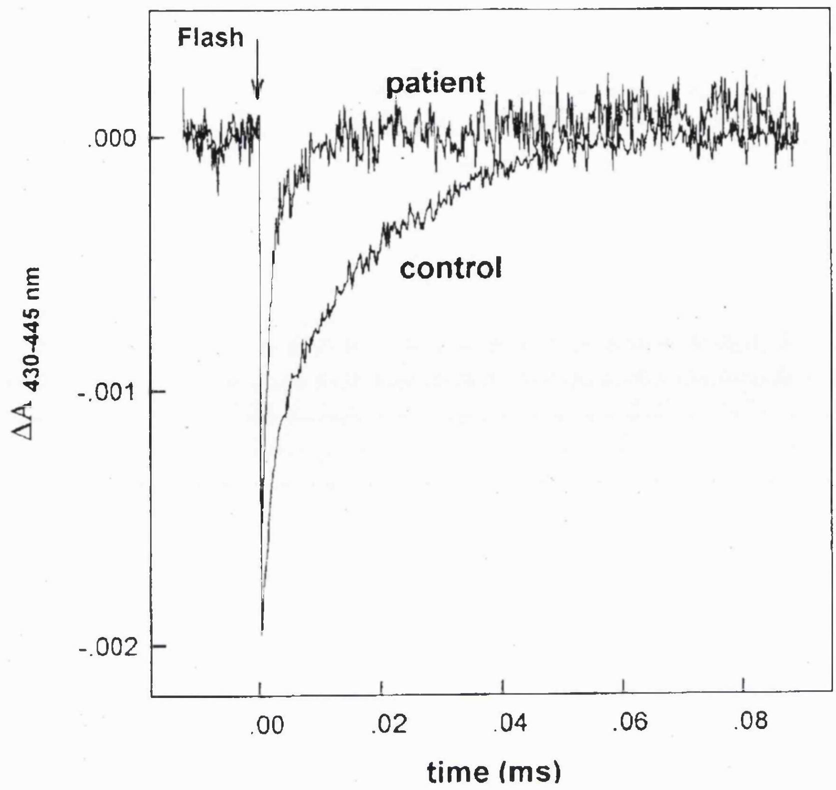


Figure 5.2 Flash photolysis and recombination spectra
Flash photolysis and recombination spectra of the CO-ferroheme a_3 compound of dissolved muscle mitochondria, from P26 and a control. After reduction by sodium dithionite and treatment with CO, laser-flash photolysis and recombination of CO was monitored at 430 - 445 nm versus time.

29

Patient	L Q D A T S	P I M E E L I T F H D H A L K I I F L I C F L V L Y A L F L T	L
Human	L Q D A T S	P I M E E L I T F H D H A L **M** I I F L I C F L V L Y A L F L T	L
Gorilla	L Q D A T S	P I M E E L I I F H D H A L **M** I I F L I C F L V L Y A L F L T	L
Equine	F Q D A T S	P I M E E L L H F H D H T L **M** I V F L I S S L V L Y I I S S M	L
Bovine	F Q D A T S	P I M E E L L H F H D H T L **M** I V F L I S S L V L Y I I S L M	L
Feline	F Q D A T S	P I M E E L L H F H D H T L **M** I V F L I S S L V L Y I I S L M	L
Murine	L Q D A T S	P I M E E L M N F H D H T L **M** I V F L I S S L V L Y I I S L M	L
Xenopus	F Q D A A S	P I M E E L L H F H D H T L **M** A V F L I S T L V L Y I I T I M	M
Chicken	F Q D A S S	P I M E E L V E F H D H A L **M** V A L A I C S L V L Y L L T L M	L
Honeybee	F Q E S N S	Y Y A D N L I S F H N M V M **M** I I I M I S T L T V Y I I L D L	F

Figure 5.3 Evolutionary comparison of COX subunit II
Alignment of amino acid sequence in COX subunit II (mitochondrial genome sequence data obtained from the Organelle Genome Database GOBASE (http://megasun.bch.umontreal.ca/gobase/gobase.html). The large box represents the first α-helical region in the bovine structure. The mutation in P26 involves amino acid residue 29 (M29K).

analysis of mtDNA was performed. Direct sequence analysis (see Chapter 4) revealed a novel thymine to adenine transversion at np 7671 of mtDNA (T7671A), within the gene encoding COX II (Figure 4.11 in Chapter 4), resulting in a missense substitution of lysine for methionine at amino acid residue 29 of the polypeptide (M29K). Comparative sequence data demonstrated a high degree of conservation of the first α-helix, including methionine 29, in COX subunit II of vertebrates and the honeybee (Figure 5.3). Other base changes from the Cambridge reference sequence (Anderson et al. 1981) identified in P26 were 6 previously reported polymorphisms and a novel silent polymorphism (see Table 4.6 in Chapter 4 for full list of polymorphisms identified in P26).

Heteroplasmy of the T7671A mutation in P26, with a high mutant load in skeletal muscle, was demonstrated by mismatch PCR amplification followed by RFLP analysis with *Tru*9 I (Figure 5.4). The T7671A mutation was present at 90% in DNA extracted from both skeletal muscle biopsies (at ages 11 and 14 years) but at only low percentage (4.5 - 6%) in the patient's blood (Table 5.2). The mutation was not detected in the mother's blood, nor in 110 normal controls nor 15 individuals with mitochondrial disease in whom the underlying mutation was not known.

The percentage of mutant mtDNA in individual muscle fibres was correlated with COX activity by use of a single fibre PCR assay. This single fibre PCR analysis revealed a significantly higher mean mutant load in COX-negative muscle fibres (81%, n=13) than in COX-positive fibres (45%, n=4) ($p=0.01$, Mann-Whitney U test) and is illustrated in Figure 5.5.

5.7 Cell culture studies

No mutation was detected in cultured myoblasts or fibroblasts at early cell passage and staining for COX activity and COX I immunoreactivity (Figure 5.6) was normal in these cultured cells. Spectrophomometric assays of COX activity were also normal in these cultures (assays were performed by Dr Mark Cooper in the Department of Clinical Neuroscience, Royal Free Hospital - data not shown). Immunoblot analysis of COX subunits was normal in cell protein extracts prepared from myoblast and fibroblast cell pellets (data not shown).

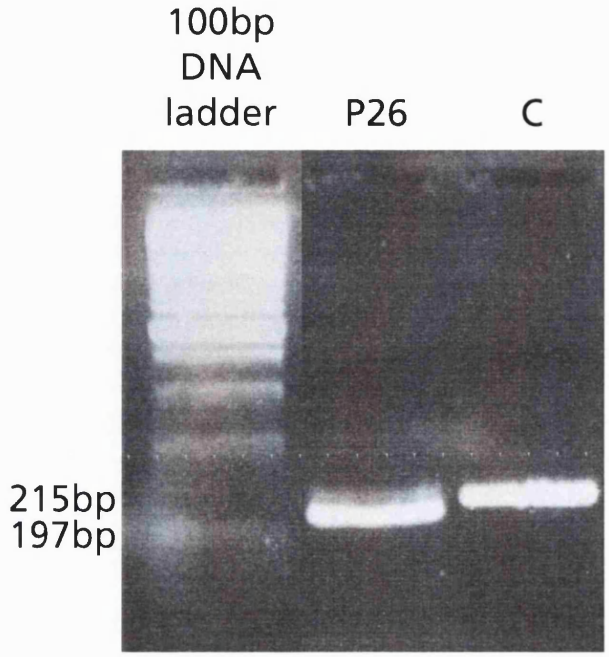


Figure 5.4 RFLP analysis of the T7671A transversion in COX subunit II
Ethidium bromide stained agarose gel showing restriction analysis with *Tru*9 I of a 215 bp PCR product obtained from muscle DNA. After digestion with *Tru*9 I the PCR product from the mutant allele in P26 yields two fragments of 197 and 18 bp (the 18 bp band is not seen in this photograph), whilst the wild type allele remains uncut. C = control.

Tissue	% Mutant Load
muscle (at 11y)	90.4%
muscle (at 14y)	90.5%
blood (at 11y)	6%
blood (at 14y)	4.5%
cultured myoblasts	0%
cultured fibroblasts	0%
mother s blood	0%

Table 5.2
T7671A mutation load in tissues of P26

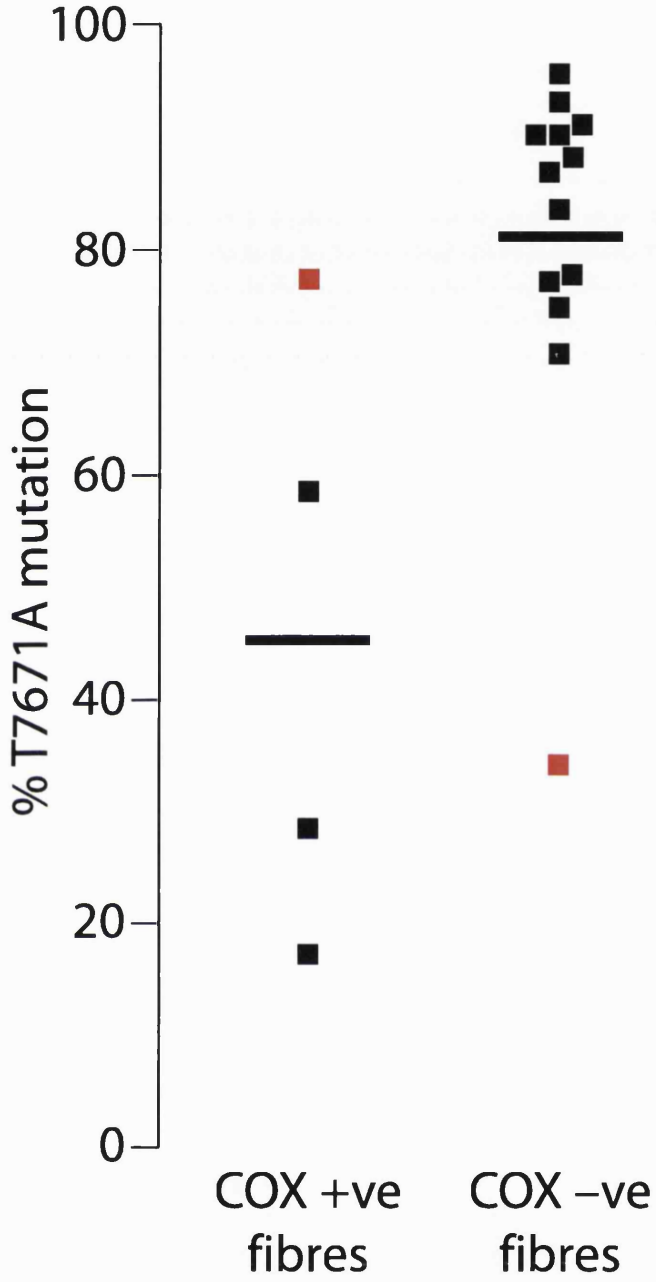


Figure 5.5 Single fibre PCR analysis
Distribution of the percentage of T7671A mutation in single COX-negative and COX-positive muscle fibres in skeletal muscle biopsied from P26 at 11 years of age. The mean proportion of mutant mtDNA in each fibre type is indicated by a horizontal line. Outlying values are indicated in red.

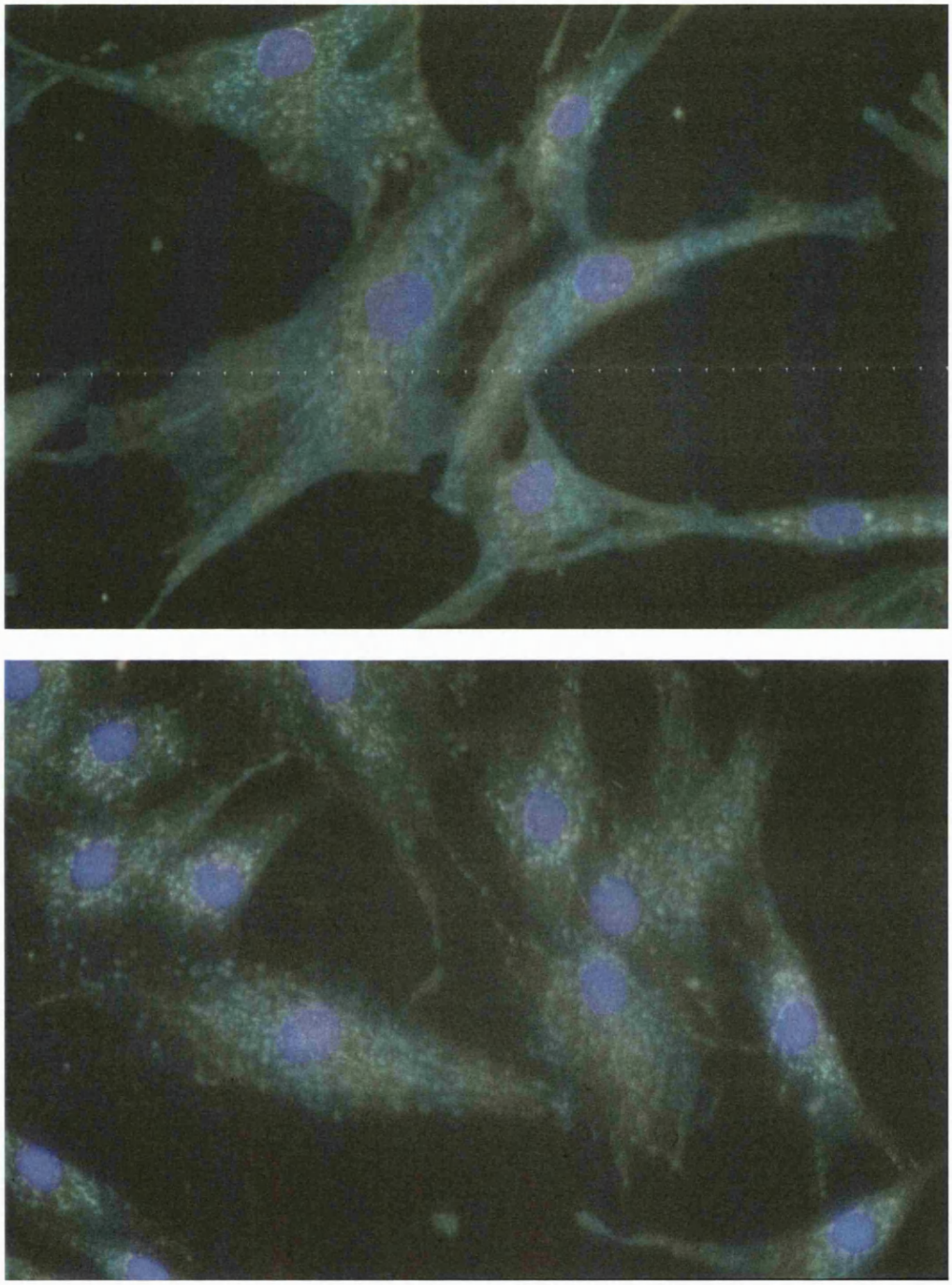


Figure 5.6 Immunocytochemistry of COX subunit I in myoblasts from P26
Green fluorescence represents immunostaining with monoclonal antibody directed against COX subunit I in cultured myoblasts from a control (upper panel) and P26 (lower panel).

5.8 Discussion

5.8.1 The T7671A mutation is pathogenic

This chapter describes the findings in patient P26 who presented with isolated COX deficiency. Biochemical, immunohistochemical and immunoblot analyses strongly suggested a mutation in the mtDNA gene for COX II. A missense thymine to adenine transversion at nucleotide position 7671 (T7671A) was identified in *CO II* and evidence for an aetiological role of this mutation is presented. Sequencing of the remaining mtDNA molecule did not identify any other base changes of likely pathogenic relevance (see section 4.3.4.6 in Chapter 4).

Pathogenicity of the T7671A mutation is supported by several lines of evidence. Firstly it is heteroplasmic and present at high mutant load (90%) in skeletal muscle, the only clinically affected tissue, but at very low mutant load ($\leq$ 6%) in blood. This genotype-phenotype correlation is further supported by the single fibre PCR studies that demonstrated significant correlation between mutant load and COX activity in individual muscle fibres. Thirdly, the mutation alters an amino acid residue that is relatively conserved in vertebrates (Figure 5.3). A non-charged methionine residue is replaced by a basically-charged lysine in the middle of the first N-terminal membrane-spanning domain of COX II (Tsukihara et al. 1996). Fourthly, the mutation was not identified in a large number of ethnically matched control subjects.

5.8.2 Functional effect of T7671A on COX assembly

The mechanism of pathogenesis of the T7671A (M29K) mutation may be inferred from analogy with known yeast mutations. Yeast studies have allowed classification of COX mutations into two major groups: 'activity' mutations and 'assembly' mutations that affect the assembly and/or stability of the COX holoenzyme (Meunier and Rich, 1998b). Activity mutations are generally clustered in COX I and affect the electron transfer or proton pumping functions of the enzyme. COX II is anchored to the mitochondrial inner membrane with an N-terminal helix-hairpin, while its large C-terminal hydrophilic domain, which protrudes into the intermembrane space, contains the mixed-valence binuclear Cu_A centre and serves as the docking site for cytochrome *c* (Capaldi, 1990b; Tsukihara et al. 1996). The M29K mutation in P26

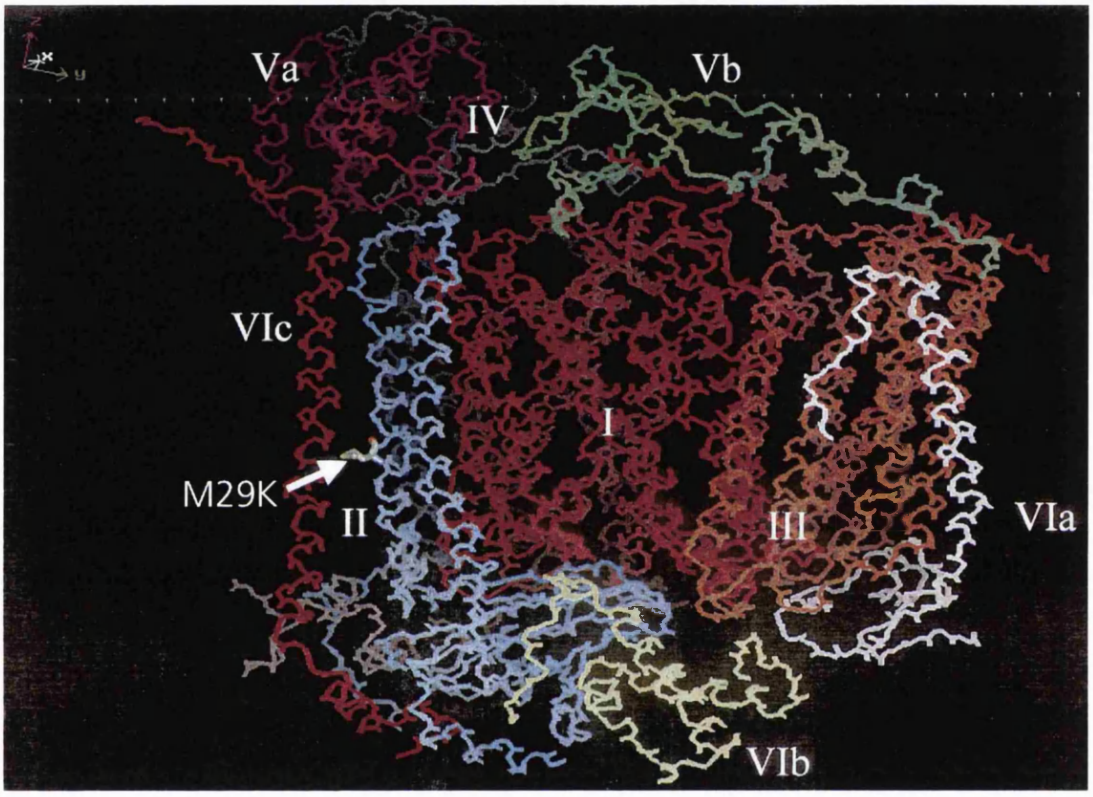


Figure 5.7 Structure of monomer of bovine cytochrome oxidase indicating the theoretical position of the M29K mutation
The diagram shows the assembled bovine COX holoenzyme, with an arrow indicating the site in subunit II that is mutated in P26. The diagram was constructed with use of the data published by Tsukihara et al 1996 in the Quanta program.

converts an uncharged amino acid residue in the highly conserved transmembrane segment of the COX II polypeptide to a basic charged residue (Figure 5.7). The mutation is therefore predicted to interfere with the anchoring of COX II within the mitochondrial membrane and to affect the assembly or stability of the COX holoenzyme rather than the electron transfer function of the subunit.

Studies of the assembly of COX in cultured human cells have identified assembly intermediates of the enzyme (Nijtmans et al. 1998; see Figure 1.5 in Chapter 1). Intermediate S2 contains subunits I and IV. Subunits II and III are subsequently added, together with subunits Va, Vb, VIb, VIc, VIIa, VIIc and VIII. Finally subunits VIa and VIIb are incorporated to complete the assembly of the holoenzyme. Results of the immunohistochemical and immunoblot analyses performed on P26 indicate that the COX subunit II mutation identified here arrests assembly of the COX holoenzyme after formation of S2 (Figure 5.8), since the components of this intermediate (subunits I and IV) are relatively preserved, as determined by immunoblotting, compared to the other subunits investigated.

The studies of Nijtmans et al. did not address the formation of the metal centres of COX. To determine the steady state levels of subunit I-associated haem a_3 in biopsied muscle from P26, the CO flash-photolysis and recombination signals were followed spectrophotometrically (Meunier and Rich, 1998a). CO reacts with reduced COX and produces the CO-ferrohaem a_3 compound. CO can be photolysed by a high intensity short duration laser flash and will then recombine to the haem on a millisecond time scale. CO photolysis and rebinding can be monitored optically and provide a sensitive means of quantitation of COX. Because of the high photolysis yield (>95%) and the high extinction coefficient of the CO-COX compound (ε=113 $mM^{-1}.cm^{-1}$ at 430-445 nm), it is possible to measure accurately COX content even in samples with low level enzyme. Quantitation of COX can be hindered by the presence of other haemoproteins such as haemoglobin or myoglobin which also react with CO. The optical signal induced by the binding of CO on these haemoproteins overlaps that of the CO compound of COX. However, the kinetics of CO recombination to haemoglobin and myoglobin are significantly faster (k_{obs} >500 s^{-1}) than the kinetics of CO recombination to COX (around 60 s^{-1}), allowing the deconvolution of the contribution of COX from the haemoglobin and myoglobin, especially when their concentration is low.

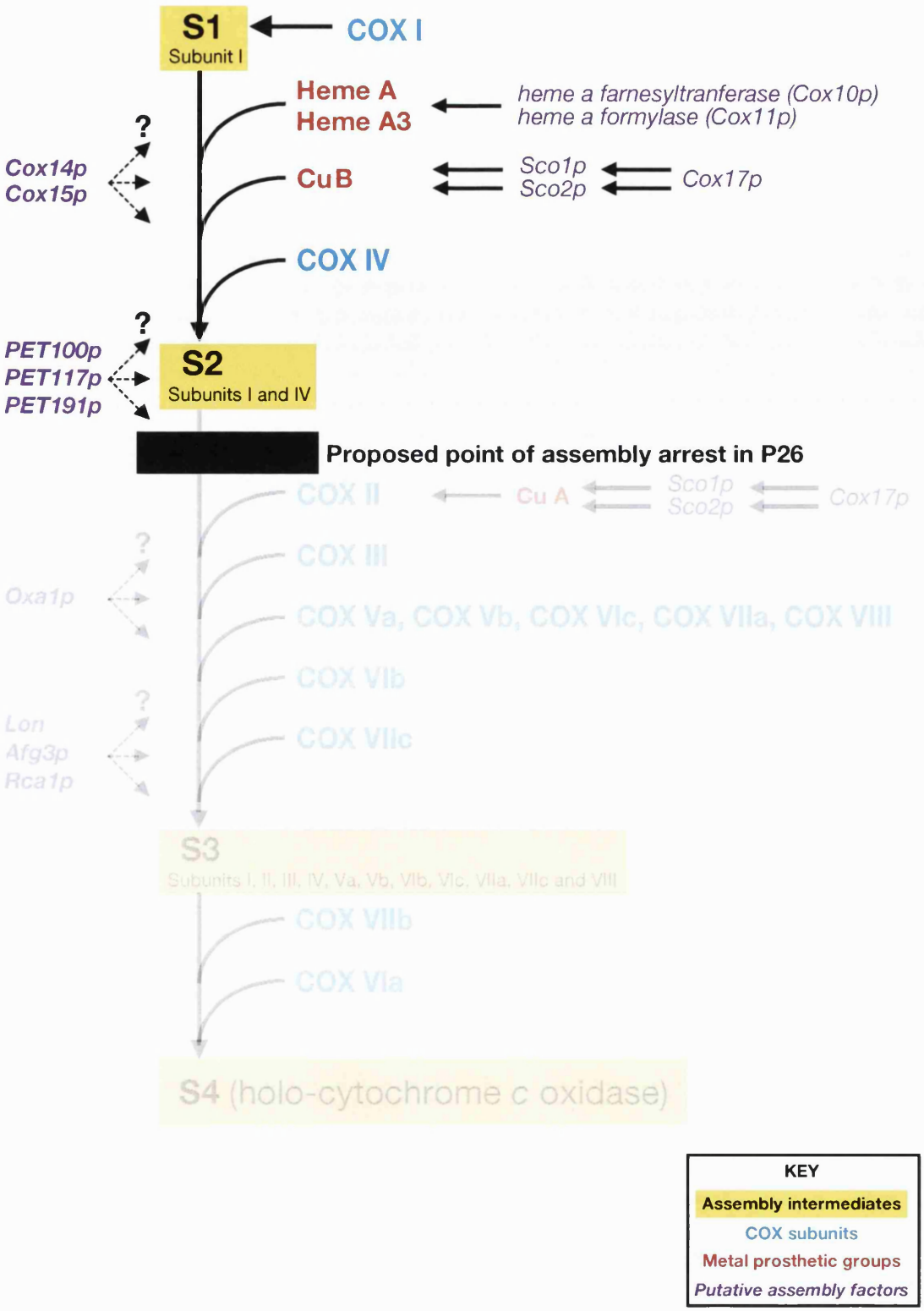


Figure 5.8 Proposed pathway of COX assembly arrest in P26
COX assembly arrest is postulated to occur after formation of assembly intermediate S2, since the M29K (T7671A) mutation is predicted to interfere with COX II anchoring within the mitochondrial membrane.

Immunohistochemical (Figure 3.11) and immunoblot (Figure 5.1) analyses showed that the apo-subunit I levels in P26 were only marginally affected. In contrast, subunit I-associated haem a_3 levels were markedly decreased in P26 (Figure 5.2). Taken together, these data suggest that a structural association of subunit II with subunit I is required to secure binding of the haem a_3 prosthetic group to apo-subunit I.

Further support for the above findings has come from recent studies of the aa_3-type COX of the proteobacterium *Rhodobacter sphaeroides* (Bratton et al. 2000). This aa_3-type COX has three subunits and its structure is virtually identical to the catalytic core of the mitochondrial COX. Genetic manipulation studies in *Rb sphaeroides* revealed that in the absence of COX subunit II an early assembly intermediate was formed, in which subunit I had incorporated a single haem A molecule in a distorted binding site. Subunit II was found to be necessary for the stable addition of a second haem A and the Cu_B centre. This second assembly intermediate had maximal COX activity. The authors concluded that subunit II but not subunit III is necessary for formation of the metal centres in COX subunit I.

5.8.3 Phenotypic considerations

The surprisingly mild phenotype associated with the T7671A mutation in patient P26 probably reflects the tissue distribution of the mutation. It is likely that skeletal muscle, the only tissue affected clinically, is also the only tissue with a mutant load above the threshold required to reduce OXPHOS capacity. This threshold is generally about 85% for mitochondrial tRNA mutations (Bentlage and Attardi, 1996; Schon et al. 1997). However the threshold for phenotypic expression of mutations in mtDNA polypeptide-coding genes appears to be lower than that reported in tRNA mutations (Hanna et al. 1998b). For example, transmitochondrial cybrids harbouring 35-65% mutant load of the G930A nonsense mutation in COX subunit I were COX deficient (Bruno et al. 1999). Furthermore a dinucleotide deletion 8042delAT resulting in frameshift and truncation of the COX II polypeptide was present at ≤20% of total mtDNA in several tissues of a premature infant who died of severe lactic acidosis associated with apnoea and bradycardia (Wong et al. 2001). These findings might be explained by tight posttranscriptional or translational regulation of respiratory chain polypeptide synthesis, compared to relative overproduction of the mitochondrial tRNAs (Bruno et al. 1999). Another possibility is that unassembled

respiratory chain subunits might be degraded rapidly by mitochondrial proteases (Rep and Grivell, 1996). Finally, since functional COX exists as a dimer, a truncated COX subunit might exert a dominant negative effect by binding to a normal COX monomer to form a nonfunctional dimer. This means that a mutant load of 15%, for example, would lead to 28% defective COX holoenzyme (Wong et al. 2001).

Mutations involving mtDNA polypeptide-coding genes seem to present fairly frequently with isolated myopathy or even exercise intolerance alone. Such mutations include two other mutations in COX subunit genes (Keightley et al. 1996; Karadimas et al. 2000), two mutations in ND genes of complex I (Andreu et al. 1999b; Musumeci et al. 2000) and 9 mutations in the cytochrome b subunit of complex III (reviewed by DiMauro and Andreu, 2000). Mutations in tRNA genes, on the other hand, are usually associated with multisystem features such as MELAS and MERRF syndromes (Goto et al. 1990; Shoffner et al. 1990). Exceptions to these generalisations include a *CO III* mutation associated with MELAS (Manfredi et al. 1995a) and two mutations in *CO II*: T7587C, which presented as an encephalomyopathy with ataxia, dementia and optic atrophy (Clark et al. 1999); and G7896A, which was associated with an early onset multisystem disorder including encephalopathy and hypertrophic cardiomyopathy (Campos et al. 2001). Therefore there does not appear to be any correlation between which COX subunit is mutated and the clinical phenotype. The tissue distribution of the mutation is likely to be more important.

The lack of RRF in patient P26 is also of interest. The first mutations described in mtDNA polypeptide-coding genes were associated with LHON and NARP (neurogenic muscle weakness, ataxia and retinitis pigmentosa) syndrome (Wallace et al. 1988; Holt et al. 1990). These patients did not have RRF and it was initially thought that lack of mitochondrial proliferation might be a general characteristic of polypeptide-coding mutations (Schon et al. 1997). Accordingly, there were no or very few RRF in some of the patients reported to have COX mutations (Manfredi et al. 1995a; Hanna et al. 1998b; Bruno et al. 1999). However, although P26 does not have RRF, he does have evidence of muscle mitochondrial proliferation with the SDH stain. Furthermore RRF were observed in two patients with mutations involving COX subunit genes (Keightley et al. 1996; Comi et al. 1998). Both these patients had microdeletions but the nature of the mutation cannot explain the difference in

muscle morphology since both groups contain mutations resulting in premature termination of translation (Hanna et al. 1998b; Comi et al. 1998; Bruno et al. 1999). The overall mutant load in skeletal muscle in patient P26 has not changed over a period of three years, and this is mirrored by clinical stability over this same period. Stability of mutant load was also observed in the patient with the stop mutation at nucleotide position 9952 in the mitochondrial *CO III* gene (Hanna et al. 1998b; see Chapter 4), and may be a common feature of mtDNA COX gene mutations. In contrast mutant load of mtDNA tRNA mutations has frequently been observed to increase with time in skeletal muscle (Fu et al. 1996; Weber et al. 1997). The explanation for this is not clear. Demonstration of low levels of mutation in peripheral blood cells from our patient may reflect clearance of mutation in this rapidly dividing tissue (Rahman et al. 2001). This is supported by the finding of lower mutant load (4.5%) in blood at 14 years compared to 6% at 11 years.

Absence of the T7671A mutation in maternal blood but presence in both muscle and blood of P26 suggests that the mutation may have arisen sporadically in early embryogenesis of this patient. However the possibility of presence of the mutation in the maternal germline cannot be excluded. The apparently sporadic nature of the T7671A mutation concurs with almost all other reported COX subunit mutations. An exception is the T7587C mutation in COX subunit II that was maternally inherited (Clark et al. 1999). Other maternally inherited polypeptide-coding mtDNA mutations include homoplasmic ND mutations associated with LHON and heteroplasmic mutations in the ATPase gene associated with NARP and maternally inherited Leigh syndrome. It is important to note the lack of maternal inheritance of most COX mutations, as defined by muscle mtDNA analysis, because this may lead to a delay in achieving a molecular diagnosis, and because it has significant implications for genetic counselling.

The T7671A mutation was not detected in cultured myoblasts, despite the high mutant load in mature skeletal muscle. Although it is possible that there was selection against satellite cells (undifferentiated muscle precursor cells) and myoblasts containing high levels of mutation during the tissue culture process if they had a growth disadvantage, lack of mutation in satellite muscle cells has previously been reported with another COX point mutation (Hanna et al. 1998b) and also with tRNA point mutations (Fu et al. 1996; Weber et al. 1997). Absence of the mutation

from satellite cells has potential therapeutic implications. Previous studies have demonstrated that in patients with strong segregation of mutation between satellite and mature muscle cells, induction of muscle necrosis, either by bupivicaine local anaesthesia (Clark et al. 1997) or by local trauma (Shoubridge et al. 1997), was followed by repopulation of the muscle with cells containing only wild type mtDNA. It remains to be seen if induction of widespread muscle necrosis will be a viable therapeutic option for such patients. Eccentric exercise has been suggested as a therapeutic rationale to attempt to induce widespread muscle necrosis (Taivassalo et al. 1999) but so far there have not been any clinical trials of this treatment.

5.8.4 Conclusions

The T7671A mutation was one of the first mutations to be identified in the mtDNA gene for COX subunit II (Rahman et al. 1999). The data derived from this study and specifically the coexisting severe deficiency of COX subunit II and haem a_3 indicate that COX subunit II is required for stability of haem a_3 attachment to the holoenzyme. Further evidence for this role of COX subunit II in assembling the metal centres of the enzyme has come from studies of the aa_3-type COX in the prokaryote *Rhodobacter sphaeroides* (Bratton et al. 2000). Thus the clinical, morphological and biochemical consequences of the T7671A mutation provide valuable information about structure/function relationships within the COX holoenzyme.

Chapter 6

Studies of Cultured Cells

Chapter 6 Studies of Cultured Cells

6.1 Introduction

In the immunohistochemical analysis described in Chapter 3, 13 patients with COX deficiency displayed a global reduction of COX subunit immunostaining and a further 10 had normal staining of all COX subunits despite reduced COX activity (see section 3.4.3 and Table 3.3). These two patterns of immunostaining are hypothesised to arise from nuclear gene defects. Chapter 6 describes studies of cultured skin fibroblasts obtained from some of the patients whose muscle biopsies appeared to have a 'nuclear' pattern of COX subunit immunostaining in Chapter 3. These studies were COX staining, COX subunit immunocytochemistry, Western blot analysis and sequencing of the putative candidate gene *SURF1*. Mutations in the COX assembly gene *SURF1* were first described in patients with COX-deficient Leigh syndrome at the end of 1998 (Zhu et al. 1998; Tiranti et al. 1998b), towards the end of the experimental period of this thesis.

6.2 Fibroblasts

Patient identification numbers are those used in Table 3.1 in Chapter 3, except for P37 whose muscle was not studied by immunohistochemistry. Cultured skin fibroblast lines were available from 25 of the 36 patients described in Chapter 3 (see Table 3.1), including 17 of the 23 patients with presumed 'nuclear' patterns of COX subunit immunostaining. The other 8 cell lines were from patients with selective reduction of immunostaining to mtDNA-encoded COX subunits. Seven of these have not been analysed further but studies on the eighth cell line, from P26, are described in Chapter 5 (see Section 5.7). Of the 16 patients with presumed nuclear gene defects whose fibroblasts were available for study, one had mtDNA depletion (P29) and this cell line has not been studied further in this thesis. Analysis of 8 of the remaining 15 cell lines (from P2, P4, P17, P20, P27, P28, P30 and P36), as well as fibroblasts from P37, is presented below. Time constraints prevented the systematic analysis of all 15 cell lines during this thesis.

Fibroblast COX activities, assayed in the routine laboratory for 6 patients, are shown in Table 6.1. P4 had 50% residual COX activity in skeletal muscle but normal activity

in fibroblasts. P37 however had a more severe defect in fibroblasts (<1% residual COX activity) than in muscle (29% residual COX activity). The other 4 patients also expressed severe COX deficiency in fibroblasts, but muscle enzyme assay data was not available.

Table 6.1 Fibroblast COX activities

	P4	P20	P27	P28	P36	P37
Fibroblast COX	N	2	2.3	0	5	<1
Fibroblast COX (% lowest control)	>100%	7%	8%	0%	17%	<3%
Muscle COX	0.007	ND	ND	ND	ND	0.004
Muscle COX (% lowest control)	50%	-	-	-	-	29%

Fibroblast COX activity nmole/mg (reference range 30 – 90)
Muscle COX activity expressed as COX/CS ratio (reference range 0.014 - 0.034)
N=normal; ND= not determined

6.3 Case histories

Only case histories of patients whose *SURF1* gene was sequenced are presented below.

6.3.1 P20 Leigh-like syndrome, with congenital lactic acidosis, multicystic kidney, hypertrophic cardiomyopathy and dysmorphism

This boy, the first child of distantly related Turkish parents, was born at 37 weeks gestation weighing 1.97 kg (<3rd centile). Antenatal ultrasound at 36 weeks gestation demonstrated a multicystic right kidney, but the pregnancy was otherwise uncomplicated. There was a family history of 6 early infant deaths in his cousins.

Immediate neonatal problems included hypoglycaemia (blood glucose 0.8 mmol/L), which resolved rapidly with treatment, and oliguria with raised urea (4.7 mmol/L) and creatinine (130 μmol/L). Hypospadias, undescended testes and a palpable cystic mass in the right flank were noted at birth. Postnatal renal ultrasound confirmed the presence of a multicystic dysplastic right kidney, which was found to be nonfunctioning on a MAG3 scan. He failed to thrive from birth.

Development was normal for the first 3 months, but he subsequently regressed. Metabolic acidosis was noted during an emergency admission with respiratory

difficulties at 5 months. At 6 months he was no longer fixing and following and reacted only to sudden noise. Physical examination at 6 months revealed microcephaly and dysmorphic features, including coarse facies, large ears, bilateral small preauricular pits, a supernumerary nipple, and deep palmar and plantar creases suggestive of trisomy 8. However karyotype analysis was normal. He had profound hypotonia, with no head control or any purposeful movements. There were bilateral pyramidal tract signs in the upper and lower limbs, and also bilateral extensor plantar responses and limb stiffness suggestive of extrapyramidal involvement.

Investigations revealed lactic acidosis, with plasma lactate 10 mmol/L, plasma pyruvate 0.235 mol/L (lactate/pyruvate ratio 43) and CSF lactate 9.3 mmol/L. Urine organic acid analysis revealed high excretion of 2-oxoglutarate, with raised fumarate and mildly raised lactate and pyruvate excretion. Hepatic transaminases were mildly elevated (AST 103 U/L, reference range 20 to 60). MRI of the brain demonstrated lesions suggestive of Leigh syndrome, with extensive high signal in the corona radiata, subthalamic regions, crura cerebri, central midbrain, lower pons and anterior medulla. EEG was nonspecifically abnormal with no focal or paroxysmal features. Echocardiogram showed thickening of the left ventricle. Skeletal muscle biopsy performed at 6 months was normal other than mild fibre type variation, and COX staining was noted to be present.

He was treated with sodium bicarbonate, ubiquinone and high carbohydrate feeds, without any obvious clinical response. He had recurrent episodes of acute decompensation with decreased level of consciousness, tachypnoea and worsening acidosis. He died at home at 7 months age. A limited post mortem was performed, with liver and muscle biopsies. The brain was not examined, so it was not possible to confirm a diagnosis of Leigh syndrome. Histology of the post mortem specimens revealed severe reduction of COX staining in the liver, together with some fatty change. COX staining was also reduced in muscle, but less severely than in liver. Activities of respiratory chain enzyme complexes were not determined.

6.3.2 P27 Leigh syndrome

The patient was the first child of first cousin parents of Sri Lankan origin and was delivered by forceps at 38 weeks gestation after a normal pregnancy. Development

was normal until the age of 9 months, at which time she was able to sit unsupported and pull to stand. At 9 months, after a febrile illness, she developed problems with swallowing and was noted to be failing to thrive. She was subsequently fed nasogastrically until the age of 22 months. At 18 months her mother noted that she had a tremor on reaching and at 23 months, after a further febrile illness, she lost skills including sitting and speech, and suffered a respiratory arrest from which she was successfully resuscitated. She required mechanical ventilation for a period of 24 hours. A provisional diagnosis of 'ataxic cerebral palsy' was made.

She was first seen in the metabolic unit at the age of 2 years 3 months, when physical examination revealed coarse intermittent nystagmus, generalised hypotonia, an ataxic gait with truncal ataxia and intention tremor, and symmetrically brisk reflexes with extensor plantar responses. Investigations at this time revealed a mild lactic acidosis of 2.3 mmol/L, with an elevated lactate/pyruvate ratio of 39. MRI of the brain revealed typical features of Leigh syndrome, with high T2 signal in the lentiform nuclei, medial thalami, central midbrain and dentate nuclei (Figure 6.1). Nerve conduction studies demonstrated a mixed motor and sensory peripheral neuropathy. Echocardiography was normal.

Needle muscle biopsy demonstrated almost complete absence of COX activity, together with type 2 fibre atrophy and an increase in lipid droplets within muscle fibres. Respiratory chain enzyme assays were not performed. Routine screening for mtDNA rearrangements and point mutations at nucleotide 8993 was negative.

Her clinical course was marked by repeated respiratory arrests, once during an intercurrent infection with respiratory syncytial virus. From the age of 4 years she received intermittent positive pressure ventilation via a face mask at home. She continued to lose skills progressively and at 6 years 11 months she had no speech, could not fix and follow, was dependent on nasogastric feeds and could only swallow saliva with difficulty. On examination she had irregular respiration with intermittent sighing. Neurological examination revealed generalised tremor, cogwheel rigidity of the upper limbs and variable muscle tone of the lower limbs.

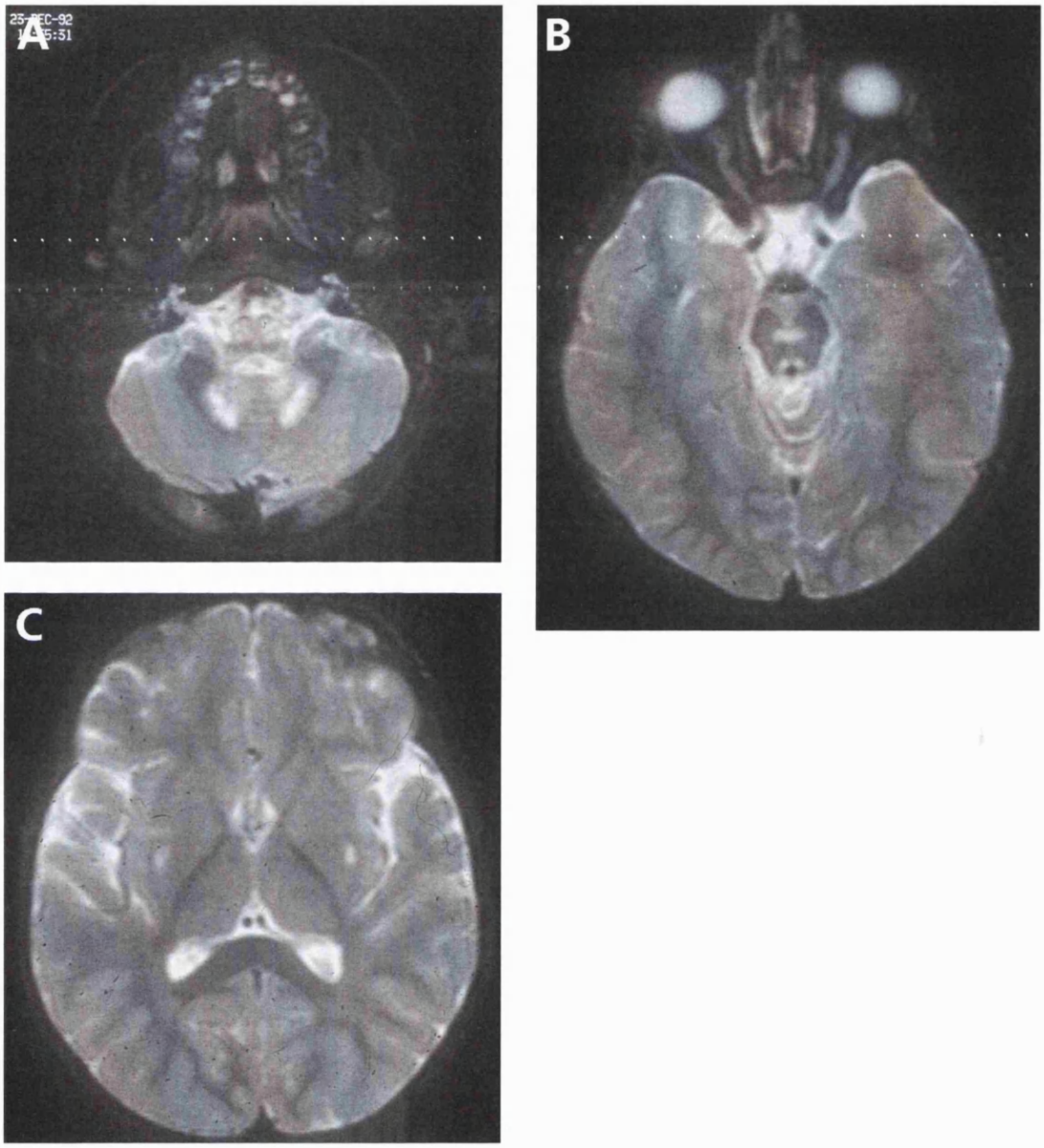


Figure 6.1 MRI brain of P27
T_2-weighted axial magnetic resonance images of the patient's brain at age 2 years 3 months, demonstrating bilateral symmetrical areas of abnormal high signal in the cerebellar white matter (A), mid-brain (B) and putamina (C). These lesions are typical of Leigh syndrome.

Deep tendon reflexes were absent in the lower limbs but preserved in the upper limbs. She had roving eye movements and fundoscopy demonstrated bilateral optic atrophy.

She continued to deteriorate and died at 7 years 3 months. Post mortem examination was not performed.

6.3.3 P28 Leigh syndrome

Jacobs et al. previously reported this patient as Case 1 (Jacobs et al. 1990). She was the first child of unrelated East African Asian parents and was born at 41 weeks gestation after a normal pregnancy. She initially presented at 2 years 10 months with a history of progressive hirsutism from 9 months. Endocrine investigations were normal. She also had gross motor delay; she first walked at 18 months and had a wide-based unsteady gait.

At 3.5 years she had an episode of 'encephalitis', after which she developed dysarthria, ataxia and ophthalmoplegia. Investigations at 5 years revealed lactic acidosis. CT of the brain, EEG, ERG and VER were normal. Nerve conduction studies demonstrated a mixed demyelinating polyneuropathy. Muscle biopsy showed increased variability of fibre diameters and excess fat in type I fibres. There were no ragged red fibres. Histochemical staining revealed severe reduction of COX activity.

At 9 years she developed dysphagia, emotional incontinence and sleep disturbance. Physical examination revealed jerky nystagmus on lateral and upward gaze, truncal and limb ataxia, hypotonia and depressed deep tendon reflexes. Repeat CT of the brain demonstrated symmetrical low attenuation lesions in the globus pallidus and cerebellar atrophy. In the last year of her life she was no longer able to walk and developed irregular respiration and swallowing difficulties. She died at 11 years 9 months, and post mortem examination confirmed the diagnosis of Leigh syndrome.

A younger sister of P28 was also affected (Case 2 in Jacobs et al. 1990). She presented with lack of developmental progress following rotavirus infection at 10 months, and was hirsute by 1 year of age. At 23 months she also had a mixed

demyelinating polyneuropathy. CT of the brain was normal. She developed increasing ataxia, and at 3 years had jerky nystagmus on upward and lateral gaze, intention tremor, a broad-based gait, proximal muscle weakness and absent tendon reflexes. Enzyme assays in cultured skin fibroblasts revealed COX deficiency and normal PDHC activity. From 4 years she had increasing problems with swallowing difficulties, persistent vomiting and unintelligible speech. She developed an irregular breathing pattern and died suddenly at 6.5 years. Post mortem demonstrated typical neuropathological changes of Leigh syndrome, together with muscle COX deficiency.

6.3.4 P36 Lactic acidosis with failure to thrive, vomiting, myopathy, peripheral neuropathy and proximal renal tubular acidosis

The first child of healthy unrelated parents, P36 was born by spontaneous vaginal delivery at 41 weeks gestation. He first presented at the age of 2 months with a history of failure to gain weight since birth. This was associated with intermittent episodes of vomiting from the ages of 2 to 11 months. He sat unsupported at 7 months but subsequently there was little progression in his motor milestones. Investigations at 11 months age revealed a renal tubular acidosis, with low levels of bicarbonate and high urinary pH. Oral bicarbonate replacement therapy was initially associated with some improvement in weight gain and reduction of vomiting.

Assessment in the metabolic unit at the age of 14 months revealed marked central and peripheral hypotonia, with muscle wasting and global reduction of muscle power. Deep tendon reflexes were absent, but plantar responses were normal. Investigations at this time revealed a persistent lactic acidosis. Plasma lactate was 4.06 mmol/L and CSF lactate 3.74 mmol/L. Renal tubular reabsorption of phosphate was normal. EMG demonstrated a mild predominantly sensory axonal polyneuropathy. CT of the brain revealed no abnormalities. Muscle biopsy demonstrated small fibres with very low levels of COX activity. Respiratory chain enzyme complex activities were not assayed. Electron microscopy was unremarkable.

Increasing oral bicarbonate supplements to 50 mmol/day (8 mmol/kg/day) led to an improvement in appetite and in his general wellbeing. At 20 months he developed

horizontal nystagmus and at 22 months difficulty in swallowing. No further cerebral imaging was performed. His clinical condition continued to deteriorate and he died before his second birthday.

A younger sister was also affected. She presented with failure to thrive, intermittent hypotonia and abnormal eye movements from the age of 6 weeks. At 5 months blood and CSF lactates were mildly elevated and CT of the brain demonstrated widening of the subarachnoid spaces anteriorly. At 9 months she was noted to be hirsute. Her clinical course was marked by recurrent vomiting; regression of developmental skills with intercurrent infections, followed by partial recovery; marked hypotonia and muscle weakness; deteriorating vision with optic atrophy; and episodes of irregular respiration. She died at 4 years of age. Post mortem examination was not performed on either child, nor was neuro-imaging performed late in the course of their illness, so it was not possible to confirm the clinical impression of Leigh syndrome.

6.3.5 P37 Leukodystrophy

This girl was referred at 2 years of age for investigation of failure to thrive associated with metabolic acidosis. The second daughter of healthy consanguineous Bengali parents, she was born after a normal pregnancy by vaginal delivery. There were no neonatal problems but poor growth was noted from 9 months. At 11 months she had an episode of cyanosis and floppiness, without preceding intercurrent illness, and subsequently her developmental milestones slowed. At a year of age she was able to pull to stand, cruise around the furniture and crawl. An older sister is well and there is no family history of neurological disease. Examination at two years revealed weight, height and head circumference all below the third centile. There were no dysmorphic features. She had mild hypotonia with normal deep tendon reflexes. Initial investigations revealed lactic acidosis (plasma lactate 3.2-5.7 mmol/L and lactate/pyruvate ratios 22-26). Urine metabolic screen revealed mildly raised levels of lactate, pyruvate, alanine, 2-methyl-3-hydroxybutyrate and 3-hydroxybutyrate.

MRI of the brain performed at 2 years 5 months demonstrated abnormal signal in the cerebral white matter (particularly posteriorly), posterior limbs of the internal capsule, corpus callosum (particularly the splenium), dentate nuclei and adjacent

cerebellar white matter (Figure 6.2A and B). Areas within the more confluent white matter abnormalities appeared to be cyst-like (Figure 6.2C and D). No abnormalities were seen in the caudate or lentiform nuclei. The appearances were considered to be those of leukoencephalopathy with involvement of the corticospinal tracts. EEG revealed mild nonspecific abnormality, with excess fast activity over the anterior half of the head and rhythmic intermediate slow activity posteriorly. A search for known causes of leukodystrophy revealed no vacuolated lymphocytes in the blood film, and activities of leukocyte lysosomal enzymes were all within the normal range. Plasma very long chain fatty acid concentrations and transferrin isoelectric focusing were normal.

Further assessment at 2 years 8 months revealed that she had lost skills. Her floppiness had progressed and she was barely able to sit without support. She had fewer words and her speech was less clear. On examination she was continuing to fail to thrive and was tachypnoeic. She was hirsute. She had poor to moderate visual acuity, being able to fix and follow both a bright light and a finger. Eye movements were full, with a few beats of nystagmus intermittently. There was no evidence of optic atrophy or pigmentary retinopathy. She had generalised symmetrical hypotonia and pathologically brisk reflexes with up-going plantar responses.

Repeat blood lactate was 6.8 mmol/L and CSF lactate was 7.65 mmol/L, with CSF pyruvate 0.24 mmol/L (CSF lactate/pyruvate ratio 32). CSF protein was mildly elevated at 0.58 g/L (reference range 0-0.3). Further investigations revealed slightly abnormal renal tubular function with a mildly elevated urinary N-acetylglucosaminidase/creatinine ratio of 83 units/mmol (reference range 3.5 - 27.3), normal liver function and normal echocardiogram with no evidence of cardiomyopathy. Neurophysiological studies demonstrated normal electroretinogram but marked post-retinal dysfunction on visual evoked potentials. Intact parathyroid hormone level was low at 0.3 pcmol/L (reference range 1.1-5.4), but plasma calcium and magnesium levels were within the normal range. Histology of an open quadriceps muscle biopsy showed only a mild increase in intracytoplasmic fat and atrophy of type II fibres. No ragged red fibres were seen. Respiratory chain enzyme assays on the biopsied muscle revealed a severe isolated deficiency of cytochrome oxidase (COX/CS ratio 0.004, reference range 0.014 - 0.034– see Table 6.1) with

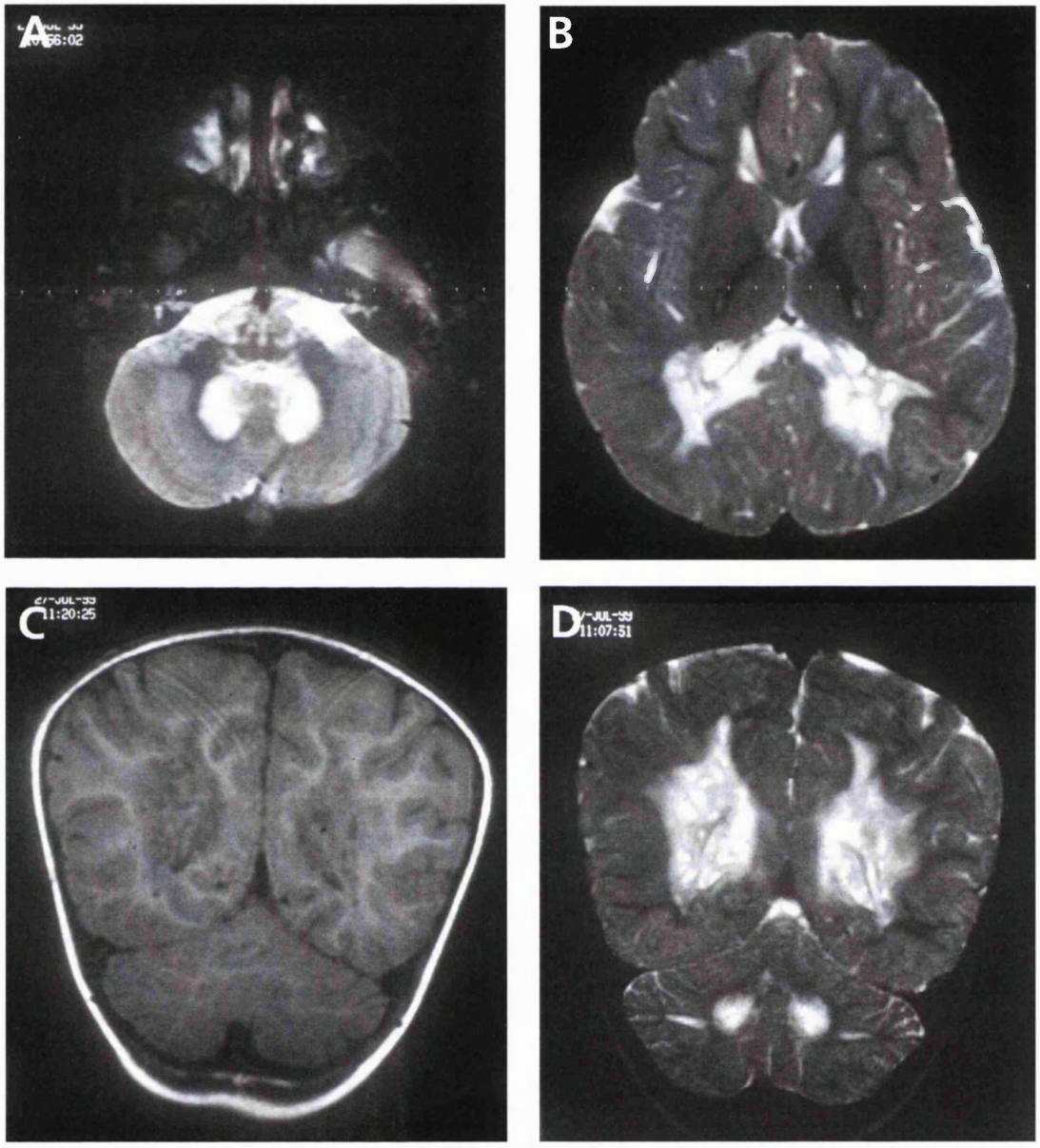


Figure 6.2 MRI brain of P37
Magnetic resonance images of the patient's brain at age 2 years 5 months.
A Axial T2-weighted image demonstrating abnormal high signal in the cerebellar white matter.
B Axial T2-weighted image demonstrating abnormal high signal in the splenium and internal capsule. No lesions are visible in the basal ganglia.
C Coronal T1-weighted image demonstrating cyst-like lesions in the white matter.
D Cyst-like lesions in the white matter are also seen in this coronal T2-weighted image, which also demonstrates abnormal high signal in the cerebral and cerebellar white matter.

normal activities of complexes I (0.113, reference range 0.104-0.268) and II/III (0.049, reference range 0.040-0.204).

6.4 Immunocytochemistry

Fluorescent staining of mitochondrial membrane potential, nuclear DNA and COX subunit I in control fibroblasts is illustrated in Figure 6.3. Co-localisation of COX subunit I immunostaining and mitochondrial staining is demonstrated (Figure 6.3C). Immunocytochemical staining of fibroblasts from patients P20 and P27 demonstrated marked reduction of staining of COX subunits I, IV and VIc compared to controls (results for P27 are shown in Figure 6.4). Immunocytochemical staining of COX subunits was normal in fibroblasts from other patients (P4, P17 and P30 – data not shown).

6.5 Western blotting

Immunoblot analysis confirmed the immunocytochemical observations that the steady state levels of COX subunits I, IV and VIc were greatly reduced in fibroblasts from patients P20 and P27 (Figure 6.5). Patients P17 and P30 apparently had normal steady state levels of these subunits, but subunit IV was slightly reduced in P2.

6.6 Sequence analysis of the *SURF1* gene

Sequence analysis of the *SURF1* gene was performed in collaboration with Dr Garry Brown and Ruth Brown at the University of Oxford. Patients P20, P36 and P37 were sequenced by myself, and P27 and P28 were sequenced by Ruth Brown. Results of the sequence analysis are illustrated in Figures 6.6 to 6.8 and summarised in Table 6.2. Figure 6.10 is a diagrammatic representation of the *SURF1* gene, showing the sites of the mutations observed in this thesis. Numbering of the *SURF1* gene is from the ATG start codon, according to the nomenclature of Antonarakis 1998.

6.6.1 Results of *SURF1* sequencing in P20

The only base change identified in P20 was a missense mutation 736A>G in exon 7 (Figure 6.6), leading to a substitution of valine for an isoleucine at residue 246. This

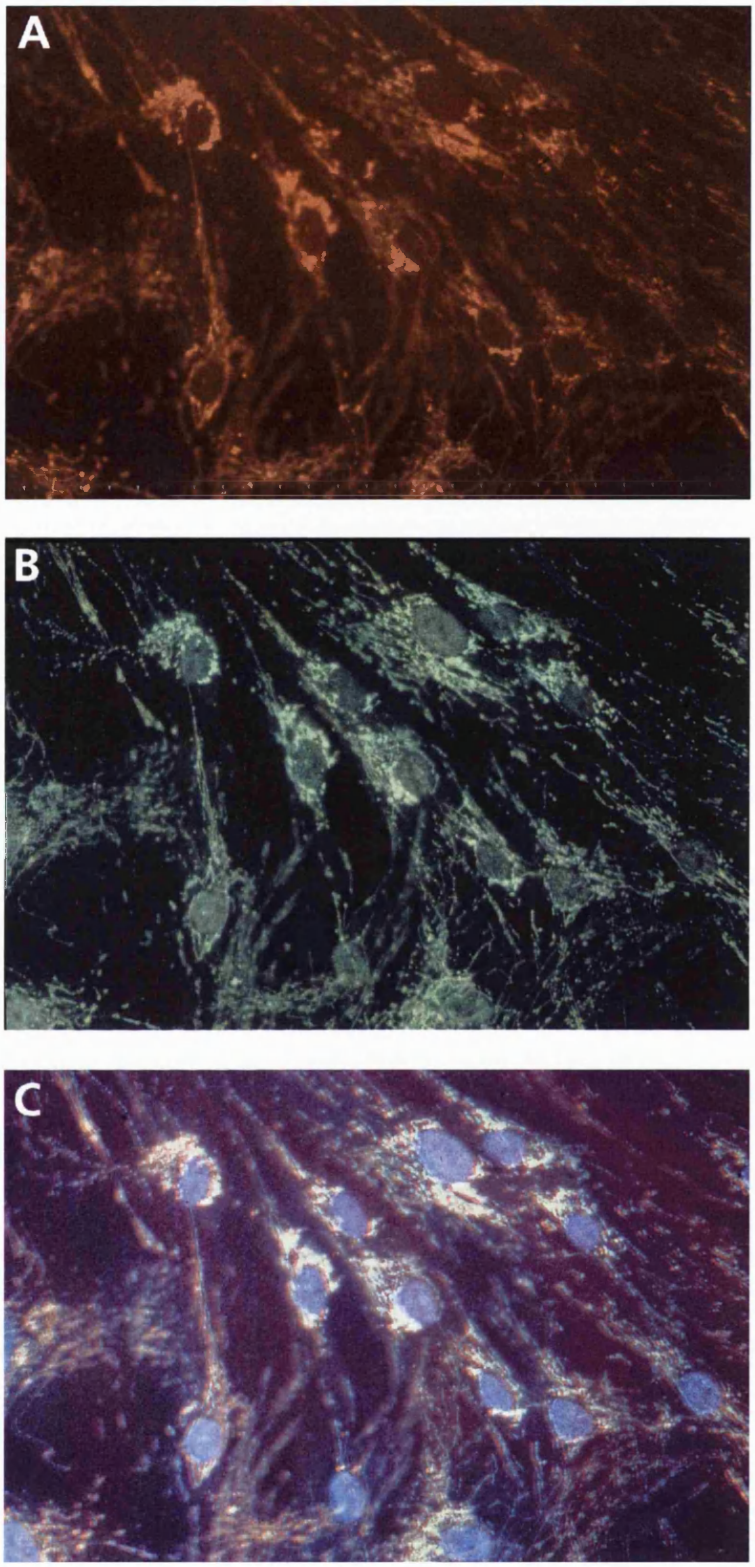


Figure 6.3 Immunocytochemistry of COX subunits in control fibroblasts
Cultured skin fibroblasts from a control triple-stained for mitochondrial membrane potential (red fluorescence), nuclear DNA (blue fluorescence) and COX subunit I (green fluorescence). Cells were cultured in the presence of MitoTracker™ CMXRos-H_2 to label mitochondria and subsequently fixed, permeabilised and immunostained with monoclonal antibodies against COX subunit I and bis-benzimide to reveal nuclei.

A Mitochondrial staining
B COX subunit I immunostaining
C Triple exposure illustrates co-localisation of COX subunit I with mitotracker but not with the nuclear stain.

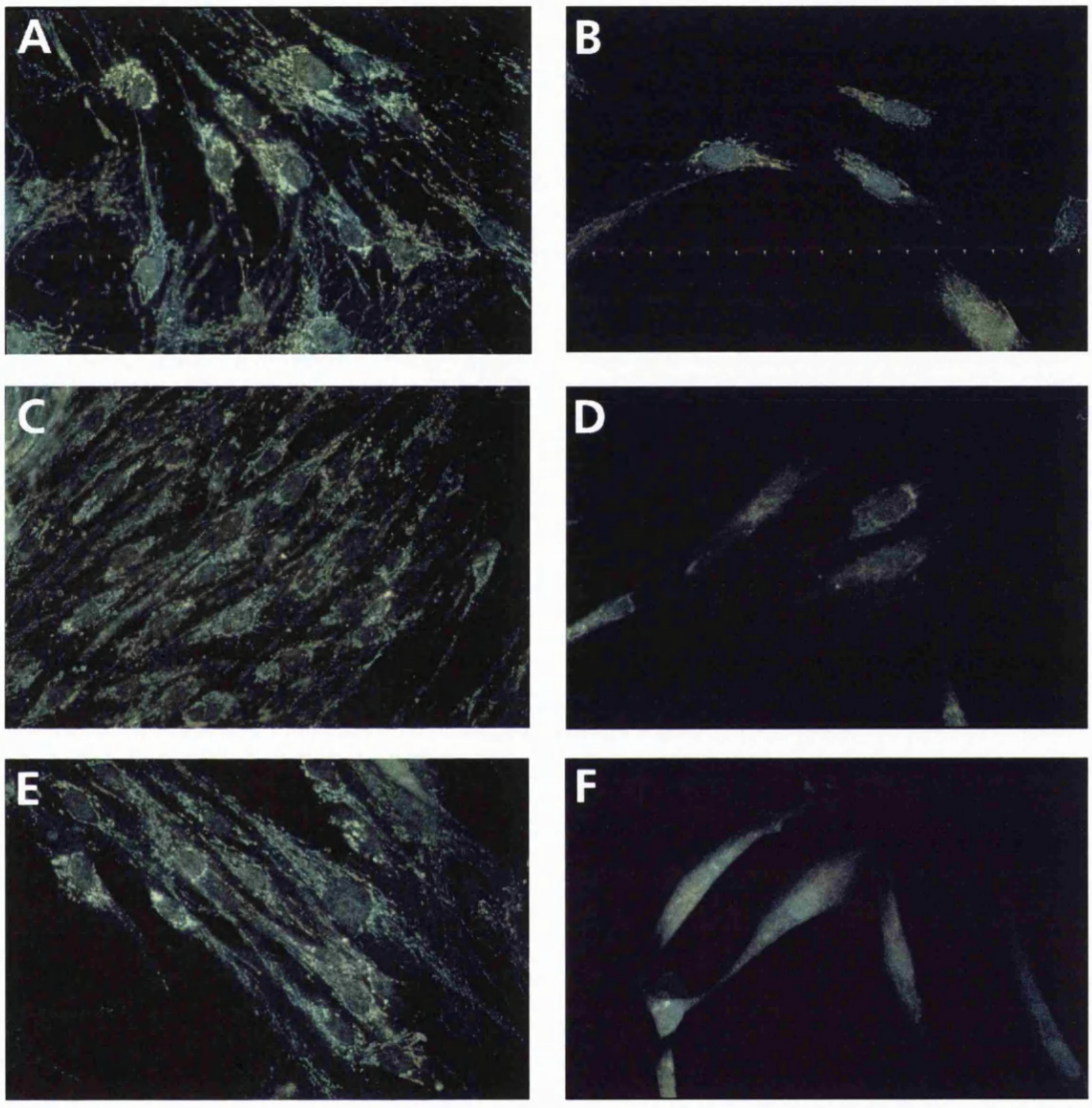


Figure 6.4 Immunocytochemistry of COX subunits in fibroblasts from P27
COX subunit I immunostaining in A) control fibroblasts and B) P27 fibroblasts; COX subunit IV immunostaining in C) control fibroblasts and D) P27 fibroblasts; COX subunit VIc immunostaining in E) control fibroblasts and F) P27 fibroblasts. Immunostaining of all 3 COX subunits is reduced in P27 fibroblasts compared to control.

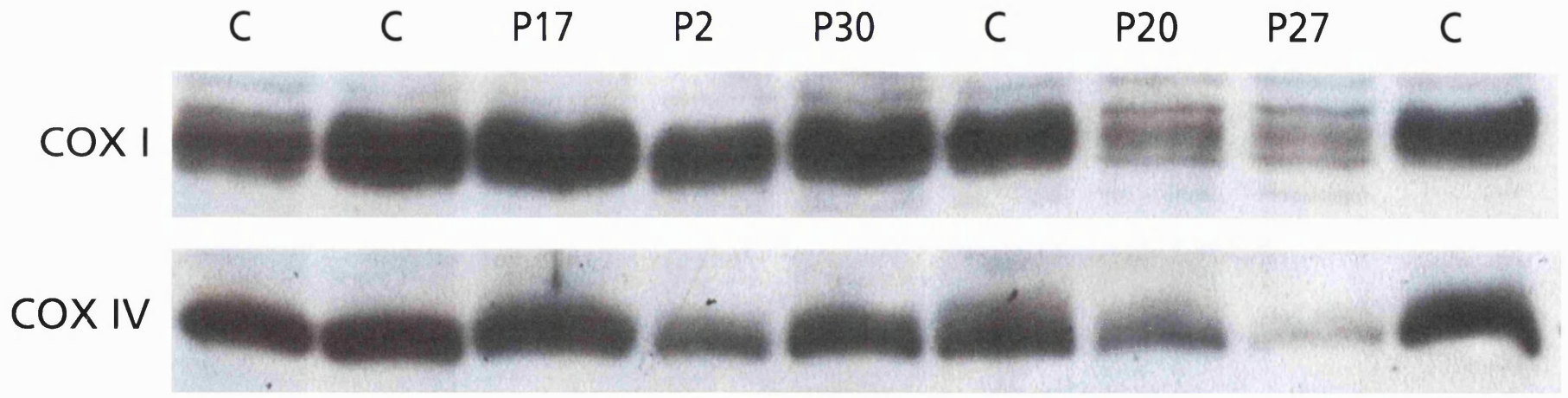


Figure 6.5 Western blot
Immunoblot analysis of COX subunits I and IV in fibroblast protein extracts from P2, P17, P20, P27, P30 and 4 controls (C). Steady state levels of COX I are reduced in P20 and P27 but appear normal in the other patients compared to control samples. Steady state levels of COX IV are reduced in P2, P20 and P27.

was heterozygous and the other strand had completely normal sequence of coding DNA.

6.6.2 Results of *SURF1* sequencing in P27

Patient P27 appeared to be homozygous for a nonsense mutation 751C>T at the end of exon 7. The mutation is associated with loss of a *Bsi* WI restriction site and RFLP analysis with *Bsi* WI was used to confirm homozygosity in the patient's genomic DNA (data not shown).

6.6.3 Results of *SURF1* sequencing in P28

Patient P28 was found to have a splice site mutation leading to exon skipping. Initial sequence analysis demonstrated a 72 bp deletion of her cDNA, encompassing the whole of exon 6. Further sequencing studies of her genomic DNA revealed a homozygous A to G mutation at the end of intron 5, 516-2A>G.

6.6.4 Results of *SURF1* sequencing in P36

Sequence analysis of *SURF1* cDNA from P36 revealed an apparently homozygous deletion of 10 bp together with an insertion of an AT dinucleotide at encoded amino acid position 104 in exon 4. This mutation is referred to as 312_321del10,311_312insAT or312del10/insAT and is illustrated in Figure 6.7. The mutation results in a frameshift and a truncated protein product. RFLP analysis was used to confirm homozygosity of this mutation in the patient's genomic DNA (data not shown).

6.6.5 Results of *SURF1* sequencing in P37

Sequence analysis of the coding region of the *SURF1* gene of P37 revealed an apparently homozygous 2 bp deletion of one of two AG repeats in exon 8 after nucleotide 789 (designated 790_791delAG, Figure 6.8). This mutation leads to a frameshift after threonine 263 and the generation of a new stop codon after a further 26 amino acids. Restriction endonuclease analysis of genomic DNA with *Bsr*I

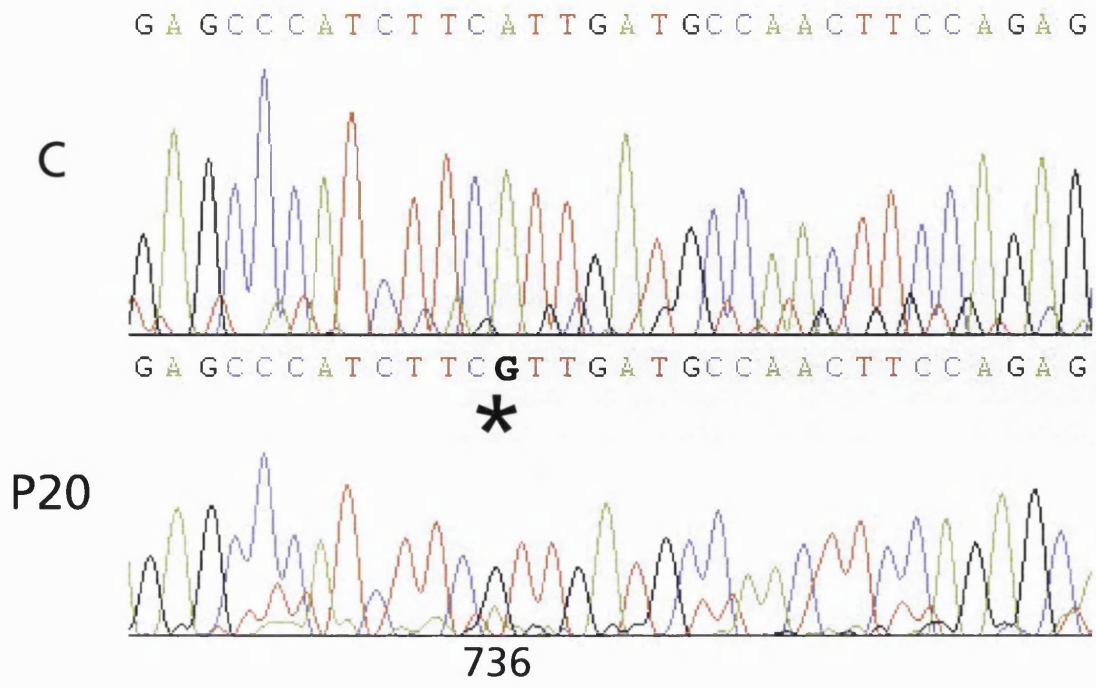


Figure 6.6 Sequence analysis of SURF1 exon 7 in P20
A section of the cDNA sequence from P20 demonstrates a heterozygous base substitution 736A>G in exon 7 of the SURF1 gene.

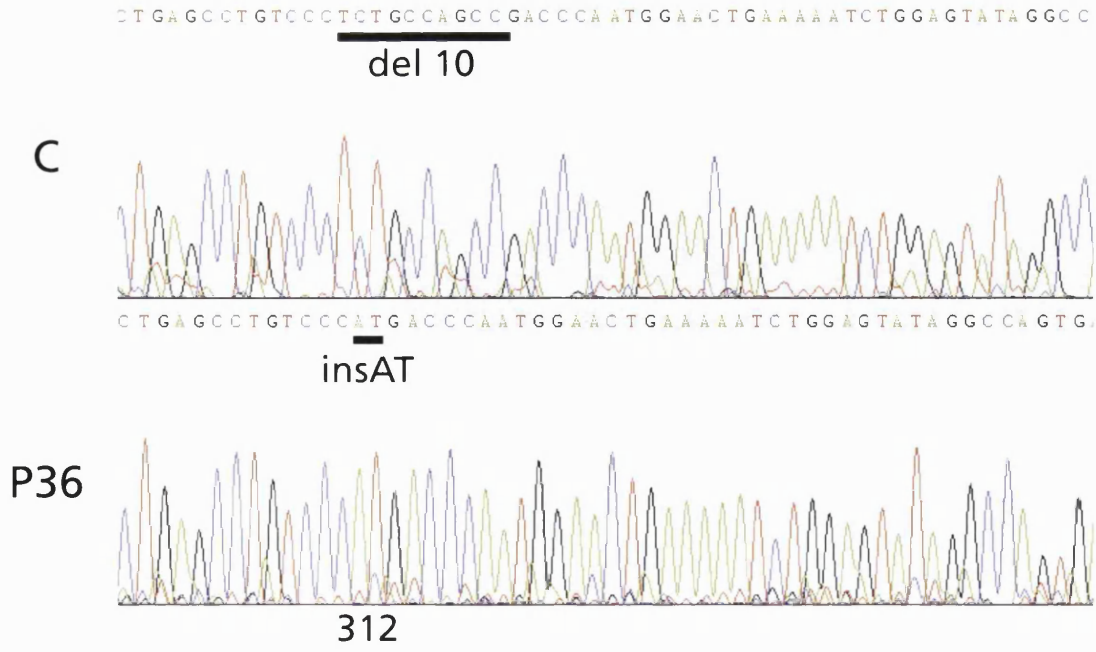


Figure 6.7 Sequence analysis of SURF1 exon 4 in P36
A section of the cDNA sequence from P36 demonstrates that he has a homozygous deletion of 10 bp together with an insertion of an AT dinucleotide at np 312 in exon 4 of the *SURF1* gene.

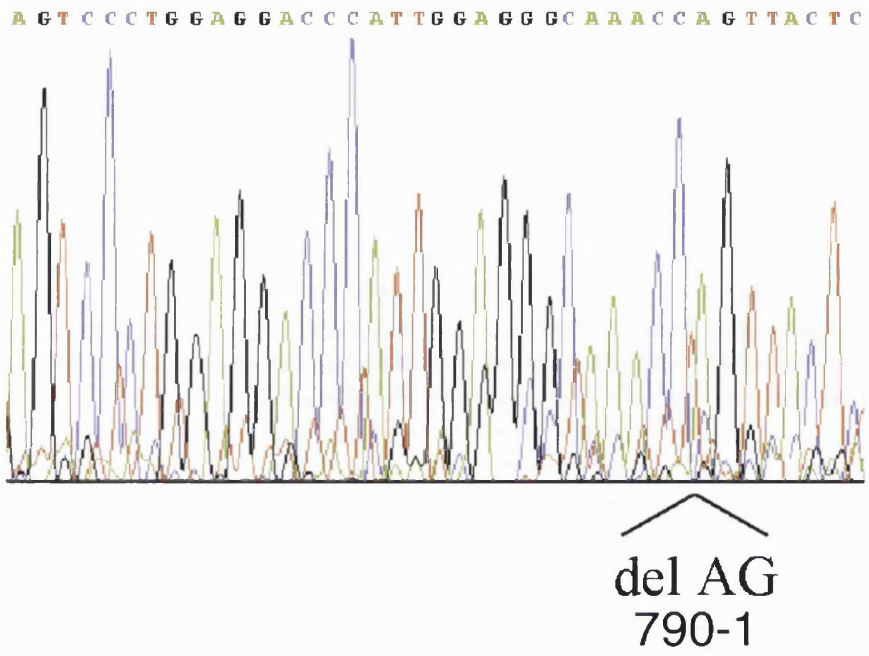


Figure 6.8 Sequence analysis of SURF1 exon 8 in P37
A section of the cDNA sequence from P37 demonstrates that she is homozygous for a 2 bp (AG) deletion 790_791delAG in exon 8 of the *SURF1* gene.

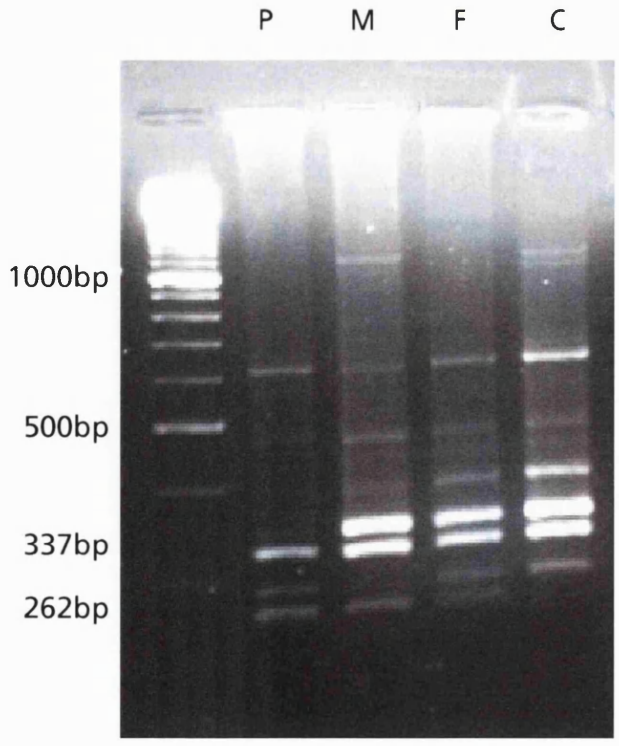


Figure 6.9 Demonstration of the SURF1 exon 8 AG deletion in genomic DNA
A segment of the *SURF*1 gene encompassing exons 6-9 and digested with the restriction enzyme *Bsr* I is shown. The 790_791delAG deletion in exon 8 leads to formation of a new *Bsr* I site with the result that the 337 bp restriction fragment is further digested into 262 and 75 bp fragments. The patient P37 (P) is homozygous for this deletion, while both parents (M and F) are heterozygous. A normal control (C) is shown for comparison. The left hand lane is a 100 bp molecular size ladder with the 500 and 1000 bp fragments indicated.

demonstrated that the patient was homozygous for this mutation and that both parents were heterozygous (Figure 6.9).

Table 6.2 Results of *SURF1* Mutation Analysis

Patient	Mutation	Exon	Effect on Protein	Comments
P20	736A>G Heterozygous	7	I246V	Polymorphism
P27	751C>T Homozygous	7	Q251X	Common pathogenic mutation
P28	516-2A>G Homozygous	5-6	Truncated: deletion of exon 6	Novel splice site mutation (exon skipping)
P36	312_321del10, 311_312insAT Homozygous	4	Frameshift	Common pathogenic mutation
P37	790_791delAG Homozygous	8	Frameshift	Pathogenic

6.7 Discussion

This chapter has described the clinical findings and biochemical and molecular genetic observations in cultured skin fibroblasts of a group of patients who appear to have nuclear-encoded defects of COX assembly or maintenance.

6.7.1 Immunological studies

Immunocytochemistry and Western blot analysis confirmed the findings of muscle immunohistochemistry in P20 and P27, with reduction of all COX subunits analysed in each case. P2, however, had reduction of all COX subunits on muscle immunohistochemistry but relatively normal levels of COX I and only a modest reduction of COX IV steady state levels on immunoblot analysis of fibroblast proteins. This is likely to reflect a difference in tissue expression in this patient.

6.7.2 *SURF1* molecular genetics

6.7.2.1 SURF1 sequence in P20

Sequence analysis of the *SURF1* coding sequence failed to identify a pathogenic mutation in P20. The 736A>G mutation is unlikely to be pathogenic, as substitution of valine for isoleucine is a relatively minor amino acid change. Both amino acids

contain a branched aliphatic side chain and it is unlikely that substitution will affect the tertiary structure of the protein. The 736A>G substitution did not appear in a list of polymorphisms reported in the *SURF1* gene (Pequignot et al. 2001b) and so should be added to the list of known polymorphisms in this gene, although linked to disease unless it is subsequently reported in a healthy control. It is possible that P20 may have a mutation in the noncoding region of the *SURF1* gene, but his clinical features are significantly different to those of all other patients reported to have *SURF1* mutations and therefore this seems unlikely.

6.7.2.2 SURF1 sequence in P27

The nonsense mutation 751C>T in exon 7 is predicted to produce a truncated SURF1 protein. This mutation has been reported to be pathogenic previously and is the second most commonly reported *SURF1* mutation (Tiranti et al. 1998b; Zhu et al. 1998). It has also been reported in a pair of monozygotic twins with uniparental disomy of chromosome 9 (Tiranti et al. 1999d).

6.7.2.3 SURF1 sequence in P28

The splice junction mutation 516-2A>G in P28 is a novel mutation. This mutation abolishes the splice acceptor site AG in intron 5, so that the next downstream splice acceptor has to be employed, leading to the deletion of exon 6. The mutation is therefore very likely to be pathogenic as it results in skipping of exon 6 and thus a shortened SURF1 protein. The mRNA transcript and/or truncated protein product may be unstable (Culbertson, 1999), resulting in reduced steady state levels of SURF1 and consequently reduced assembly of the COX holoenzyme. Splice mutations appear to be relatively common causes of SURF1 deficiency. Several other *SURF1* splice mutations have been reported (see Table 1.7 in Chapter 1), including 2 which led to skipping of exon 6 (Pequignot et al. 2001b). All three affected patients had classical Leigh syndrome with a severe disease course (Tiranti et al. 1998b; Pequignot et al. 2001a).

6.7.2.4 SURF1 sequence in P36

On sequence analysis P36 appeared to be homozygous for the 312del10/insAT mutation in exon 4 (Figure 6.7). However to exclude the possibility of a complete

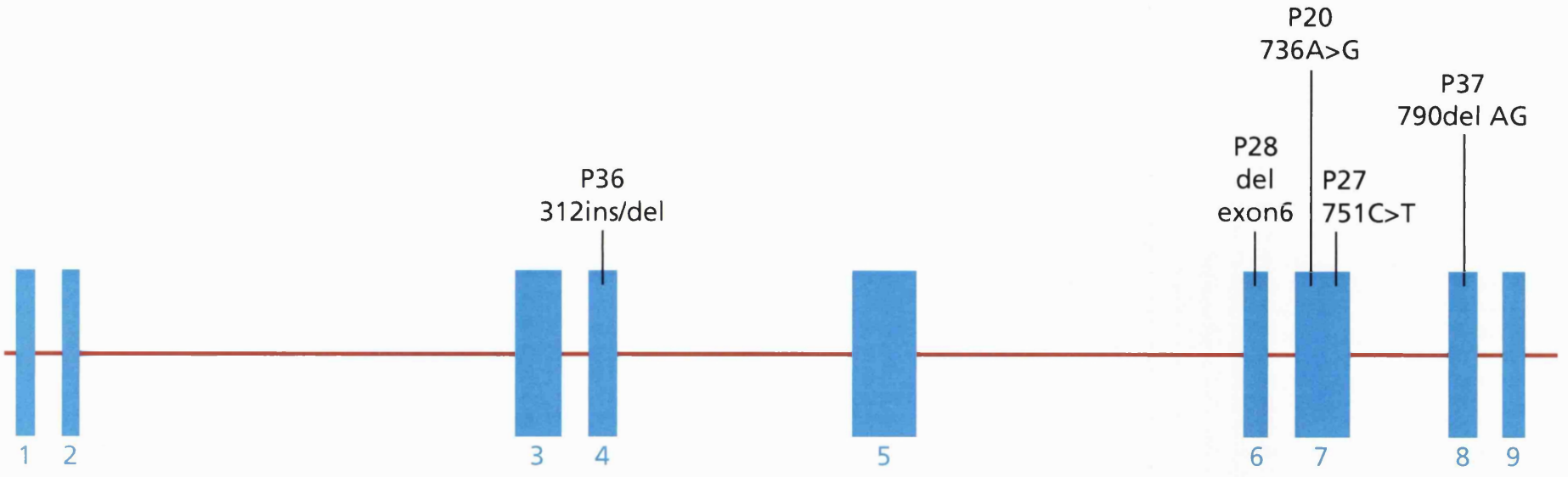


Figure 6.10 Diagrammatic representation of the SURF1 gene showing sites of mutations
Introns (red lines) and exons (blue rectangles) are drawn to scale. The positions of the mutations identified in patients P20, P27, P28, P36 and P37 are indicated.

deletion of the second *SURF1* allele, it would be helpful to examine DNA from the parents to confirm that both are heterozygous for this mutation. The 312del10/insAT mutation is a known mutation and is the most commonly reported *SURF1* mutation in COX-deficient Leigh syndrome (Zhu et al. 1998; Tiranti et al. 1999c). It is not known whether this locus is a hotspot for mutations in the *SURF1* gene, or if a 'founder' effect has occurred (Robinson, 2000).

6.7.2.5 SURF1 sequence in P37

The 790_791delAG microdeletion in P37 has been reported only once previously in a patient with Leigh syndrome (Teraoka et al. 1999). The previous patient was heterozygous for this mutation and had a second missense mutation 820T>G (Y274D), also in exon 8.

The findings in this thesis (Figure 6.10) thus confirm the reports of other groups that most *SURF1* mutations appear to be small-scale rearrangements, leading to a frameshift and predicting a truncated protein product (see Table 1.7 in Chapter 1).

6.7.3 The clinical phenotype associated with *SURF1* mutations

The patients studied in this chapter help to characterise the clinical phenotype of SURF1 deficiency. Until very recently all patients with *SURF1* mutations in the literature had Leigh syndrome, and this is probably true of three of the patients in the present series. However only one of these (P28) had a post mortem and neuropathological confirmation of Leigh syndrome. A clinical diagnosis of Leigh syndrome was made in P27 and P36, with radiological corroboration in the former. These three patients also had peripheral neuropathy, which has been reported previously patients with Leigh syndrome and *SURF1* mutations (Santoro et al. 2000; Pequignot et al. 2001a).

P37 also had predominantly neurological symptoms and signs, but her radiological features were those of leukodystrophy rather than typical Leigh syndrome. She had extensive white matter changes in the cerebrum and cerebellum. Multiple, small cyst-like white matter lesions, similar to those described in two of five families previously reported with leukodystrophy and respiratory chain defects (de Lonlay-Debeney et al. 2000), were observed within the more confluent white matter

abnormalities. It is possible that these cyst-like lesions may be a specific feature of white matter disease due to respiratory chain defects.

It has been suggested that loss of function mutations of *SURF1* are exclusively associated with COX-deficient Leigh syndrome (Tiranti et al. 1999c). A significant finding of the present study is the demonstration that *SURF1* mutations may be associated with more than one neuroradiological pattern, i.e. diffuse leukodystrophy as well as Leigh syndrome. Levels of residual COX activity in skeletal muscle and cultured skin fibroblasts in P37 were similar to those in patients with *SURF1* mutations and Leigh syndrome (Pequignot et al. 2001b). Furthermore there is a report of typical Leigh syndrome in a patient heterozygous for the same AG deletion in exon 8 as P37 (Teraoka et al. 1999). In Teraoka's patient, the mutation in the second *SURF1* gene resulted in substitution of aspartic acid for tyrosine 274. By contrast, P37 had no evidence of the basal ganglia necrotic lesions that are characteristic of Leigh syndrome. The reason for the different pattern of neuropathology in these two patients is unclear, but it is possible that it is related to the presence of two different *SURF1* mutations in the previously reported case. However phenotypic heterogeneity associated with identical mutations has previously been described in other single gene disorders (Wolf, 1997).

The main non-neurological manifestation of SURF1 deficiency in the present series was hypertrichosis, which was documented in the sisters P28 and P28a, patient P37 and the sister of P36 (it is possible that P36 himself had hypertrichosis that was not recorded in his case notes). Hypertrichosis has previously been reported in a patient with *SURF1* mutations without Leigh syndrome (von Kleist-Retzow et al. 2001) and may be a relatively common feature of SURF1 deficiency (Birch-Machin, 2000). The mechanism producing hypertrichosis in these patients is not known, but could possibly be related to another housekeeping role of the SURF1 protein.

Clinical characterisation of SURF1 deficiency is important, since clinical findings should help to direct the order in which molecular genetic investigations are performed. The presence of hypertrichosis and/or peripheral neuropathy in a patient with Leigh syndrome warrants early analysis of the *SURF1* gene. This may help to provide a more rapid diagnosis than has previously been possible.

Muscle immunohistochemistry was performed in 3 of the 4 patients who were found to have pathogenic *SURF1* mutations in this thesis. Two patients (P27 and P28) had reduced immunostaining of all COX subunits studied (see Figure 3.12 for results of P28 and Table 3.3 for summary of findings in P27 and P36). These findings were confirmed on fibroblast immunocytochemistry and immunoblot analysis in the case of P27. The findings in P27 and P28 agree with observations in SURF1 deficient patients reported in the literature (Sue et al. 2000; Yao and Shoubridge, 1999; Poyau et al. 2000; see section 3.5.4 in Chapter 3). Although presence of immunostaining to all COX subunits was noted in P36, desiccation artefact may have prevented correct assessment of this patient's immunostaining, and it is possible that he may actually have had reduced staining of all subunits (see section 3.4.3 in Chapter 3).

P20 did not have an identifiable *SURF1* mutation, suggesting that his clinical features, which included dysmorphism, hypertrophic cardiomyopathy and renal abnormalities, lie outside the SURF1 phenotype. In view of the presence of hypertrophic cardiomyopathy and liver dysfunction, sequencing of the *SCO2* and *SCO1* genes is indicated in P20 (Papadopoulou et al. 1999; Valnot et al. 2000a). However the pattern of COX subunit expression in P20, based on muscle immunohistochemistry and immunoblot analysis, differs significantly from that described in patients with *SCO1* and *SCO2* mutations (Papadopoulou et al. 1999; see section 3.5.2) and so it is unlikely that he had a *SCO1* or *SCO2* mutation.

6.7.4 Conclusions

Four patients were demonstrated to have pathogenic mutations in the *SURF1* gene in this chapter. The molecular basis of COX deficiency in the fifth patient P20 remains unclear. In the present series the clinical phenotype in most patients with identified *SURF1* mutations was Leigh syndrome, but one patient (P37) had additional features of leukodystrophy on MRI of her brain. Previously the search for *SURF1* mutations has focussed mainly on patients with Leigh syndrome or with "Leigh-like" features. The identification of a pathogenic *SURF1* mutation in a patient with isolated leukodystrophy leads to the suggestion that the *SURF1* gene should be analysed in all patients with isolated COX deficiency, in order to determine the range of phenotypes that may be associated with mutations in this gene.

It is possible that missense mutations in the *SURF1* gene may account for milder cases of COX deficiency or tissue-specific defects, but this remains to be proven. However, using the currently available data, there does not appear to be any obvious correlation between the position and type of mutation and the clinical phenotype observed. Differences in clinical manifestation, such as the isolated leukodystrophy observed in P37, may be attributed to other factors such as the nuclear and mitochondrial genetic background of the patient, or to environmental influences.

Chapter 7

Discussion

Chapter 7 Discussion

7.1 Introduction

This thesis contributes to the molecular characterisation of cytochrome oxidase deficiency in childhood, by providing evidence that nuclear gene defects are more likely to underlie childhood cytochrome oxidase deficiency than mitochondrial gene defects, and by describing some of these genetic defects (both mitochondrial and nuclear) at a nucleotide level.

This thesis also provides clinical information about a large series of unselected patients with cytochrome oxidase deficiency and thereby contributes to the clinical characterisation of the COX deficiencies.

7.2 Diagnostic approaches

The problem of disorders of the respiratory chain in childhood is a substantial one, and may represent the most common group of inborn errors of metabolism. Deficiency of cytochrome oxidase, either as an isolated defect or in combination with deficiencies of other respiratory chain enzymes, is the most frequent biochemical diagnosis in this group of patients (Caruso et al. 1996). Unfortunately this biochemical diagnosis fails to provide information about the likely mode of inheritance, and therefore the recurrence risk, and so other studies are required to provide the necessary genetic information for accurate counselling of affected families. Furthermore reliable prenatal diagnosis is only available for those defects known to be inherited autosomally (Ruitenbeek et al. 1996), as prenatal diagnosis of mtDNA mutations is fraught with difficulties. In particular little is known about drift of mtDNA point mutations in intrauterine life and there is a risk of maternal contamination of the sample (Harding et al. 1992a).

Identification of the precise genetic defect would be the most satisfactory method of determining the likely mode of inheritance, but most patients with COX deficiency remain uncharacterised at the molecular level. In other laboratories analysis of the obvious candidate genes (that is, the structural COX subunit genes) has been an unsuccessful approach (Adams et al. 1997; Lee et al. 1998; Jaksch et al. 1998), and

so alternative strategies are required. In the absence of an identified mtDNA mutation or a strong maternal family history of neuromuscular disease it is difficult to be certain whether the genetic defect is mitochondrial or nuclear in individual cases.

One possible approach is to use transmitochondrial cybrids, in which enucleated patient cell lines are fused with ρ^0 cells which lack mtDNA (King and Attardi, 1989). Correction of the respiratory chain deficiency by the donor ρ^0 cell nucleus in the resulting cybrid lines would be regarded as evidence of an underlying nuclear DNA defect in the patient's cells. This could be confirmed by demonstrating lack of correction of respiratory chain deficiency in cybrids generated by fusing nuclear DNA-less cytoplasts derived from normal fibroblasts with mtDNA-less (ρ^0) transformant fibroblasts derived from the patient (Tiranti et al. 1995b). However this technique relies upon the defect being expressed in cultured cells (such as fibroblasts or myoblasts), requires transformation of patient fibroblasts and is a time-consuming and expensive method that is difficult to employ on a large scale.

In this thesis an alternative screening method was investigated to distinguish mitochondrial from nuclear genetic defects. This approach involved analysis of COX subunit expression patterns detected immunohistochemically using highly specific monoclonal antibodies. This was then used to direct subsequent molecular genetic investigations. The identification of naturally occurring mutations in COX genes will provide important information about structure-function relationships in COX and about biochemical mechanisms within the mitochondrion.

7.3 Selection of patients

A large group of patients was studied in this thesis, which therefore provides much information about the presentation of COX deficiency in childhood. However a number of potential problems may have arisen from the retrospective nature of this series. A lack of existing databases recording patients with respiratory chain disorders prospectively led to difficulties in patient retrieval. Patients were retrieved by asking the relevant clinicians (consultants in paediatric metabolic medicine and neurology) to recall cases of COX deficiency which they had managed. This introduced the possibility of incomplete retrieval. To maximise the number of cases retrieved I also consulted the comprehensive records of the professor of histochemistry. These agreed well with the patient lists obtained from the clinicians.

It therefore seems likely that the vast majority of patients with identified respiratory chain defects who had muscle biopsies during the period 1985-1997 were included in this study. Fourteen of the 36 patients (39%) included in this study were recruited prospectively.

Patients with tissue-specific COX deficiency not expressed in skeletal muscle (for example, COX deficiency confined to brain, liver or cardiac muscle) would not have been detected by the retrieval methods employed in this study. Theoretically patients with heteroplasmic mtDNA mutations with low levels of heteroplasmy may also have been missed if the muscle biopsy sample analysed did not contain any fibres with sufficiently high mutant load to exceed the critical threshold required to express COX deficiency. This would be of particular concern with needle biopsy samples. However such low levels of heteroplasmy are unlikely to be clinically significant, unless there is tissue specificity, with higher levels of mutation in tissues other than muscle.

Another potential problem was ascertainment bias. Patients with severe phenotypes, those managed over a long period of time, and families with more than one affected sibling were more likely to be recalled by the clinicians involved. Patients with severe cardiomyopathies were less likely to have had open muscle biopsies (because of the risk of general anaesthesia) and consequently they may have been under-represented in the patient population studied immunohistochemically.

Diagnostic investigations evolved during the period in which the patients described in this thesis presented. In the earlier years only needle biopsies were performed and respiratory chain enzyme activities were not assayed. Therefore only a histochemical diagnosis of COX deficiency was available for patients presenting in this period, and it was not known whether they had isolated COX deficiency or a combined defect of more than one respiratory chain enzyme complex. Retrospective studies, including enzyme assays, were limited by the amount of residual tissue available for further investigations. However immunohistochemistry requires only a very small amount of tissue and was therefore an ideal technique with which to study these historical patients in more detail. Patients presenting from January 1996 had open muscle biopsies and spectrophotometric assays of

respiratory chain enzyme complexes I, II/III and IV (Heales et al. 1996) and so more detailed biochemical information was available for these patients.
Another potential problem was sample decay, particularly of historical samples. Some of the muscle samples studied had been stored at $-70^{0}C$ for greater than ten years. It is possible that individual COX subunits may not have been stable for such a long period. However samples which had obviously deteriorated during storage, with freezing artefact on sectioning, were excluded from the study. Furthermore, for the samples included in this study there was no obvious correlation between age of sample and pattern of immunostaining. Taken together, these considerations suggest that sample decay was unlikely to have been a significant problem in this study.

Despite these potential difficulties, this is the largest systematic study of COX deficiency in childhood to date and provides valuable clinical, biochemical and molecular genetic information about COX deficiency in an ethnically diverse population.

7.4 Clinical phenotypes observed

The phenotypes observed in this group of patients were heterogeneous. Lactic acidosis was noted in 94% of cases. Most patients had myopathy (86%) and/or encephalopathy (64%). 17% had cardiomyopathy, 33% had liver disease, and 19% had a renal tubular defect. Nine patients (25%) had Leigh syndrome. A wide range of other clinical features were noted in this series of patients, including dysmorphic features, corneal opacification, hypertrichosis and peripheral neuropathy.

Robinson described five phenotypes of nuclear-encoded human isolated COX deficiency: Leigh syndrome, fatal infantile lactic acidosis, cardiomyopathy with hypotonia, ataxia with leukodystrophy and renal tubulopathy, and the Saguenay Lac St Jean variant of Leigh syndrome (Robinson, 2000). The group of patients studied in this thesis does not conform exactly to Robinson's description. There are various explanations for this. The higher proportion of patients with lactic acidosis may reflect the pattern of referrals to the metabolic team at Great Ormond Street Hospital. An ethnically diverse patient population is seen at Great Ormond Street Hospital and it therefore may be anticipated that novel phenotypes may be encountered. Finally, not all the patients studied in this thesis had isolated COX

deficiency. Some had COX deficiency combined with other defects of the respiratory chain, particularly complex I deficiency, and this might explain the observation of different phenotypes.

7.5 COX subunit expression patterns

In Chapter 3 muscle immunohistochemistry with monoclonal antibodies directed against COX subunits was used to demonstrate a specific pattern of COX subunit staining in 5 patients with known mtDNA mutations. Selective and marked reduction of mtDNA-encoded COX subunits I and II restricted to COX-deficient fibres was observed in all these patients, together with reduced COX subunit VIc staining in the same fibres. Two patients with mtDNA depletion also had this pattern of immunostaining, although a third mtDNA depletion patient had reduced staining of all subunits. Eight of 10 patients with the same 'mtDNA' pattern of immunostaining, but without a known molecular cause of their COX deficiency, were selected for mtDNA sequencing studies. The results are discussed in detail in Chapter 4 and summarised below.

The remaining 23 patients whose muscle was examined immunohistochemically either had intact staining of all COX subunits tested (n=10), or reduced staining of all subunits (n=13). The group of 10 patients with near normal COX subunit steady state levels despite severe reduction in COX activity is particularly interesting. These patients appear to have a defect in catalytic activity of the enzyme that does not affect assembly or stability of the enzyme complex. Kinetic studies may be helpful in further delineating the defect in these patients, but are hampered by lack of available tissue as many of these patients are deceased. Cultured fibroblast cell lines are available from 7 of these patients. If these cells express COX deficiency in culture, then kinetic studies would be worthwhile, as part of a future project. Kinetic defects of COX have been described previously in 5 patients with COX deficiency (Nijtmans et al. 1995a; Glerum et al. 1987; Zimmermann and Kadenbach, 1992; Hanson et al. 2001).

No patient expressed loss of a single nuclear-encoded COX subunit, suggesting a mutation in the gene encoding that subunit. Although only three nuclear-encoded COX subunits (IV, Va and VIc) were studied in this thesis, these results correlate well with the findings of other groups who have systematically sequenced all 12

nuclear-encoded COX subunit genes in patients with isolated COX deficiency and failed to find any mutations (Adams et al. 1997; Lee et al. 1998; Jaksch et al. 1998).

7.6 Role of mtDNA mutations

7.6.1 Detection of new pathogenic mtDNA mutations

The highly polymorphic nature of mtDNA means that it is not unusual to find sequence differences between individuals, particularly if they are from different ethnic groups. In this thesis 10 putative pathogenic base changes were identified, in addition to 28 known or silent polymorphisms. Caution must be employed before ascribing pathogenicity to novel sequence changes.

7.6.2 Mutations in COX subunit genes

This thesis confirms that mutations in the mtDNA-encoded subunits of COX appear to be particularly uncommon in childhood. Only one such mutation was identified in this study, a novel thymine to adenine transversion at nucleotide position 7671 of mtDNA (T7671A), within the gene encoding COX II. This mutation is particularly interesting for two reasons. Firstly it appears to cause defective assembly of the COX holoenzyme and thereby adds to the available information about the COX assembly sequence. The second interesting feature of this mutation is the segregation of mutant load between mature muscle cells and satellite cells. This introduces the possibility of therapeutic intervention, by promoting regeneration of muscle from the satellite cell pool with low mutant load following microscopic muscle necrosis induced either pharmacologically or by physical training, as has previously been demonstrated for two other mtDNA mutations (Clark et al. 1997; Taivassalo et al. 1999).

7.6.3 tRNA mutations

Mutations in tRNA genes usually cause a combined deficiency of complexes I and IV, but isolated defects of complex IV are well recognised in patients with tRNA mutations (Silvestri et al. 1993; Silvestri et al. 1998). There are two reasons for this. Firstly there is a higher rate of synthesis of COX subunits compared to subunits of complexes I and III (Hanna et al. 1995b). Secondly some amino acids, for example tryptophan, are present in relatively larger proportions in COX subunits I and II than

in subunits of other respiratory chain complexes (Silvestri et al. 1998; Silvestri et al. 2000).

The secondary structure of the wild type human tRNA molecules is not well characterised and so it is difficult to predict the effect of base changes on this secondary structure. Hence establishing pathogenicity is particularly difficult for tRNA mutations. This is well illustrated by the three potentially pathogenic tRNA mutations identified in this thesis. These included two base changes that have previously been reported to be pathogenic (the A10044G transition in tRNA glycine and the 15940-4delT microdeletion in tRNA threonine) (Santorelli et al. 1996b; Seneca et al. 1998), but which now appear to be found in clinically unaffected family members. The third mutation was homoplasmic and from an African patient and may represent an ethnic variant (C5773T transition in tRNA cysteine).

7.6.4 The role of the background mtDNA haplotype

The extent to which background mtDNA variation might interact with and influence the expression of mtDNA disease mutations is not known. However Wallace has shown that in the case of LHON certain mtDNA haplotypes are more prone to expression of LHON than others (Wallace et al. 1999). It is quite possible that this may also apply to other mtDNA diseases. In this thesis it is possible that some of the base changes identified that do not appear to be primarily pathogenic may nevertheless be contributing to the disease phenotype. Furthermore the mtDNA haplotype might modulate the expression of COX deficiency due to nuclear gene mutations.

7.7 The role of nuclear genes

One of the unsolved mysteries of human COX deficiency is why no mutations have been identified to date in the genes encoding nuclear subunits of the enzyme. It is possible that such mutations are lethal in utero (Sue and Schon, 2000). However a number of mutations have been reported in nuclear-encoded polypeptides of complexes I and II (Bourgeron et al. 1995; Loeffen et al. 1998; van den Heuvel et al. 1998; Triepels et al. 1999; Schuelke et al. 1999; Parfait et al. 2000; Baysal et al. 2000; Budde et al. 2000; Petruzzella et al. 2001; Benit et al. 2001; Loeffen et al. 2001). An intriguing suggestion to explain this apparent paradox is that, because

both complex I and complex II feed into ubiquinone 'in parallel', the loss of either complex may not be immediately fatal (Sue and Schon, 2000). Complex IV, however, is distal to ubiquinone and acts 'in series' in the respiratory chain, so that defects may be less well tolerated by the affected individual.

Further support for this is the finding that even patients with the most severe COX-deficient phenotypes have some residual COX activity. Presumably mutations leading to complete loss of COX activity are lethal in embryonic or fetal life. However the converse could also be true: it is possible that some nuclear subunit mutations are well tolerated and result in mild or even subclinical phenotypes. This could be the case if a subunit is not essential for COX function or stability. However this hypothesis has not yet been tested as so far only patients with severe COX-deficient phenotypes have had their nuclear COX subunit genes sequenced (Adams et al. 1997; Lee et al. 1998; Jaksch et al. 1998).

Some nuclear gene mutations have been identified in human COX deficiency. These mutations are in genes responsible for assembly and/or maintenance of the COX holoenzyme.

7.7.1 SURF1

SURF1 is a housekeeping gene of unknown function that appears to be involved at an early stage in the assembly of COX (Zhu et al. 1998). In this thesis pathogenic mutations were identified in the *SURF1* gene of 4 patients. These patients had isolated COX deficiency with an apparently 'non-mtDNA' and thus 'nuclear' pattern of subunit staining on muscle immunohistochemistry. The clinical phenotype in 3 of these 4 patients with *SURF1* mutations was Leigh syndrome, but one patient (P37) had isolated leukodystrophy. This means that clinical features alone cannot be used to reliably identify all patients with *SURF1* mutations.

The range of phenotypes associated with *SURF1* mutations has not yet been established. So far the search for *SURF1* mutations has focussed mainly on patients with Leigh syndrome or with 'Leigh-like' features (Tiranti et al. 1999c). Three of the patients with *SURF1* mutations described in this thesis were noted to have hypertrichosis. One also had peripheral neuropathy. However peripheral neuropathy does not appear to be a discriminatory feature as patients with

peripheral neuropathy in this thesis were found to have all 3 patterns of COX subunit immunostaining in Chapter 3 (i.e. reduction of mtDNA subunits only, reduced staining of all subunits or normal staining of all subunits).

The majority of reported *SURF1* mutations result in a frameshift and are predicted to result in a truncated protein product (see Table 1.7 in Chapter 1). Almost all patients reported in the literature with these mutations had Leigh syndrome (Pequignot et al. 2001b). It is possible that missense mutations in *SURF1* might result in a milder phenotype. These observations, together with the identification of a pathogenic *SURF1* mutation in a patient with isolated leukodystrophy in this thesis, leads to the conclusion that the *SURF1* gene should be analysed in all patients with isolated COX deficiency, in order to determine the range of phenotypes that might be associated with mutations in this gene.

7.7.2 Other nuclear genes

A large number of nuclear-encoded ancillary proteins is required for the correct assembly and function of COX. However so far mutations have only been found in three other nuclear genes (*SCO2*, *SCO1* and *COX10*) in patients with isolated COX deficiency (Papadopoulou et al. 1999; Valnot et al. 2000a; Valnot et al. 2000b). The clinical phenotype of patients with mutations in these genes differs significantly from that observed in patients with *SURF1* mutations, in that none of these patients had Leigh syndrome. Furthermore early-onset hypertrophic cardiomyopathy was a prominent feature in SCO2-deficient patients (Papadopoulou et al. 1999; Sue et al. 2000), whilst liver and renal disease were notable findings in patients with *SCO1* and *COX10* mutations respectively (Valnot et al. 2000a; Valnot et al. 2000b).

One of the most important unanswered questions, and one which will benefit patients by directing clinical investigations and potentially allow a molecular diagnosis to be achieved more rapidly, is whether there is any genotype-phenotype correlation in COX deficiency. So far most patients with *SURF1* mutations had Leigh syndrome, whilst those with *SCO2* mutations had a fatal syndrome of hypertrophic cardiomyopathy and encephalopathy. Neuropathology of patients with *SCO2* mutations did not show the typical features of Leigh syndrome (Sue et al. 2000). However this apparent genotype-phenotype correlation might be related to a bias in selection of patients for sequencing studies of these genes. Furthermore as only

single families with *SCO1* and *COX10* mutations have been reported, it is difficult to predict whether homogeneous phenotypes will be associated with mutations in this genes. Characterisation of further patients with mutations in these and other, as yet undiscovered, genes is required to determine whether the early suggestion of genotype-phenotype correlation is indeed correct.

7.8 Proposed diagnostic cascade

At present patients with *SURF1* mutations represent the largest subset of COX deficiency with an identified molecular cause. Any diagnostic strategy should therefore aim to identify these patients as efficiently as possible, since these families could then be offered accurate genetic counselling and first trimester prenatal diagnosis. Based on the findings in this thesis and reports in the literature, patients with *SURF1* mutations appear to have the following biochemical features:

1. isolated COX deficiency in muscle (Zhu et al. 1998; Tiranti et al. 1998b)
2. a homogeneous appearance on COX immunohistochemistry in muscle, with reduced staining of both mtDNA and nuclear DNA-encoded COX subunits (Sue et al. 2000)
3. expression of COX deficiency in cultured skin fibroblasts (Tiranti et al. 1999c).

Taking this information into account, a diagnostic cascade can be proposed for the investigation of such patients in future (Figure 7.1). The first phase of investigation would involve muscle histochemistry and spectrophotometric respiratory chain enzyme assays. In the second phase COX subunit immunohistochemistry would be performed on muscle from patients with isolated COX deficiency. This could be used to select patients with reduced immunostaining of all COX subunits. COX enzyme assays could then be performed on cultured skin fibroblasts from these patients and the *SURF1* gene sequenced in those expressing COX deficiency in fibroblasts, i.e. those with 'systemic' COX deficiency.

Patients with selective reduction of mtDNA-encoded COX subunits on muscle immunohistochemical studies would follow a different diagnostic path. In these patients mtDNA sequence analysis might reveal a causative mutation. If no pathogenic mtDNA mutation is identified, it is possible that these patients might have a mutation in a gene involved in the delivery of prosthetic groups to the mtDNA-encoded COX subunits, such as *SCO1* or *SCO2* (Sue et al. 2000). These

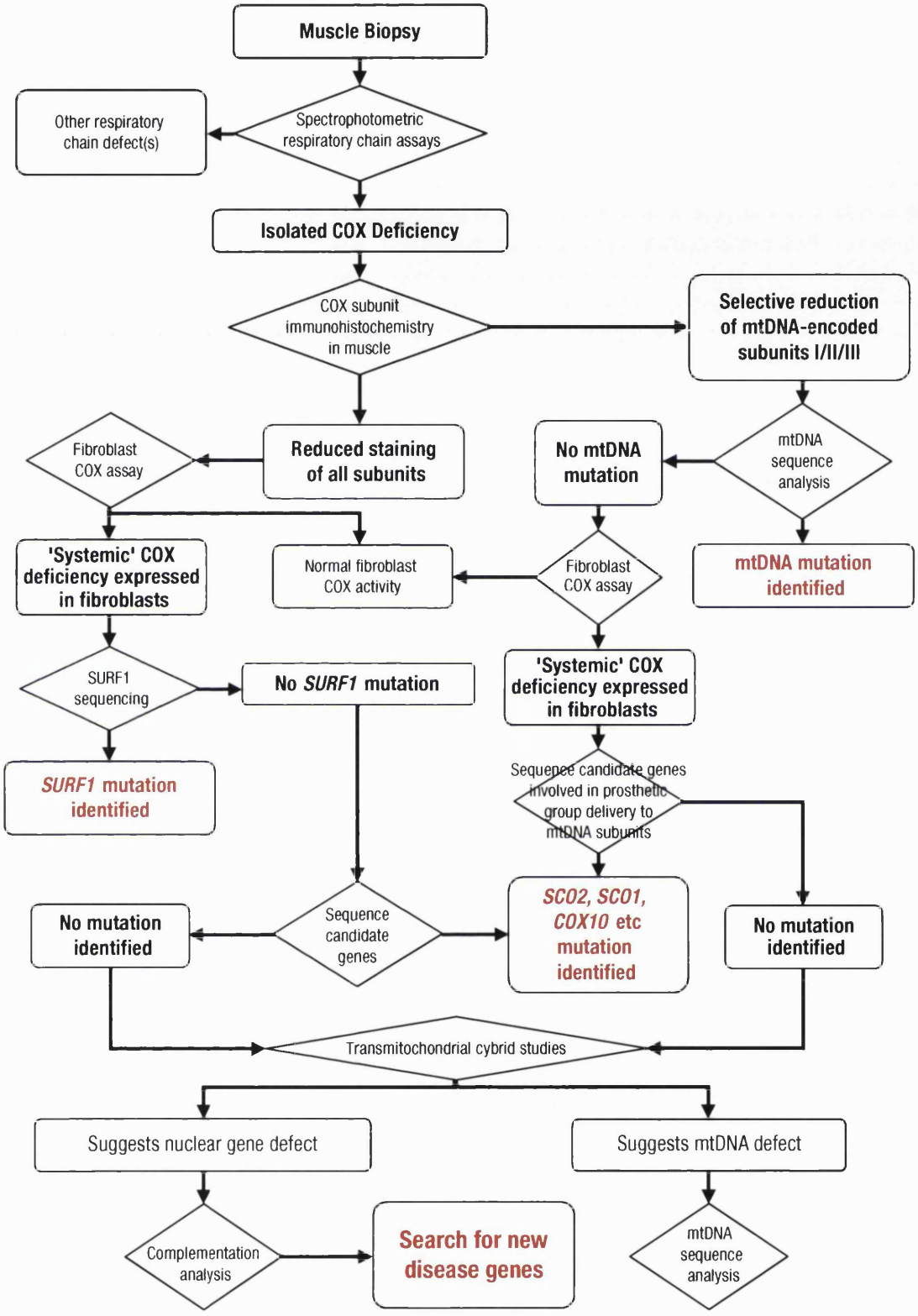


Figure 7.1 Proposed diagnostic pathway for cytochrome oxidase deficiency

genes should therefore be sequenced in these patients, together with other candidate genes as they are discovered.

7.9 Future directions of research

Further studies are required in patients with an apparently 'mitochondrial' pattern of COX subunit immunostaining but in whom a pathogenic COX or tRNA gene mutation has not been identified. These studies include mtDNA-encoded complex I subunit (ND) gene sequencing in selected patients. Another approach would be to screen for mutations in genes which deliver prosthetic groups to the mtDNA-encoded COX subunits, for example *SCO2* which provides copper to subunits I and II. Transmitochondrial cybrid studies could also be used to determine whether these patients do have an underlying mtDNA defect. This has been proven for one patient, P31 (Louise Turner, personal communication).

Sequencing *SURF1* in the remaining patients from whom cultured cell lines are available will allow further characterisation of the SURF1-deficient phenotype. *SCO2*, *SCO1* and *COX10* should be also be sequenced in those patients without an identifiable *SURF1* mutation. Further studies could be directed at elucidating the mechanism of SURF1 function, perhaps by using yeast 2 hybrid analysis (Fields and Song, 1989; Chien et al. 1991) to search for protein partners that co-function with SURF1. These would clearly represent additional candidate genes for COX deficiency. Establishing the function of the *SURF1* gene might also explain the different phenotypes observed in patients with *SURF1* mutations compared to those with *SCO2* mutations, for example why cardiac involvement is apparently not observed in patients with *SURF1* mutations but is the predominant clinical feature in patients with *SCO2* mutations. Generalised COX deficiency is observed in both these diseases, suggesting ubiquitous expression of both genes, at least in postnatal life. It is possible that differential tissue expression of these genes occurs in fetal life (Sue and Schon, 2000), and further studies could address this issue.

There is evidence for genetic heterogeneity in some of the clinical phenotypes associated with isolated COX deficiency. For example at least three complementation groups exist for COX-deficient Leigh syndrome (Brown and Brown, 1996; Munaro et al. 1997). In accordance with this, *SURF1* mutations have

been identified in only 23 to 75% of these patients (Tiranti et al. 1999c; Sue et al. 2000). Similarly not all patients with isolated COX deficiency and cardiomyopathy have *SCO2* mutations. These observations suggest that other genes are likely to be involved. Protein partners that co-function with SURF1 might be mutated in patients with Leigh syndrome, and other genes in the copper delivery pathway may be involved in COX-deficient cardiomyopathy. These avenues need to be explored. Furthermore some clinical and biochemical phenotypes have not yet been matched to specific genes, for example fatal infantile COX deficiency, benign reversible COX deficiency and the French Canadian Saguenay Lac St Jean variant of COX deficiency.

Transmitochondrial cybrid studies could be used in our patients to establish complementation groups for the remaining patients without identified mtDNA, *SURF1* or other mutations. Gene mapping strategies could then be used to identify the disease genes responsible for COX deficiency in each of these complementation groups. A large number of yeast nuclear genes have been reported to cause a respiratory deficient or *petite* phenotype when mutated and their human homologues are obvious candidates for causing human mitochondrial respiratory chain disease. Two approaches could be used to search for further candidate genes: 1) traditional gene mapping or 2) computerised search techniques. The former would include homozygosity mapping in consanguineous pedigrees and in island populations with specific disease variants. Computerised searching for human homologues of these yeast genes, for example the BLAST similarity searching program, is a useful tool to identify candidate genes causing human mitochondrial respiratory chain disease. Recently a systematic comparison of yeast protein sequences to the GenBank dbEST database identified 102 groups of human EST likely to represent orthologues of yeast genes (Rotig et al. 2000). Another strategy for computer-based searching is to identify genes whose protein products have presequences for mitochondrial targeting.

Yeast mutants are a useful tool with which to study the effects of identified human mutations in vitro. Unfortunately methionine 29 in COX subunit II is not conserved in yeast, so it was not possible to study the effects of the T7671A mutation identified in this thesis in a yeast model. However this would be a potentially powerful method to

demonstrate pathogenicity of any other mutations identified in future. This would apply to both mtDNA and nuclear gene mutations.

In conclusion this thesis has demonstrated molecular defects in two genomes in patients with COX deficiency and has suggested a diagnostic cascade to accelerate the achievement of a molecular genetic diagnosis in patients presenting with COX deficiency in the future.

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Electronic Database Information

The URLs for data in this thesis are as follows:

GENATLAS http://www.citi2.fr/GENATLAS/

National Centre for Biotechnology Information (NCBI) **GenBank** nucleotide sequence database http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide

GOBASE Organelle Genome Database http://megasun.bch.umontreal.ca

Mitomap http://www.gen.emory.edu/mitomap.html

MITOP http://www.mips.biochem.mpg.de/proj/medgen/mitop/

NCBI **LocusLink** http://www.ncbi.nlm.nih.gov/LocusLink/

NCBI **Protein** sequence database
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein

SWISS-PROT http://www.expasy.ch/sprot/

tRNA sequence database
http://www.uni-bayreuth.de/departments/biochemie/sprinzl/trna/

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Cytochrome c Oxidase Deficiency Associated with the First Stop-Codon Point Mutation in Human mtDNA

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Summary

We have identified the first stop-codon point mutation in mtDNA to be reported in association with human disease. A 36-year-old woman experienced episodes of encephalopathy accompanied by lactic acidemia and had exercise intolerance and proximal myopathy. Histochemical analysis showed that 90% of muscle fibers exhibited decreased or absent cytochrome c oxidase (COX) activity. Biochemical studies confirmed a severe isolated reduction in COX activity. Muscle immunocytochemistry revealed a pattern suggestive of a primary mtDNA defect in the COX-deficient fibers and was consistent with either reduced stability or impaired assembly of the holoenzyme. Sequence analysis of mtDNA identified a novel heteroplasmic G→A point mutation at position 9952 in the patient's skeletal muscle, which was not detected in her leukocyte mtDNA or in that of 120 healthy controls or 60 additional patients with mitochondrial disease. This point mutation is located in the 3′ end of the gene for subunit III of COX and is predicted to result in the loss of the last 13 amino acids of the highly conserved C-terminal region of this subunit. It was not detected in mtDNA extracted from leukocytes, skeletal muscle, or myoblasts of the patient's mother or her two sons, indicating that this mutation is not maternally transmitted. Single-fiber PCR studies provided direct evidence for an association between this point mutation and COX deficiency and indicated that the proportion of mutant mtDNA required to induce COX deficiency is lower than that reported for tRNA-gene point mutations. The findings reported here represent only the second case of isolated COX deficiency to be defined at the molecular genetic level and reveal a new mutational mechanism in mitochondrial disease.

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Introduction

A large number of primary defects of mtDNA have been described in association with a group of human diseases known as the mitochondrial myopathies and encephalomyopathies (Morgan-Hughes 1994). This is a group of clinically and biochemically heterogeneous disorders that frequently have the common feature of mitochondrial proliferation at the level of the single muscle fiber (Morgan-Hughes 1994). The primary mtDNA defects described fall broadly into two groups: large-scale rearrangements and point mutations (Holt et al. 1988; Nelson et al. 1989; Moraes 1996; Schon et al. 1997). Large-scale rearrangements, which include deletions and duplications, are frequently associated with chronic progressive ophthalmoplegia, Pearson syndrome, and Kearns-Sayre syndrome (Morgan-Hughes 1994; Poulton et al. 1989). Point mutations may occur either in tRNA genes or in structural genes. There are now >50 tRNA-gene point mutations that have been described (Moraes 1996), the most common being at nucleotide positions 3243 and 8344, and these frequently are associated with MELAS (mitochondrial encephalomyopathy with lactic acidosis and strokelike episodes) syndrome and MERRF (myoclonus epilepsy and ragged red fibers) syndrome, respectively. Missense point mutations in protein-coding genes are associated with Leber hereditary optic neuropathy (Harding and Sweeney 1994). However, unlike observations made in nuclear-genetic disorders, point mutations leading to premature stop codons (null mutations) in protein-coding genes have not been described in human mtDNA. This is perhaps surprising, in view of the high polymorphic rate of human mtDNA (Brown et al. 1979). Point mutations are inherited in the matrilineal line, whereas single deletions of mtDNA are sporadic and are rarely, if ever, transmitted (Morgan-Hughes 1994).

Cytochrome c oxidase (COX), complex IV of the respiratory chain, catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen, forming water, and has proton-pumping activity (Capaldi 1990). It comprises 13 subunits, 3 encoded by mtDNA and the

remainder encoded in the nucleus. Isolated complex IV deficiency is not infrequently associated with pediatric disorders such as subacute necrotizing encephalomyelopathy, fatal infantile myopathy, myopathy with cardiomyopathy, and benign reversible COX deficiency (DiMauro et al. 1994). These disorders generally develop during the first few years of life and are thought to be autosomal recessive, but, to date, no nuclear-gene defect has been identified (Adams et al. 1997; Munaro et al. 1997).

We now report an adult patient with recurrent encephalopathy, myopathy, and exercise-induced myalgia who had isolated COX deficiency demonstrated biochemically and histochemically. A new stop-codon point mutation in the COX III gene has been identified, and we provide evidence that, uniquely, this point mutation is not inherited in the matrilineal line.

Patients and Methods

Patients

The proband is a 36-year-old woman who was a normal full-term delivery and who had normal early motor and cognitive development. She was good at sports at school and excelled in middle-distance running. At the age of 17 years she collapsed at the end of a 1.5-km race and was found to be confused and complaining of a headache. She recovered during the next few hours and remained well for the next 4 years. In retrospect, this may have been an episode of exercise-induced lactic acidosis. At the age of 21 years she began to experience symptoms of fatigue after exertion and, soon after exertion, would develop generalized muscle aches that could last for $\leqslant$24 h but were not accompanied by pigmenturia. She stopped drinking alcohol, which exacerbated her myalgia, gave up athletics, and became less active. Her symptoms became much worse during her three pregnancies, although her labors were uneventful. At the age of 28 years, 3 mo after her third child was born, she developed a marked increase in fatigue, with headaches, over the course of 1 wk. She subsequently became drowsy and confused and was admitted to hospital in a stuporous state.

On clinical examination she had a reduced level of consciousness. She exhibited decorticate posturing on painful stimulation, but there was no spontaneous movement. There were roving eye movements. All four limbs exhibited increased tone in a spastic fashion, and there was generalized hyperreflexia with extensor-plantar responses. Laboratory investigations showed an increased anion gap and metabolic acidosis that were due to an elevated level of plasma lactate (7 mmol/liter). Serum creatine kinase was elevated, at 860 IU (normal <150 IU/liter). An electroencephalogram showed widespread slow-wave abnormalities, which resolved over the next 72 h. Results of both computed-tomography brain scan on admission and subsequent magnetic-resonance–imaging brain scans have been normal. She recovered from this episode but had two further identical presentations over the next year. There was no evidence of intercurrent illness preceding any of these episodes. Since the age of 29 years her symptoms and signs have been static. She experiences fatigue and myalgia and frequent common migraine-type headaches. Current clinical examination shows symmetrical proximal (mild) muscle weakness involving all four limbs. Results of electromyogram and nerve conduction studies have been normal. Exercise testing by a bicycle ergometer, as described elsewhere (Petty et al. 1986), has demonstrated an abnormal lactate response to sub–anaerobic-threshold exercise, consistent with a defect in respiratory-chain function. The patient has been unable to tolerate phosphorous magnetic-resonance spectroscopy (MRS) studies of her muscle or proton MRS of her brain.

Her mother had a hypertensive intracerebral hemorrhage at the age of 50 years, from which she recovered, and she is otherwise well. The patient has three sons, ages 16, 15, and 10 years. The youngest son is well. The two older sons describe episodes of myalgia lasting $\leqslant$24 h that are not clearly related to exertion, but they have normal muscle function and, indeed, are both good athletes. The eldest son has a history of predominantly nocturnal episodes in which he becomes confused and describes vivid visual hallucinations. Despite extensive investigation, no abnormality has been identified in these two boys, and, in particular, there is no evidence of respiratory-chain dysfunction (see Results section). Our current hypothesis is that these two sons are exhibiting psychologically driven symptoms reinforced by their mother's illness behavior; they are currently undergoing family therapy.

Methods

Histochemical and immunocytochemical analysis.—Ten-micron cryostat sections of frozen muscle were stained to demonstrate the activities of COX and succinate dehydrogenase (SDH). COX activity was determined in medium containing 4 mM 3,3′-diaminobenzamidine and 100 mM cytochrome c in 0.1 mM phosphate, pH 7.0, at 25°C. SDH activity was determined by 1.5 mM nitroblue tetrazolium, 130 mM sodium succinate, 0.2 mM phenazine methosulfate, and 0.1 mM sodium azide in 0.1 mM phosphate, at pH 7.0 at 25°C. Sections were also stained with the Gomori trichrome stain (Engel and Cunningham 1963).

A battery of subunit-specific mouse monoclonal antibodies was used to identify COX subunits immunohistochemically in serial muscle sections (Taanman et al.

1996). The antibodies were directed against the following subunits: I and II (mtDNA encoded) and IV, Va, and VIc (nuclear-DNA encoded). Visualization was by the Strept ABC technique, by use of 3,3′-diaminobenzidine (Rahman et al. 1997).

Biochemical analysis.—Mitochondria were isolated from skeletal muscle removed from the left quadriceps. Polarographic determination of mitochondrial function, spectrophotometric analysis of respiratory-chain–enzyme activities, and low-temperature analysis of cytochrome content were performed as described elsewhere (Morgan-Hughes et al. 1977; Mann et al. 1992).

mtDNA analysis.—Total DNA was extracted from blood, muscle, skin, fibroblast, and myoblast cultures, by standard techniques described elsewhere (Hanna et al. 1995*b*). Southern blotting was performed on total muscle DNA (Holt et al. 1988). Oligonucleotide primers with M13 tails at the 5′ end were designed to amplify all mitochondrial tRNA genes and all three mitochondrially encoded COX subunits (sequences of primers are available, on request, from the authors). DNA extracted from muscle was used for DNA sequencing. The PCR products generated were cleaned by Centricon filters, and both strands were sequenced by a Dye Primer *Taq* cycle sequencing kit (Applied Biosystems [ABI]). The sequencing products were then separated on 10% polyacrylamide-urea gels in a 373A automated DNA sequencer (ABI). The sequence data generated were analyzed by Seq Ed software (ABI).

A mismatch PCR was designed to screen for the 9952 point mutation identified, in which a restriction site for the endonuclease, *Dra*I, was created in the presence of the mutant mtDNA. The forward primer was light strand (L) 9817–9836; and the reverse mismatch primer was heavy strand (H) 9973–9944 ATG GAG ACA TAC AGA AAT ATT (the mismatch nucleotide is underlined). Conditions for the mismatch PCR were as follows: an initial denaturing step at 94°C for 3 min, followed by 30 cycles of the following: 92°C for 30 s, 53°C for 30 s, and 72°C for 20 s. A final extension step of 72°C for 10 min was used. After digestion with *Dra*I, the fragments were separated on a 3.2% agarose gel stained with ethidium bromide and were visualized on a UV-light box. The 157-bp product was digested into two fragments—of 135 bp and 22 bp—in mutant mtDNA but not in wild-type mtDNA.

An adaptation of the same mismatch PCR was used to quantitate the proportion of mutant mtDNA in total muscle and single fibers from the patient. Fluorescently labeled deoxynucleotide was added prior to the last cycle of the mismatch PCR, followed by digestion with *Dra*I. The products were then separated on a 6% nondenaturing polyacryalmide gel on a 373A DNA sequencer (ABI) and quantified by GENESCAN software (ABI).

Single-fiber PCR.—Single-fiber PCR was performed according to methods described elsewhere (Sciacco et al. 1994; Moraes and Schon 1996). Thirty-micron transverse muscle sections were prepared and were reacted for COX. Freshly stained sections were immersed in 50% ethanol. COX-positive fibers and fibers with no visually detectable COX activity were selected. Single-fiber segments were removed by a borosilicate microcapillary tube under an inverted-light microscope. The removed segments were put into 10 μl water and immediately were centrifuged for 10 min. The water was removed, and 5 μl lysis solution was added (200 mM potassium hydroxide and 50 mM DTT). The fibers were then incubated in this lysis solution for 1 h at 65°C, and then 5 μl neutralizing solution was added (900 mM Tris-HCl, pH 8.3, and 200 mM HCl). The fluorescent mismatch PCR described above was then performed with this 10-μl product.

Human myoblast and fibroblast culture.—Mass myoblast cultures were established from the diagnostic muscle-biopsy sample, by methods described elsewhere (Hanna et al. 1995*a*). Standard growth medium for myoblasts comprised DMEM (Dulbecco's modified Eagle medium) with GLUTAMAX-1, 4.5 g glucose/liter, 110 mg sodium pyruvate/liter, 20% FCS, 2% detoxified chick-embryo extract, 50μg uridine/ml, and 10μg gentamycin/ml. Fibroblast cultures were established from skin-punch biopsy samples, by the explant method (Martin 1973). Growth medium for fibroblasts differed from that used for myoblasts, in that 10% FCS was used and chick-embryo extract was not used.

Results

Histochemical analysis of the patient's muscle-biopsy sample showed a marked reduction in COX activity, in a mosaic distribution. Ten percent of the fibers exhibited normal COX activity, whereas the remainder exhibited reduced or absent activity (fig. 1). There were no ragged red fibers, as determined by staining using the modified Gomori trichrome method. Staining for SDH activity revealed that <2% of the fibers exhibited a slightly increased staining pattern, consistent with a mild degree of mitochondrial proliferation. There were no SDH-intense blood vessels. Biochemical analysis confirmed an elevated citrate synthase activity in the muscle homogenate, compared with the control value, a result consistent with some mitochondrial proliferation (table 1). Results of histochemical analysis of the muscle-biopsy samples from the patient's two sons and from the patient's mother were entirely normal. Immunocytochemical studies using monoclonal antibodies directed against both nuclear and mitochondrially encoded COX subunits were abnormal in the patient's COX-negative fibers. There was a selective reduction in staining with antibodies against subunits I, II, and VIc. The staining pat-

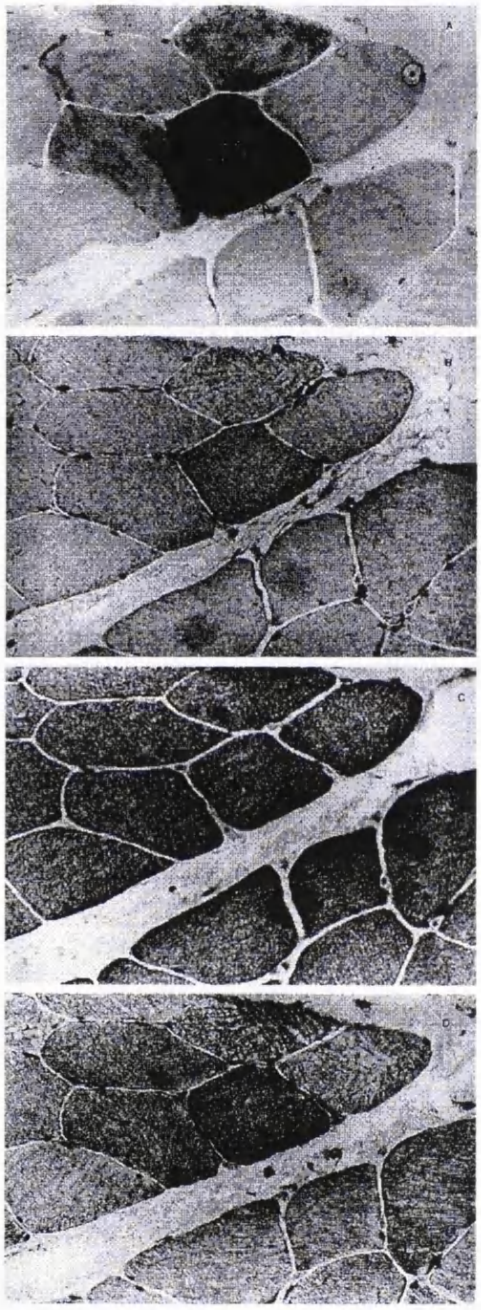

Figure 1 Muscle-biopsy serial sections from the proband, stained for COX activity (*A*) and immunostained with antibodies directed against subunits II (*B*), Va (*C*, and VIc (*D*). Panel *A* shows one type I fiber (*center*) with normal COX activity (*arrows*). The remaining fibers have markedly reduced COX staining. Fibers with reduced COX activity have reduced immunostaining with antibodies directed against COX subunits II and VIc but have normal immunostaining with antibodies directed against subunit Va. Fibers with reduced COX activity also showed reduced immunostaining with antibodies directed against subunit I and showed normal immunostaining with antibodies against subunit IV (data not shown).

tern with antibodies directed against subunits IV and Va did not differ from either that in normal controls or that of COX-positive fibers in the patient's biopsy sample (fig. 1). Biochemical analysis of respiratory-chain enzymes in freshly isolated mitochondria of the patient revealed a marked isolated decrease in both complex IV activity (20% of the control value) and cytochrome aa_3 content (17% of the control value) (table 1). Polarographic studies showed decreased oxygen consumption with the natural substrates—pyruvate + malate, glutamate + malate, and succinate—all of which require a fully functional COX. Oxygen consumption with the artificial substrate ascorbate and tetramethyl-p-phenylenediamine dihydrochloride reduces cytochrome aa_3 directly, therefore bypassing cytochrome a. Results of respiratory-chain–enzyme studies of the patient's sons and of her mother were normal (data not shown).

A large-scale rearrangement was excluded by Southern blotting. DNA sequence analysis of all 22 mitochondrial tRNA genes and of the genes for subunits I, II, and III of COX was undertaken. Three changes were identified, compared with the Anderson et al. (1981) sequence; these were T14766C in the cytochrome b gene, A1438G in the 12SrRNA gene, and G9952A in the gene for subunit III of COX. The first two changes are recognized to be neutral polymorphisms (Kobayashi et al. 1990; Tanaka and Ozawa 1994). The G→A transition at position 9952 has not been described elsewhere (fig. 2). This mutation results in the wild-type TGA codon for tryptophan being converted into a TAA stop codon. This premature stop codon is predicted to result in the loss of the last 13 amino acids of the highly conserved C-terminal region of subunit III of COX. This mutation was heteroplasmic in the patient's muscle but was not detected in her skin or blood or in the blood or muscle samples taken from her two sons and from her mother. The G9952A mutation was not present in either 120 healthy control samples or 60 additional patients with mitochondrial disease but with no known mtDNA mutation. The proportion of mutant mtDNA was the same (57%) in the patient's muscle-biopsy samples in 1990 and in 1997. Early-passage myoblasts and fibroblasts from the proband and myoblasts from her mother and her two sons were grown in medium, to support respiratorily deficient cells, as described in the Methods subsection. However, none of these cell lines harbored the G9952A mutation.

Single-fiber PCR studies were performed to investigate the relationship between the mutation identified and COX deficiency at the level of the single muscle fiber. The mean proportion of mutant mtDNA in the COX-negative fibers ($n = 24$) was 56.2% (standard error of the mean [SEM] 5.1%), and that in the COX-positive fibers ($n = 21$) was 10.1% (SEM 2.3%) ($P < .0002$, by t-test).

Discussion

Isolated COX deficiency as observed in this case is rare after the 1st decade of life, and there are few reported. Haller et al. (1989) identified a 27-year-old woman with life-long exercise intolerance and a mild proximal limb weakness. A muscle-biopsy sample showed no frank ragged red fibers by the Gomori trichrome stain but did show that >90% of the fibers had virtually absent COX activity. This defect was confirmed spectophotometrically. There was no family history, and no molecular-genetic data were reported. A recent case reported by Keightley et al. (1996) is a 15-year-old female who experienced recurrent episodes of myoglobinuria and muscle cramps with no muscle weakness on examination. A muscle-biopsy sample in this patient did show typical ragged red fibers and 64% COX-negative fibers. Results of immunoblotting and immunocytochemistry were consistent with either a lack of assembly or instability of the COX holoenzyme. A heteroplasmic in-frame 15-bp microdeletion was identified in the COX III gene. Although the patient described by Haller et al. had some clinical features in common with the patient whom we have reported, neither of these cases had evidence of CNS disease, which is the most likely cause of our patient's recurrent encephalopathy. Adult-onset Leigh syndrome has been reported in association with COX deficiency, but our patient does not have the imaging features seen in this condition (Adams et al. 1997). COX deficiency in combination with other respiratory-chain–complex deficiencies is much more commonly seen in adults, and, in such cases, a primary defect in mtDNA, resulting in impaired intramitochondrial protein synthesis, is frequently identified (Hanna et al. 1995*b*).

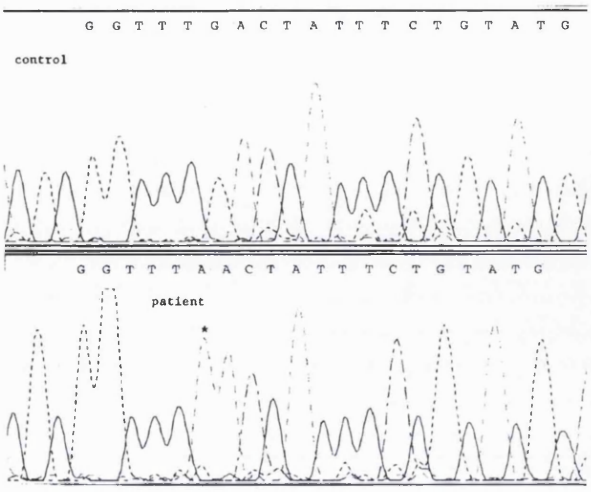

Figure 2 Electropherograms showing wild-type G nucleotide at position 9952 in control muscle mtDNA (*top*) and mutant A nucleotide (*asterisk* [*]) in the proband's muscle mtDNA (*bottom*).

Immunocytochemical studies in our patient demonstrated a decreased staining pattern in COX-negative fibers, with antibodies directed against subunits I, II, and VIc. The staining patterns with antibodies directed

Table 1

Biochemical Analyses in Isolated Muscle Mitochondria from the Proband

	Proband[a]	Mean ± SD in Controls (n = 5)
Mitochondrial state–3 respiration rates for substrates (nmol O_2/min/mg mitochondrial protein):		
Pyruvate + malate	61	106 ± 17.6
Glutamate + malate	54	107 ± 25.6
Succinate + rotenone	79	147 ± 49.6
Ascorbate +TMPD	468	305 ± 109.6
Respiratory-chain–enzyme activities:		
Rotenone-sensitive NADH CoQ reductase (nmol/min/mg mitochondrial protein)	126	182 ± 64
Succinate cytochrome c reductase (nmol/min/mg mitochondrial protein)	333	242 ± 134
COX (1st-order rate constant/min/mg/mitochondrial protein)	7.66	44.4 ± 14.6
V_{max} (nmol/min/mg mitochondrial protein) using 50 μM cytochrome c	383	2,189 ± 774
Citrate synthase:		
nmol/min/mg mitochondrial protein	1,719	1,180 ± 186
mmol/min/gm muscle homogenate	13.35	9.5 ± 1.7
Cytochrome concentrations (nmol/mg mitochondrial protein):		
b	.494	.41 ± .09
c	.646	.69 ± .10
c_1	.486	.54 ± .07
aa_3	.10	.42 ± .09

NOTE.—For methods, see Morgan-Hughes et al. (1977); Mann et al. (1992).

[a] Abnormal results are underlined.

against subunits IV and Va did not differ from those in controls. We have observed this pattern of immunostaining in COX-negative fibers in patients with a number of primary defects of mtDNA (Rahman et al. 1997). In contrast, different staining patterns are observed in COX-negative fibers from patients in whom no mtDNA defect has been identified and who are suspected to have nuclear-gene mutations as the basis of their COX deficiency (Rahman et al. 1997). The explanation for these different immunostaining patterns remains to be determined. However, it is possible that primary mtDNA defects that result in either impaired synthesis or altered amino acid sequence of subunits may impair holoenzyme assembly. This may render certain subunits more susceptible to degradation. Recent X-ray crystallographic studies in bovine heart COX suggests that subunit VIc is intimately related to subunit II, which may explain why, in patients with primary mtDNA defects, the immunostaining pattern of this nuclear subunit mirrors the other mtDNA-encoded COX components (Tsukihara et al. 1996). It is possible that some of the normally synthesized nuclear COX subunits in patients with primary mtDNA defects may be able to associate into a subcomplex that is resistant to degradation.

In view of both the mosaic pattern of COX deficiency and the pattern of immunostaining abnormalities described, we have analyzed the muscle mtDNA in this patient. Sequencing of all 22 tRNA genes and of the genes for subunits I and II of COX showed two changes from the published mtDNA sequence (Anderson et al. 1981), which are recognized polymorphisms (Kobayashi et al. 1990; Tanaka and Ozawa 1994). However, sequence analysis of the gene for subunit III of COX showed a G→A transition mutation at position 9952. In keeping with previously described mtDNA mutations associated with disease, there were two features that suggested that this mutation was likely to be pathogenic. First, it was heteroplasmic, a feature generally associated with disease-associated mtDNA mutations rather than with neutral polymorphisms. Second, it did not occur in 120 healthy control individuals. However, unlike any other mtDNA point mutation described, this mutation results in a premature stop codon being introduced at the 3′ end of the COX III gene. This predicts the loss of the final 13 amino acids of the COX III subunit. This region of COX III has been highly conserved during evolution, suggesting that it is likely to have functional importance (Desjardin and Morais 1989). Within this same region, a point mutation at position 9957, resulting in an amino acid change, has been described in a patient with MELAS, indicating that defects in this region may associate with disease (Manfredi et al. 1995).

The mutant load in our patient's most recent muscle biopsy, performed in 1997, was 57%; that in an earlier muscle biopsy, performed in 1990, was identical. This is in contrast with observations made in relation to a recently identified tRNA-gene point mutation, in which increases in both mutant load and proportion of ragged red fibers were documented over a 12-year period (Weber et al. 1997). Despite a severe decrease in COX activity in our patient's muscle, there was minimal evidence of mitochondrial proliferation, and, therefore, there were no true ragged red fibers. The absence of true ragged red fibers may, at least in part, account for the stability of the mutant load over 7 years. The precise cellular trigger for mitochondrial proliferation remains unknown. The case reported by Keightley et al. (1996), which also harbored a mutation in the COX III gene, did exhibit marked mitochondrial proliferation and ragged red fibers, indicating that it is unlikely that COX deficiency itself is the trigger for mitochondrial proliferation.

To study the relationship between the 9952 mutation and COX deficiency, we performed single-fiber PCR. We observed a clear relationship between COX deficiency and the proportion of mutant mtDNA, at the level of the single fiber. The mean proportion of mutant mtDNA in the 24 COX-negative fibers analyzed was 56.2%, whereas that in 21 COX-positive fibers was 10.1% This suggests that the threshold for expression of this mutation is lower than that reported for other mtDNA point mutations. For example, the threshold for expression of tRNA-gene point mutations is generally reported to be >85%, although this may differ in different cell types, possibly depending on mtDNA copy number (Boulet et al. 1992; Chomyn et al. 1994; Hanna et al. 1995*a*). However, there is no a priori reason why the behavior of this unique stop-codon mutation should mirror the behavior of other, previously identified point mutations. Indeed, our data suggest that the rules governing the threshold for expression of this mutation are quite different from those influencing the threshold for expression of tRNA-gene point mutations. The high threshold for expression of mtDNA tRNA-gene point mutations is thought to be due, at least in part, to intramitchondrial complementation (Boulet et al. 1992). It is possible that such complementation does not occur for defective COX subunits. Alternatively, the lower threshold may occur because functional COX is a dimer (Tsukihara et al. 1996). At a mutant level of 50%, 75% of COX holoproteins would be defective, and this may be enough to produce the biochemical phenotype observed. A functional threshold of 75% (i.e., 75% of a COX subunit is defective) is similar to that observed in some deletions of mtDNA (Hanna et al. 1998).

Although the C-terminal region of the COX III subunit is highly conserved, its precise function is not known. Indeed, the function of the entire COX III subunit remains to be elucidated. It is now established that it is not involved in the proton-pumping capability of

COX, but there is some evidence, from studies in *Paracoccus dentrificans*, that it may have a role in either energy conservation or assembly of the holoenzyme (Haltia et al. 1991; Wu et al. 1995). We were unable to study the consequences of this mutation in a cell-culture system, since neither fibroblasts nor early-passage myoblasts from the patient harbored any detectable mutation. The absence of this mutation in early-passage myoblasts may suggest that the mutation is in fact not present in the satellite-cell population from which such myoblast cultures are derived. This possibility has been suggested in relation to another tRNA-gene mutation recently identified (Weber et al. 1997), and it may have therapeutic implications (Clark et al. 1997; Shoubridge et al. 1997).

mtDNA has been shown to be virtually exclusively maternally inherited, since the spermatozoon contributes no mitochondria to the zygote (Giles et al. 1980). Primary mtDNA point mutations, as well as their associated diseases, have been shown to exhibit strict matrilineal inheritance. In contrast, deletions of mtDNA are sporadic in the vast majority of cases, and matrilineal inheritance is rarely observed (Larsson et al. 1992; Bernes et al. 1993). The absence of this COX mutation in any of the tissues examined in the maternal relatives indicates that, in our patient, it probably arose as a sporadic event. The lack of transmission to her sons is surprising and, in this respect, resembles the observations described in relation to single deletions of mtDNA. There are at least three ways in which the observed lack of transmission may be explained: (1) the mutation may not be present in the germ line; (2) it may be present in very low amounts in oocytes and be filtered out by the bottleneck thought to exist during oogenesis (Hauswirth and Laipis 1985); or (3) it may be lost during either oogenesis or early embryogenesis, possibly because of a selective disadvantage of cells harboring high proportions of the mutant molecule. Since germ-line tissues from the proband are not available for study, it is not possible to pursue this question.

In conclusion, we have identified the first stop-codon mutation in human mtDNA and have provided evidence that it is associated with adult-onset isolated COX deficiency. This mutation is likely to induce COX deficiency by a novel mechanism, possibly by affecting the assembly of complex IV. This point mutation, in contrast to others described, is not transmitted in the maternal line.

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A Missense Mutation of Cytochrome Oxidase Subunit II Causes Defective Assembly and Myopathy

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Summary

We report the first missense mutation in the mtDNA gene for subunit II of cytochrome *c* oxidase (COX). The mutation was identified in a 14-year-old boy with a proximal myopathy and lactic acidosis. Muscle histochemistry and mitochondrial respiratory-chain enzymology demonstrated a marked reduction in COX activity. Immunohistochemistry and immunoblot analyses with COX subunit–specific monoclonal antibodies showed a pattern suggestive of a primary mtDNA defect, most likely involving *CO II*, for COX subunit II (COX II). mtDNA-sequence analysis demonstrated a novel heteroplasmic T→A transversion at nucleotide position 7,671 in *CO II*. This mutation changes a methionine to a lysine residue in the middle of the first N-terminal membrane-spanning region of COX II. The immunoblot studies demonstrated a severe reduction in cross-reactivity, not only for COX II but also for the mtDNA-encoded subunit COX III and for nuclear-encoded subunits Vb, VIa, VIb, and VIc. Steady-state levels of the mtDNA-encoded subunit COX I showed a mild reduction, but spectrophotometric analysis revealed a dramatic decrease in COX I–associated heme a_3 levels. These observations suggest that, in the COX protein, a structural association of COX II with COX I is necessary to stabilize the binding of heme a_3 to COX I.

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Introduction

Cytochrome *c* oxidase (COX) is the terminal electron acceptor of the mitochondrial respiratory chain and catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen to form water (Capaldi 1990). This enzyme complex comprises 13 subunits, 3 of which (subunits I–III) constitute the catalytic core of the enzyme and are encoded by the mitochondrial genome, a circular double-stranded 16.5-kb DNA molecule present in multiple copies within mitochondria. The remaining 10 subunits are encoded by nuclear genes. The redox centers involved in electron transfer are two heme A moieties (*a* and a_3) and two copper centers (Cu_A and Cu_B). The heme *a* and the heme a_3-Cu_B binuclear centers are associated with COX I, whereas COX II contains the Cu_A center (Tsukihara et al. 1996).

COX deficiency is the most commonly recognized respiratory-chain defect in childhood and is clinically heterogeneous with phenotypes including Leigh syndrome and fatal and benign infantile myopathies (Di Mauro et al. 1983, 1994; Rahman et al. 1996). To date, mtDNA mutations have only been identified in *CO III* and *CO I:* a microdeletion in *CO III* (Keightley et al. 1996) causing cramps and recurrent myoglobinuria, and point mutations in *CO III* in two patients with encephalopathy (see Manfredi et al. 1995; Hanna et al. 1998); and a microdeletion in *CO I* in a patient with motor-neuron disease (Comi et al. 1998). In other cases, combined deficiency of COX and complex I of the respiratory chain may be caused by large-scale rearrangements of the mitochondrial genome, or by point mutations involving mitochondrial tRNA genes (Holt et al. 1989; Ciafaloni 1992). Failure to identify a mutation, even after complete sequence analysis of all COX-subunit genes in a number of patients, has led to the suggestion that many cases of COX mutations may be due to genetic defects in nuclear proteins involved in the assembly of the COX enzyme complex (Adams et al. 1997; Lee et al. 1998). Recently, mutations have been identified in

the *SURF-1* gene on chromosome 9 in patients with Leigh syndrome and COX deficiency (Tiranti et al. 1998; Zhu et al. 1998). The *SURF-1* gene product is thought to be involved in COX assembly or maintenance.

We have studied a boy with isolated COX deficiency and have identified the first heteroplasmic point mutation in the mitochondrially encoded gene for COX II. We provide evidence that this mutation affects the assembly or stability of the COX holoenzyme.

Patient and Methods

Clinical History

All studies were performed with the approval of the ethics committee of the Royal Free Hospital National Health Service Trust. A 14-year-old boy was referred for investigation of a five-year history of muscle weakness and fatigue. There was no family history of neuromuscular disease, the parents were healthy and unrelated, and four younger siblings were all asymptomatic. On examination, the patient was generally thin, but there was no focal muscle wasting. There was mild weakness of shoulder and pelvic-girdle musculature. Tendon reflexes were normal. There was no evidence of ophthalmoplegia or retinopathy. Resting-blood lactate level was elevated (4 mmol/l [reference range 0.9–1.8 mmol/l]) at age 11 years but was normal (0.97 mmol/l) at age 14 years. Mild elevation of cerebrospinal fluid lactate (2.37 mmol/l) was noted at age 14 years. Results of magnetic resonance imaging of the brain, echocardiography, electrocardiogram, blood-creatine kinase, and investigations of renal tubular function were all normal. Histochemical staining of biopsied skeletal muscle, at age 11 years, had revealed reduced COX activity, but biochemical assays of other respiratory-chain complexes were not performed. A further quadriceps-muscle biopsy was obtained at age 14 years for detailed biochemical analysis, after informed patient and parental consent was given.

Muscle Histochemistry and Immunohistochemistry

For histochemical studies, cryostat muscle samples cut into 8-μm sections were stained, to demonstrate the activities of COX and succinate dehydrogenase (SDH), with use of standard methods (Filipe and Lake 1990; Stoward and Pearse 1991). Sections were also stained with the modified Gomori trichrome. A library of subunit-specific mouse monoclonal antibodies was used to identify COX subunits immunohistochemically in 10-μm serial sections. Antibodies were directed against subunits I and II (mitochondrially encoded) and against subunits IV, Va, and VIc (nuclear encoded) (Taanman et al. 1996). Visualization was with the Strept-ABC (Dako) technique, with 3,3-diaminobenzidine hydrochloride.

Biochemical Studies

Polarographic and spectrophotometric enzyme assays of respiratory-chain complexes were performed in isolated mitochondria, as previously described by Cooper et al. (1992). For spectral analysis of laser-flash photolysis and recombination of carbon monoxide (CO), mitochondria were prepared from 10–20 mg of skeletal muscle (Darley-Usmar et al. 1983) and dissolved into 600 μl of 50 mM Tris/Cl, 10% ficoll, and 0.05% lauroyl maltoside (pH 8); 500 μl were used for optical measurements. The samples were reduced by addition of sodium dithionite and gassed for 2 min with CO to generate the CO-ferroheme a_3 compound. Room-temperature laser-flash photolysis of CO was performed as previously described (Meunier and Rich 1998*b*). Short actinic-light pulses were provided by a frequency-doubled Nd-YAG laser. Optical signals of 430 and 445 were recorded. Ten transients, at each wavelength, were signal averaged and the data were plotted as ΔA at 430 − 445 nm versus time. The observed rate constants (K_{obs}) of CO recombination were obtained by the fit of exponential decays to the traces.

Immunoblot Analysis

A mitochondrial cell fraction was prepared by the method of Darley-Usmar et al. (1983), scaled down for a small sample size of ~20 mg, and cellular proteins, including mitochondrial membrane proteins, were extracted from myoblast and fibroblast cell pellets as described by Taanman et al. (1997). Immunoblot analysis was performed with use monoclonal antibodies against COX subunits (Taanman et al. 1994, 1996), the flavoprotein subunit of SDH (Marusich et al. 1997), the voltage-dependent anion channel (VDAC) (32HL; Calbiochem), core protein 1 of complex III, and the α subunit of F_1-ATP synthase. These last two monoclonal antibodies were generated with bovine enzymes and chosen after first being screened for specificity by reaction with pure beef complex III or with ATP synthase, as well as with bovine mitochondria. Their utility in immunohistochemistry and western blotting with human enzymes was confirmed with mitochondria isolated from human skin fibroblasts.

mtDNA Amplification and Sequence Analysis

Total genomic DNA was extracted from blood, skeletal muscle biopsy, cultured myoblasts, and skin fibroblasts by established methods (Sambrook et al. 1989). Sequence analysis was performed on muscle DNA. All mitochondrial tRNA and COX genes were amplified, by use of oligonucleotide primers with bacteriophage M13 tails. PCR products were sequenced with a *Taq* FS dye primer cycle sequencing kit (PE Applied Biosystems) and

were analyzed on an automated DNA sequencer (373A; PE Applied Biosystems).

RFLP Detection of the Point Mutation

A mismatch PCR method was designed to detect the mutant mtDNA molecule by creation of a restriction site for the enzyme *Tru* 91 (Promega). The sequences of the PCR primers used were as follows: (forward) 5′(7651)-CTT TCA TGA TCA CGC CCT *TA*-(7670)3′ (mismatch nucleotide italicized), and (reverse) 5′(7865)-AGG GAT CGT TGA CCT CGT CT-(7846)3′. The PCR cycling conditions used to amplify the target sequence were as follows: 4 min denaturation at 94°C followed by hot-start addition of *Taq* polymerase, then 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 55°C and 30 s extension at 72°C. A final extension step of 10 min at 72°C was performed after the last cycle. After digestion with *Tru* 91, the 215-bp PCR product from the mutant allele yielded two fragments, of 197 bp and 18 bp, whereas the wild-type allele remained uncut.

Quantification of Mutant and Wild-Type PCR Products

The relative proportions of wild-type and mutant mtDNA were determined by the addition of fluorescent dUTP (PE Applied Biosystems) just before the final (20th) cycle of the PCR, followed by *Tru* 91 restriction digestion of the PCR product and separation onto a 5% nondenaturing polyacrylamide gel. The fluorescent products were analyzed with GENESCAN (PE Applied Biosystems).

Single-Fiber PCR Analysis

The percentage of mutant mtDNA in individual fibers was correlated with COX activity by use of a single-fiber PCR assay, as previously described (Moraes and Schon 1996). Muscle sections of 30 μm were stained for COX activity and were fixed in 50% ethanol. Individual muscle fibers were selected in accordance to COX staining (either positive or negative) and were microdissected with a borosilicate microcapillary tube under an inverted-light microscope. The removed fiber segments were placed in tubes containing 10 μl of water, centrifuged for 10 min, and then lysed at 65°C for 1 h in a solution containing 200 mM KOH and 50 mM DTT. A neutralizing solution of 5 μl (900 mM Tris-HCl [pH 8.3] and 200 mM HCl) was added, and the samples were then subjected to fluorescent mismatch PCR amplification and restriction digestion, as described above, to determine the proportion of mutant mtDNA in single-muscle fibers.

Studies in Cultured Cells

A primary myoblast culture was established, as described by Yasin et al. (1977), from skeletal muscle biopsied when the patient was 14 years of age. Myoblasts were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose and 4 mM L-glutamine and were supplemented with 20% FCS, 2% detoxified chick embryo extract, 1 mM sodium pyruvate, 200 μM uridine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cultured skin fibroblasts were grown in the same medium, but without chick embryo extract and with 10% rather than 20% FCS. Cells were cultured in a humidified 37°C incubator containing 8% CO_2 in air. Cultured myoblasts and fibroblasts were stained for COX activity and were immunostained for COX subunit I, as previously described (Taanman et al. 1997). COX and citrate synthase assays were performed in freshly harvested cell pellets, as described by Hartley et al. (1993).

Results

Morphological and Biochemical Studies

Muscle biopsy revealed severe COX deficiency in 97% of fibers (fig. 1) with increased SDH staining, but showed no frank ragged red fibers with the modified Gomori trichrome stain. Muscle morphology and histochemistry remained unchanged in two biopsies taken three years apart, at age 11 years and 14 years, respectively. A defect localized to COX was demonstrated by polarographic and enzyme assays performed on the second biopsy (table 1). Immunostaining of frozen muscle sections demonstrated almost complete absence of COX II, with preserved immunostaining of subunits I, IV, and Va, but with reduced staining of subunit VIc (fig. 1). Immunoblot analysis of a mitochondrial protein fraction, prepared from skeletal muscle, confirmed the immunohistochemical finding of a severe reduction of COX II compared to controls (fig. 2). Further immunoblot analysis with antibodies directed against other COX subunits demonstrated reduced levels of all subunits investigated. There was mild reduction of subunits I, IV, and Va, with more marked reduction of subunits III, Vb, VIa (heart/muscle isoform), VIb, and VIc (fig. 2). In addition, immunoblot analysis revealed increased steady-state levels of other respiratory-chain and oxidative phosphorylation subunits (flavin protein of SDH, core 1 subunit of complex III, and the α subunit of F_1F_0-ATP synthase). Immunoblotting for VDAC was used to demonstrate equal loading of samples (fig. 2).

Spectrophotometric Studies

To measure the COX I–associated heme a_3 levels in the muscle biopsies from controls and the COX-deficient patient, mitochondria were prepared from 10–20 mg of skeletal muscle. The samples were reduced by dithionite and treated with CO, and CO flash-photolysis and recombination signals were monitored (Meunier and Rich 1998*a*). Figure 3 shows the optical signals obtained with

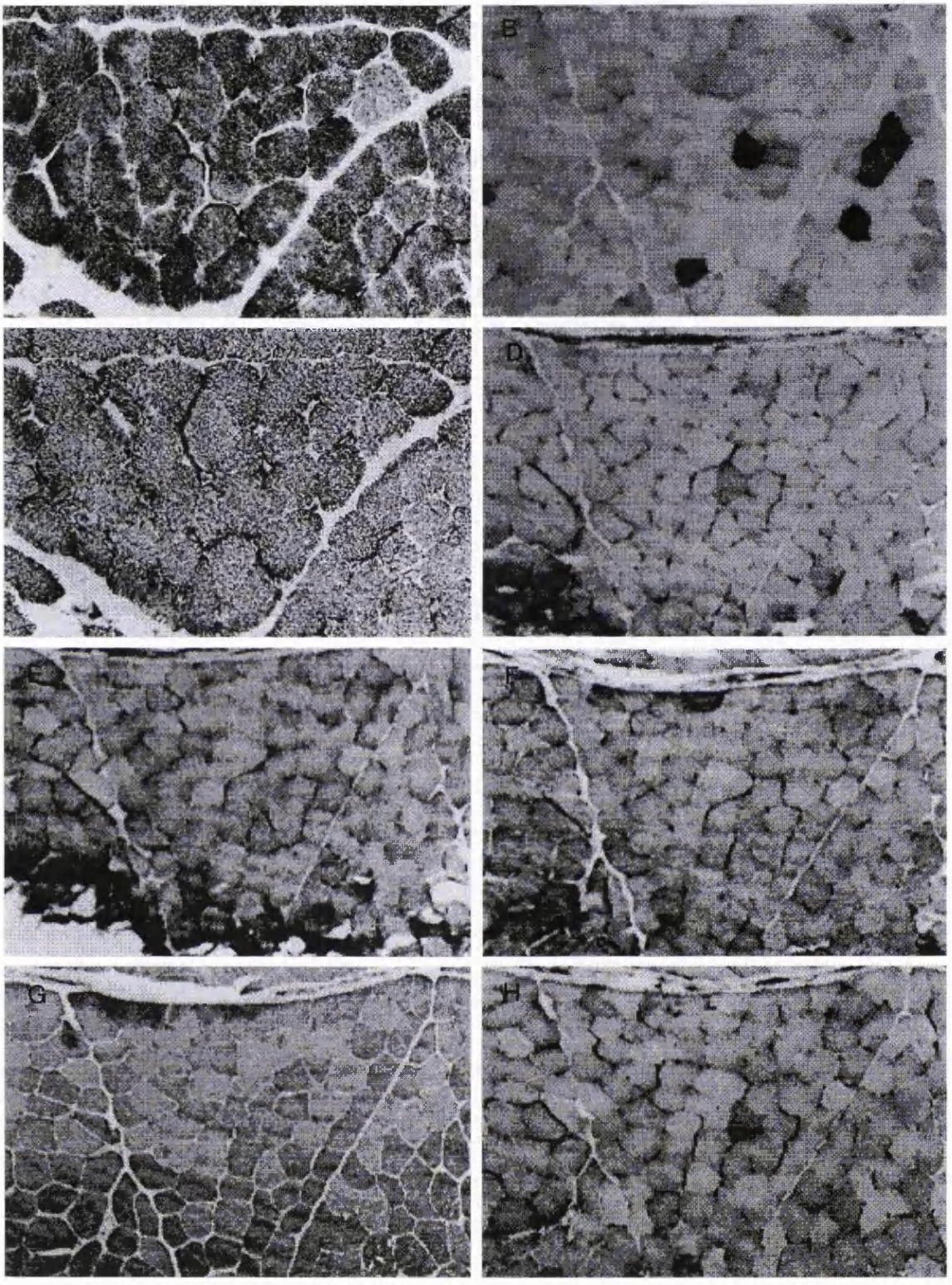

Figure 1 COX-activity staining (panels *A* and *B*) and COX immunohistochemical staining (panels *C–H*) in muscle. A = control; B = patient; C = COX subunit II (control); D = COX subunit II (patient); E = COX subunit I (patient); F = COX subunit IV (patient); G = COX subunit Va (patient); and H = COX subunit VIc (patient). Control sections stained for subunits I, IV, Va, and VIc appeared the same as section C.

Table 1

Polarography

Mitochondrial Oxygen Uptake		
Substrate	*n* Atoms (0/min/mg)	Control Range
Glutamate/malate	26.5	53.3–312.0
Succinate	34.5	68.6–376.1
TMPD-ascorbate[a]	65.4	124–467.7
Respiratory-Chain Enzyme Assays		
COX Complex	nmol/min/mg	Control Range
Complex I	167.1	(27.8–190.0)
Complex II/III	87.3	(18.3–234.3)
Complex IV[b]	2.4	(5.4–46.0)
Citrate synthase	1,745.92	(289.7–1,916.5)

[a] TMPD = N,N,N′,N′-tetramethyl-*p*-phenylenediamine.
[b] Units measured were k/min/mg.

mitochondria from a control and the patient. The traces were biphasic. The fast component (K_{obs} = 500–600 s^{-1}) was the result of CO recombination with contaminating hemoglobin (or myoglobin). The slow component (K_{obs} = 60–65 s^{-1}) arose from CO recombination with heme a_3. The major component (~95%) of the CO recombination signal obtained with the COX-deficient mitochondria was due to hemoglobin. In the control trace, the main signal (~65%) arose from CO reaction with heme a_3 (K_{obs} =~ 60 s^{-1}). The concentration of heme a_3, estimated from the slow-component photolysis signal by use of an extinction coefficient (ε) of 113 $mM^{-1}(cm^{-1})$ at 430–445 nm, was ~0.7 nM in the patient sample and ~11 nM in the control sample. heme a_3 content was measured in a second control sample at a concentration of 6 nM (data not shown). It appeared, therefore, that CO-reactive heme a_3 content was significantly decreased in the mitochondria from the patient.

mtDNA studies

Southern blot and long-range PCR analyses excluded the presence of large-scale rearrangements of mtDNA in total genomic DNA extracted from the patient's muscle (data not shown). In view of the immunohistochemical and immunoblot findings, we performed further analysis of mtDNA (Rahman et al. 1997). Direct-sequence analysis revealed a novel thymine to adenine transversion at nucleotide position 7,671 of mtDNA (T7671A), within the gene encoding COX II (fig. 4), resulting in a missense substitution of a lysine for a methionine at amino acid residue 29 of the polypeptide. Other base changes from the Anderson sequence (Anderson et al. 1981) were either previously reported or were silent polymorphisms, except for a previously unreported change in the D-loop (G468A), which appeared homoplasmic in the sequence chromatogram.

The T7671A mutation was present at 90% in DNA extracted from both skeletal muscle biopsies (done at age 11 years and age 14 years) but at only a low percentage in the patient's blood (4.5%–6%). Single-fiber PCR analysis in muscle samples revealed a significantly higher mean mutant load in COX-negative muscle fibers (81%, n = 13) than in COX-positive fibers (45%, n = 4) (P = .01, Mann-Whitney U test). The mutation was not detected in the mother's blood, nor in 110 normal controls, nor in 15 individuals with mitochondrial disease in whom the underlying mutation was not known.

Cell-Culture Studies

No mutation was detected in cultured myoblasts or fibroblasts at early cell passage, and staining for COX activity and COX I immunoreactivity and spectrophotometric assays of COX activity were normal in these cultures (data not shown). Immunoblot analysis of COX subunits was also normal in cell protein extracts, prepared from myoblast and fibroblast cell pellets (data not shown)

Discussion

We describe a patient with isolated COX deficiency. Biochemical, immunohistochemical, and immunoblot analyses strongly suggested a mutation in the mtDNA gene for COX II. A missense thymine to adenine transversion at nucleotide position 7,671 was identified in *CO II*, and evidence for an etiologic role of this mutation is presented. Sequencing of the remaining mtDNA molecule did not identify any other base changes of likely pathogenic relevance.

Pathogenicity of the T7671A mutation is supported by several lines of evidence. First, the mutation is het-

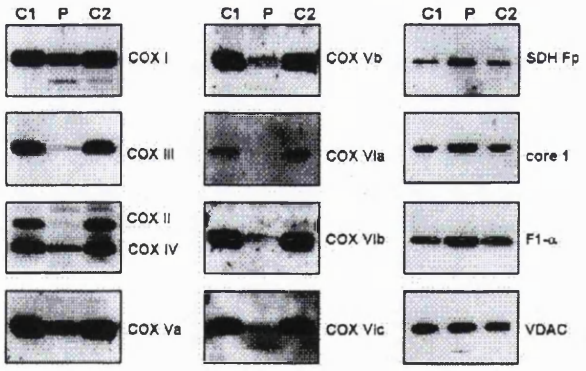

Figure 2 Immunoblot analysis of skeletal muscle mitochondrial fractions, from the patient (P) and two controls (C). Blots were developed with subunit-specific monoclonal antibodies to COX subunits, the flavoprotein subunit of SDH (SDH F_p), core protein 1 of complex III (core 1), the α subunit of F_1-ATP synthase (F_1-α), and VDAC.

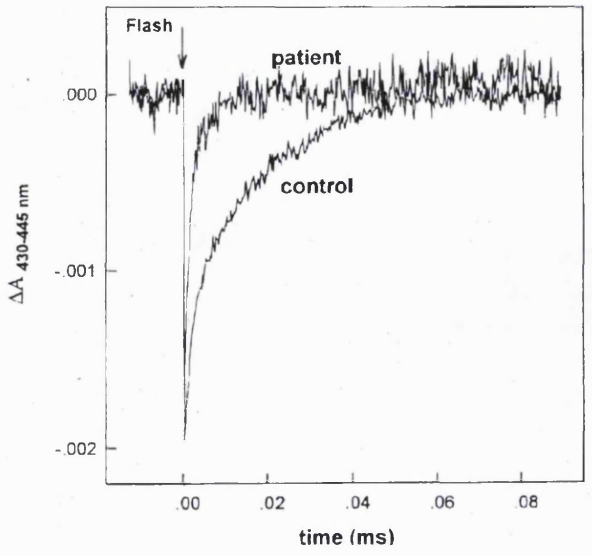

Figure 3 Flash photolysis and recombination spectra of the CO-ferroheme a_3 compound of dissolved muscle mitochondria, from the patient and a control. After reduction by sodium dithionite and treatment with CO, laser-flash photolysis and recombination of CO was monitored at 430 – 445 nm versus time.

eroplasmic and present at high levels of mutant load (90%) in skeletal muscle, the only clinically affected tissue, but at very low levels of mutant load (~6%) in blood. Second, this genotype-phenotype correlation is further supported by the single-fiber PCR studies, which demonstrated significant correlation between mutant-load level and COX activity in individual muscle fibers. Third, the mutation alters an amino acid residue that is relatively conserved in vertebrates (fig. 5). A noncharged methionine residue is replaced by a basically charged lysine, in the middle of the first N-terminal membrane-spanning domain of COX II (Tsukihura et al. 1996). Although the noncharged methionine residue is replaced by other noncharged amino acid residues, in some species, there is no known case in which it is substituted for a charged amino acid residue. Fourth, the mutation was not identified in a large number of ethnically matched control subjects.

The mechanism of pathogenesis of the T7671A mutation may be inferred from analogy with known yeast mutations. Yeast studies have allowed classification of COX mutations into two major groups: "activity" mutations, and "assembly" mutations that affect the assembly and/or stability of the COX holoenzyme (Meunier and Rich 1998*b*). Activity mutations are generally clustered in *CO I* and affect the electron-transfer function or the proton-pumping function of the enzyme. COX II is anchored to the mitochondrial inner membrane with an N-terminal helix-hairpin, whereas its

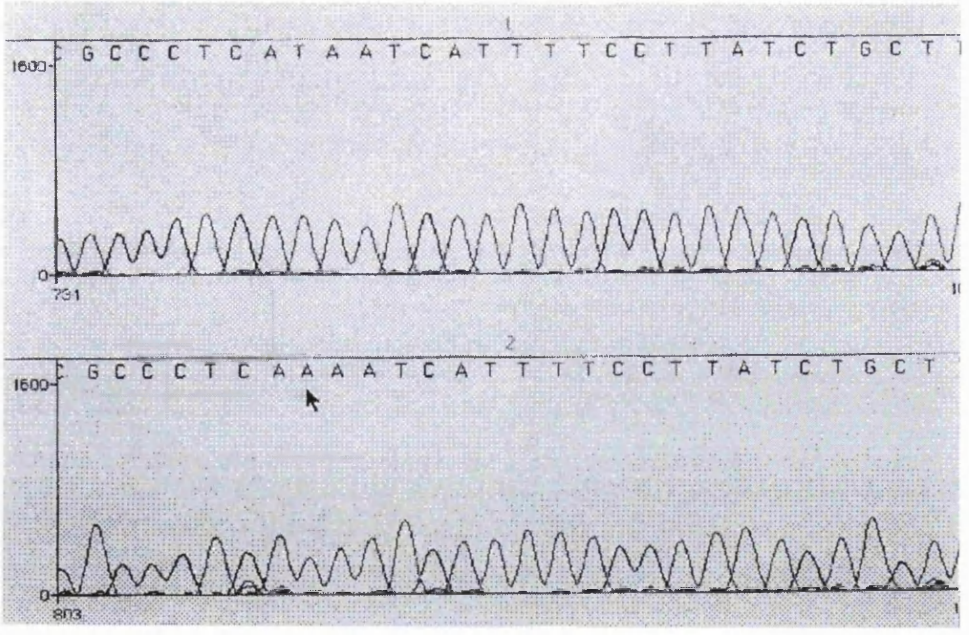

Figure 4 Sequence chromatogram of mitochondrial COX II gene, in control sequence (top) and patient's sequence (bottom). The arrow indicates mutated base (adenine in place of wild-type thymine).

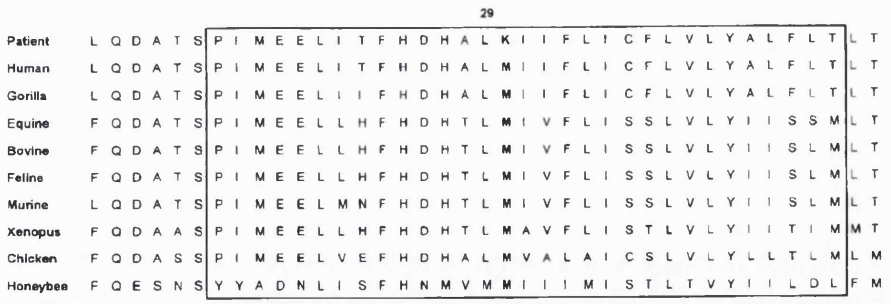

Figure 5 Alignment of amino acid sequence in COX subunit II (mitochondrial genome sequence data obtained from gopher directory at the Molecular Evolution and Organelle Genomics web site). The large box represents the first α-helical region in the bovine structure (Tsukihara et al. 1996). The mutation in our patients involves amino acid residue 29.

large C-terminal hydrophilic domain, which protrudes into the intermembrane space, contains the mixed-valence binuclear Cu_A center and serves as the docking site for cytochrome *c* (Capaldi 1990; Tsukihara et al. 1996). The mutation, in our patient, converts an uncharged amino acid residue in the highly conserved transmembrane segment of the COX II polypeptide into a basic charged residue (fig. 6). The mutation is, therefore, predicted to interfere with the anchoring of COX II within the mitochondrial membrane and to affect the assembly or stability of the COX holoenzyme, rather than the electron-transfer function of the subunit.

Studies of the assembly of COX in cultured human cells have identified two assembly intermediates of the

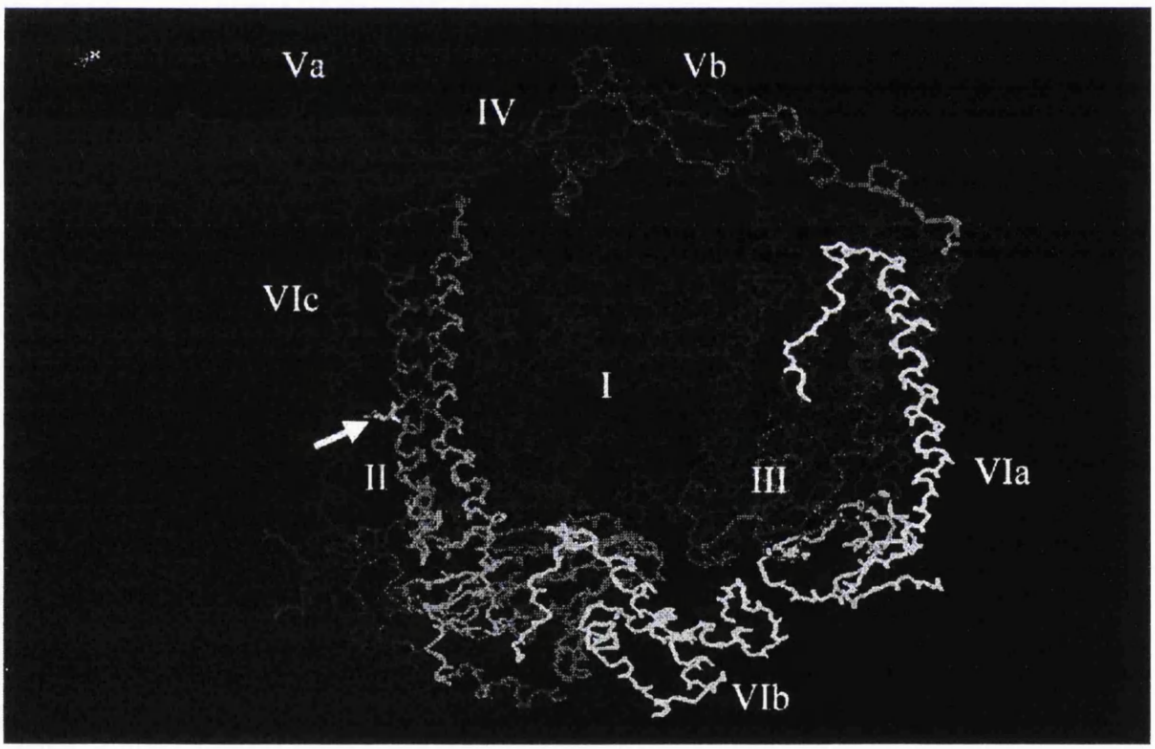

Figure 6 Structure of monomer of bovine cytochrome *c* oxidase. The diagram shows the assembled bovine holoenzyme, with an arrow indicating the site, in subunit II, that is mutated in our patient. The diagram was constructed with use of the data published by Tsukihara et al. (1996) in the Quanta program.

enzyme (Nijtmans et al. 1998). The first contains subunits I and IV. Subunits II and III are subsequently added, together with subunits Va, Vb, VIb, VIc, VIIa or VIIb, VIIc, and VIII. Finally, subunits VIa and VIIa or VIIb are incorporated, to complete the assembly of the holoenzyme. Results of the immunohistochemical and immunoblot analyses performed in our patient indicate that the COX subunit II mutation identified here arrests assembly of the COX holoenzyme after formation of the first assembly intermediate, because the components of this intermediate (subunits I and IV) are relatively preserved (as determined by immunoblotting) compared to the other subunits investigated.

To determine the steady-state levels of subunit I–associated heme a_3, we followed the CO flash-photolysis and recombination signals spectrophotometrically (Meunier and Rich 1998*a*). CO reacts with reduced COX and produces the CO-ferroheme a_3 compound. CO can be photolyzed by a high-intensity short-duration laser flash and will then recombine with the heme on a millisecond time-scale. CO photolysis and rebinding can be monitored optically and provide a sensitive means of quantitation of COX. Because of the high photolysis yield (>95%) and the high ε of the CO-COX compound (ε = 113 $mM^{-1}(cm^{-1})$ at 430–445 nm), it is possible to measure accurately COX content even in samples with low levels of enzyme. Quantitation of COX can be hindered by the presence of other hemoproteins, such as hemoglobin or myoglobin, which also react with CO. The optical signal induced by the binding of CO to these hemoproteins overlaps that of the CO compound of COX. However, the kinetics of CO recombination with hemoglobin and myoglobin are significantly faster ($K_{obs} > 500\ s^{-1}$) than the kinetics of CO recombination with COX (~60 s^{-1}), allowing the deconvolution of the contribution of COX from hemoglobin and myoglobin, especially when their concentration is low.

Immunohistochemical (fig. 1) and immunoblot (fig. 2) analyses showed that the apo–subunit I levels in the patient were only marginally affected. In contrast, subunit I–associated heme a_3 levels were markedly decreased in the patient (fig. 3). Taken together, our data suggest that a structural association of subunit II with subunit I is required to secure binding of the heme a_3 prosthetic group to apo-subunit I.

The surprisingly mild phenotype associated with the mutation in our patient probably reflects the tissue distribution of the mutation. It is likely that skeletal muscle, the only tissue affected clinically, is also the only tissue with a mutant-load level above the threshold required to reduce oxidative phosphorylation capacity. This threshold is generally ~85% for mitochondrial tRNA mutations but may be lower for protein-coding mutations (Hanna et al. 1998). The mutant load in skeletal muscle in our patient has not changed over a period of three years, and this is mirrored by clinical stability over this same period. Stability of mutant load was also observed in our patient with the stop mutation at nucleotide position 9,952 in the mitochondrial *CO III* gene (Hanna et al. 1998), and may be a common feature of mtDNA COX gene mutations. In contrast, mutant load has frequently been observed to increase with time in mtDNA tRNA mutations (Fu et al. 1996; Weber et al. 1997). The explanation for this is not clear. Demonstration of low levels of mutation in peripheral blood cells from our patient may reflect clearance of mutation in this rapidly dividing tissue, and this would be supported by the lower mutant load (4.5%) at 14 years compared to a mutant load level of 6% at 11 years.

The absence of the mutation in maternal blood, with the presence of mutation in both muscle and blood of the patient, suggests that the mutation may have arisen sporadically in early embryogenesis. However, the possibility of the presence of the mutation in the maternal germline cannot be excluded.

The T7671A mutation was not detected in cultured myoblasts, despite the high mutant load in mature skeletal muscle. The lack of mutation in satellite muscle cells has previously been reported with another COX point mutation (Hanna et al. 1998) and also with tRNA point mutations (Fu et al. 1996; Weber et al. 1997). Absence of the mutation from satellite cells has potential therapeutic implications. Previous studies have demonstrated that, in patients with strong segregation of mutation between satellite and mature muscle cells, induction of muscle necrosis—either by bupivicaine local anaesthesia (Clark et al. 1997) or by local trauma (Shoubridge et al. 1997)—was followed by repopulation of the muscle with cells containing only wild-type mtDNA. It remains to be seen if induction of widespread muscle necrosis will be a viable therapeutic option for such patients.

Acknowledgments

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Electronic-Database Information

The URL for data in this article is as follows:

Molecular Evolution and Organelle Genomics, http://megasun.bch.umontreal.ca (for COX subunit II mitochondrial genome sequence data)

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Cytochrome oxidase immunohistochemistry: clues for genetic mechanisms

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Summary

Cytochrome *c* oxidase (COX) is encoded by three mitochondrial and nine nuclear genes. COX deficiency is genetically heterogeneous but current diagnostic methods cannot easily distinguish between mitochondrial and nuclear defects. We hypothesized that there may be differential expression of COX subunits depending on the underlying mutation. COX subunit expression was investigated in five patients with known mtDNA mutations. Severe and selective reduction of mtDNA-encoded COX subunits I and II was consistently observed in all these patients and was restricted to COX-deficient fibres. Immunostaining of nuclear-encoded subunits COX IV and Va was normal, whilst subunit VIc, also nuclear-encoded, was decreased. Twelve of 36 additional patients with histochemically defined COX deficiency also had this pattern of staining, suggesting that they had mtDNA defects. Clinical features in this group were heterogeneous, including infantile encephalopathy, multisystem disease, cardiomyopathy and childhood-onset isolated myopathy. The remaining patients did not have the same pattern of immunostaining. Fourteen had reduced staining of all subunits, whilst 10 had normal staining of all subunits despite reduced enzyme activity. Patients with COX deficiency secondary to mtDNA mutations have a specific pattern of subunit loss, but the majority of children with COX deficiency do not have this pattern of subunit loss and are likely to have nuclear gene defects.

Keywords: cytochrome oxidase; immunohistochemistry; mitochondria; mtDNA

Abbreviations: COX = cytochrome *c* oxidase; DAB = 3,3′-diaminobenzidine hydrochloride; MELAS = mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERRF = myoclonus epilepsy and ragged red fibres; mtDNA = mitochondrial DNA; PDHC = pyruvate dehydrogenase complex; PBS = phosphate-buffered saline; SDH = succinate dehydrogenase; tRNA = transfer RNA

Introduction

Cytochrome *c* oxidase (COX), or complex IV of the mitochondrial respiratory chain, catalyses the transfer of electrons from reduced cytochrome *c* to molecular oxygen, and couples this reaction to proton pumping across the inner mitochondrial membrane. The human enzyme is composed of 13 polypeptide subunits that are of dual genetic origin. The three major subunits (I, II and III) constitute the catalytic core of the enzyme (Capaldi, 1990) and are encoded by mitochondrial DNA (mtDNA). The mitochondrial genome is a maternally inherited circular 16 569 bp (base pair) double-stranded DNA molecule encoding 22 transfer RNAs (tRNAs), two ribosomal RNAs and 10 other respiratory chain enzyme subunits in addition to the three subunits of COX (Anderson *et al.*, 1981). The remaining subunits of COX (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII) are nuclear-encoded, synthesized on cytosolic ribosomes and subsequently transported into the mitochondria. It is postulated that the nuclear subunits may be involved in the assembly or stability of the holoenzyme complex, and/or may have regulatory functions by binding ligands that modulate the catalytic function of the enzyme.

COX deficiency, either total or partial, is the most commonly recognized respiratory chain defect in childhood (Caruso *et al.*, 1996). This may be an isolated defect or may be combined with deficiencies of other components of the respiratory chain. The availability of a reliable cytochemical method to visualize COX activity (Seligman *et al.*, 1968) means that COX deficiency can be detected in very small muscle biopsies, even when there is insufficient tissue for measurement of enzyme activity. Clinical presentations are

Table 1 *Clinical and biochemical features of patients studied*

Patient	Sex	Age at onset	Age at biopsy	Family history	Myo-pathy	Encephalo-myopathy	Cardio-myopathy	Liver disease	Renal tubular defect
Known mtDNA mutations									
C1	F	11 y	16 y	–	+	–	–	–	–
C2	F	45 y	49 y	+	+	–	–	–	–
C3	M	Birth	7 y 10 m	–	+	+	–	+	+
C4	F	17 y	29 y	–	+	+	–	–	–
C5	M	7 y	18 y	+	–	+	–	–	–
Other patients									
P1	M	3 y	10 y 3 m	+	+	–	–	–	–
P2	M	Birth	2 y 6 m	– C	+	+	–	–	–
P3	M	9 m	8 y 8 m	+	–	+	–	–	–
P4	M	Birth	2.5 m	+ C	+	+	–	–	–
P5	M	1.5 m	4 m	– C	+	–	–	+	+
P6	M	Birth	2 m	–	–	+	–	+	–
P7	F	Birth	5 d	– C	+	–	+	–	–
P8	M	1.5 m	3.5 m	–	+	+	+	–	–
P9	M	6 m	2 y 1 m	–	+	+	–	+	–
P10	F	6 m	1 y 8 m	–	+	+	–	–	–
P11	M	Birth	1 y 2 m	–	+	+	–	+	+
P12	F	Birth	9 m	+	+	+	–	+	+
P13	M	5 m	2 y 2 m	+	+	+	–	–	–
P14	F	8 m	3 y 10 m	–	+	+	–	–	–
P15	M	Birth	15 y 7 m	+	+	+	–	–	–
P16	M	1 y	15 y 10 m	–	+	–	–	+	–
P17	M	Birth	16 d	+	+	+	–	–	–
P18	F	9 m	3 y 1 m	–	+	+	–	–	–
P19	M	Birth	11.5 m	–	+	–	–	–	–
P20	M	Birth	6 m	+ C	+	+	+	+	–
P21	M	1 y 1 m	1 y 9 m	+	+	+	–	–	–
P22	M	3 m	1 y 1 m	+ C	+	–	+	+	–
P23	F	2 m	5 m	+	+	+	–	+	–
P24	M	3 m	1 y 6 m	– C	+	+	–	–	–
P25	M	Birth	6 d	–	+	+	+	–	+
P26	M	8 y	11 y	–	+	–	–	–	–
P27	F	9 m	2 y 3 m	– C	+	+	–	–	–
P28	F	9 m	11 y 9 m	+	+	+	–	–	–
P29	F	15 d	4.5 m	+	+	–	–	+	+
P30	M	3 m	9.5 m	+ C	+	+	–	–	–
P31	F	Birth	17 d	+	–	–	+	+	+
P32	F	1 y	1 y 7 m	–	–	+	–	–	–
P33	F	1 y 5 m	7 y 2 m	+	+	–	–	–	–
P34	F	3 y	10 y 6 m	+	+	–	–	–	–
P35	M	Birth	2.5 m	–	–	–	–	+	–
P36	M	1 m	1 y 4 m	+	+	–	–	–	+

very heterogeneous and include fatal infantile myopathy with or without a Fanconi-type renal tubulopathy (DiMauro *et al.*, 1980), Leigh syndrome (subacute necrotizing encephalomyelopathy) (Willems *et al.*, 1977; Rahman *et al.*, 1996), cardiomyopathy and myopathy (Zeviani *et al.*, 1986), recurrent myoglobinuria (Saunier *et al.*, 1995; Keightley *et al.*, 1996) and a 'benign' spontaneously reversible COX-deficient myopathy (DiMauro *et al.*, 1983). In addition, partial COX deficiency may be seen in certain well-recognized mitochondrial syndromes, such as the Kearns–Sayre, MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes) and MERRF (myoclonus epilepsy and ragged red fibres) syndromes (Johnson *et al.*, 1983; Hammans *et al.*, 1995; Taanman, 1997). These syndromes are associated with mtDNA mutations, and the COX deficiency is patchy with some fibres deficient and others apparently normal, reflecting the heteroplasmy of the mutations. A critical mutant load is required to produce a biochemical defect, and fibres with mutant loads below this threshold appear histochemically normal (Schon *et al.*, 1997).

Mutations have been identified in all three mtDNA-encoded COX subunit genes. Most reported mutations are in the COX subunit III gene: a 15 bp microdeletion in a highly conserved region of the gene was associated with recurrent myoglobinuria and myopathy in a 15-year-old girl (Keightley *et al.*, 1996); a missense mutation at position 9957 was found

Table 1 *Continued*

Patient	Hypo glycaemia	Lactate in blood mmol/l (<1.8)	Lactate in CSF mmol/l (<2.0)	Lactate : pyruvate ratio (<20)	Other features	Respiratory chain enzyme assays in skeletal muscle
C1	–	ND	ND	ND		↓CI, CII/III + CIV
C2	–	1.9	ND	ND	Diabetes	↓CI + CIV
C3	–	2.2–3.0	5.9	26		ND
C4	–	3.0	ND	ND		↓CIV
C5	–	4.1	2.9	ND	LS, PN	↓CI + CIV
P1	–	3.2	2.1	ND	PN	ND
P2	–	4.4–8.5	6.4	32–35		ND
P3	–	0.9–2.0	1.9	14		Normal*
P4	–	2.8–11.8	4.3–6.5	18	LS	↓CIV
P5	+	3.0–14.2	ND	66		↓CIV
P6	–	6.8–38.1	ND	62	LS	ND
P7	+	18.3–27.3	ND	ND		ND
P8	–	10.1–16.8	15.8	55		↓CI, CII/III + CIV
P9	+	0.7–9.0	3.3–4.1	22–30	LS	↓CI + CIV
P10	–	4.3	2.3	ND	LS	↓CI, CII/III + CIV
P11	+	2.3–8.1	1.9–3.8	ND		↓CI + CIV
P12	+	3.9–14.6	ND	ND		↓CI + CIV
P13	–	8.2	3.4	ND	PN	↓CII/III + CIV
P14	–	2.3–8.9	8.2	38–42		ND
P15	–	2.0	ND	ND	PN	Normal*
P16	–	1.3–4.0	1.6	9–13		Normal†
P17	–	2.9–20.3	5.4–6.1	28–51	Dysmorphic	ND
P18	–	2.1–9.7	4.1–11.4	ND	LS	↓CI + CIV
P19	+	0.3	ND	ND	Dysmorphic	↓CII/III*
P20	+	5.7–11.1	9.3	43	Dysmorphic	ND
P21	–	3.7–4.5	5.2	ND	LS, PN	ND
P22	–	ND	ND	ND		ND
P23	–	5.8–8.5	ND	25		ND
P24	–	1.7	2.2–3.5	ND		↓CI, CII/III + CIV
P25	–	5.9–12.2	5.2	71	Clouded cornea	ND
P26	–	4.1	ND	ND		↓CIV
P27	–	2.3–.38	4.0	24–39	LS, clouded cornea, PN	ND
P28	–	1.1–4.4	ND	14–21	LS, PN, hirsutism	ND
P29	–	4.2–8.6	ND	59	Cataracts	ND
P30	–	8.6	4.2	ND	LS	↓CI + CIV
P31	+	5.3–27.0	21.6	40–86		↓CI + CIV‡
P32	–	3.55	7.0	24		↓CII/III + CIV
P33	–	0.8–4.1	1.9	ND	Cataracts	ND
P34	–	2.3–5.6	2.0	36	Cataracts	↓CI + CIV
P35	–	10.9–20.0	ND	ND		ND
P36	–	2.1–4.1	3.7–4.0	ND	PN	ND

F = female; M = male; y = year(s); m = month(s); d = day(s); C = consanguineous; ND = not determined; LS = Leigh syndrome; PN = peripheral neuropathy. The lactate range refers to measurements from the same patient on different occasions. Reference ranges for blood and CSF lactate and lactate : pyruvate ratios are given in brackets. ↓ = Respiratory chain enzyme complex activity below the control range. * Patients with COX activity at the bottom of the normal range but with some COX-negative fibres on histochemistry; † patient with COX activity at the bottom end of the normal range and without COX-negative fibres, but in whom histochemical staining for COX was weaker than in a control biopsy stained alongside (on the same slide); ‡ assays performed in cultured skin fibroblasts.

in an adult with MELAS (Manfredi *et al.*, 1995); and a stop mutation at nucleotide 9952 was described in a 36-year-old woman suffering from episodes of encephalopathy associated with lactic acidaemia, exercise intolerance and proximal myopathy (Hanna *et al.*, 1998*b*). A heteroplasmic 5 bp microdeletion in the mitochondrial COX subunit I gene has been described in association with severe isolated muscle COX deficiency in a patient with atypical motor neuron disease (Comi *et al.*, 1998). Recently, a point mutation in the initiation codon of the COX subunit II gene was reported in a patient with encephalomyopathy (Clark *et al.*, 1999).

As most subunits of COX are nuclear-encoded and additional nuclear-encoded factors are essential for assembly of the holoenzyme complex, it is likely that most cases of COX deficiency are caused by nuclear gene mutations. However, mutations have yet to be described in the nuclear

COX subunit genes, despite extensive sequence analysis (Adams *et al.*, 1997; Lee *et al.*, 1998). Characterization of human nuclear-encoded assembly and import factors and other nuclear regulatory genes for COX is still in its infancy. Recently, mutations have been identified in the *SURF1* gene on chromosome 9 in patients with Leigh syndrome and COX deficiency (Tiranti *et al.*, 1998; Zhu *et al.*, 1998). The *SURF1* gene product is thought to be involved in COX assembly or maintenance.

Thus, most patients with COX deficiency remain uncharacterized at the molecular level. In the absence of an identified mtDNA mutation or a strong maternal family history of neuromuscular disease it is difficult to be certain whether the genetic defect is mitochondrial or nuclear in individual cases. This leads to difficulties in genetic counselling. We sought to determine whether COX subunit expression patterns detected immunohistochemically, using highly specific monoclonal antibodies, can distinguish between mtDNA defects and nuclear DNA defects in COX deficiency. This would be an invaluable aid to genetic counselling and would help to direct subsequent molecular genetic investigations. In addition, studying patterns of subunit expression in COX-deficient patients is fundamental to understanding the pathogenesis of respiratory chain enzyme deficiencies.

Patients

Muscle biopsies with decreased or absent COX activity, as determined by histochemistry, were identified retrospectively from the histopathological records at Great Ormond Street Hospital for Children. These muscle biopsies had been obtained, after informed parental consent, for diagnostic purposes from children with suspected respiratory chain disorders over a 12-year period (1985–97). Patients were excluded from the study only if no frozen muscle remained. We studied 36 children with reduced COX staining in biopsied skeletal muscle in whom the molecular defect was uncertain. Clinical features are summarized in Table 1 (P1 to P36). Patients were aged between 5 days and 15 years 10 months (median 1 year 5 months) at the time of biopsy, the majority being infants. There was a male preponderance of 1.8 : 1. Lactate levels were raised in the blood and/or CSF in 34 of 35 patients. Lactate : pyruvate ratios were determined in 19 patients and were elevated in 16 of these. Nine patients had Leigh syndrome, defined by clinical features, lactic acidosis and neuroimaging.

We also examined muscle from five patients with known mtDNA defects (C1 to C5 in Table 1). Three had point mutations involving tRNA genes: insertion of a cytosine at nucleotide position 7472 in patient C1 (Hanna *et al.*, 1998*a*); T14709C in patient C2 (Hanna *et al.*, 1995); and A8344G in patient C5 (Sweeney *et al.*, 1994). Patient C4 had a point mutation in the COX subunit III gene (G9952A) (Hanna *et al.*, 1998*b*) whilst patient C3 had the 'common' 4.9 kb mtDNA deletion in association with clinical features of the Pearson and Kearns–Sayre syndromes (McShane *et al.*, 1991).

Three normal control biopsies had no histological evidence of muscle disease. Three other biopsies with normal quantitative COX activity were obtained from patients with congenital lactic acidosis associated with isolated deficiencies of pyruvate carboxylase, the pyruvate dehydrogenase complex (PDHC) and complex I of the respiratory chain.

Material and methods

Open or needle muscle biopsies were performed under general anaesthesia and snap frozen immediately in hexane maintained in an acetone/CO_2 bath at –80°C. After sectioning for diagnostic purposes, they were stored at –50°C until the time of this study.

Histochemistry

For histochemical studies, cryostat sections cut at 10 μm were stained to demonstrate the activities of succinate dehydrogenase (SDH) and COX using standard methods (Filipe and Lake, 1990; Stoward and Pearse, 1991). Sections were also stained with the modified Gomori trichrome, and for glycogen and fat. Muscle fibres were typed using standard ATPase techniques, with and without acid preincubation.

Antibodies

A battery of subunit-specific monoclonal antibodies was used to identify COX subunits immunohistochemically. Mouse monoclonal antibodies directed against subunits I and II (mitochondrial-encoded) and IV, Va and VIc (nuclear-encoded) were used (Taanman *et al.*, 1996). A mouse monoclonal antibody directed against fast myosin (Novocastra, Newcastle-upon-Tyne, UK) was used to identify type I and type II muscle fibres.

Immunohistochemistry

Serial 8-μm cryostat sections were cut from patient and control biopsies, mounted adjacently on polysine slides (BDH, Poole, UK) and air-dried for 1 h. Mouse monoclonal antibodies directed against COX subunits and fast myosin were optimally diluted in phosphate-buffered saline (PBS), pH 7.4, and 200 μl of each was applied to serial sections for each patient and control. Sections were incubated with primary antibody overnight in a humidified chamber to prevent dehydration. One set of sections was incubated in PBS alone, without primary antibody, as a control. All sections were washed in PBS and then incubated for 45 min in biotinylated rabbit anti-mouse IgG secondary antibody (Dako, Glostrup, Denmark). The immunoreaction was visualized after a 45 min incubation in streptavidin–biotinylated horseradish peroxidase complex (ABC complex, Dako) by developing the peroxidase activity in a solution

containing 0.05% 3,3′-diaminobenzidine hydrochloride (DAB) and 0.03% hydrogen peroxide in PBS for 10 min. Sections were washed with PBS between each step of the procedure, and finally in tap water for 10 min after incubating in DAB. Carazzi's haematoxylin was used as a nuclear counterstain. Sections were dehydrated, cleared and mounted in DPX. All incubations were at room temperature.

Results

Histochemistry

All patients had reduced COX activity on histochemical staining. In some cases there was complete absence of COX staining, whilst in others there was a more patchy loss, with variation in staining intensity unrelated to fibre type (Table 2). In 15 patients, the decreased COX staining activity shown histochemically was associated with COX enzyme activity below the normal range on biochemical assay (Table 1). In four patients, COX activity was at the bottom end of the normal range. In three of these there were some COX-negative fibres on histochemistry, whilst the fourth had weaker histochemical staining for COX than a control biopsy stained alongside on the same slide. There was insufficient material for performing enzyme assays in the other patients. All but two patients (P14 and P20) had increased neutral lipid droplets within their muscle fibres. Only three cases (P1, P9 and P15) had classical ragged red fibres on Gomori trichrome staining, but 20 had evidence of mitochondrial proliferation suggested by increased SDH staining.

Immunohistochemistry

Clear particulate immunoreactivity consistent with mitochondrial staining was observed in muscle fibres from normal control biopsies with all antibodies directed against COX subunits. Permeabilization, as used by Johnson and colleagues (Johnson *et al.*, 1988), had no effect on the staining intensity in control samples and was not used in this study. Immunoreactivity was more prominent in type I fibres, which have a higher mitochondrial content. No immunostaining was observed in sections incubated with PBS, pH 7.4, in place of primary antibody, as a negative control. Patients with proven pyruvate carboxylase, PDHC and isolated complex I deficiency also had normal immunostaining of all COX subunits as well as normal COX activity.

mtDNA mutations

All five patients with known mtDNA mutations had an identical pattern of COX subunit immunostaining, with selective loss of staining of mitochondrial subunits I and II, together with reduced staining of subunit VIc (Fig. 1). This reduction was limited to COX-deficient fibres. All subunits stained normally in COX-positive fibres. The other 36 patients fell into two groups, defined by their immunostaining. In those biopsies in which fibres were COX-positive by histochemistry, these same fibres stained positively with the range of COX monoclonal antibodies used. For COX-negative fibres, however, the pattern of immunostaining varied according to the classification outlined.

Group 1 (Table 2)

Ten of the COX-deficient patients with unknown aetiology had a pattern of COX immunostaining similar or identical to that of the five patients with mtDNA mutations (Fig. 1). One of these patients (P26) had reduction of subunit II only, with normal staining of subunit I. Subunit VIc was also reduced in six of these 10 patients, including the patient with isolated reduced staining of subunit II. This group of patients was clinically heterogeneous, including patients with encephalopathy in infancy, multisystem disease, cardiomyopathy and isolated myopathy. Screening for mtDNA deletions, point mutations at nucleotides 3243, 8344 and 8993 and for mtDNA depletion was negative in these 10 patients. Two other patients, with mtDNA depletion on Southern blot analysis of muscle DNA, also had selective loss of mtDNA-encoded subunits (patients P9 and P23 in Table 2), whereas a third patient with mtDNA depletion had patchy loss of staining of all subunits (P29).

Group 2 (Table 2)

The majority of patients did not have the same pattern of immunostaining as those with known mtDNA mutations. Thirteen patients had reduced staining of all subunits (group 2A in Table 2), whilst 10 had normal staining of all subunits despite reduced COX activity on histochemical staining (group 2B in Table 2).

Discussion

As neither clinical, biochemical nor histochemical features enable patients with mtDNA or nuclear mutations to be differentiated reliably, we sought to determine whether COX subunit expression patterns detected immunohistochemically can distinguish between mtDNA defects and nuclear defects in COX deficiency.

COX subunit expression pattern in patients with mtDNA mutations

Selective and marked reduction of mtDNA-encoded COX subunits I and II was observed in all five patients with a known mtDNA mutation. This reduction was restricted to COX-deficient fibres, all subunits appearing normal in COX-positive fibres. In addition, less marked reduction of the nuclear-encoded COX subunit VIc was also observed in COX-deficient fibres in these patients. Immunostaining with

Table 2. *Results of COX immunohistochemistry*

Patient	COX staining	Immunohistochemistry to COX subunits				
		I	II	IV	Va	VIc
Normal muscle						
Known mtDNA mutations						
C1 7472insC	patchy					
C2 T14709C	patchy					
C3 4.9 kb deletion	Patchy					
C4 G9952A	patchy					
C5 A8344G	patchy					
mtDNA depletion						
P9	patchy					
P23						
P29	patchy					
Group 1 Selective reduction of mtDNA-encoded subunits +/- VIc						
P1	patchy					
P6						
P8						
P10	patchy					
P14	patchy					
P15	patchy					
P25	patchy					
P26	patchy					
P31						
P35	patchy					
Group 2A Reduction of all subunits						
P2	patchy					
P3	patchy					
P7						
P13	patchy					
P16						
P18						
P20						
P24	patchy					
P27						
P28						
P30	patchy					
P33	patchy					
P34	patchy					
Group 2B All subunits stain normally						
P4	patchy					
P5						
P11	patchy					
P12						
P17						
P19	patchy					
P21						
P22						
P32						
P36						

normal staining
mildly reduced staining
reduced staining
severely reduced or absent staining

This refers to intensity of staining, either histochemical for COX, or immunohistochemical with COX subunit antibodies. 'Patchy' refers to variation in staining intensity unrelated to fibre typing.

antibodies directed against nuclear-encoded subunits COX IV and Va, however, was normal in all muscle fibres from all five patients with known mtDNA mutations.

Selective loss of mitochondrial-encoded COX subunits has been observed previously in patients with mtDNA deletions (Mita *et al.*, 1989; Moraes *et al.*, 1992; Taanman *et al.*, 1996), a point mutation in the mitochondrial $tRNA^{leu(CUN)}$ gene (Fu *et al.*, 1996) and a 15-bp microdeletion in the COX III gene (Keightley *et al.*, 1996). Levels of COX subunit IV were preserved in the patients studied by Mita and colleagues (Mita *et al.*, 1989), Moraes and colleagues (Moraes *et al.*, 1992) and Fu and colleagues (Fu *et al.*, 1996). However no

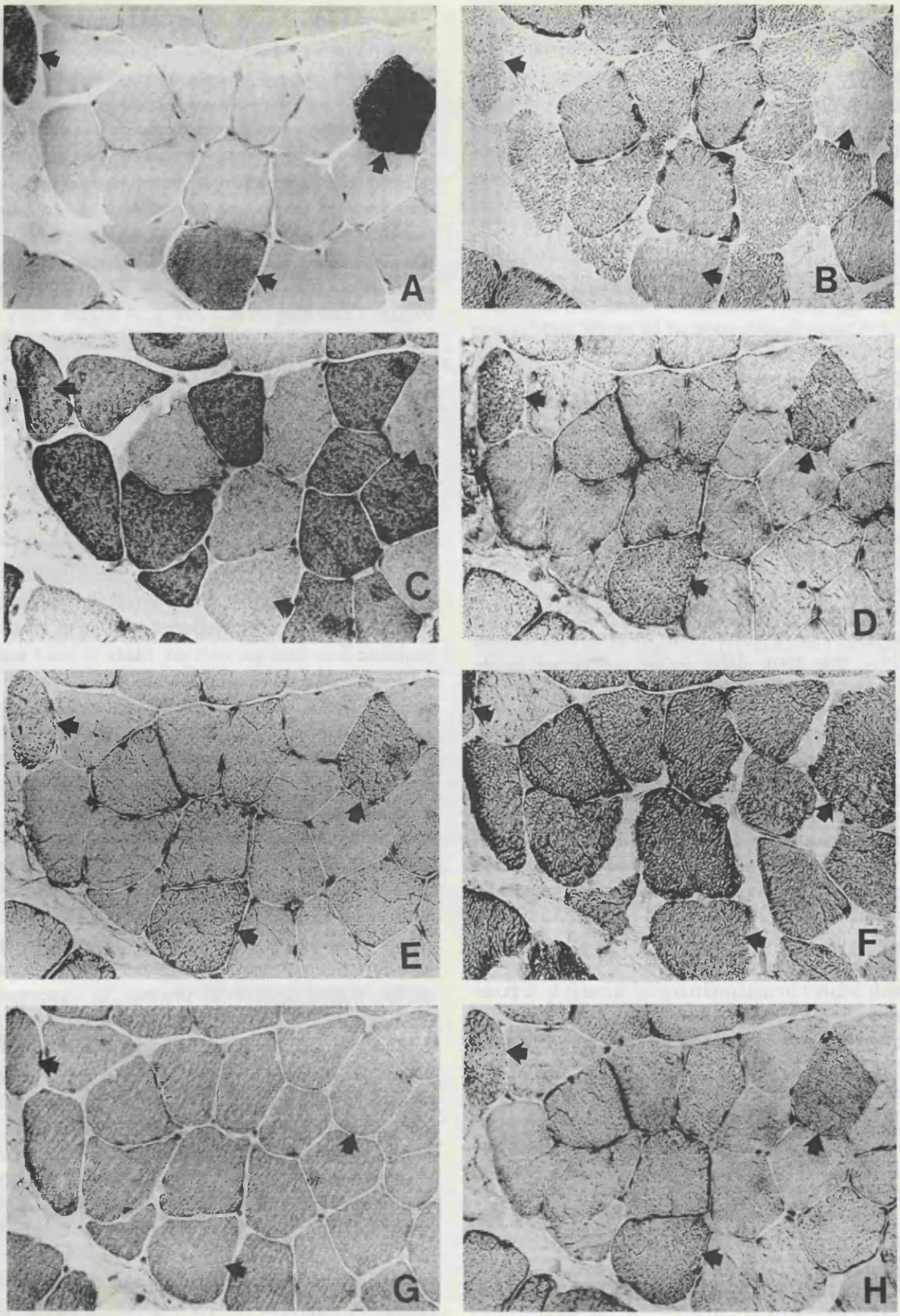

Fig. 1 Histochemistry and immunohistochemistry of muscle from patient C1. Serial cryostat sections. (**A**) Stained to show COX activity. Arrows indicate three positive fibres. The remaining fibres are totally deficient. (**B**) Stained to show SDH activity. Note that fibres with absent COX activity have enhanced SDH activity. (**C**) Fast myosin immunostaining. Two of the COX-positive fibres are type I; the third is type II. (**D**) COX subunit I immunostaining. (**E**) COX subunit II immunostaining. (**F**) COX subunit IV immunostaining. (**G**) COX subunit Va immunostaining. (**H**) COX subunit VIc immunostaining. Sections **A** and **C**–**H** have a light nuclear counterstain. Arrows indicate the same (COX-positive) fibres in each section. All photographs were exposed and printed under the same conditions. Magnification = ×200.

antibodies against other nuclear-encoded subunits were used in these studies and so the fate of other nuclear-encoded COX subunits was not determined in these patients. Taanman and colleagues examined immunostaining of COX Va, Vb and VIc as well as IV in a patient with chronic progressive external ophthalmoplegia harbouring a mtDNA deletion, and found greatly reduced levels of COX I, II, Va, Vb and VIc in COX-negative fibres, with relatively preserved levels of COX IV (Taanman *et al.*, 1996). The patient with the COX III microdeletion had little or no reactivity with antibodies to COX I and VIc but intact immunostaining of subunit IV (Keightley *et al.*, 1996). Immunoblot analyses have also demonstrated relative preservation of COX IV and Va and reduction of COX VIc in ρ^0 cells lacking mtDNA-encoded subunits (Taanman *et al.*, 1996; Marusich *et al.*, 1997).

Differential loss of immunostaining of nuclear-encoded COX subunits in patients with known mtDNA mutations may be related to the quaternary structure of the holoenzyme, which was recently determined for the bovine enzyme (Tsukihara *et al.*, 1996). Subunit VIc forms a dumb-bell, with a transmembrane helix in contact with helix I of subunit II. It is possible that the stability of subunit VIc may be impaired when there are reduced levels of subunit II, since the two subunits are intimately related. Subunits IV and Va, on the other hand, have interactions with several nuclear subunits. These may form stable partial complexes in the absence of mitochondrial subunits.

In the present study, the COX-deficient patients who had loss of subunits I and II were not typical of the group of patients usually associated with mtDNA defects. Some of the patients presented in the neonatal period with congenital lactic acidosis, variably associated with encephalomyopathy, cardiomyopathy, liver disease and renal tubulopathy. Two had Leigh syndrome confirmed neuropathologically. In the past these patients may have been assumed to have nuclear defects. Three patients did have a ragged-red fibre myopathy presenting late in childhood, a common phenotype associated with mtDNA defects. One of these had selective loss of subunit II with normal immunostaining of subunit I. A point mutation has been identified in the COX subunit II gene in this patient, who is described in detail elsewhere (Rahman *et al.*, 1999). Loss of COX II immunostaining in this patient was associated with reduced immunostaining of subunit VIc. This may be seen as further evidence that subunit II is required for stability of subunit of VIc. None of the 12 patients with selective loss of mtDNA-encoded COX subunits in this study has the common mtDNA point mutations at nucleotides 3243, 8344 or 8993. Southern blot analysis has revealed mtDNA depletion in two of these patients, including one with pathologically proven Leigh syndrome. Direct sequencing of mtDNA is in progress for the remaining nine patients.

COX subunit expression patterns in the mtDNA depletion syndrome

COX subunit expression has been studied previously in 10 patients with the mtDNA depletion syndrome (Moraes *et al.*, 1991; Tritschler *et al.*, 1992; Macmillan and Shoubridge, 1996; Taanman *et al.*, 1997; Marusich *et al.*, 1997). Eight had selective loss of mitochondrial-encoded subunits (I and/or II), with variable loss of nuclear subunits. Two patients, however, had homogeneous reduction of all COX subunits compared with controls. In our study, two patients with mtDNA depletion had loss of mitochondrial-encoded subunits whilst a third had loss of all subunits. We therefore confirm that different patterns of subunit expression may be seen in this syndrome. This may reflect genetic heterogeneity or, alternatively, may be related to the stage in the course of the disease (Taanman *et al.*, 1997). A recent study of human leukaemic cells (Molt-cells) which lack mitochondrial protein synthesis suggested that all COX subunits are lost if the mitochondrial translation defect is sufficiently severe (Nijtmans *et al.*, 1995*b*). Studies of ρ^0 cells, which lack mtDNA, have yielded similar results (Taanman *et al.*, 1996; Marusich *et al.*, 1997).

Other COX subunit expression patterns

The remainder of our group of patients had COX subunit patterns falling into one of two broad groups: intact staining of all subunits tested, or reduced staining of all subunits. We postulate that these patients are likely to have nuclear DNA defects. To date no mutations have been described in nuclear-encoded COX subunits; therefore, patients with known mutations cannot be used for comparison. However, pedigree analysis indicates autosomal recessive inheritance in some distinct clinical entities associated with COX deficiency, such as fatal infantile myopathy and Leigh syndrome (Rahman *et al.*, 1996). The present study suggests that Leigh syndrome associated with COX deficiency is aetiologically heterogeneous. Two patients had loss of immunostaining of mitochondrial subunits (including one with the mtDNA depletion syndrome) whilst others had loss of staining of all subunits or normal staining of all subunits. Sequence analysis of the *SURF1* gene is in progress in these patients with COX-deficient Leigh syndrome.

In patients with intact immunostaining of all subunits despite reduced or absent COX staining, there seems to be reduced function despite apparently normal assembly of the holoenzyme. These patients may have a kinetic defect. A single patient with COX deficiency has been described who had an altered K_m (Michaelis constant) for reduced cytochrome *c* (Nijtmans *et al.*, 1995*a*). Studies in cultured fibroblasts from this patient revealed decreased COX activity but normal synthesis, assembly and stability of both mitochondrial and nuclear-encoded COX subunits. This patient was thought to have a defect in one of the nuclear subunits of the enzyme, as mtDNA analysis failed to identify a mutation and the parents were consanguineous. In other patients it is theoretically possible that a mtDNA defect involving a COX gene could alter the kinetic properties of the enzyme but allow normal assembly and therefore normal immunostaining of COX subunits.

Conclusions

We have identified a specific pattern of COX subunit loss in COX deficiency secondary to mtDNA mutations. We now use COX subunit immunohistochemistry to identify patients likely to have mtDNA defects and, after excluding mtDNA depletion by Southern blot analysis, selectively sequence mtDNA in these patients. The majority of children with COX deficiency do not have selective loss of mitochondrially encoded subunits. It therefore seems likely that nuclear gene defects account for a large proportion of childhood-onset COX deficiency.

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A SURF1 Gene Mutation Presenting as Isolated Leukodystrophy

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Mitochondrial respiratory chain defects are increasingly recognized in patients with leukodystrophy. We report the first case of leukodystrophy with systemic cytochrome oxidase deficiency caused by a loss of function mutation in the SURF1 gene in a 2-year-old girl presenting with failure to thrive, global neurodevelopmental regression, and lactic acidosis. Although all previously reported mutations in the SURF1 gene have been found in patients with cytochrome oxidase (COX)-deficient Leigh syndrome, the phenotype associated with SURF1 protein deficiency should be extended to include leukodystrophy.

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The inherited leukodystrophies comprise a heterogeneous group of neurodegenerative disorders principally affecting the white matter of the brain.[1] Clinical features are extremely variable, but motor manifestations usually predominate. A period of normal development usually precedes onset of neurological symptoms and signs and loss of skills. Seizures are rare and cognitive deterioration usually occurs late in the clinical course. Characteristically neuroimaging demonstrates bilateral symmetrical signal abnormality in the white matter. Current biochemical classifications are incomplete as the underlying metabolic defect is unknown in many cases.

The isolated finding of diffuse leukodystrophy is a rare but increasingly recognized manifestation of mitochondrial respiratory chain defects.[2–6] Among the respiratory chain deficiencies presenting in infancy and early childhood, cytochrome oxidase (COX) deficiency is the most commonly diagnosed.[7] At present, it appears that mutations in the SURF1 gene, which encodes a protein involved in COX assembly, are responsible in a significant proportion of patients.[8,9] All patients previously reported with SURF1 mutations have had Leigh syndrome, a neurodegenerative disorder with characteristic basal ganglia and brainstem lesions. Although white matter lesions may be observed in Leigh syndrome, they are not a major neuropathological feature of this disorder. We now report a homozygous mutation in the SURF1 gene in a child with isolated leukodystrophy.

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Patient Details

A two-year-old girl was referred for investigation of failure to thrive associated with metabolic acidosis. The second daughter of healthy consanguineous Bengali parents, she was born after a normal pregnancy by vaginal delivery. There were no neonatal problems, but poor growth was noted from 9 months. At 11 months she had an episode of cyanosis and floppiness, without preceding intercurrent illness, and subsequently her developmental milestones slowed. At 1 year of age she was able to pull to stand, cruise around furniture, and crawl. An older sister is well and there is no family history of neurological disease. Examination at 2 years revealed weight, height, and head circumference all below the third centile. There were no dysmorphic features. She had mild hypotonia with normal deep tendon reflexes. Initial investigations revealed lactic acidosis with plasma lactate between 3.2 and 5.7 mmol/l (normal <1.8) and lactate/pyruvate ratios between 22 and 26 (normal <20).

Magnetic resonance imaging (MRI) of the brain performed at 2 years and 5 months demonstrated abnormal signal in the cerebral white matter, particularly posteriorly, with signal change in the posterior limbs of the internal capsule, corpus callosum (particularly the splenium), dentate nuclei, and adjacent cerebellar white matter (Fig 1A, 1B). Areas within the more confluent white matter abnormalities appeared to be cystlike (Fig 1C, 1D). No abnormalities were seen in the caudate or lentiform nuclei. There were small bilateral slightly asymmetric lesions in the medulla, predominantly involving the olives and inferior cerebral peduncles. With the predominant abnormalities being in the white matter, the appearances were considered to be those of leukoencephalopathy with involvement of the corticospinal tracts. Electroencephalogram revealed mild nonspecific abnormality, with excess fast activity over the anterior half of the head and rhythmic intermediate slow activity posteriorly. There were no vacuolated lymphocytes in the blood film and activities of leukocyte lysosomal enzymes were all within the normal range. Plasma very long chain fatty acid levels were also normal, and transferrin isoelectric focusing showed a normal pattern.

At 2 years 8 months, she had lost skills. She had fewer words and less clear speech. Her floppiness had

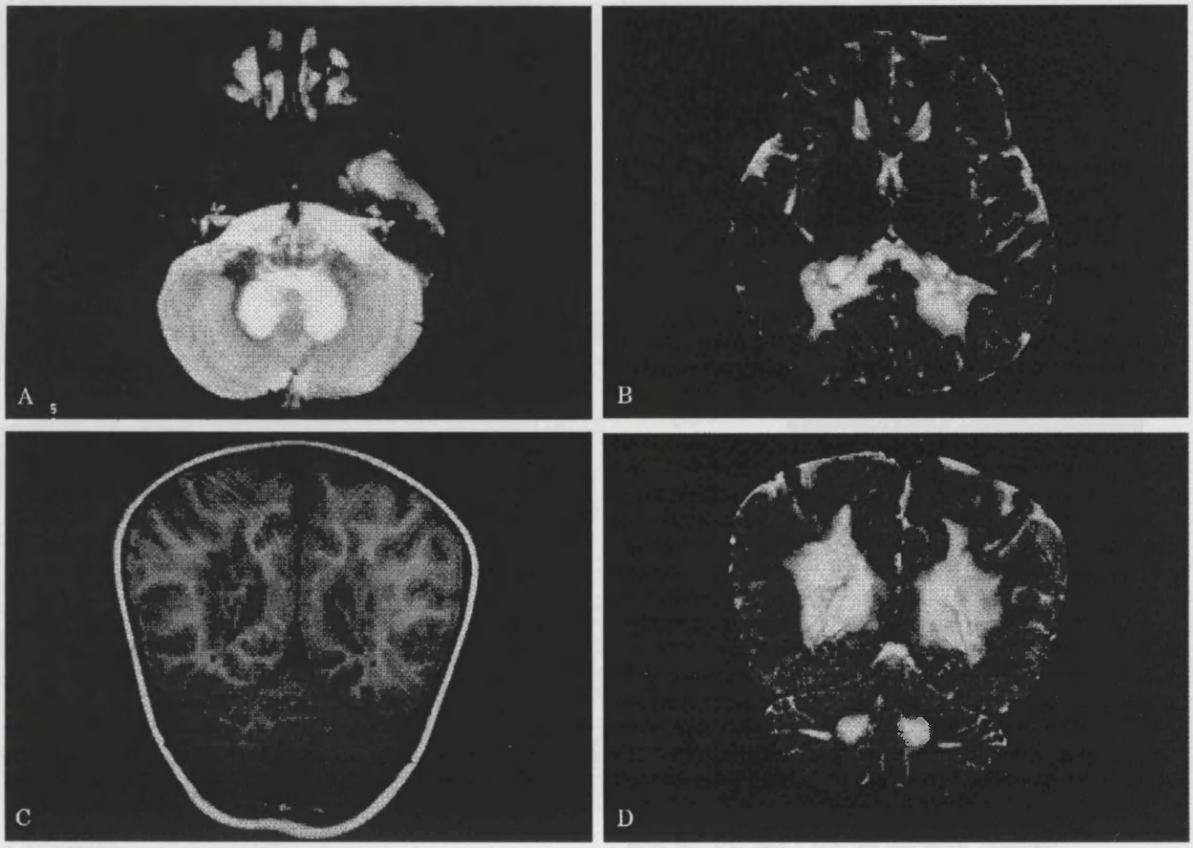

Fig 1. Magnetic resonance images of the patient's brain at age 2 years 5 months. (A) Axial T2-weighted image demonstrating abnormal high signal in the cerebellar white matter. (B) Axial T2-weighted image demonstrating abnormal high signal in the splenium and internal capsule. No lesions are visible in the basal ganglia. (C) Coronal T1-weighted image demonstrating cystlike lesions in the white matter. (D) Cystlike lesions in the white matter are also seen in this coronal T2-weighted image, which also demonstrates abnormal high signal in the cerebral and cerebellar white matter.

progressed and she was barely able to sit without support. She had generalized symmetrical hypotonia and pathologically brisk reflexes with upgoing plantar responses. She continued to fail to thrive and was tachypnoeic. She was hirsute. She had poor to moderate visual acuity; eye movements were full with intermittent beats of nystagmus, and there was no evidence of optic atrophy or pigmentary retinopathy. Neurophysiological studies demonstrated normal electroretinogram but marked postretinal dysfunction on visual evoked potentials.

Paired blood and CSF lactates were 6.8 and 7.65 mmol/l respectively (normal for CSF <2). CSF protein was mildly elevated at 0.58 g/l (reference range 0–0.3). Further investigations revealed slightly abnormal renal tubular function with a mildly elevated urinary N-acetylglucosaminidase/creatinine ratio of 83 units/mmol (reference range 3.5–27.3), normal liver function, and normal echocardiogram with no evidence of cardiomyopathy.

Histology of an open quadriceps muscle biopsy showed only a mild increase in cytoplasmic fat and atrophy of Type II fibers. No ragged red fibers were seen. Respiratory chain enzyme assays on the biopsied muscle revealed a severe isolated deficiency of COX (COX/CS ratio 0.004, reference range 0.014–0.034) with normal activities of complexes I (0.113, reference range 0.104–0.268) and II/III (0.049, reference range 0.040–0.204). Severe COX deficiency was also expressed in cultured skin fibroblasts with activity <1 nmole/mg protein/min compared with a normal range of 30 to 90.

Methods

Messenger RNA was isolated from cultured skin fibroblasts using the High Pure RNA isolation kit (Boehringer Mannheim) and cDNA was synthesized using the Omniscript RT kit (Qiagen). SURF1 cDNA was amplified using oligonucleotide primers 5′-AGGAGCGTCCTCAGGGTC-3′ (forward) and 5′-CATGATCCAGCATAAAGGCA-3′ (reverse). The amplified fragment covers all of the SURF1 coding region apart from exon 1 and the first part of exon 2.

Genomic DNA was extracted from cultured skin fibroblasts using the Nucleon BACC2 kit (Nucleon) and a DNA fragment encompassing exons 6 to 9 was amplified using oligonucleotide primers 5′-TGCCTGAGTGACCATGAGTG-3′ (forward) and 5′-TGGGAAAGTTCTTTGGACTGA-3′ (reverse). Sequence analysis was performed in an automated ABI 377 sequencer using the cDNA PCR primers and Big Dye™ Terminator Cycle Sequencing kit (Applied Biosystems). The mutation in exon 8 was confirmed by restriction endonuclease digestion of the exons 6 to 9 genomic fragment with *BsrI* as the mutation creates an additional restriction site for this enzyme.

Results

Sequence analysis of the coding region of the SURF1 gene of the patient revealed only a single sequence in which there was a 2-bp deletion in exon 8. The deletion involves one of a pair of AG dinucleotides after nucleotide 789 (numbering from the ATG start codon), and as it is not possible to determine which pair is deleted, the mutation has been arbitrarily designated del AG 790–791. This mutation would lead to a frameshift after threonine 263 and the generation of a new stop codon after a further 26 amino acids. Restriction endonuclease analysis of genomic DNA with BsrI demonstrated that the patient was homozygous for this mutation and that both parents were heterozygous (Fig 2).

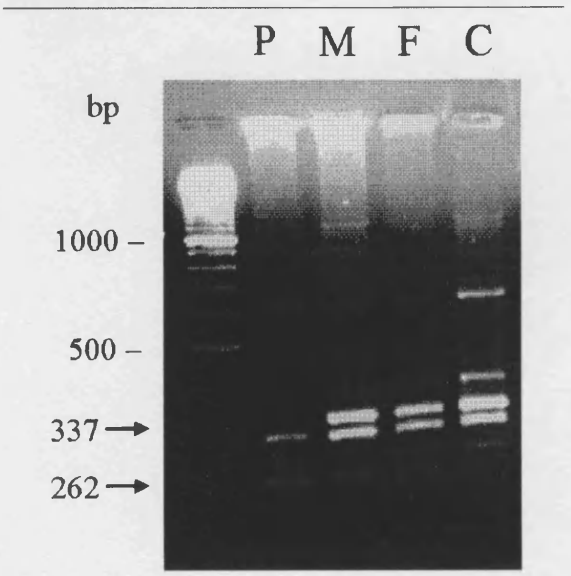

Fig 2. Demonstration of the SURF1 mutation in genomic DNA. A segment of the SURF1 gene encompassing exons 6 to 9 and digested with the restriction enzyme BsrI is shown. The AG deletion in exon 8 leads to formation of a new BsrI site with the result that the 337-bp restriction fragment is further digested into 262 and 75 bp fragments. The patient (P) is homozygous for this deletion, while both parents (M and F) are heterozygous. A normal control (C) is shown for comparison. The left-hand track is a 100 bp molecular size ladder with the 500 and 1,000 bp fragments indicated.

Discussion

We report a patient with a mutation in the SURF1 gene, predicted to produce a truncated protein and complete deficiency of SURF1 protein. The patient had significant neurological dysfunction associated with extensive white matter changes in the cerebrum and cerebellum. Multiple, small, cystlike white matter lesions, similar to those described in 2 of 5 families previously reported with leukodystrophy and respiratory chain defects, were observed within the more confluent white matter abnormalities.[10] It is possible that these cystlike lesions may be a specific feature of white matter disease caused by respiratory chain defects.

All patients with SURF1 mutations previously reported have had Leigh syndrome and COX deficiency, leading to the suggestion that loss of function mutations of SURF1 are exclusively associated with this phenotype.[11] We now demonstrate that SURF1 mutations may be associated with more than one neuroradiological pattern, ie, diffuse leukodystrophy as well as Leigh syndrome. Levels of residual COX activity in skeletal muscle and cultured skin fibroblasts in our patient were similar to those in patients with SURF1 mutations and Leigh syndrome.[11] Of particular note is a report of typical Leigh syndrome in a patient heterozygous for the same AG deletion in exon 8 as our patient. In the previous patient, the mutation in the second SURF1 gene resulted in substitution of aspartic acid for tyrosine 274.[12] By contrast, the patient we describe had no evidence of the basal ganglia necrotic lesions that are characteristic of Leigh syndrome. The reason for the different pattern of neuropathology in these 2 patients is unclear, but it is possible that it is related to the presence of two different SURF1 mutations in the previously reported case. However phenotypic heterogeneity associated with identical mutations has previously been described in other single gene disorders.[13]

The search for SURF1 mutations has focused mainly on patients with Leigh syndrome or with "Leigh-like" features. The identification of a pathogenic SURF1 mutation in a patient with isolated leukodystrophy leads us to suggest that mitochondrial respiratory chain enzymes should be assayed in patients with leukodystrophy and lactic acidosis, and the SURF1 gene sequenced in those with isolated COX deficiency. Furthermore, the SURF1 gene should be analyzed in all patients with isolated COX deficiency, to determine the range of phenotypes that may be associated with mutations in this gene.

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