GENETIC ANALYSIS OF HUMAN CYTOCHROME *C* OXIDASE DEFICIENCY

by
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ABSTRACT

Cytochrome *c* oxidase (COX) deficiency is a disorder of the respiratory chain resulting in defects of energy metabolism. The genetic defects causing isolated COX deficiency are unknown. This thesis describes the molecular analyses of COX deficiency in several patients and the characterization of a 15bp deletion in the mtDNA encoded subunit III of COX (COX III) identified in a patient with recurrent episodes of myoglobinuria and isolated cytochrome *c* oxidase deficiency (Keightley *et al.*, 1996, see appendix 1). The deletion, which removes five amino acids from a conserved transmembrane region of the COX III polypeptide, is the first example of a mutation in any of the COX subunits (encoded by the mitochondrial DNA (mtDNA) or nuclear DNA) to be associated with cytochrome *c* oxidase deficiency. To investigate the consequences of this mutation for assembly and enzymatic function of COX, transmitochondrial cell lines harboring 0%, 97% and 100% of the COX III mutation were created by fusing human cells lacking mitochondrial DNA (ρ^0 cells) with an isolated platelet/lymphocyte fraction from the patient. The COX III mutation resulted in a severe respiratory chain defect in mutant cell lines. Cell lines homoplasmic for the mutation had no detectable levels of COX activity or ATP synthesis, and required uridine and pyruvate supplementation for growth, a phenotype similar to the ρ^0 cell line. Analysis of mitochondrial protein synthesis revealed that the COX III polypeptide was translated in mutant cell lines, but the mutant COX III polypeptide had decreased stability, presumably resulting in significantly lower steady-state levels of the polypeptide. There was no evidence for COX assembly in cell lines homoplasmic for the mutation. These data indicated that homoplasmic levels of the COX III deletion resulted in a complete loss of COX activity due to the absence of assembled COX in mutant cell lines. Assembled COX I and COX II polypeptide complexes were detected in the 97% mutant cell line suggesting that low levels of COX III polypeptide may

promote partial assembly of COX. However, the partially assembled enzyme has little or no catalytic activity.

During my investigations, a T9205C mutation was identified in the mtDNA encoded A6 gene in the patient with the COX III deletion. The T9205C mutation (TAA→CAA) changed the A6 stop codon to glutamine and was present in the mtDNA of both the patient and her unaffected mother. It was of interest to study the biochemical phenotype of this mutation to determine if this mutation contributed to the respiratory defect observed in transmitochondrial cell lines with the COX III mutation. These studies revealed that the T9205C mutation did not affect ATP synthesis or overall respiratory function in the transmitochondrial cell lines with the COX III mutation. The T9205C mutation resulted in the translation of an A6 polypeptide which had a higher molecular weight than the wild-type A6 polypeptide. Two dimensional electrophoretic analysis indicated that this aberrant A6 polypeptide was probably derived from the translation of a partially processed mRNA transcript derived from the A6 and COX III genes.

CHAPTER 1

INTRODUCTION

Mitochondria were first recognized as cellular components in the late nineteenth century (for review see Ernster and Schatz, 1981). Originally termed "bioblasts", the name mitochondria was coined from two Greek words; mitos [thread] and chondros [granule] (Benda, 1898). The first detailed pictures of mitochondria in the 1950's (for review see Ernster and Schatz, 1981) revealed a double membrane structure, an outer membrane and inner membrane, enclosing a compartment called the matrix (Fig 1.1). The space between the inner and outer membranes is referred as the inter-membrane space. The composition and the permeability of the two membranes differ considerably, the inner membrane having a much higher ratio of protein to lipid content and being virtually impermeable. Typically, mitochondria have numerous invaginations of the inner membrane, termed cristae. Cristae dramatically increase the internal surface area of the inner membrane and the number of cristae directly correlates with respiratory capacity of the mitochondrion (Ernster and Schatz, 1981).

Oxidative phosphorylation

As early as 1912, mitochondria were proposed sites for cellular respiration (for review see Ernster and Schatz, 1981). Although based solely on morphological evidence, this prediction was surprisingly accurate. Oxidative phosphorylation, the citric acid cycle and β-oxidation of fatty acids, all metabolic activities vitally important for aerobic respiration, are located in mitochondria. Mitochondria have been nicknamed the "powerhouse" of the cell, because the majority of ATP synthesis in the cell is generated by oxidative phosphorylation (respiratory chain and ATP synthetase).

The anaerobic metabolism of glucose, fatty acids and amino acids results in the production of acetyl CoA. In the mitochondria, acetyl CoA is oxidized by the citric acid

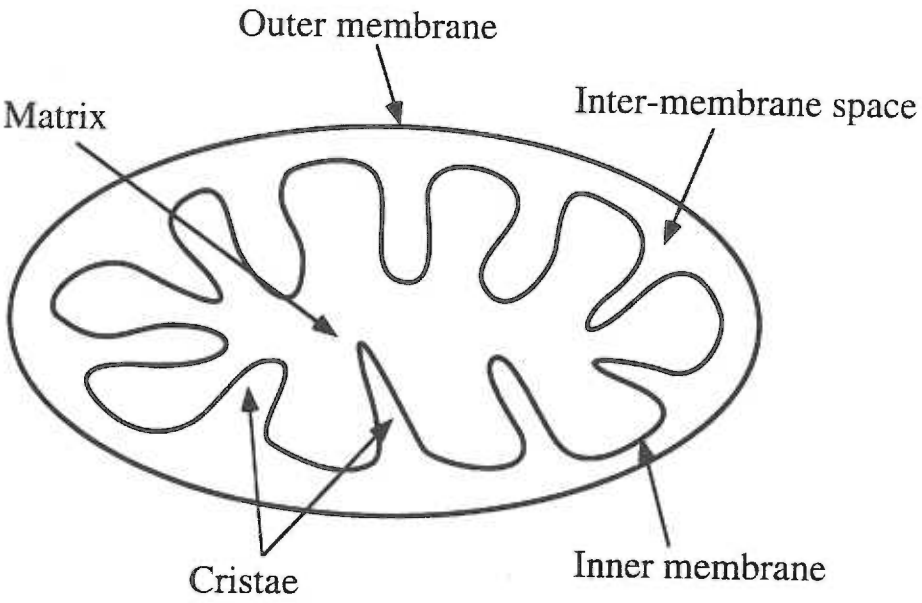
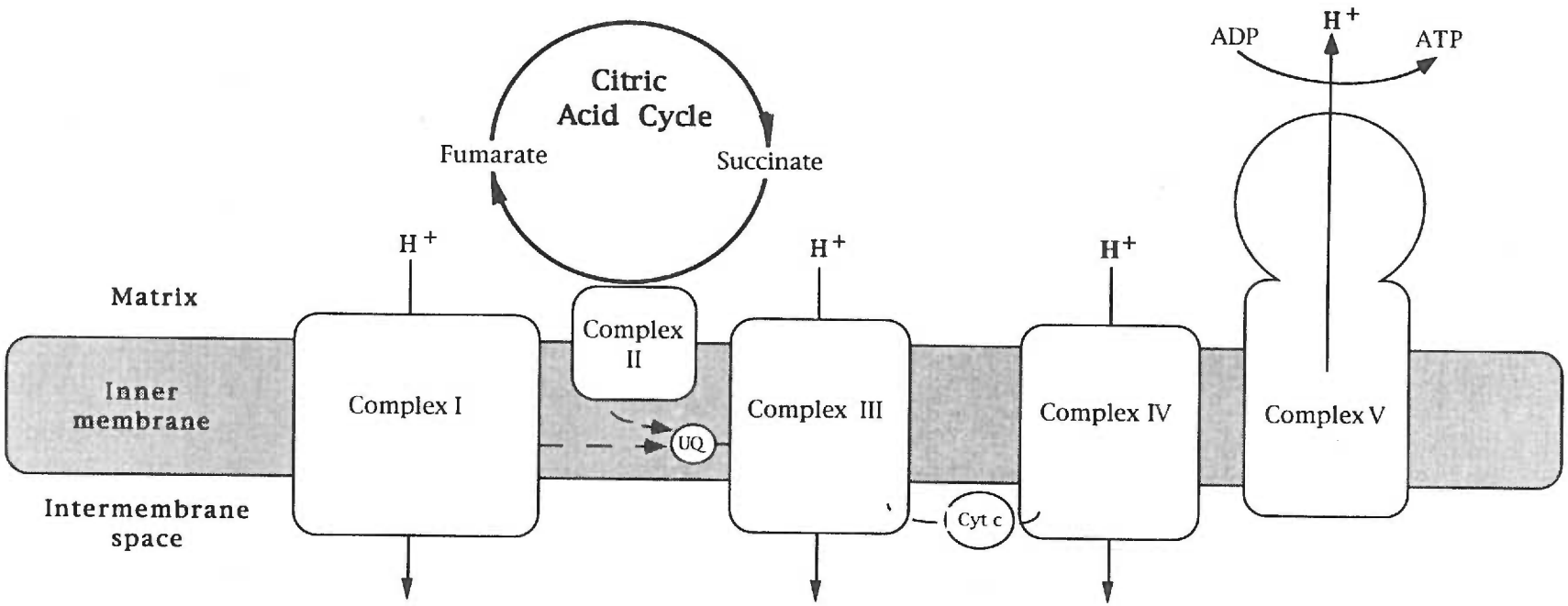


Figure 1.1. Diagram of a mitochondrion

cycle releasing energy in the form of NADH, $FADH_2$ and GTP. The oxidation of NADH and $FADH_2$ by the respiratory chain is coupled to the reduction of molecular oxygen to water and to the synthesis of ATP. The respiratory chain consists of a series of redox reactions in which electrons are transferred from compounds with low reduction potentials to those with higher potentials. The energy generated from these reactions is stored in the form of a proton motive force which is utilized by ATP synthetase for ATP production. Four multi-subunit, membrane bound enzyme complexes catalyze electron transfer: NADH-ubiquinone oxidoreductase (NADH dehydrogenase or complex I), succinate-ubiquinone reductase (complex II), ubiquinol-cytochrome *c* oxidoreductase (complex III), cytochrome *c* oxidase (complex IV). Although all four enzyme complexes catalyze electron transfer, only three pump protons across the membrane [complex I, complex III and complex IV]. Electron flow between complexes is diagrammed in Fig. 1.2. Electrons are transferred from either complex I or complex II to complex III and then to complex IV, terminating with the reduction of molecular oxygen to water. Two mobile electron carriers ubiquinone and cytochrome *c* shuttle electrons between the enzyme complexes. Prosthetic groups, such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), iron sulfur clusters (FeS), cytochromes and Cu atoms, serve as redox centers within the enzyme complexes

The chemiosmotic theory first proposed that energy generated from redox reactions in the respiratory chain was conserved as a proton motive force and provided a mechanism for the coupling of ATP synthesis to the reduction of oxygen (Mitchell, 1961). The three oxidoreductase complexes of the respiratory chain generate a proton gradient by translocating protons across the inner membrane, approximately three to four protons by each enzyme complex. Mechanisms of proton translocation are debated but evidence supports the existence of both proton loops which involve hydrogen carrying prosthetic groups and actual proton pumps (Senior, 1988). Because the inner membrane is highly impermeable to ions, the high concentration of protons within the inter-membrane space

Figure 1.2. Diagram of the respiratory chain. The respiratory chain consists of five multi-subunit membrane bound enzyme complexes. The first four complexes catalyze electron transfer in a series of reduction oxidation reactions. Complex I (NADH dehydrogenase), Complex III (ubiquinol-cytochrome *c* oxidoreductase) and Complex IV (cytochrome *c* oxidase) conserve the energy generated from the redox reactions to pump protons into the inter-membrane space, creating both a chemical and electrical gradient. Energy from the proton gradient is utilized by ATP synthetase for ATP production. All the enzyme complexes of the respiratory chain except complex II are composed of subunits encoded by both the mitochondrial DNA and nuclear DNA.



	Complex I	Complex II	Complex III	Complex IV	Complex V
nDNA-encoded subunits	35	4	10	10	12
mtDNA-encoded subunits	7	0	1	3	2

and the low concentration in the matrix creates both an electrical and a chemical gradient. The energy stored within the proton gradient is utilized by ATP synthetase (complex V) for ATP production. ATP synthetase also termed F_1F_0-ATPase is composed of two complexes, F_1 and F_0. The membrane embedded complex F_0 is a proton pore which allows the movement of protons back into the matrix. F_1, the catalytic complex captures the energy released from proton movement to drive ATP synthesis. The reverse reaction, the hydrolysis of ATP will occur in the absence of a proton gradient or separation of the F_1 from subunit F_0 (Senior, 1988; Cox *et al.*, 1992).

ATP synthetase and the four other respiratory enzymes are multi-subunit enzyme complexes. The complexity of these enzymes is exemplified by their dual genetic origin. Four of the five enzyme complexes have components encoded by two genomes, mitochondrial DNA (mtDNA) and nuclear DNA. Nuclear subunits and prosthetic groups of these complexes are synthesized in the cytoplasm and imported into the mitochondria. The number of subunits varies among complexes, but the largest complex, complex I, is composed of more than forty individual subunits (7 encoded by mtDNA and more than 32 subunits encoded by nuclear DNA) (Fig. 1.2). The structure and composition of cytochrome c oxidase, the terminal component of the respiratory chain, is reviewed in the following paragraphs.

Cytochrome c oxidase

Cytochrome c oxidase (COX) catalyzes the reduction of oxygen to water. COX is composed of thirteen subunits, the three largest are encoded by the mtDNA [COX I, COX II and COX III] and ten are encoded by nuclear DNA. The enzyme complex has four redox-active metal centers, two coppers, Cu_A and Cu_B, and two types of heme, a and a_3. The Cu_A metal center located in COX II, accepts electrons from cytochrome *c*. Electron flow proceeds from Cu_A in subunit COX II to heme a and then terminates at the heme a_3-Cu_B center, both located in COX I. The reduction of O_2 to water coupled to the pumping

of four protons across the membrane occurs at the bimetallic heme a_3-Cu_B center located in COX I. Analysis of the crystal structure of COX from both *P. denitrificans* (Iwata *et al.*, 1995) and bovine heart (Tsukihara *et al.*, 1996) has indicated that the bimetallic heme a_3-Cu_B center is buried deep within COX I and has revealed two possible routes for proton movement within COX I and a putative channel for the diffusion of O_2 (Iwata *et al.*, 1995; Riistama *et al.*, 1996; Ostermeier *et al.*, 1996). COX I, with 12 transmembrane segments, forms the center of the complex with COX II associated at one side and COX III bound to the other. COX II is composed of two transmembrane segments which are firmly bound to COX I and a polar globular domain protruding into the inter-membrane space which serves as the cytochrome *c* docking site. On the other side of COX I, the seven transmembrane segments of COX III are arranged in a V shape with 2 segments on one side and five on the other. This cleft may serve as a channel for O_2 because of its hydrophobic interior (Iwata *et al.*, 1995; Riistama *et al.*, 1996; Ostermeier *et al.*, 1996; Tsukihara *et al.*, 1996). Early work with COX in *P. denitrificans* had suggested that COX III may be responsible for proton pumping because loss of the subunit or dicyclohexylcarbodiimide (DCCD) modification at E98, a conserved residue in COX III, resulted in the reduction of proton translocation across the inner membrane (Saraste *et al.*, 1981; Casey *et al.*, 1980; Prochaska *et al.*, 1981). However, later experiments demonstrated that proton translocation still occurred in *P. denitrificans* strains mutated at the E98 residue and in strains deleted for the COX III gene [COX activity and proton translocation were severely reduced in the deletion strains] (Haltia *et al.*, 1989; Haltia *et al.*, 1991). Although COX III is not directly involved in proton translocation, it has been suggested that this subunit may modulate proton pumping activity (Brunori *et al.*, 1987). A more likely role for COX III may be structural and/or regulatory. Not much is known about the assembly of COX: how hemes and coppers are inserted into subunits COX I and COX II, what proteins are assembled first and what types of proteins regulate assembly and turnover of the

holoenzyme. COX III may have a dual function, regulating some stage of assembly and stabilizing the structure within the membrane (Brunori *et al.*, 1987; Haltia *et al.*, 1989).

Regulatory and structural roles also have been proposed for the ten subunits of the mammalian COX which are encoded in the nucleus and transported into the mitochondrion (Kadenbach 1986; Capaldi 1990; Kennaway *et al.*, 1990). Because of the difficulties working with mammalian COX, yeast has been the system of choice for mutagenesis studies. Nine COX structural genes have been identified in S. *cerevisiae* which are homologous to their nuclear counterpart in mammals (Dowhan *et al.*, 1985; Poyton *et al.*, 1988; Aggeler and Capaldi 1990; LaMarche *et al.*, 1992; Taanman and Capaldi, 1993; Poyton and McEwen, 1996). Null mutations in six of the COX genes produced severe reductions in COX activity, cytochromes aa_3 and cellular respiration. COX assembly was inhibited or greatly reduced in four null strains (COX 4, COX 6, COX 7 and COX 7a), indicating these nuclear subunits may be required for assembly or stability of COX (Dowhan *et al.*, 1985; Poyton *et al.*, 1988; Aggeler and Capaldi 1990; Calder and McEwen 1991; Poyton and McEwen, 1996). Although it is evident from yeast studies that most of the nuclear subunits are necessary for enzymatic activity, functions for many remain obscure. The crystallization of COX from bovine heart is an important step towards delineating roles for each of the nuclear subunits. Already, analysis has revealed that a nuclear subunit is responsible for dimerization of the enzyme complex (Tsukihara *et al.*, 1996). Subunit VIa bridges the two monomers through subunits COX III and COX I. Mammals have tissue specific isoforms of subunits VIa, VIIa and VIII. In human tissues, a heart specific isoform of VIa and VIIa is expressed only in heart and skeletal muscle. The liver isoform of VIa and VIIa is found in all other tissues, and it's expression overlaps with the heart isoform in adult heart and in muscle and heart during early developmental stages. (Van Beeumen *et al.*, 1990; Arnaudo *et al.*, 1992; Fabrizi *et al.*, 1992; Ewart *et al.*, 1991; Taanman *et al.*, 1992; Taanman *et al.*, 1993). Isoforms are hypothesized to fine-tune COX activity to meet the energy demands of a specific tissue. Isoforms of COX V in yeast and

COX VII in slime mold are differentially expressed depending on the stage of growth; aerobic versus anaerobic in yeast and exponential versus stationary in slime mold (Poyton *et al.*, 1988; Bisson and Schiavo, 1988). Characterization of the yeast enzyme has revealed kinetic differences in heme a oxidation and turnover rates of the enzyme complex with different isoforms (Waterland *et al.*, 1991). Functional roles for isoforms in the mammalian enzyme are not as clear. An ATP/ADP binding site located in subunit VIa of bovine heart but not liver has been shown to differentially regulate the H^+/e^- stoichiometry in mitochondria (Taanman and Capaldi 1994; Kadenbach *et al.*, 1995; Frank and Kadenbach, 1996). It is interesting to speculate that the VIa heart isoform may modulate proton pumping of the enzyme and regulate dimerization of COX. The orientation of the other COX subunits encoded by the nuclear genome has been revealed from analysis of the crystal structure (Tsukihara *et al.*, 1996). Six subunits [IV, VIc, VIIa, VIIb, VIIc, VIII] have one transmembrane segment and all except VIc and VIIa interact with COX I. Three subunits [Va, Vb, VIb] lack transmembrane segments. Functional roles for many of these nuclear subunits may be determined with further analysis of the crystal structures. Nuclear subunits with transmembrane segments probably serve a structural role within the enzyme complex. Functions for subunits without transmembrane domains are less obvious. Subunit VIb, a subunit on the cytosolic side of the membrane and subunits COX I and COX II may form a probable site for interaction with cytochrome *c* (Tsukihara *et al.*, 1996).

Import of nuclear encoded subunits

Nuclear subunits of COX and other respiratory enzymes, in addition to matrix enzymes and other polypeptides required for mitochondria function, are imported into the mitochondria. Mechanisms involving mitochondrial import are similar to those of import of proteins into the endoplasmic reticulum of the cell and import of polypeptides into chloroplasts of plant cells (Schatz and Dobberstein, 1996). N-terminal signal sequences

specify import of nuclear proteins into the ER, chloroplast or mitochondrion. Signal sequences for mitochondrial proteins range in length from 10-70 amino acids and primarily are composed of basic amino acids which fold into an amphiphilic alpha helix (Rusch and Kendall, 1995; Schatz and Dobberstein, 1996). The N-terminal region functions as a signal to cellular chaperones to transport proteins to receptors on the mitochondrial outer membrane. The signal sequence may specifically bind to the membrane import machinery (a transmembrane channel) and facilitate the translocation of the polypeptide across both outer and inner mitochondrial membranes. Once in the matrix, the signal sequence is cleaved from the polypeptide by a matrix processing peptidase. Proteins which function within the inter-membrane space or have transmembrane domains within the outer or inner membrane, such as subunits of the respiratory chain, have a second signal sequence which specifies export of the protein. Transmembrane proteins are exported until a stop transfer sequence within the protein is recognized causing protein to remain within the membrane (Schatz and Dobberstein, 1996).

THE MITOCHONDRIAL GENOME

Mitochondria are the only organelles apart from the nucleus in mammalian cells which contain their own DNA. The human mitochondrial genome is a double stranded circular DNA comprised of 37 genes; 2 rRNAs, 22 tRNAs and 13 genes encoding polypeptides necessary for respiration (Fig. 1.3). Four of the five enzyme complexes of the respiratory chain have components encoded by mtDNA. These include genes for seven subunits of NADH ubiquinone oxidoreductase (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), one subunit of ubiquinol-cytochrome *c* oxidoreductase (cytb), three subunits of cytochrome *c* oxidase (COX I, COX II, COX III) and two subunits of ATP synthetase (ATPase 6 and ATPase 8). The majority of genes are encoded by the heavy strand, but sequences for eight tRNAs and one peptide, ND6, are located on the light strand. With a total of 16.6 kb, the genome is an example of conservation. None of the genes encoding

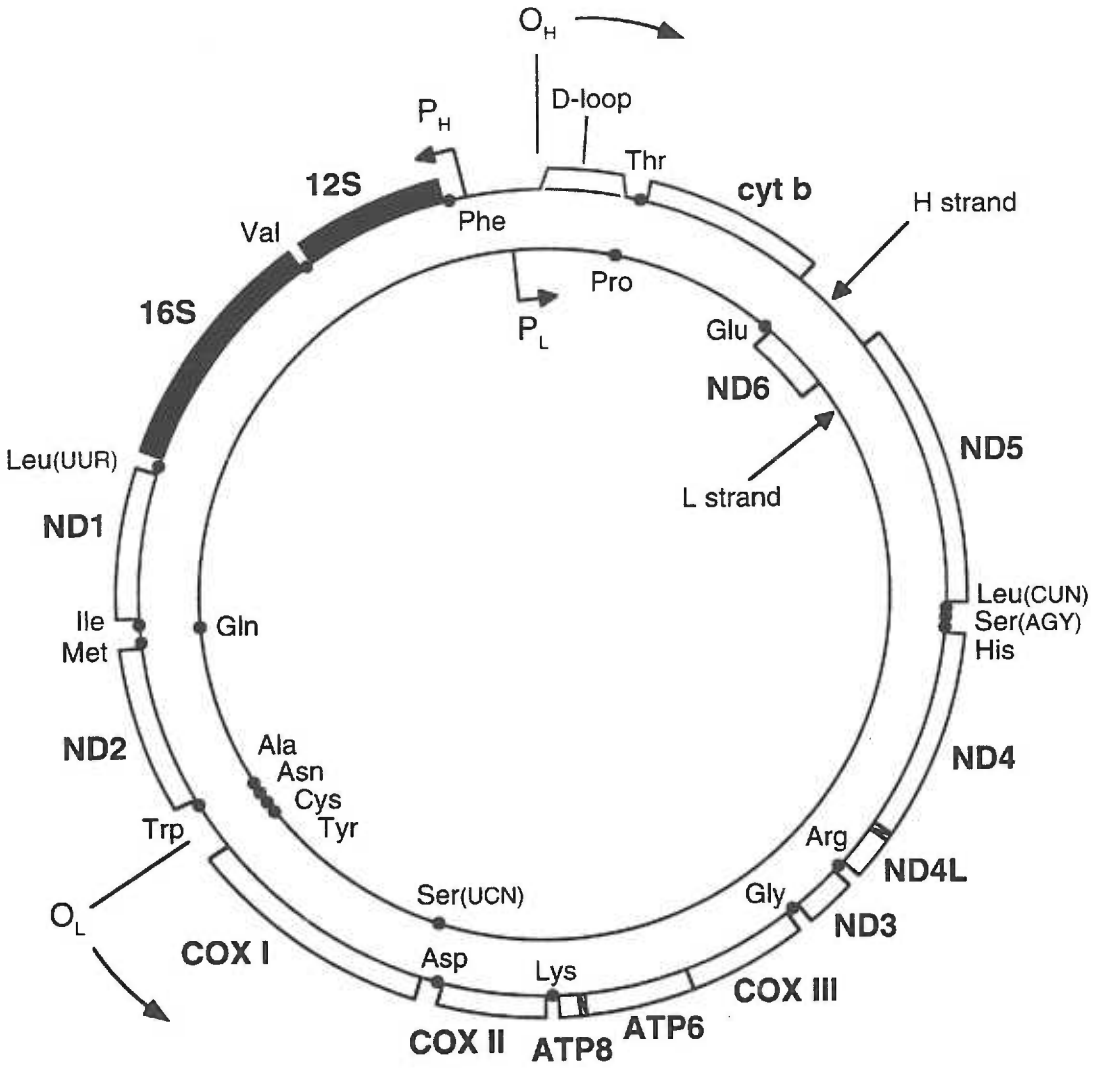
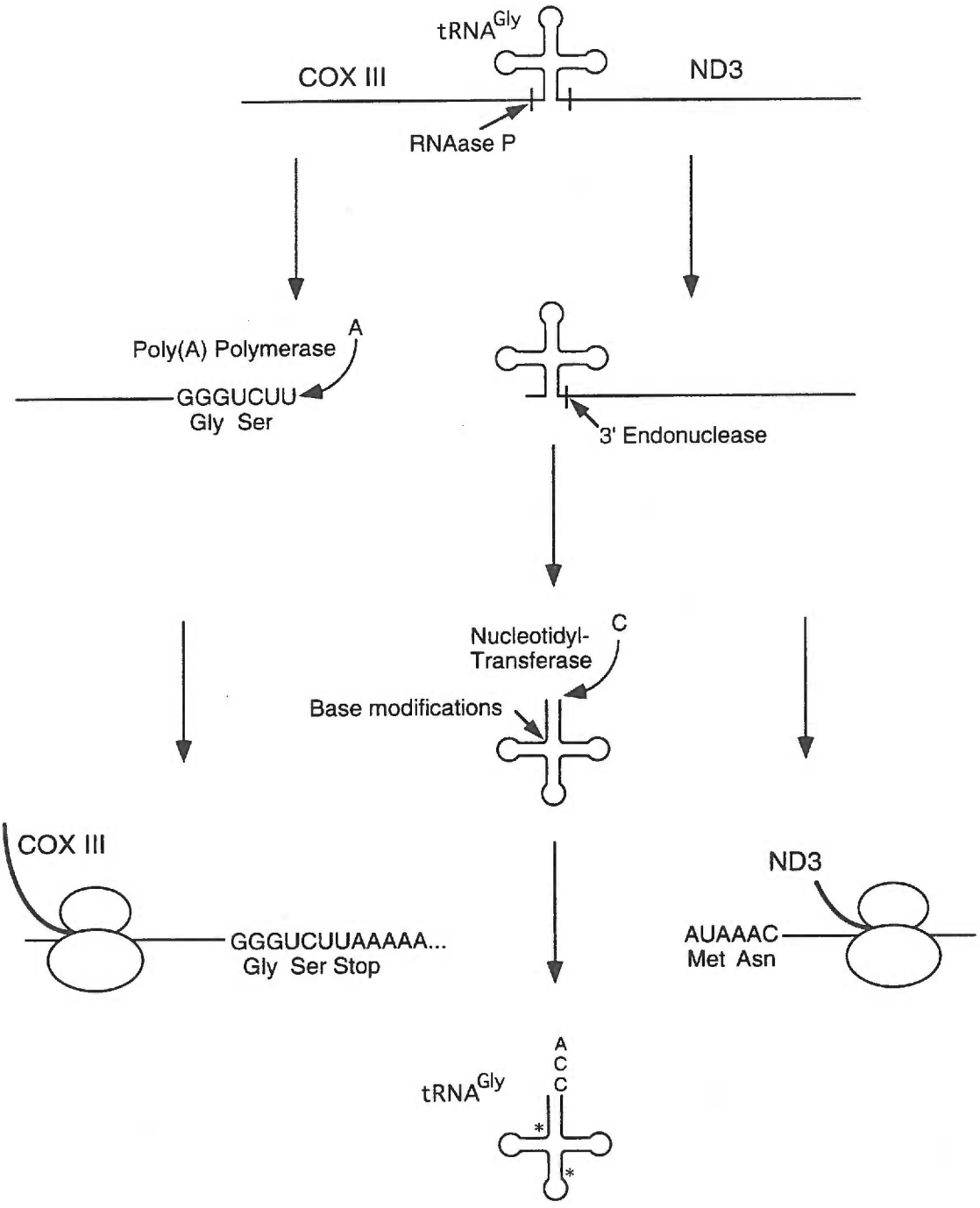


Figure 1.3. The human mitochondrial genome. A double stranded, 16.5 kb, circular DNA molecular encoding 2 rRNAs (black boxes), 13 polypeptides (white boxes) and 22 tRNAs (black circles). Genes are located on both the heavy strand (H-strand, outer circle) and the light strand (L strand, inner circle). Replication of the mitochondrial genome initiates at the origin for heavy strand synthesis, O_H, and proceeds clockwise until the origin for light strand synthesis, O_L, is encountered, three-quarters around the genome. Figure was copied with permission from M. King (Davidson and King, 1997).

polypeptides contain introns. The only large noncoding region, termed the displacement loop (D-loop), contains the origin of replication for the heavy strand (O_H) and promoters for transcription of both the heavy strand and light strand (Fig. 1.3) (Anderson *et al.*, 1981). Replication of mtDNA is initiated within the D-loop at O_H. Initially, replication is unidirectional only with elongation of the heavy strand, until the origin for replication of the light strand is exposed, three quarters around the genome from the D-loop (Clayton, 1982; Clayton, 1991).

Both heavy and light strands of mtDNA are transcribed as polycistronic messages which are processed into individual mRNA species. Within the genome, tRNAs are interspersed between most of the genes coding for rRNAs or proteins and thought to serve as RNA processing signals (Ojala *et al.*, 1981). The tRNA structure is recognized by RNAse P which cleaves at the 5' end of the sequence and by a 3' endonuclease which cleaves the 3' end (Rossmanith *et al.*, 1995). The processing occurs precisely at the 5' and 3' end of the tRNAs because in many cases, the coding region is immediately adjacent to the tRNA sequence (Fig. 1.4). Another striking feature of mtDNA transcripts is the absence of termination codons. More than half of the transcripts have just the U or UA of the termination codon. The complete codon is formed when the mRNA transcripts are polyadenylated (Anderson *et al.*, 1981; Ojala *et al.*, 1981). Steady state levels of RNA transcripts differ considerably; rRNAs are 50 times more abundant in mitochondria compared to mRNA transcripts (Gelfand and Attardi, 1981). Transcripts encompassing only the two rRNAs and the tRNAs interspersed between the rRNAs are initiated from a second heavy strand initiation site (H_1) and terminated at a specific termination region within the $tRNA^{leu(UUR)}$, the downstream gene immediately adjacent to 16s rRNA (Montoya *et al.*, 1983; Christianson and Clayton, 1988). The higher transcription rates observed from the H_1 initiation site and the premature termination results in the high steady state levels of the rRNA transcripts, and represents one main mechanism of differential

Figure 1.4. Processing of mitochondrial polycistronic transcripts. Shown above is a portion of mitochondrial polycistronic message encompassing COX III, $tRNA^{gly}$ and ND3 genes. MtDNA is transcribed as a polycistronic transcript. tRNAs are interspersed between a majority of the protein coding genes and the tertiary structures of tRNAs serve as processing signals and are recognized by RNA processing enzymes. RNAase P cleaves the 5' end of the tRNA, and a 3' endonuclease cleaves the 3' end of the tRNA, precisely at the boundary between the coding genes COX III and ND3 and $tRNA^{gly}$. Many of the mitochondrial transcripts lack a complete termination codon, having just a U or a UA as in the case with COX III. A UAA termination codon is created by polyadenylation of the transcript. mRNA transcripts lack non-translated leader sequences, beginning immediately with the initiation codon AUA or AUG as shown by ND3. Post-transcriptional modifications of tRNAs include a CCA addition on the 3' end of the tRNA by nucleotidyl-transferase and base modifications which are shown by asterisks for $tRNA^{gly}$. Figure was copied with permission from M. King (Davidson and King, 1997).


tRNA^Gly
COX III
ND3
RNAase P
Poly(A) Polymerase
A
GGGUCUU
Gly Ser
3' Endonuclease
Nucleotidyl-
Transferase
C
Base modifications
COX III
GGGUCUUAAAAA...
Gly Ser Stop
ND3
AUAAAC
Met Asn
tRNA^Gly
A
C
C
*
*

gene expression in mammalian mitochondria (Montoya *et al.*, 1983; Christianson and Clayton, 1988; Attardi *et al.,* 1989; Clayton, 1991)

MtDNA only codes for 22 tRNAs (ten fewer than nuclear DNA), but the number of tRNAs is sufficient for protein translation within mitochondria because of differences in the genetic code. Stop codon UGA is recognized as a tryptophan in mitochondria, arginine codons AGA and AGG are recognized as stop codons and isoleucine, and AUA as methionine. Even the initiation codon is not universal; AUA or AUU can be present instead of AUG (Anderson *et al.*, 1981). In addition to having different initiation codons, non-translated leader sequences are absent from mRNA transcripts, suggesting that mitochondrial ribosomes don't require the presence of other sequences besides the initiation codon for recognition (Anderson *et al.*, 1981).

Mitochondria and mtDNA are maternally inherited; thus children inherit mtDNA only from their mother. This non-Mendelian pattern of inheritance is illustrated particularly well by the maternal transmission of mtDNA mutations in human pedigrees. The genetics of mtDNA and mtDNA diseases are complicated by the high copy numbers of mtDNA molecules in cells. Mitochondria contain an average of five mtDNA molecules, which multiplies to thousands within individual cells. Populations of mtDNA molecules within cells and tissues of an individual are usually homoplasmic [same genetic sequence]. Heteroplasmy, the coexistence of two populations of mtDNA, is rare but often is observed in mitochondrial diseases where there is a selective advantage for the maintenance of a small percentage of wild type DNA. Heteroplasmy can exist within the same tissue, same cell or even within the same mitochondrion. Because segregation of mitochondria during mitosis and meiosis is a random process, the percentage of a certain population of mtDNA may change in successive generations. Dramatic shifts have been observed in the percentages of mtDNA mutations in different generations of human pedigrees and in segregation of neutral polymorphisms in successive generations of Holstein cows (Howell *et al.*, 1994; Santorelli *et al.*, 1994; Harding *et al.*, 1995; Olivo *et al.*, 1983; Laipis *et al.*,

1988). In one such human pedigree, the percentage of mutation shifted from 7% in the first generation to a range of 0-88% within descendants of the third generation (Santorelli *et al.*, 1994). This amplification of the mtDNA mutation is not explained solely by random segregation. At some stage of oogenesis, the population of mtDNA is drastically reduced which may disproportionately increase the percentage of one population of mtDNA compared to the other. This 'bottleneck" results in the rapid fixation of new mtDNA mutations and polymorphisms. The maintenance of new mutations in the population and the high mutation rate for mtDNA (5-10 times faster than nuclear DNA) contributes to the variability of mtDNA in populations (Brown *et al.*, 1979). MtDNA is highly polymorphic in populations, differing by approximately 0.32% between randomly selected individuals (Cann *et al.*, 1987). The largest non-coding region within the human mtDNA genome, the D-loop, is a hot spot for polymorphisms, thus providing an ideal system for population mapping and phylogeny studies (Wallace, 1995).

MITOCHONDRIAL DISEASES

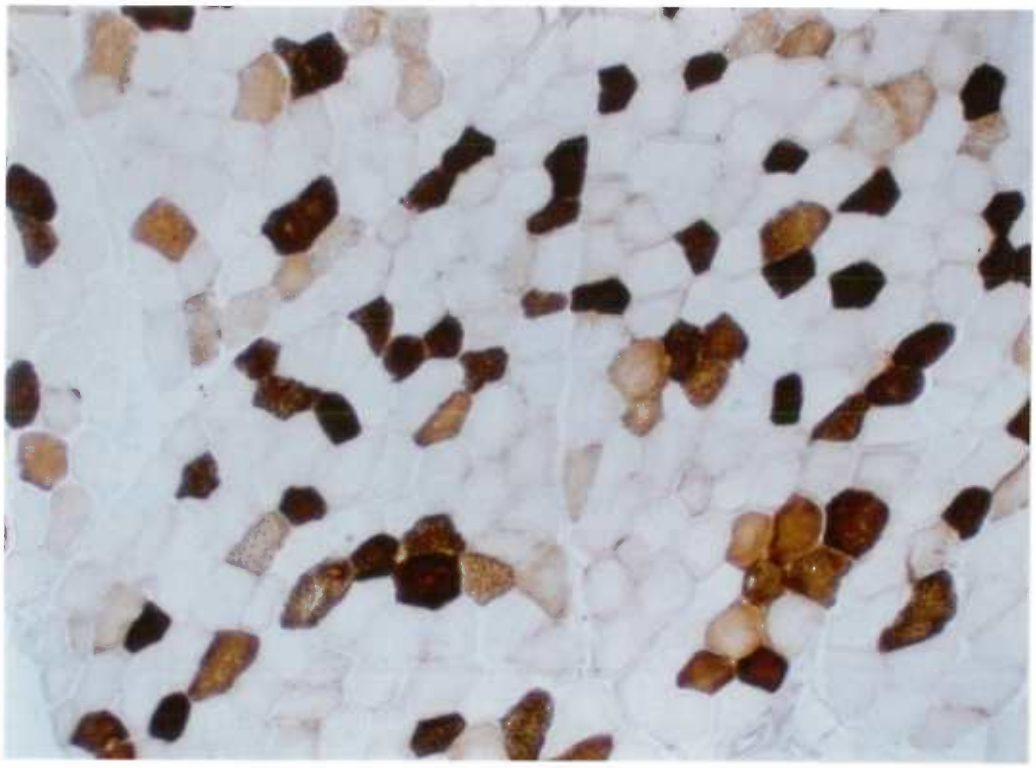
In 1962, Luft and colleagues described a novel disease involving biochemical and structural abnormalities of mitochondria (Luft *et al.*, 1962). Over the last 35 years, deficiencies in every complex within the respiratory chain have been observed in patients, in addition to the documentation of more than 30 pathogenic mutations within mtDNA genome (for reviews see Wallace, 1995; Shoffner and Wallace, 1994; Larsson and Clayton, 1995). Recently, it was estimated that disorders involving energy metabolism affect one in 5000 in the population (Robinson, 1993). Although most of these disorders are rare, together they provide important insights about energy production in cells, especially in tissues with high energy demands such as muscle and brain. Mitochondrial diseases present with a broad spectrum of clinical features with variable severity. A description of a typical presentation is difficult since the involvement of almost every organ has been documented (Munnich *et al.*, 1992). Cardiomyopathy, myopathy, exercise

intolerance, ataxia, diabetes, lactic acidemia, hearing loss and optic atrophy are commonly reported complications of oxidative metabolism disorders (Munnich *et al.*, 1992; Breningstall, 1993). Lactic acidemia is a typical feature in patients with mitochondrial diseases in addition to isolated or multiple respiratory enzyme deficiencies. Muscle fibers may have histochemical abnormalities when stained for oxidative enzymes such as COX. Fibers can show a complete lack of staining or a mosaic of COX positive and negative staining (Fig. 1.5a). Patients with mtDNA mutations may have abnormal accumulations of mitochondria in muscle fibers which are termed ragged red fibers because of their red appearance upon Gomori trichrome staining (Fig 1.5b). A combination of a mosaic of COX positive and negative fibers and ragged red fibers is suggestive of a mtDNA mutation. At the EM level, mitochondria are frequently abnormal in size and structure and may contain concentric or paracrystalline inclusions (Breningstall, 1993).

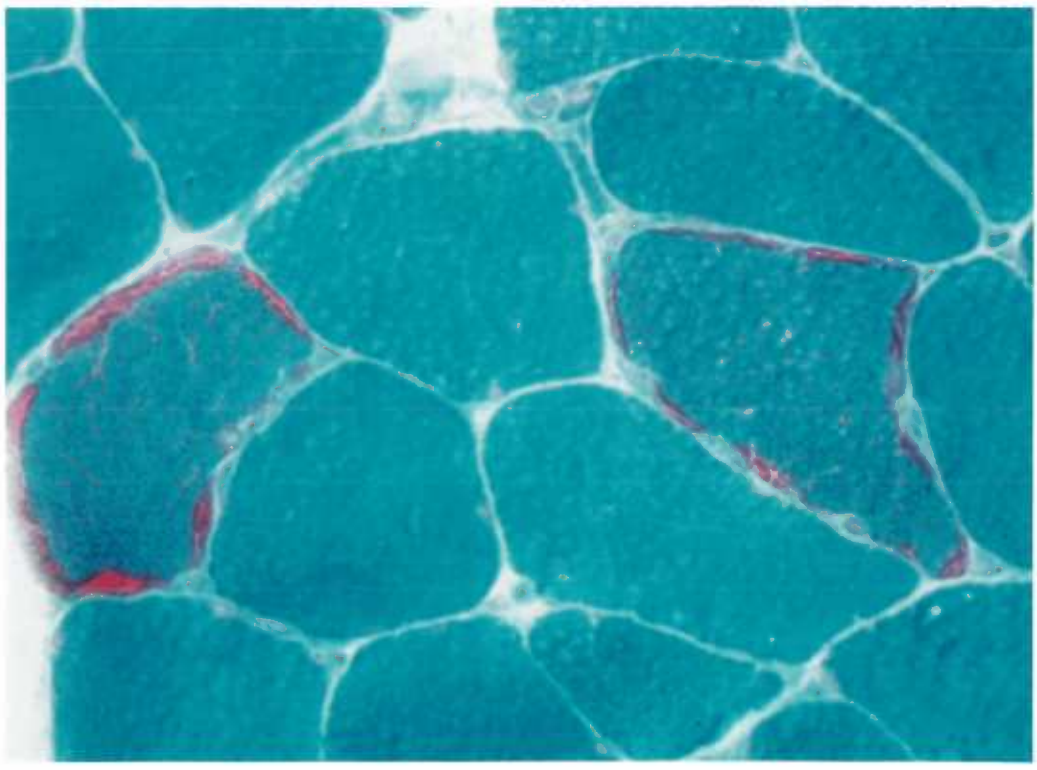
The molecular genetics of mitochondrial diseases is complicated. In addition to the division of respiratory genes between two genomes, numerous processes are involved in the transcriptional and translational control of both the mtDNA and nuclear encoded peptides, import and processing of nuclear proteins into the mitochondria and assembly of enzyme complexes. The presence of multiple polymorphisms in mtDNA from any one individual contributes to the difficulty in identification of pathogenic mutations [an estimated 50 base changes considering the 0.32% sequence variability between random individuals] (Davidson and King, 1997; Cann *et al.*, 1987). Correlations between clinical phenotype and genotype also can be difficult because of heteroplasmy and the variability of mutation levels in different tissues. Some of these limitations have been overcome with the development of a transmitochondrial cell system which allows the transfer of mitochondria from a patient's cell line to a ρ^0 cell line, a human cell line completely lacking mtDNA (King and Attardi, 1989). ρ^0 cell lines are created by exposure to low concentrations of ethidium bromide (Et BR). Et BR inhibits mtDNA replication but has no effect on nuclear replication (King, 1996). ρ^0 cells depend solely on glycolysis for energy and require

Figure 1.5. COX positive and negative fibers and ragged red fibers. a. Cross section of a muscle fiber stained for COX activity, shows a mosaic of dark staining fibers (COX positive fibers) and fibers with a lack of staining (COX negative fibers). **b.** Cross section of a muscle fiber stained with Gomori trichrome. Ragged red fibers are those with red staining areas around the muscle fiber.

a.



b.

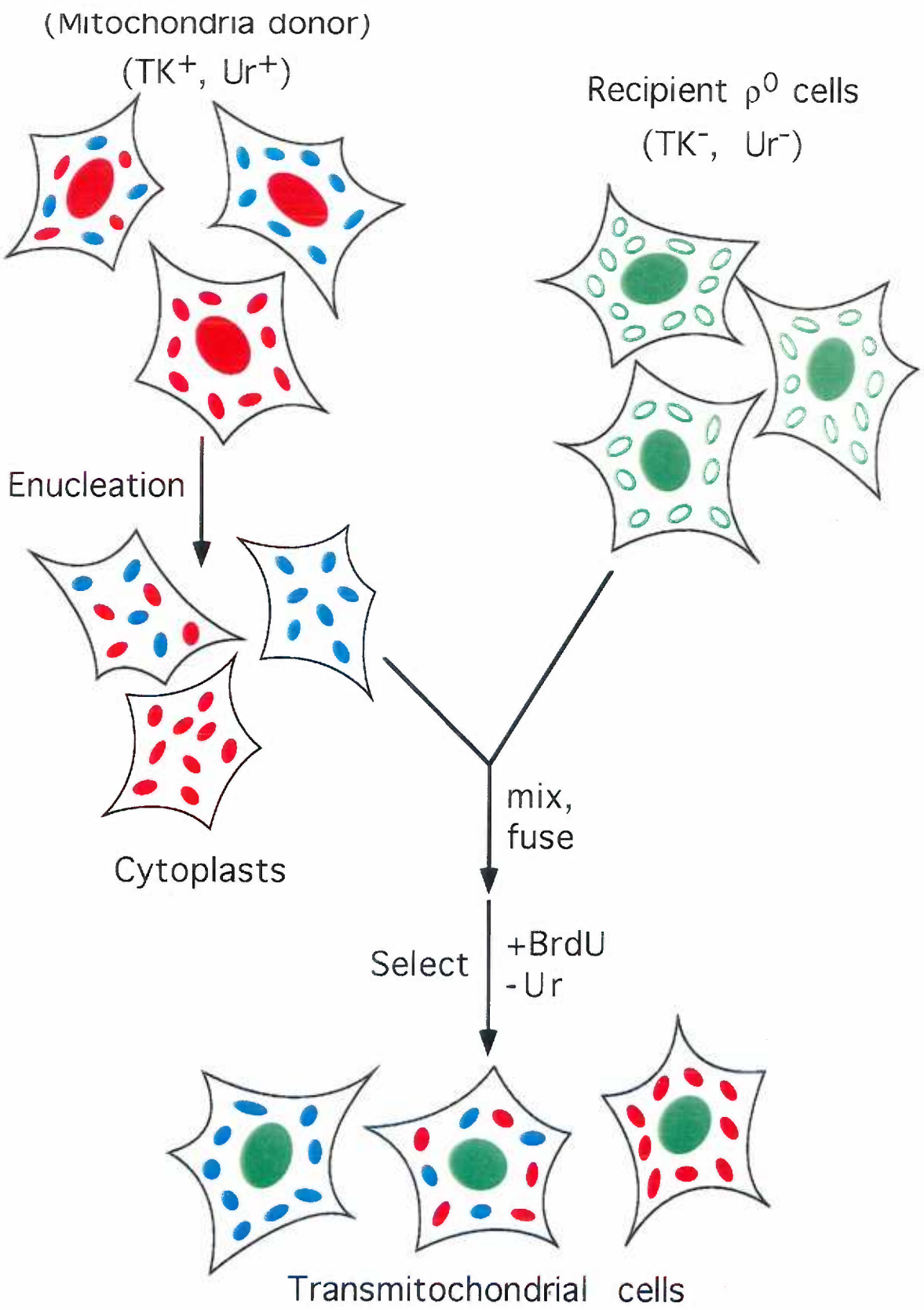


supplementation with pyrimidine and pyruvate for growth. Transfer of mitochondria is accomplished by PEG mediated fusion of enucleated donor cells (patient cells) with the ρ^0 cell line. After fusion, selection of transmitochondrial cell lines which contain mtDNA from the donor cell line and nuclear DNA from the ρ^0 cell line is achieved by growth in medium lacking uridine and containing bromodeoxyuridine [ρ^0 are TK^- and donor cells are TK^+], (Fig. 1.6) (King and Attardi, 1989). This system allows the study of mtDNA mutations in the presence of a neutral nuclear background in addition to the creation of cell lines homoplasmic for patient wild-type and mutated mtDNA. Homoplasmic cell lines for either wild-type or mutated DNA are derived from the same patient cell line and should only differ at one position (the mutation) between mtDNA genomes, thus providing a powerful system for evaluation of pathogenic mtDNA mutations (Davidson and King, 1997). This system has been used successfully to study tRNA mutations, mtDNA rearrangements and mutations within protein encoding genes (Chomyn *et al.,* 1991; Masucci *et al.,* 1995; King *et al.,* 1992; Chomyn *et al.,* 1992; Hayashi *et al.,* 1991; Jun *et al.*, 1996)

Transfer RNA mutations

Transfer RNA mutations are the most prevalent type of mtDNA abnormality, more than 20 different tRNA mutations having been reported to be associated with disease, nine within the $tRNA^{leu(UUR)}$ (Shoffner and Wallace, 1994; Schon *et al.,* 1994; Larsson and Clayton, 1995). Two heteroplasmic mutations in $tRNA^{leu(UUR)}$, A3243G or T3271C, are the predominant mtDNA abnormalities found in patients with a multi-system disorder featuring mitochondrial encephalopathy, lactic acidosis and stroke like episodes (MELAS) (Goto *et al.*, 1990; Goto *et al.*, 1991). Two other mutations in the $tRNA^{leu(UUR)}$ and a missense mutation in protein encoding gene COX III have been documented in patients with MELAS (Sweeny *et al.*, 1993; Morten *et al.,* 1993; Manfredi *et al.*, 1995). These mutations are maternally inherited and heteroplasmic, with levels of mutation in skeletal muscle exceeding 80% (Goto *et al.*, 1992; Ciafaloni *et al.*, 1992). Two heteroplasmic

Figure 1.6. Creation of transmitochondrial cell lines. The mitochondrial donor cells, which are thymidine kinase positive (TK^+) and are able to synthesize uridine (Ur^+), are enucleated by centrifugation in the presence of cytochalasin B. The enucleated cells (cytoplasts) are mixed with the recipient 143B206 ρ^0 cells, which are TK^- and Ur^-, and membrane fusion is promoted by the addition of polyethylene glycol. The transmitochondrial cells, containing mtDNA from the donor cells and nuclear DNA from the ρ^0 cells, are selected by their ability to grow in the absence of uridine (to select for mitochondrial function) and in the presence of bromodeoxyuridine. The ρ^0 cells are not able to grow in this medium and eventually die. Growth in the presence of bromodeoxyuridine selects against contaminating donor cells and cell hybrids which contain a functional thymidine kinase.


(Mitochondria donor)
(TK+, Ur+)
Recipient ρ0 cells
(TK-, Ur-)
Enucleation
Cytoplasts
mix,
fuse
Select
+BrdU
-Ur
Transmitochondrial cells

mutations in the $tRNA^{lys}$, A8344G and T8356C, are associated with MERRF, a neurological disease characterized by myoclonic epilepsy, ataxia, myopathy and ragged red fibers (Shoffner *et al.,* 1990; Silvestri *et al.,* 1992). Both mutations are heteroplasmic with high levels of mutation (73-100%) documented in skeletal muscle of MERRF patients (Shoffner *et al.,* 1990; Silvestri *et al.,* 1992; Silvestri *et al.,* 1993).

The high levels of mutation in affected tissue of MELAS and MERRF patients and the lower levels in their asymptomatic maternal relatives suggested these tRNA mutations were functionally recessive. In vitro studies with the MELAS A3243G mutation and the MERRF A8344G confirmed that high levels of mutation were required for expression of respiratory deficiencies, and these studies revealed these mutations exhibited a sharp threshold of expression (Chomyn *et al.,* 1992; Boulet *et al.*, 1992; Yoneda *et al.,* 1994). In transmitochondrial cell lines and cultured myoblasts, mutation levels greater than 85-90% resulted in a severe reduction of translation of mtDNA encoded polypeptides and subsequent enzyme deficiency of respiratory complexes. Cell lines containing less than 85% mutated mtDNA displayed almost normal rates of translation and enzyme activity, indicating that tRNAs may not become limiting for mtDNA translation until their levels are reduced by approximately 85-90% (Boulet *et al.*, 1992; Chomyn *et al.,* 1992; Yoneda *et al.,* 1994). Similar thresholds were documented at the single fiber level. Ragged red and COX negative fibers from MELAS patients had high levels of mutation, exceeding 85-90% while non-ragged red fibers and COX positive fibers had much lower percentages of mutation (Moraes *et al.,* 1992; Petruzzella *et al.,* 1994). Similar observations have been described with other tRNA mutations (Moraes *et al.*, 1993a; Moraes *et al.*, 1993b).

Mitochondrial DNA rearrangements: deletions and duplications

Several distinct clinical syndromes are associated with large scale deletions of mtDNA: these include progressive external ophthalmoplegia (PEO), Kearns-Sayre syndrome (KSS) and Pearson marrow pancreas syndrome. Onset of both PEO and KSS

occurs in adolescence. PEO is the less severe of the two, presenting only as an ocular myopathy while KSS is characterized by PEO, in addition to heart conduction block and cerebellar syndrome. Pearson syndrome is a fatal infantile disorder associated with sideroblastic anemia and exocrine pancreatic dysfunction (Holt *et al.*, 1989; Shoffner and Wallace, 1994). Rare familial cases of PEO have been described which are characterized by either dominant or recessive mode of inheritance and multiple deletions. Linkage analysis has localized two different nuclear loci associated with dominant PEO (Suomalainen *et al.*, 1995; Kaukonen *et al.*, 1996). Most mtDNA deletions are sporadic, with no evidence of maternal or familial inheritance. Typically only one type of deletion is present in sporadic cases indicating that the deletion probably arose from clonal expansion in oogenesis or early embryogenesis (Schon, 1994). MtDNA deletions vary in location, and range in size from 1.3kb to 10 kb. Like tRNA mutations, both wild-type and deleted mtDNA molecules are present in affected tissues with variable percentages in different tissues of an individual. Often, muscle histochemistry is characterized by the presence of ragged red fibers and a mosaic of COX positive and negative fibers. Thresholds for phenotypic expression also have been observed in transmitochondrial cell lines harboring deletions (Hayashi *et al.*, 1991; Sancho *et al.*, 1992). Reductions in mitochondrial respiratory chain activities occurred only in cell lines with greater than 80% level of deleted mtDNA genomes, and deficiencies coupled with generalized reductions in protein translation occurred in cell lines containing greater than 90% deleted mtDNA (Davidson and King, 1997). Duplications (composed of two mtDNA molecules; one wild type and one deleted mtDNA, arranged head to tail) also have been documented in KSS but their association with neuromuscular disease is not clear (Poulton *et al.*, 1994).

Mutations within protein encoding genes

Lebers hereditary optic neuropathy (LHON), maternally inherited Leigh syndrome, and neuropathy, ataxia and retinitis pigmentosa (NARP) are all clinical syndromes

associated with point mutations within coding genes of mtDNA. More than ten different mutations have been described in patients with the permanent visual loss characteristic of LHON. Approximately 90% of all cases are caused by three mutations within ND4, ND1 and ND6, all subunits of complex I; in most cases these mutations are homoplasmic although heteroplasmy has been described in some families (Newman, 1993; Shoffner and Wallace, 1994; Wallace, 1995; Harding *et al.*, 1995). Other mutations associated with LHON are secondary because of their association with additional mutations or environmental factors and their prevalence in the normal population (Wallace, 1995; Shoffner and Wallace, 1994).

Two mutations at position 8993 (T→C or T→G) within ATPase subunit 6 are documented in cases of both maternally inherited Leigh syndrome and NARP (Holt *et al.*, 1990; Tatuch *et al.*, 1992). Proportions of the 8993 mutation differ between syndromes, but higher levels of mutation correlate with phenotypic severity of the two diseases. Leigh syndrome, a multi-system disorder involving the brainstem and basal ganglia, is expressed in patients with greater than 85% levels of mutation. The milder presentation of NARP, predominantly characterized by ataxia and retinitis pigmentosa, is associated with 75-85% levels of mutation. The 8993 mutation changes a highly conserved amino acid in the ATPase subunit 6 which is a component of the F_0 complex of F_1F_0 ATP synthetase. Cultured lymphoblast lines and transmitochondrial cells with high levels of mutation displayed decreased rates of ATP synthesis (Tatuch and Robinson, 1993; Trounce *et al.*, 1994; Davidson and King, 1997). Analysis of patient tissues revealed a reduction in fully assembled F_1F_0 ATP synthetase, indicating the mutation probably reduces the stability of the complex resulting in the decline of ATP production (Houstek *et al.*, 1995).

CYTOCHROME *C* OXIDASE (COX) DEFICIENCY

COX deficiency comprises a heterogeneous group of disorders with equally complicated molecular etiologies. Although tRNA mutations and deletions within mtDNA,

such as MELAS, MERRF and Kearns Sayre syndrome, are associated with multiple respiratory chain deficiencies including COX deficiency, the majority of cases of isolated COX deficiency have not been defined at the molecular level (Holt *et al.*, 1989; Zeviani *et al.*, 1991, Wallace *et al.*, 1988; Robinson, 1993). Pedigree analysis has indicated autosomal recessive patterns of inheritance in many families, suggesting that both defects of nuclear DNA and mtDNA are responsible for COX deficiency (DiMauro *et al.*, 1990; Robinson, 1993). Four to five types of isolated COX deficiency have been described including a fatal infantile form presenting as a myopathy or cardiomyopathy and two forms of Leigh disease specifically associated with isolated COX deficiency. Myopathy is the predominant manifestation of fatal infantile COX deficiency, but the involvement of heart, kidney and liver are commonly reported complications. Children with the fatal form have severe weakness, lactic acidosis and die of respiratory failure before age of one year (DiMauro *et al.*, 1990; Robinson, 1993). In rare instances, children may have a benign form where symptoms spontaneously improve with no evidence of COX deficiency or lactic acidosis by the age of two years (DiMauro *et al.*, 1983; Zeviani *et al.*, 1987; Kennaway, unpublished results). Multi-system disorders involving COX deficiency typically present as Leigh syndrome. Children with pyruvate dehydrogenase (PDH) deficiency or high levels of 8993 mutation in A6 also may have clinical symptoms of Leigh disease. Leigh syndrome is dominated by brain dysfunction, with ataxia, dystonia and respiratory abnormalities (Van-Coster *et al.*, 1991; DiMauro *et al.*, 1990; Robinson, 1993). COX deficiency in Leigh disease may involve a severe reduction in enzyme activity in all tissues or a tissue specific reduction predominantly confined to brain and liver. The tissue specific form of Leigh syndrome with COX deficiency is rare and shows a strong founder effect, with all reported cases from the Saguenay-Lac-Saint-Jean region of Quebec (Merante *et al.*, 1993).

No mutations have been identified in any case of fatal COX deficiency or Leigh syndrome associated with COX deficiency. In some patients many or all of the COX

structural genes have been sequenced, indicating that the defects causing these diseases probably involve proteins regulating COX assembly or biogenesis (Merante *et al.*, 1993; Adams *et al.*, 1997). Complementation studies with Leigh cell lines suggest that the pathogenesis of this disease may be monogenic (Munaro *et al.*, 1997). In yeast, 13 nuclear encoded proteins in addition to the COX structural subunits are required for assembly of COX. A null mutation in any one of these genes can lead to reduced assembly of COX and reduced steady state levels of many COX subunits (Dowhan *et al.,* 1985; Calder and McEwen, 1991; Poyton and McEwen, 1996; Church *et al.*, 1996)

A few mutations within the mtDNA encoded COX subunits have been documented, but their pathogenicity has been debated because of the occurrence of some of these mutations in the normal population and/ or the lack of COX deficiency in patient tissues. Three homoplasmic point mutations within mtDNA encoded COX genes, at position 7444 within COX I and positions 9438 and 9804 within COX III have been found in a minority of LHON cases. None of these mutations are associated with COX deficiency, and the presence of the 7444 and 9438 mutations has been described in normal controls at a lower frequency (Brown *et al.*, 1992; Johns and Neufeld, 1993; Oostra *et al.,* 1995). These mutations are probably rare polymorphisms, although functional roles have been proposed such as serving as secondary risk factors for LHON. Another missense mutation within the COX III gene, T9957C, was identified in a patient with MELAS (Manfredi *et al.,* 1995). This mutation had some of the hallmarks of pathogenic mtDNA mutations such as heteroplasmy with higher amounts in muscle (80%) and absence in over 100 controls. But no COX deficiency was observed by histochemical staining of muscle sections or by biochemical assay of purified mitochondria isolated from muscle. The patient had severe encephalopathy with no symptoms of myopathy which may explain the near normal levels of COX activity in muscle. Studies with transmitochondrial cell lines will help determine the pathogenicity of this mutation.

The molecular analysis of COX deficiency in numerous cases will be described in the following chapters. Recently, our laboratory identified a 15 bp, in frame, deletion in the COX III gene, encoding subunit III of cytochrome *c* oxidase in a patient with recurrent episodes of myoglobinuria and isolated COX deficiency (Keightley *et. al*, 1996, see appendix 1). This was the first documentation of any mutation in a COX subunit (encoded by mtDNA or nuclear DNA) to be associated with COX deficiency. The patient had high levels of mutant mtDNA in her skeletal muscle (93%) and barely detectable levels of mutant mtDNA in her leukocytes. Single muscle fiber analysis revealed a strong correlation between percentage of mutation and COX negative fibers. Ragged red COX negative fibers had a much higher percentage of mutation ($98.8 \pm 1.3\%$) compared to COX positive fibers ($25 \pm 25\%$). The immunoreactivity for COX subunits I, II, IV, Va, Vb, and VIc was significantly reduced in ragged red, COX negative muscle fibers as compared to COX positive muscle fibers. Western analysis also revealed a reduction in the steady state levels of COX subunits II, IV, Va, Vb and VIc, indicating that assembly or stability of the holoenzyme may be compromised by the 15 bp deletion in COX subunit III.

The deletion removes five amino acids from a conserved transmembrane region of the COX III polypeptide which is one of two helices of COX III which make contact with COX I, as indicated by the crystal structure of bovine heart (Fig. 1.7.) This segment may play an important structural role in COX, in addition to forming part of a putative hydrophobic channel for diffusion of oxygen (Iwata *et al.,* 1995; Riistama *et al.*, 1996; Ostermeier *et al.,* 1996; Tsukihara *et al.,* 1996). To investigate the consequences of the 15 bp deletion for COX assembly and enzymatic function, the mutation was transferred to the ρ^0 cell line developed by King and Attardi (1989). A direct relationship was found between the 15 bp deletion in the COX III gene and the expression of isolated COX deficiency.

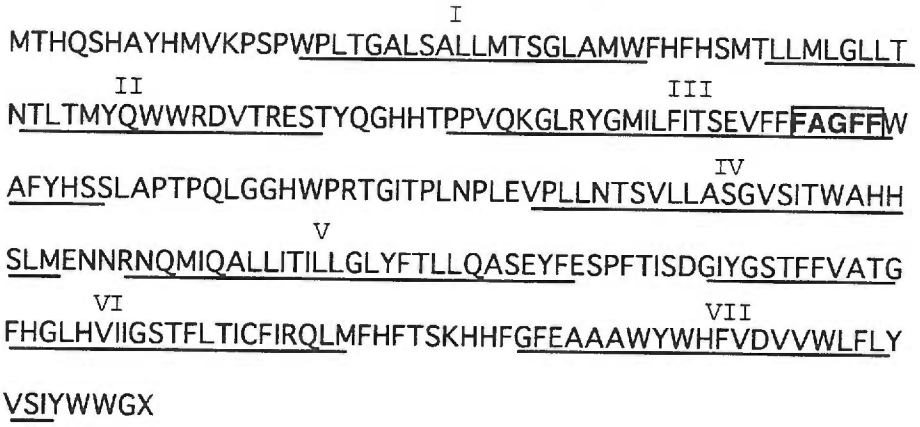


Figure 1.7. The amino acid sequence of COX III polypeptide. The location of the seven transmembrane segments of COX III polypeptide are indicated in the diagram (underlined amino acids), and are based on analysis of COX crystal structure of bovine heart (Tsukihara *et al.,* 1996). The COX III mutation deletes five amino acids from the third transmembrane domain (shown bold type, inside box).

CHAPTER 2

MOLECULAR ANALYSIS OF COX DEFICIENCY

COX deficiency can be divided into several sub-types. COX deficiency can be expressed as a secondary phenotype in diseases such as Menkes disease which involves a defect of copper transport (French *et al.*, 1972), and often is observed along with other defects of the respiratory chain in mitochondrial diseases which are caused by mtDNA deletions or tRNA mutations (Holt *et al.*, 1989; Zeviani *et al.*, 1991; Wallace *et al.*, 1988). Isolated COX deficiency usually presents as Leigh disease, involving a generalized COX deficiency in all tissues, or as a tissue specific form which is characterized by a severe reduction in COX activity in skeletal muscle, heart or both tissues. The genetics of isolated COX deficiency are poorly characterized and only one case, reported by our laboratory, has been defined at the molecular level (Keightley *et al.*, 1996, see appendix 1). In this patient, a 15 bp deletion was identified in mtDNA encoded subunit III of COX in a patient with recurrent myoglobinuria. Pedigree analysis, in many families has indicated autosomal recessive patterns of inheritance, suggesting that some forms of isolated COX deficiency may be caused by mutations in one or more nuclear genes (DiMauro *et al.*, 1990; Robinson, 1993, Tiranti *et al.*, 1995).

Three subunits of COX are encoded by the mtDNA genome and the remaining ten are encoded by the nuclear genome. Tissue specific isoforms of two COX nuclear subunits, COX VIa and VIIa, exist in human tissues. Heart specific isoforms (VIaH and VIIaH) are expressed only in heart and skeletal muscle whereas the liver isoforms (VIaL and VIIaL) are expressed in all tissues. Human heart is composed of approximately equal proportions of heart and liver tissue specific isoforms while skeletal muscle is composed almost entirely of the heart isoforms (Van Beeumen *et al.*, 1990; Arnaudo *et al.*, 1992; Fabrizi *et al.*, 1992; Ewart *et al.*, 1991; Taanman *et al.*, 1992; Taanman *et al.*, 1993). In

many cases, the pattern of expression of either the heart or the liver isoforms of COX subunits VIa and VIIa in human tissues parallels the expression of COX deficiency (DiMauro *et al.*, 1990; Robinson, 1993).

Both the tissue specific isoforms of COX subunits VIa and VIIa and the mitochondrial encoded COX genes were analyzed in five patients with tissue specific expression of either isolated COX deficiency or combined complex I and COX deficiency. Defects in either the tissue specific isoforms or mtDNA encoded COX genes could be consistent with COX deficiency which is limited to one or a few tissues.

MATERIALS AND METHODS

Clinical presentation. All five patients included in this study had a myopathy and/or cardiomyopathy associated with either isolated COX deficiency or combined COX and complex I deficiency. A summary of clinical symptoms and biochemical studies for each patient is shown in Table 2.1 and Table 2.2.

N.J. presented after birth with lactic acidosis and hypotonia which gradually improved. She had a normal echocardiogram at day 6 of age, but died at 22 days of age from a massive biventricular hypertrophic cardiomyopathy. At autopsy, COX activity in heart mitochondria was 7% of control values while COX activity in muscle mitochondria was in the low normal range. COX activities in kidney and liver also were reduced, 25% of control values (Kennaway *et al.*, 1990).

K.B. presented at six months of age with episodic lactic acidosis and muscle weakness, and was diagnosed with a lipid storage myopathy. K.B. experienced poor growth and progressive muscle weakness. Skeletal muscle, biopsied at 3 years of age, showed a specific and severe COX deficiency (16% of control values). K.B. died at 6 years of age; COX activity in autopsied muscle was 20% of control values.

Table 2.1. Clinical presentation

	N.J.	K.B.	A.C.	A.CU.	M.L.
Sex	F	M	M	M	F
Onset	birth	6 months	infancy	birth	birth
Family history	-	-	+	+	+
Hypotonia	+	+	-	+	+
Renal failure	-	-	+	-	-
Lactic Acidosis	+	+	+	+	+
Myopathy	±	+	+	+	+
Cardiomyopathy	+	-	+	+	+
Ragged Red Fibers	-	-	-	+	+
Seizures	+	+	-	-	+
Death	22 days	6 years	7 weeks	17 days	1 month

Table 2.2. Biochemical profile of patients

	PATIENTS				
SKELETAL MUSCLE	N.J.	K.B.	A.C.	A.CU.	M.L.
Complex I	63	126	35	**ND**	**8**
Cytochrome *c* oxidase	52	**16**	34	**2**	**3**
Citrate synthase	-	87*	40	39	-
Succinate Dehydrogenase	89	121	55	129	62
CARDIAC MUSCLE					
Complex I	67*	-	**ND***	-	-
Cytochrome *c* oxidase	**7***	-	**<6***	-	-
Citrate synthase	-	-	63*	-	-
Succinate Dehydrogenase	87*	-	52*	-	-

The enzymatic activities are expressed as percentage of mean control values. Citrate synthase and succinate dehydrogenase are citric acid cycle enzymes. Activities of the other respiratory complexes were measured and were unremarkable (not shown). Activities denoted with a * were measurements of autopsy samples. ND = not detectable. Abnormal values are shown in bold type.

The other patients included in this study had a combined deficiency of COX and complex I. A.C. was diagnosed in infancy with lactic acidosis and a myopathy and died at 7 weeks of age from a progressive hypertrophic cardiomyopathy. A combined deficiency of both complex I and COX activities was detected in autopsied heart tissue but not autopsied skeletal muscle tissue. A.C. had a positive family history, having a brother who died at 42 days of age from a similar mitochondrial problem.

A.CU. presented after birth with lactic acidosis and hypotonia. An echo cardiogram at 2 days was normal. A muscle biopsy at 10 days after birth demonstrated low complex I and COX activities. Ragged red fibers and COX negative fibers were detected with histochemical staining of biopsied muscle. A.C. died 17 days after birth from cardiomyopathy. A.CU. had a positive family history, with a sister (M.L.) who presented at birth with similar complications. A combined deficiency of both complex I and COX activities was detected in biopsied skeletal muscle. M.L. died 1 month after birth from a cardiomyopathy.

Cell culture. Fibroblast cultures had been established previously for patients N.J., K.B. and M.L., by described methods (Kennaway *et al.*, 1989). Fibroblasts were maintained in Eagles minimum essential medium (MEM) with nonessential amino acids, 10% FCS, penicillin (100U/ml) and streptomycin (100mg/liter).

RNA isolation and cDNA synthesis. Total RNA was isolated from exponentially growing fibroblast cell lines from approximately 10^6 cells or from 0.2 - 0.5g of skeletal muscle or heart autopsy tissue using a commercial RNA isolation reagent, RNAzol B (Tel-test Inc.). RNA was resuspended in RNAse free H_2O for cDNA synthesis or formazol (Tel-test Inc.), a commercial brand of purified formamide, for northern analysis and was quantitated by 260/280 UV absorbance. Complimentary DNA was reverse transcribed from approximately 1-6 μg of total RNA, utilizing a non-specific oligo dT (17mer) and M-MuLv

reverse transcriptase (BRL), following the manufacturer's directions and diluted approximately 1-5 for PCR reactions.

PCR. Complimentary DNA was amplified by PCR using 10 pmoles of forward and reverse primers, in a 25 µl reaction volume containing 50mM KCl, 10mM Tris Cl, 1.5mM $MgCl_2$, 0.01% gelatin, 160 µM dNTP, and 0.2 units of a modified Taq polymerase (kindly provided by Dr. Robb Moses, Oregon Health Sciences University). The reactions were denatured for 1 min. at 94°C, annealed for 1 min. at a temperature ranging between 55-65°C depending on primer pair used, and extended for 1 min. at 72°C. Reactions were cycled 30 times. For SSCP, 1-2µCi of ^{32}P alpha dCTP (3000 Ci/mmol) was added at the beginning of the reaction for incorporation throughout the PCR cycles (the amount of cold dCTP was reduced in these reactions to a concentration of 0.25 µM). Primer pairs used in PCR reactions were as follows: for COX nuclear subunit VIaL 1. forward 5' GTGGTAGTTGGTGTGTCCTCGG 3', reverse 5' CGGCTTGGTCCTGATGCGGAG 3' 2. forward 5' GTACCTGAAGTCGCACCACG 3', reverse 5' GCCATCTCCTGCCACAAGCAAAG 3'; VIaH forward 5' CGCACCATCATGGCTTTGCCT 3', reverse 5' CTTCACACCTTTATTGTGTCC 3'; VIIaL forward 5' GTAACAGCCAAGATGCTGCGGAAT 3', reverse 5' TGGTGGCAGTTACTACAAGTCAAT 3'; VIIaH forward 5' GGAATTCAGGAGAAGGGAGGTGAC 3', reverse 5' GGAATTCCAGAGGCCAGCGTTTATTGAC 3'.

Single-stranded conformation polymorphism (SSCP) Analysis. SSCP analysis was performed using a modified version of the procedure first described by Orita *et al.* (1989). To yield fragments small enough for analysis, COX VIaH, VIIaL and VIIaH PCR products were digested with restriction enzymes, *Sac I*, *Msp 1* and *Sau 3A 1*, respectively. Eight µl of stop solution (95% formamide, 10mm NaOH, 0.1% bromophenol blue and 0.1% xylene cyanol) was added to 1.5 µl of the PCR reactions or restriction digestions and denatured at 95°C for 2 minutes. Three µl of this mixture was

loaded on two types of electrophoretic gels: a 5% non-denaturing 0.4mm polyacrylamide containing 10% glycerol or a 5% non-denaturing polyacrylamide gel without glycerol. Non-denatured samples of each PCR product or restriction digestions were loaded on the gels to help identify the double stranded products and the single stranded conformers. Electrophoresis conditions were 3 watts for 16 hours at room temperature with constant cooling (gel with glycerol) and 15 watts for 3-4 hours at 4°C (gel without glycerol). Gels were dried and exposed to X-ray film for 1-4 days with intensifying screen at -70°C.

Northern analysis. Patient and control total RNA (10 - 15 μg) was electrophoresed through a 1% agarose gel containing 0.66M formaldehyde and transferred to Hybond-N membrane (Amersham) using blotting conditions as described by Fourney *et al.* (1988). Probes for northern analysis were labeled by random priming using the Prime It kit (Stratagene), following manufacturer's directions and unincorporated radioactive nucleotides were removed using nuctrap columns (Stratagene). Templates for random priming reactions were prepared by restriction digestion of vectors containing cDNAs corresponding to tissue specific subunits and K-1 tubulin or PCR amplification (mitochondrial encoded COX genes). Probes were: 1. *EcoRI - HindIII* fragment of pCOX6AHM.01, the muscle isoform of human COX VIa (gift from Dr. M. Lomax, Detroit, Michigan) 2. *EcoRI* fragment of pCOX6A.60, the liver isoform of human COX VIa 3. *EcoRI* fragment of pCOX7aM.541, the muscle isoform of COX VIIa 4. *EcoRI* fragment of pCOX7aL.22, the liver isoform of human COX VIIa 5. *PstI* fragment of ptub.01, cDNA corresponding to K-1 tubulin (gift Dr. M. Thayer, Portland, Or.) 6. A PCR amplified fragment corresponding to nt 5898 - 7014 of mitochondrial encoded COX I gene 7. A 810bp XbaI fragment of PCR product (mtDNA 7476-8783), encompassing COX II gene, $tRNA^{Asp}$ and $tRNA^{Ser}$ 8. A PCR amplified fragment corresponding to nt 9438-9950 of mtDNA encoded COX III gene. Vectors, pCOX6A.60, pCOX7aM.541 and

pCOX7aL.22 were generously provided by Dr.Eric Schon, Columbia University, New York.

Blots were pre-hybridized in 50% formamide, 0.47 X Denhardts, 4.7 X SSPE, 0.1% SDS, 10% dextran sulfate for 4 hours at 42°C and then were hybridized for 20 hours at 42°C. Probes were denatured with 250mg/ml salmon sperm at 95°C for 2 minutes and added to hybridization solution. Wash conditions were followed as described by Fourney *et al.* (1988), briefly 2 washes in 1 X SSC and 0.1% SDS, at room temperature and two washes at 55°C in 0.1 X SSC and 0.1% SDS. Blots were exposed 1-5 days to X-ray film with an intensifying screen at -70°C. Blots were stripped by boiling in 0.1% SDS.

Genomic DNA isolation and direct sequencing of mtDNA. Total DNA was isolated from exponentially growing cells according to established protocols (Davis *et al.*, 1986) or from skeletal muscle or heart tissue using DNA isolation kit (Puregene). PCR fragments of 1.4 -3.0 kb, generated from genomic DNA, were purified using Qiaex gel extraction resin (Qiagen) and sequenced directly using a combination of automated sequencing (Applied Biosytems at Columbia University Sequencing Facility and Oregon Health Sciences University Sequencing Facility) and cycle sequencing (New England biolabs) with ^{32}P labeled primer. Sequencing reactions were electrophoresed through 6% polyacrylamide 8 M urea gels, dried and exposed to X-ray film for 24-48 hours. A total of 3500 bp of mtDNA was sequenced for patient N.J. with primers spaced approximately 300 bp apart. The boundaries of the sequenced regions for N.J. were nt 3190-3310, 5890-8365, 9206-10060.

RESULTS

Five patients diagnosed with either a myopathy and/or cardiomyopathy and COX deficiency were selected for molecular studies. The clinical presentation of each patient is

summarized in Table 2.1 and the biochemical studies in Table 2.2. The biochemical profile of three patients, diagnosed with a cardiomyopathy (A.C.) or both a myopathy and cardiomyopathy (siblings, A.CU. and M.L), was characterized by a combined complex I and COX deficiency. At the time of the study, it was not clear whether our measurements of complex I activities accurately reflected deficiencies in muscle, because of the lability of the assay and the condition of the tissue, which in many cases was autopsy material.

The tissue specific expression of isolated COX deficiency was particularly evident in two patients (N.J., K.B.). The COX deficiency in patient N.J., which was characterized by a severe reduction in COX activity in heart, and a less severe reduction in COX activity in kidney and liver, was unusual compared to other documented cases of COX deficiency (Kennaway *et al.*, 1990), and could be consistent with a defect in the liver isoform COX VIaL or VIIaL or a defect in a mitochondrial encoded COX gene. In patient (K.B.), the predominant myopathy without associated cardiomyopathy could be consistent with a defect in one of the muscle specific isoforms of COX subunits VIa or VIIa.

All of the patients, including N.J. and K.B., were negative for mtDNA deletions and the most common mtDNA mutations A3243G in $tRNA^{Leu(UUR)}$, and A8344G in $tRNA^{Lys}$.

SSCP Analysis. Since the respiratory defect in all five patients was characterized by a tissue specific expression of COX deficiency or combined COX and complex I deficiency, the tissue specific isoforms of COX (VIaH, VIaL, VIIaH, and VIIaL) were likely candidates for mutation analysis. Single-stranded conformation polymorphism (SSCP) analysis was the method chosen for mutation screening because the full length cDNAs of the COX tissue specific isoforms were relatively small. To evaluate the sensitivity of my SSCP method for detecting mutations, the $tRNA^{lys}$ gene was amplified by PCR from two patients with the MERRF A8344G mutation and three normal controls, and analyzed by SSCP. SSCP analysis also was performed on PCR fragments encompassing the

$tRNA^{leu(UUR)}$ gene, amplified from one patient with the MELAS A3243G mutation and three normal controls. SSCP was performed using two electrophoretic conditions: PCR fragments were denatured and either electrophoresed at room temperature in non-denaturing polyacrylamide gels containing 10% glycerol or were electrophoresed at 4°C in non-denaturing polyacrylamide gels containing no glycerol. The MERRF A8344G mutation was detected by SSCP in both patients, but none of the controls when SSCP was performed at room temperature (4°C with no glycerol was not performed). The MELAS A3243G mutation was detected only when SSCP was performed at 4°C; the room temperature SSCP analysis did not show any differences between the patient and controls. Others have reported that the efficiency of mutation detection by SSCP is dependent on electrophoretic conditions (Orita *et al.*, 1989; Sheffield *et al.*, 1993). Based on these results, to maximize detection of mutations in the COX tissue specific subunits, two types of electrophoretic conditions (room temperature with glycerol and 4°C with no glycerol) were used routinely to screen for mutations.

For SSCP analysis of the tissue specific isoforms of COX, total RNA was isolated from either skeletal muscle and/or heart tissue from four patients (N.J., K.B., A.C., A.CU.) or primary fibroblast cell lines from three patients (N.J., K.B., M.L.) and two controls, reversed transcribed and PCR amplified. SSCP analysis was performed on fragments of 300 bp or less. Since the full lenght cDNAs ranged in size from 370bp - 550bp, smaller fragments for SSCP analysis were obtained by designing multiple primer pairs for cDNA amplification or by restriction digestion of the PCR products prior to electrophoresis. SSCP analysis of COX subunits VIaH, VIaL, VIIaH, and VIIaL revealed no differences in migration of single-stranded conformers between patients and controls. Because of tissue availability, only the muscle specific subunits could be screened in patients A.C. and A.CU. and the liver specific subunits in M.L. (sibling of A.CU.).

Northern Analysis. The steady state levels of the mRNA transcripts of COX tissue specific isoforms and the mtDNA encoded subunits of COX were investigated by Northern analysis. Total RNA was isolated from skeletal muscle and/or heart tissues from four patients (N.J., K.B., A.C., A.CU.) and cultured fibroblast cell lines from three patients (N.J., K.B., M.L.) and was examined for differences in steady state levels. Total RNA isolated from autopsied heart and skeletal muscle tissues, and cultured fibroblasts of two patients who had no evidence of COX deficiency were included on blots as controls. The RNA was hybridized with seven probes which identified three mtDNA encoded COX genes (COX I, COX II, COX III) and five nuclear genes (COX VIaL, VIaH, VIIaL and VIIaH and K- tubulin). The steady state levels of K- tubulin were examined to control for equal loading of RNA on blots.

Northern analysis revealed no obvious differences in the mobility of COX transcripts or in the steady state levels of COX transcripts between patients and controls. A summary of the molecular studies which were performed on each patient is shown in Table 2.3.

Sequence Analysis of mtDNA. The mitochondrial encoded $tRNA^{Leu\ (UUR)}$ seems to be a genetic hot spot for mutations; nine different mutations within the gene have been reported to be associated with disease, of which three were specifically documented in patients with cardiomyopathies (for review, see Shoffner and Wallace, 1994; Schon *et al.*, 1994; Larsson and Clayton, 1995). The $tRNA^{Leu\ (UUR)}$ gene was PCR amplified and analyzed for mutations by SSCP in 4 patients (N.J., K.B., A.C., A.CU.). A different single-stranded conformer was observed in two patients (N.J. and A.CU.). The mtDNA of these two patients was cycle sequenced and a T→C transition was identified at position 3197 in the 16s rRNA gene in both patients (the 16s rRNA gene lies immediately upstream of $tRNA^{Leu\ (UUR)}$). This mutation is most likely a polymorphism and was present in the unaffected mother of patient A.CU.

The three mitochondrial encoded COX genes (COX I, COX II and COX III) were sequenced in cardic muscle from one patient with tissue specific expression of isolated COX deficiency (N.J.). Five differences from the Cambridge sequence (Anderson *et al.*, 1981) were identified (Table 2.4); however, these coding changes were presumably polymorphisms for two reasons. The mutation either did not change the amino acid or the amino acid change was conservative and had been reported previously in the normal population (Table 2.4) Four mtDNA encoded tRNAs ($tRNA^{Ser}$, $tRNA^{Asp}$, $tRNA^{Lys}$ and $tRNA^{Gly}$) also were sequenced and no differences were detected.

Table 2.3. Summary of molecular analysis

		Patients				
Type of analysis	COX subunits	N.J.	K.B.	A.C.	A.CU.	M.L.
SSCP						
	VIa liver	X	X			X
	VIa heart	X	X	X	X	
	VIIa liver	X	X			X
	VIIa heart	X	X	X	X	
Northern						
	VIa liver	X	X			X
	VIa heart	X	X	X	X	
	VIIa liver	X	X			X
	VIIa heart	X	X	X	X	
	COX I	X	X	X	X	
	COX II	X	X	X	X	
	COX III	X	X	X	X	

RNA isolated from skeletal muscle or heart tissue and fibroblasts was used in SSCP and northern analysis. Both types of tissue (muscle and fibroblasts) was available for analysis for patients N.J. and K.B. Cardiac tissue was the only tissue available for analysis for patient A.C., cardiac and skeletal muscle for patient A.CU. and fibroblasts for patient M.L. Patients A.CU. and M.L. were siblings.

Table 2.4. MtDNA polymorphisms identified in patient N.J.

Gene	nt position	amino acid
16s rRNA	T3197C	N/A
COX I	T7193C	no change
COX II	A7768G	no change
COX III	G9477A	Val 91 Ile, previously reported
COX III	G9559C	Arg 118 Pro
		error in Cambridge sequence[1]
COX III	G9966A	Val 254 Ile, previously reported

1. Monnab and Loeb, 1985.

DISCUSSION

Nuclear and mitochondrial subunits of COX were analyzed for mutations in five patients with COX deficiency. The clinical phenotypes of all the patients in this study were characterized predominantly by a myopathy, cardiomyopathy or both. In particular, the tissue specific expression of COX deficiency was evident in two patients whose clinical phenotype was consistent with a defect in either the heart (K.B.) or the liver (N.J.) tissue specific isoforms of COX or one of the mitochondrial encoded COX genes. No mutations were detected in the liver and heart isoforms of COX subunits VIa and VIIa, and no differences were found in mRNA expression of these subunits or in the expression of the mitochondrial encoded COX genes (COX I, COX II and COX III) in patients and controls. The mtDNA encoded COX genes were sequenced in one patient with a well characterized tissue specific form of isolated COX deficiency. Although five coding differences were identified, all were most likely polymorphisms due to their occurrence in the normal population or their neutral effect on amino acid identity.

These results were surprising because a mutation in a tissue specific isoform or mtDNA encoded subunit seemed to be a likely cause for COX deficiency in at least one of these patients (N.J.). The tissue specific isoforms were screened for mutations by SSCP analysis. The level of mutation detection for SSCP has been reported to be as high as 90% on fragments less than 250bp (Orita *et al.*, 1989). However, recent reports have suggested that the sensitivity of SSCP is highly dependent on the target sequence being analyzed, and the level of mutation detection can vary between 40-80% (Sheffield *et al.*, 1993; Liu and Sommer, 1994). Obviously, one of the limitations when using a mutation screening procedure such as SSCP is that it is impossible to determine the sensitivity level of SSCP when positive controls are not available.

Only one case of a nuclear mutation causing a mitochondrial disease has been described. The patient had Leigh disease associated with complex II deficiency (Bourgeron

et al., 1995). Studies with transmitochondrial cell lines recently have indicated that Leigh disease associated with COX deficiency also is the result of a nuclear defect and a single disease locus may be responsible for the majority of cases (Tiranti *et al.*, 1995; Munaro *et al.*, 1997). Recently, a tissue specific form of Leigh disease was documented in patients with low levels of COX activity in liver and brain but not in heart, kidney or skeletal muscle. No defects were identified in coding regions of the liver specific isoforms of VIa and VIIa or in the levels of mRNA expression (Merante *et al.*, 1993). Another report described the sequencing of all thirteen COX structural subunits in a patient with Leigh disease and generalized COX deficiency. No mutations were identified, indicating the COX deficiency in this patient was caused by a defect in a gene other than a COX structural gene (Adams *et al.*, 1997).

COX is composed of thirteen polypeptide subunits whose expression, processing and assembly must be coordinated by other cellular factors. Studies in yeast have demonstrated the existence of mitochondrial chaperonins, members of the hsp 70 family which assist the refolding of imported nuclear encoded proteins and assembly of membrane enzyme complexes (Hartl and Martin, 1992; Poyton and McEwen, 1996). At least eight nuclear gene products are specifically required for the assembly of cytochrome c oxidase, and are not involved in the assembly of the other respiratory enzyme complexes (for review, see Poyton and McEwen, 1996). Functions for some of these factors include components of the heme A biosynthesis pathway (COX10 and COX11) and transportation of copper to the mitochondrion, COX17 (Tzagoloff *et al.*, 1993; Glerum *et al.*, 1996). Human homologues exist for at least two COX specific assembly factors and presumably defects in any of these assembly factors could result in COX deficiency (Glerum *et al.*, 1994; Bonnefoy *et al.*, 1994).

Regulation of COX nuclear subunits also occurs at the level of mRNA expression. The heart isoforms of subunits VIa and VIIa are regulated at the level of transcription, resulting in the expression of these subunits only in heart and skeletal muscle. Transcripts

for the liver isoforms are detectable in all tissues, but are post-transcriptionally regulated (Fabrizi *et al.*, 1992; Arnaudo *et al.*, 1992). Recently, an RNA binding protein specific for the 3' end of the liver transcript was identified in bovine liver homogenates and human liver Hep G2 cell homogenates (Preiss and Lightowlers, 1993; Preiss *et al.*, 1994). The binding activity of the L-form transcript-binding protein (COLBP) was tissue specific and consistent with the expression of the VIa and VIIa liver polypeptides, indicating that the presence of the COLBP protein probably promotes stability of the transcript (Preiss and Lightowlers, 1993; Preiss *et al.*, 1994). Other unidentified factors must regulate the expression of the heart isoforms. Defects in any of these factors could contribute to the pathogenesis of COX deficiency, especially the tissue specific forms, and are good candidates for further studies aimed at identifying the molecular defects in these patients.

CHAPTER 3

A PATHOGENIC 15BP DELETION IN MITOCHONDRIAL DNA ENCODED CYTOCHROME *C* OXIDASE SUBUNIT III (COX III) RESULTS IN THE ABSENCE OF FUNCTIONAL CYTOCHROME *C* OXIDASE

Cytochrome *c* oxidase (COX), an essential component of the respiratory chain, transfers electrons from reduced cytochrome *c* to molecular oxygen, conserving the energy in the form of an electrochemical gradient. The human enzyme is composed of thirteen subunits. The three largest are encoded by the mtDNA (COX I, COX II and COX III) and the remainder are encoded by the nuclear DNA. COX I and COX II form the catalytic core of the enzyme while COX III, the only mtDNA encoded COX subunit without prosthetic groups, may serve a structural or regulatory role within the complex. Functions for the ten nuclear subunits are unclear but they also are hypothesized to be structural or regulatory subunits. COX is a complex enzyme whose biogenesis not only involves coordinating the expression of two genomes, but also numerous factors controlling the import of nuclear subunits, insertion of hemes, correct folding of the subunits, and assembly of the holoenzyme. While some of these processes have been delineated in yeast, most have not been identified. Examining the molecular basis of human COX deficiencies is one approach towards understanding the biosynthesis of COX.

COX deficiency has been documented in numerous cases of neuromuscular diseases, presenting either in combination with other respiratory enzyme deficiencies or as an isolated deficiency (French *et al.*, 1972; Wallace *et al.*, 1988; Holt *et al.*, 1989; DiMauro *et al.*, 1990; Zeviani *et al.*, 1991,). The molecular causes of COX deficiencies are variable. Mutations within mtDNA encoded tRNA genes and rearrangements within mtDNA can result in COX deficiencies; however, patients with these mutations are characterized typically by deficiencies in more than one respiratory chain enzyme. Genetic analysis of

isolated COX deficiency, which often presents as Leigh syndrome or as a muscle specific form, characterized by cardiomyopathy or myopathy, has suggested an autosomal recessive mode of inheritance (DiMauro *et al.*, 1990; Robinson, 1993; Tiranti *et al.*, 1995 Munaro *et al.*, 1997). No mutations have been identified in any case of COX deficiency or Leigh syndrome associated with COX deficiency, even though in some patients many or all of the COX structural genes have been sequenced (Merante *et al.*, 1993; Adams *et al.*, 1997).

The first documentation of any mutation in a COX subunit (encoded by mtDNA or nuclear DNA) to be associated with COX deficiency was described by our laboratory (see appendix 1, Keightley *et al*, 1996). A 15 bp, in frame, deletion in the COX III gene, encoding subunit III of COX was identified in a patient with recurrent episodes of myoglobinuria and an isolated COX deficiency. The deletion removed five amino acids (PheAlaGlyPhePhe) from a conserved transmembrane region of the COX III polypeptide, one of two regions which contact COX I in the holoenzyme (Tsukihara *et al.*, 1996). The patient had high levels of mutation in her skeletal muscle (93%) and barely detectable levels (<1%) in her leukocytes. Single muscle fiber analysis revealed a strong correlation between percentage of mutation and COX negative fibers, with ragged-red COX negative fibers having a much higher percentage of mutation (98.8 $\pm$ 1.3%) than COX positive fibers (25 $\pm$ 25%). Western analysis revealed a reduction in the steady state levels of COX subunits II, IV, Va, Vb and VIc, indicating that assembly or stability of the holoenzyme may be compromised by the 15 bp deletion in COX III.

To investigate the consequences of the 15 bp deletion for COX assembly and enzymatic function, the mutation was transferred to a cell line devoid of mtDNA (ρ^0 cell line) developed by King and Attardi (1989). This cellular system allows the study of mtDNA mutations without the contribution of the patient's nuclear background and has been used successfully to investigate mutations in mitochondrial tRNA and protein coding genes and rearrangements of mtDNA (Chomyn *et al.*, 1991; Masucci *et al.*, 1995; King *et al.*, 1992; Chomyn *et al.*, 1992; Hayashi *et al.*, 1991; Jun *et al.*, 1996).

MATERIALS AND METHODS

Genomic DNA isolation and direct sequencing of mtDNA. Total DNA was isolated from skeletal muscle of patient C.S. using DNA isolation kit (Puregene). PCR fragments of 1.4 -3.0 kb spanning the COX genes, were generated from genomic DNA, purified using Qiaex gel extraction resin (Qiagen) and sequenced directly using a combination of automated sequencing (Applied Biosystems at Columbia University Sequencing Facility and Oregon Health Sciences University Sequencing Facility) and cycle sequencing (New England Biolabs) with appropriate ^{32}P labeled primers. Sequencing reactions were electrophoresed through 6% polyacrylamide, 8 M urea gels, dried, and exposed to X-ray film for 24-48 hours. A total of 4150 bp of mtDNA was sequenced for patient C.S. with primers spaced approximately 300 bp apart. The boundaries of the sequenced regions for C.S. were nt 5903-10060.

Cell culture. Human osteosarcoma cell lines 143B and 143B206 (King and Attardi, 1989), were grown in Dulbecco's modified Eagle medium containing 4.5 mg of glucose per ml and 110 μg of pyruvate per ml (DMEM) supplemented with 5% fetal bovine serum (FBS). The medium for cell line 143B206 also was supplemented with 50 μg of uridine per ml. Myoblast cultures were established from a needle biopsy of patient, C.S. as previously described (Miranda *et al.*, 1988). Myoblasts were grown in DMEM supplemented with 50μg of uridine per ml and 15% FBS.

Transformation of the 143B206 cell line with patient mitochondria by cytoplast fusion of enucleated myoblasts was performed as previously described (King and Attardi, 1996b). Briefly, myoblasts were enucleated by exposure to cytochalasin B (1 mg/per ml in DMEM) and centrifugation. The enucleated myoblasts (cytoplasts) were mixed with the recipient 143B206 ρ^0 cells (1.5 X 10^6 cells) and membrane fusion was promoted by the addition of polyethylene glycol. Cybrids were plated in selective medium 24h after fusion

in DMEM supplemented with 100μg of 5-bromodeoxyuridine (BrdU) per ml and 5% dialyzed FBS. This medium selects only for the growth of transmitochondrial cell lines containing mtDNA from the myoblasts and ρ^0 nuclear DNA. ρ^0 cells depleted of mtDNA are not able to proliferate without the uridine supplementation. Non-enucleated myoblasts or hybrids between these and ρ^0 cells will be selected against because endogenous thymidine kinase will convert BrdU into the toxic phosphorylated derivative (King and Attardi, 1996b). Cybrids from the myoblast fusions were grown in selective medium for approximately two to three weeks until colony formation at which time colonies were isolated with glass cloning cylinders. Cells were subsequently cultured in DMEM supplemented with 5% FBS.

Transformation of the 143B206 cell line with patient mitochondria obtained from a platelet/leukocyte fraction was performed as previously described (Chomyn, 1996a). A platelet enriched fraction and a leukocyte fraction were isolated from whole blood by differential centrifugation (Chomyn, 1996a). The platelet and leukocyte fractions were mixed, washed with DMEM and centrifuged. The pellet was mixed with the recipient 143B206 ρ^0 cells (1.5 X 10^6 cells) and membrane fusion was promoted by the addition of polyethylene glycol. Cybrids were plated in selective medium 24h after fusion in DMEM supplemented with 100μg of 5-bromodeoxyuridine (BrdU) per ml and 5% dialyzed FBS. Mass cultures were isolated after two weeks of growth in selective medium and were replated at low density, approximately 100 cells per plate, in non-selective medium (DMEM supplemented with 5% fetal bovine serum (FBS) and 50 μg of uridine per ml) for two to three weeks until colony formation.

Ethidium bromide was used to manipulate the mtDNA content in two cell lines (51.129 and 51.112) using previously described methods (King, 1996). For each cell line, two 6cm plates of exponentially growing cells were grown in DMEM supplemented with 5% FBS, 50ng/ml ethidium bromide and 50μg/ml uridine. The cells were grown in the presence of ethidium bromide for 14 cell generations. After treatment, cells were replated

in DMEM supplemented with 5% FBS and 50μg/ml uridine and grown for 3 days. At this time, cells were replated at low density in either DMEM supplemented with 5% FBS or DMEM supplemented with 5% FBS and 50μg/ml uridine. Two - four weeks later, individual colonies were selected with glass cloning cylinders and subsequently cultured for genetic analysis

PCR Analysis. Total DNA was isolated from exponentially growing cells according to established protocols (Davis *et al.*, 1986). PCR reactions were performed as described previously (Keightley *et al.*, 1996). Briefly, genomic DNA was amplified by PCR using 10 pmol of forward and reverse primers, in a 25 μl reaction volume containing 50mM KCL, 10mM Tris-Cl, 1.5mM $MgCl_2$, 0.01% gelatin, 160 μM dNTP, and 0.2 units Taq polymerase. The reactions were denatured for 1 min. at 94°C, annealed for 1 min. at 60°C and extended for 1 min. at 72°C. Reactions were cycled 25 times. The PCR products were labeled by adding 1-2μCi of ^{32}P alpha dATP (3000 Ci/mmol) in the last cycle of the PCR reaction.

PCR fragments of the wild-type (85bp) or the mutated mtDNA (70bp) were differentiated by size by electrophoresis through 10% polyacrylamide gels. Gels were dried and quantitated by phosphorimaging using Molecular Analyst software (Molecular Imager, Biorad). Boxes of equal area were drawn around the wild-type and mutant PCR bands. Each PCR band was quantitated by calculating the volume of the box (representing the mean pixel density multiplied by the area of the box), and then subtracting the background (volume of a box of equal area) and dividing by the number of ^{32}P alpha dATP nucleotides which would be incorporated into the wild-type PCR product (27 dATP) or the mutant PCR product (17 dATP).

Isolation of Mitochondria. Mitochondria was isolated from 4-5 15cm plates of exponentially growing cells by differential centrifugation as previously described

(Chomyn, 1996b). After trypsinization, cells were resuspended twice in 10-20 ml of cold NKM buffer (0.005M KCl, 0.13M NaCl, 0.0075M $MgCl_2$). After the second NKM wash, the cell pellet was collected by centrifugation at 800 rpm, and cells were resuspended in 5 ml of special RSB buffer (0.01M Tris-Cl, pH 6.7, 0.01M KCl, 1.5 x 10^{-4}M $MgCl_2$). Cells were incubated in RSB buffer on ice for 4 min. and then homogenized until approximately 70-80% of the cells were broken. 2M sucrose was added to cell lysates to a concentration of 0.25M. Mitochondria were isolated by differential centrifugation. Cell lysates were centrifuged at 2300 rpm to remove the unbroken cells and the nuclei. The supernatant was centrifuged at 8000 rpm for 10 min. to pellet the fraction containing the mitochondria. Mitochondria were resuspended in 200µl of RSB buffer (0.25M sucrose, 0.01M Tris, pH 6.7,1.5 x 10^{-4}M $MgCl_2$). Total mitochondrial protein was determined by DC protein assay (Biorad).

O_2 Consumption And Enzyme Activity

O_2 consumption was performed as described previously (King *et al.*, 1992) utilizing samples of 4-5 X 10^6 cells resuspended in 1.65 ml of DMEM.

Cytochrome *c* oxidase activity of isolated mitochondria was measured as previously described (Wharton and Tzagoloff, 1967), except that the oxidation of cytochrome *c* was performed at 30°C using 0.1% laurylmaltoside in the assay buffer. Citrate synthase activity was measured at 30°C according to the method of Shepherd and Garland (1969) after resuspending mitochondria in Triton X-100. Complex III activity was assayed according to Birch-Machin *et al.* (1989), except *n*-decyl coenzyme Q (2,3-dimethoxy-5-methyl-6-*n*-decyl 1,4-benzoquinone: DB) was used as a substrate in place of UQ_1.

ATP synthesis assay. Mitochondria were prepared according to Millis and Pious (1973) after scraping monolayer cultures from five 15 cm culture dishes (approximately 5 x 10^6 cells) into 30 ml of an isolation buffer consisting of 0.27M mannitol, 0.1mM EDTA,

0.05% bovine serum albumin (BSA) and 10mM Tris-HCl pH7.3. After a brief treatment with protease (protease XIV, Sigma), cells were disrupted by four passes in a teflon/glass homogenizer, and the mitochondrial fraction was then isolated by differential centrifugation.

The ability of isolated mitochondria to synthesize ATP by oxidative phosphorylation was assayed using succinate as a substrate. ATP synthesis was assayed indirectly by measuring the incorporation of ^{32}P into glucose-6-phosphate following phosphorylation of ADP by inorganic phosphate in the presence of the myokinase inhibitor adenosine pentaphosphate (Tuena de Gomez-Puyou *et al.*, 1984). Freshly isolated mitochondria (100-200 µg protein) were incubated in 0.3 ml of 30 mM Tris-acetate pH 7.4, 10 mM $MgCl_2$, 10 mM phosphate-tris (0.5µCi ^{32}P-orthophosphate NEN), 6 mM substrate (succinate), 20 mM glucose, 20 U of hexokinase, 1 mM ADP and 50 µM adenosine pentaphosphate in a shaking water bath at 30°C. Control reactions contained the same plus 5µg of the F_0 inhibitor oligomycin. The reaction was terminated after 35 min. by adding 30% trichloroacetic acid to a final concentration of 10%. The reaction mixture was then centrifuged, and a 0.3ml aliquot was removed, to which was sequentially added 1ml H_2O, 1ml 3.3% ammonium molybdate in 3.75N H_2SO_4, and 200µl of acetone. Unincorporated phosphate was removed by extracting 6 times with 1ml of benzene:isobutanol mixture (50:50, vol:vol, H_2O saturated), after vortexing vigorously for 45 seconds each time. The amount of radioactivity present in an aliquot of the extracted reaction mix, determined by scintillation counting, was taken to represent incorporation of radioactive phosphate.

RNA Isolation and Northern Analysis. Total RNA was isolated from exponentially growing cell lines from approximately 1.5 x 10^7 cells using the commercial RNA isolation reagent, RNAzol B (Tel-test Inc.). RNA was resuspended in RNAse free H_2O for cDNA

synthesis or formazol (Tel-test Inc.), a commercial brand of purified formamide and was quantitated by 260/280 UV absorbance.

Total RNA (10 - 15μg) was electrophoresed through a 1% agarose gel containing 0.66M formaldehyde, transferred to Zeta probe (Biorad) using blotting conditions as described by Fourney *et. al.* (1988) and hybridized according to manufacturer's suggested directions. The following probes were labeled by random priming (Feinberg and Vogelstein, 1983) following manufacturer's directions (Random prime labeling kit, Boehringer Mannheim Co): pHFBA-1, a plasmid containing the human cytoplasmic β-actin gene (Gunning *et al.*, 1983); a PCR amplified fragment corresponding to nt 9268-9645 of mtDNA encoded COX III gene; a PCR amplified fragment corresponding to nt 7640-8051 of mtDNA encoded COX II gene. Strand specific probes for the following were obtained by extension of the universal M13 primer according to the method of Sucov *et al.* (1987): mp9.M9 (mtDNA 1-739) which hybridizes to 12S rRNA; a Pst digest of OP-5 (mtDNA 8592-9020) which hybridizes to A8/A6. RNA hybridization signals were quantitated with Biorad phosphor-imager (Molecular Imager) and visualized by autoradiography.

Western Analysis. Equal amounts of total mitochondrial protein were separated by 10% tricine SDS-PAGE (Schagger and von Jagow, 1987) and transferred to nitrocellulose by electroblotting, following standard protocols (Towbin *et al.*, 1979) except with the addition of 0.1% SDS to the transfer buffer. Blots were incubated with 1 X PBS and 0.3% Tween to prevent non-specific binding and were incubated with the 1-1000 dilution of the appropriate antibody. The bound antibody was visualized by chemiluminescent detection (CDP Star Kit, Tropix). Mouse monoclonal antibodies against subunits I, II IV of COX were generously provided by R. Capaldi, University of Oregon.

Analysis Of Mitochondrial Protein Synthesis. For pulse labelings, 6-10 10 cm plates of exponentially growing cells were incubated for 60-120 minutes in methionine free DMEM containing 250-500μCi ^{35}S (> 1,000 Ci/mmol) labeled methionine, 5% dialyzed FBS, and 100μg emetine per ml, according to previously described methods (Chomyn, 1996b). A modified labeling protocol was used for the short pulse labelings. Cells were labeled for 15min., 1h or 2h in methionine free DMEM containing 500μCi ^{35}S (> 1,000 Ci/mmol) labeled methionine, 5% dialyzed FBS, and 100μg emetine per ml. Immediately after labeling, plates were washed with ice cold DMEM containing 2mM unlabeled methionine, and 100μg emetine per ml and five types of protease inhibitors; PMSF 174μg/ml, leupeptin 10μg/ml, pepstatin A 3.4μg/ml, E-64 3.6μg/ml, benzamidine HCl 56μg/ml. Cells were washed once with ice cold NKM buffer (0.005M KCl, 0.13M NaCl, 0.0075M $MgCl_2$), incubated in 1ml of special RSB buffer (0.01M Tris-Cl, pH 6.7, 0.01M KCl, 1.5 X 10^{-4}M $MgCl_2$) and homogenized; mitochondria were isolated by differential centrifugation.

For pulse-chase labelings, 8-15 10 cm plates of exponentially growing cells were labeled with 250-500μCi ^{35}S (> 1,000 Ci/mmol) methionine in the presence 100μg emetine per ml for 30-120 minutes. After labeling, cells were washed twice with DMEM containing 100μg emetine per ml and 2mM unlabeled methionine, washed once with DMEM containing 2mM unlabeled methionine and then incubated in DMEM containing 2mM unlabeled methionine and 5% dialyzed FBS at 37°C. At each chase time point, cells were lysed by addition of 1% SDS, 10mM Tris-HCl, pH 7.4 directly to the cells (1 10cm plate) or the cells were trypsinized for mitochondrial isolation (3-4 10cm plates). Some pulse and pulse-chase labelings were performed in the presence of 100 μg/ml of cycloheximide.

Labeled polypeptides from cell lysates or an isolated mitochondrial fraction were analyzed by 10% tricine SDS-PAGE (Schagger and von Jagow, 1987) subjected to fluorography (NEN Intensify, Dupont) on Kodax Biomax MS film or were analyzed with

Biorad phosphor-imager (Molecular Imager). The assignment of mtDNA-encoded translation products was based on similarity to those described by Chomyn and Lai (1990). Translation products were quantitated using Molecular Analyst software (Molecular Imager, Biorad). Each translation product was quantitated by drawing a box around the band and calculating the volume of the box (representing the mean pixel density multiplied by the area of the box), subtracting background (volume of a box of equal area) and then dividing by the volume of the A6 polypeptide (to compare levels of translation products in different cell lines).

Immunoprecipitations. Immunoprecipitations were performed as previously described by Mariottini *et al.* (1986). A mitochondrial fraction (90-150µg) isolated from pulse labeled or pulse-chase labeled cells was lysed in 0.5% Triton X-100 for 30 min. at 4°C in a total volume of 80µl. Samples were then diluted 5 fold with IB buffer (0.005M Tris-HCL, pH 6.7, 0.001M PMSF, 0.5% Triton X-100, 1mg/ml BSA and 0.002 M methionine) and pre-absorbed for 1h at 4°C with 125µl of 10%v/v zysorbin (formaldehyde fixed protein A positive *S. aureus*, Zymed Laboratories) which was resuspended in IB buffer. After centrifugation, supernatants were removed and incubated for 1.5-2h with 6-10µl of antiserum or normal rabbit serum at 4°C, under rotation. Samples were then incubated with 300-500µg of a mitochondrial fraction isolated from unlabeled cells and lysed in 0.5% Triton X-100 and 125 µl of 10% v/v zysorbin for 30 min.-2h at 4°C. Samples were centrifuged and pellets were washed once with IB buffer containing 0.1% SDS, once with IB buffer and once with 0.01M Tris-HCL, pH 6.7. The immuno-complexes were released by incubation at 37°C in 30 µl of sample buffer and analyzed by 10% tricine SDS-PAGE (Schagger and von Jagow, 1987) subjected to fluorography (NEN Intensify, Dupont) on Kodax Biomax MS film. Two controls was performed in all immunoprecipitations, one using normal rabbit serum and another using a protein A-negative, formaldehyde fixed *Staphylococcus aureus* strain (Wood 46, Zymed Laboratories). The rabbit polyclonal

antibodies against COX II have been generously provided by R. Doolittle, University of California.

RESULTS

Mutation Analysis. To confirm that no other mutation in mtDNA encoded COX genes was co-segregating with the 15 bp deletion, the genes for COX I, COX II and COX III were sequenced in their entirety. In addition, the genes for ATPase 6, ATPase 8, $tRNA^{Ser(UCN)}$, $tRNA^{Asp}$, $tRNA^{Lys}$, $tRNA^{Glu}$ were also analyzed (A total of 4150bp was sequenced). Four differences from the Cambridge sequence (Anderson *et al.*, 1981) were detected in mtDNA from the patient's muscle and are listed in Table 3.1. Two sequence changes were identified in the COX III gene, a G→C transversion at nt 9559 substituted a proline for an arginine, and has been reported to be a polymorphism which normally occurs in human populations (Monnab and Loeb, 1985). A T→C transition which does not change the amino acid identity was detected at nt 9836. A G→A transition was identified at nt 8387 within ATP synthetase subunit 8 (A8) changing a methionine to a valine and a T→C transition at nt 9205 in ATP synthetase subunit 6 (A6) changed the stop codon to a glutamine. Both of these changes were present in mtDNA from the patient and her unaffected mother. There was no evidence of heteroplasmy in patient mtDNA for either of these coding changes from sequencing analysis or from RFLP analysis of the T9205C mutation. These changes are presumably polymorphisms, not related to the disease phenotype. Further studies with transmitochondrial cell lines indicated that the A6 and A8 mutations did not affect the rate of ATP synthesis (see chapter 4).

Creation and Genetic Characterization Of Transmitochondrial Cell Lines. To investigate the consequences of the COX III mutation on COX function and structure, mitochondria from the patient with the COX III mutation were introduced into a human cell

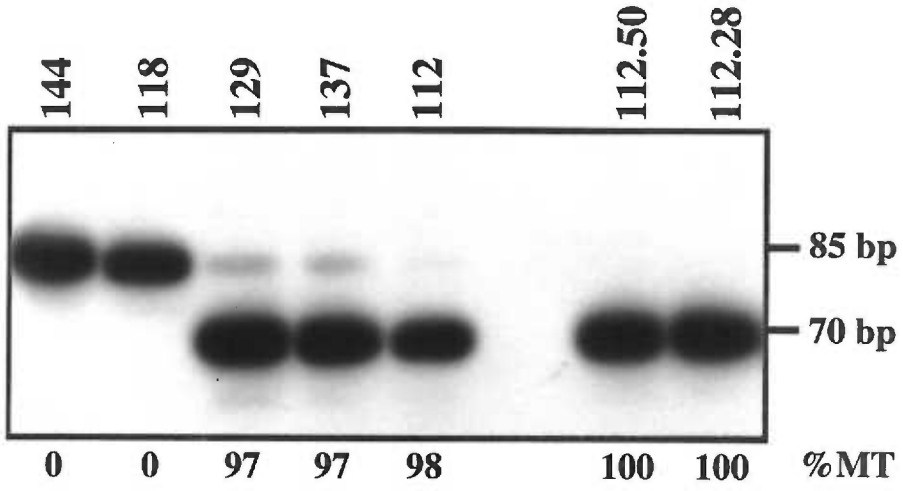
Table 3.1. MtDNA polymorphisms identified in patient C.S.

Gene	nt position	Coding change
A8	G8387A	Met 167 Val
A6	T9205C	Ochre 226 Gln
COX III	G9559C	Arg 118 Pro
		error in Cambridge sequence[1]
COX III	T9836C	no change

1. Monnab and Loeb, 1985.

line devoid of mtDNA. Cytoplasts derived from enucleated cultured myoblasts and from a platelet/leukocyte fraction were fused with an excess of cells from the ρ^0 cell line 143B206 (King and Attardi, 1989). Fusions were plated in selective medium, containing bromodeoxyuridine and lacking uridine, which supports the growth of only the transmitochondrial cell lines containing the mtDNA from the patient and the nuclear DNA from the ρ^0 cell line. Since ρ^0 cells are auxotrophic for pyrimidines, ρ^0 cells which had not fused with cytoplasts or platelets would not grow in this selective medium. Because ρ^0 cells are also thymidine kinase deficient, medium containing bromodeoxyuridine only permits the growth of ρ^0 cells fused with enucleated donor cells. Bromodeoxyuridine prevents the growth of nucleated donor cells which did not fuse with ρ^0 cells or intact cells that fused with ρ^0 cells. Individual clones were isolated from both myoblast and platelet fusions and were screened by PCR for the presence of the COX III mutation. PCR fragments containing either the wild-type mtDNA or mtDNA with the 15 bp deletion were differentiated by size after electrophoresis through 10% polyacrylamide gels (Fig. 3.1). Transmitochondrial cell lines containing high levels of the COX III mutation were obtained from the platelet/leukocyte fusions but not from the myoblast fusions. Of a total of 102 clones that were analyzed from the platelet/leukocyte fusions, 99 were homoplasmic for

Figure 3.1 Quantitation of mutated mtDNA in transmitochondrial cell lines. Transmitochondrial cell lines were created by fusing a platelet/leukocyte fraction from the patient with an excess of ρ^0 cells from cell line 143B206 (King and Attardi, 1989). A total of 102 clonal cell lines were analyzed; 99 cell lines were homoplasmic for wild-type mtDNA and three cell lines harbored 97% mutated mtDNA. Shown above are the two cell lines (cell lines 52.144 and 51.118) harboring 0% mutation and the three cell lines harboring 97% mutated mtDNA (cell lines 51.129, 51.112, 52.139) which were used in subsequent biochemical and molecular studies. Cell lines homoplasmic for the COX III mutation were created by manipulating levels of the mutation in two 97% mutant cell lines (51.129, 51.112) by growth in 50ng/ml of ethidium bromide. Cell lines were grown in ethidium bromide for 14 cell generations to reduce the mtDNA content to approximately one molecule per cell. Ethidium bromide was then removed to allow the rapid repopulation of mtDNA. Cells were plated at low density and a total of 62 clonal cell lines were analyzed. Two viable cell lines homoplasmic for the mutation were obtained (cell lines 51.112.50, 51.112.28). Clonal cell lines were screened for the presence of the COX III mutation by PCR. The wild-type mtDNA is amplified as an 85bp PCR product while the PCR product with the COX III deletion is 70 bp in size. PCR amplified fragments were electrophoresed through 10% polyacrylamide gels and quantitated by phosphorimaging. %MT= % mutation.


144
118
129
137
112
112.50
112.28
85 bp
70 bp
0
0
97
97
98
100
100
%MT

wild-type mtDNA and three contained approximately 96-98% of mutated mtDNA. The sharp dichotomy between mutation levels in cell clones suggested that the majority of platelets were homoplasmic wild-type with a few cells containing high levels of mutation. This is entirely consistent with the level of mutation determined for the patient's leukocytes (1%) (Keightley *et al.*, 1996). All clonal cell lines derived from fusions with muscle satellite cells (78 total) were homoplasmic for wild-type mtDNA. This result was surprising since skeletal muscle contained high levels of mutated mtDNA (93%). It is possible that myoblasts containing high levels of mutation were selected against during culture because of a growth disadvantage. However, when pools of uncloned myoblasts and 30 individual myoblast clones were screened for the mutation after 3-4 weeks in culture, the mutation was not detected in any clones or in the pooled myoblasts (with a sensitivity of < 1%). These results suggested that muscle satellite cells from the patient may contain much lower levels of mutation compared to differentiated skeletal muscle.

To obtain cell lines homoplasmic for the COX III deletion, the ratio of wild-type to mutated mtDNA was manipulated using ethidium bromide (King, 1996). MtDNA replication is inhibited by low concentrations of ethidium bromide. When cells are grown in the presence of ethidium bromide, mtDNA is progressively diluted with each cell division, but the remaining mtDNA rapidly repopulates cells when the drug is removed. Stability of mtDNA, nuclear replication, and cell division are not affected by ethidium bromide (King, 1996). Two cell lines containing high levels of the mutation (approximately 96-98%; 51.112 and 51.129) were grown in the presence of ethidium bromide until mtDNA levels were reduced to approximately one molecule per cell (14 cell generations). Ethidium bromide was then removed to allow the rapid repopulation of mtDNA within cells. A total of 62 individual colonies were picked. To avoid selection against clonal cell lines with a severe respiratory chain deficiency, approximately half of the clonal cell lines were grown in non-selective medium conditions with uridine. Five clonal cell lines homoplasmic for the mutation were identified by PCR, but only two could be

maintained in culture. When growth properties of these two homoplasmic mutant cell lines (51.112.50 and 51.112.28) were examined, both uridine and pyruvate supplementation were required for growth, a growth phenotype similar to human cell lines lacking mtDNA. The other three homoplasmic mutant cell lines that could not be maintained in culture were clonal cell lines that were initially grown in medium without uridine.

The cell lines chosen for further studies included the two cell lines homoplasmic for the COX III mutation (51.112.50 and 51.112.28), two cell lines with high levels of mutation (ranging between 96-98% mutated mtDNA; 51.112 and 51.129), and two cell lines homoplasmic for the patient's wild-type mtDNA (52.144 and 51.118), Fig. 3.1. In this chapter, cell lines homoplasmic for the mutation will be referred to as the 100% mutant cell lines and the cell lines with mutation levels ranging between 96-98% will be referred to as the 97% mutant cell lines. Mutation levels in all cell lines were periodically checked by PCR and were stable over a period of several months

Mutant Transmitochondrial Cell Lines Exhibit a Severe Respiratory Chain Defect. The capacity of the entire respiratory chain in transmitochondrial cell lines was determined by measuring the rates of oxygen consumption of intact cells (Fig. 3.2). The rates of oxygen consumption were severely reduced in 100% mutant cell lines (mean = 0.21 $\pm$ 0.02 [1SE] fmol per cell per min.) compared to wild-type cell lines (mean = 5.5 $\pm$ 0.26 fmol per cell per min.), approximately a 95% reduction in oxygen consumption. The rate of oxygen consumption observed for 100% mutant cell lines was similar to the rate determined for ρ^0 cell line 143B206 cells (data not shown). Rates of oxygen consumption in 97% mutant cell lines were 20% of wild-type (mean = 1.1 $\pm$ 0.12 fmol per cell per min).

A severe deficiency of COX activity was observed in all mutant cell lines (Fig. 3.2). COX activity was not detectable in 100% mutant cell lines. In 97% mutant cell lines, activities were decreased by approximately 94% (mean = 0.62 $\pm$ 0.069 k/min/mg)

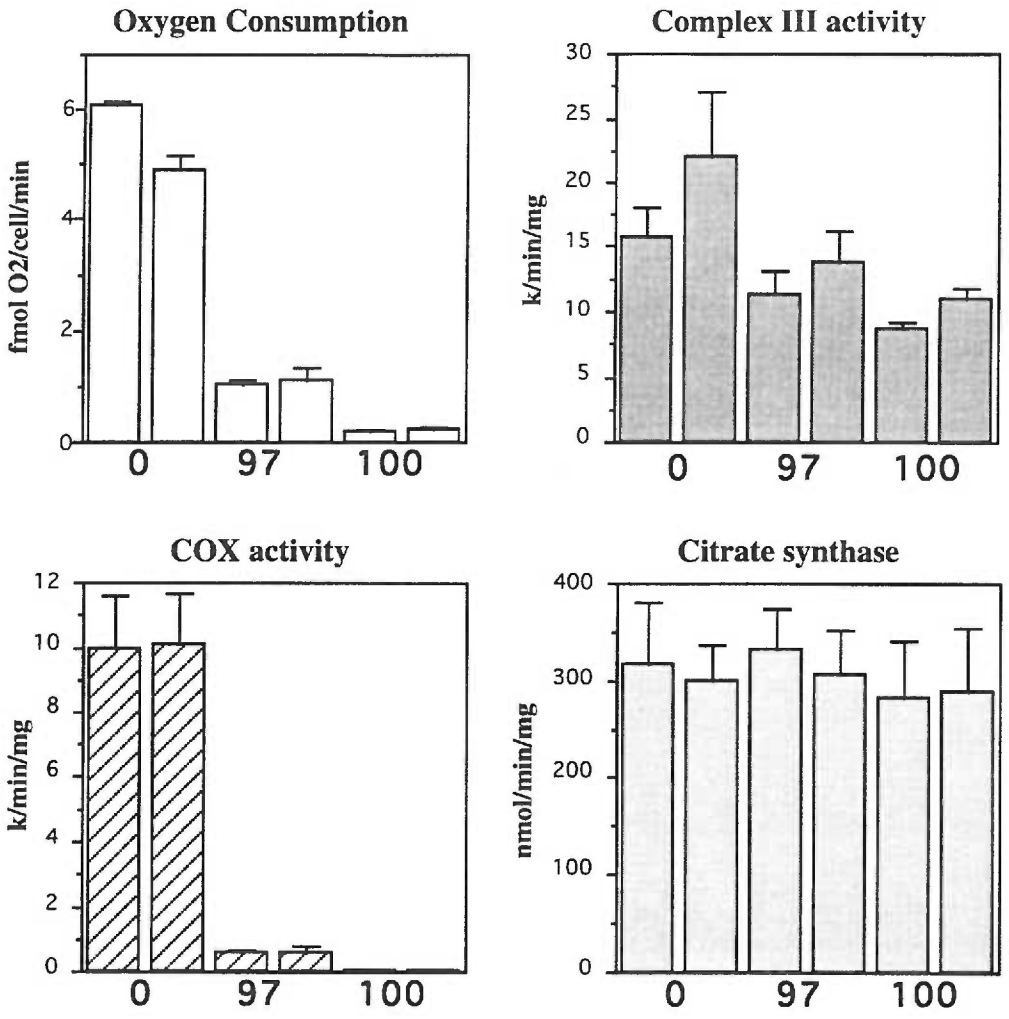


Figure 3.2. Biochemical characterization of transmitochondrial cell lines harboring 0%, 97% and 100% COX III mutation. Biochemical measurements were performed on two wild-type cell lines (52.144, 5.118), two 97% mutant cell lines (51.129, 51.112) and two 100% mutant cell lines (51.112.50, 51.112.28). The rate of oxygen consumption of intact cells, a measure of the entire respiratory chain, is shown in the upper left panel. Activities of two respiratory chain enzymes, COX and Complex III, and a citric acid cycle enzyme, citrate synthase, were determined by spectrophotometric methods in isolated mitochondria. The activities of COX and Complex III are expressed as apparent first order rate constants. Error bars represent 1 standard error.

compared to wild-type cell lines (mean = 10.0 ± 1.1 k/min/mg). Activities of citrate synthase, an enzyme in the mitochondrial matrix, were similar among mutant and wild-type cell lines, indicating there were no significant differences in mitochondrial content among cell lines. Activities of complex III, the enzyme in the respiratory chain responsible for the reduction of cytochrome *c*, were reduced 50% in 100% mutant cell lines, (mean = 9.9 ± 0.75 k/min/mg) and reduced 32% in 97% mutant cell lines (mean = 12.6 ± 1.4 k/min/mg) compared to wild-type (mean =18.9 ± 2.9 k/min/mg). The lower complex III activities in mutant cell lines, particularly the 100% mutant cell lines, suggested that a severe COX defect may affect the stability or activity of other enzymes in the respiratory chain.

ATP synthesis was examined in cell line 143B, wild-type cell line 52.144 and the 100% mutant cell line 51.112.50 (Fig. 3.3). The ability of isolated mitochondria to synthesize ATP during oxidative phosphorylation was assayed using succinate as a substrate. ATP synthesis was assayed indirectly by measuring the incorporation of ^{32}P into glucose-6-phosphate following phosphorylation of ADP by inorganic phosphate (Tuena de Gomez-Puyou *et al.*, 1984). The rate of ATP synthesis in the 100% mutant cell line (7.84 ± 1.10 [1 SE] nmol ATP/hr/mg protein) was approximately 0.1% the rate of ATP synthesis in the wild-type cell line (7060 ± 660 nmoles ATP/hr/mg protein) and the rate in 143B (5110 ± 292 [1 SE] nmol ATP/hr/mg protein).

RNA Analysis. Northern blot analysis was performed to determine if steady-state levels of COX III mRNA transcripts in mutant cell lines were altered by the 15 bp deletion. Total RNA was isolated from exponentially growing mutant and wild-type cells and examined by northern analysis (Fig. 3.4). The RNA was hybridized with five probes which identify five mtDNA-encoded genes (COX III, A6, A8, COX II and 12s rRNA) and one nuclear encoded gene (β-actin). Two wild-type cell lines (52.144 and 51.118) and two 100% mutant cell lines (51.112.50 and 51.112.28) were analyzed. RNA from 143B, transmitochondrial cell line AT153 and ρ^0 cell line 143B/206 also were included in these

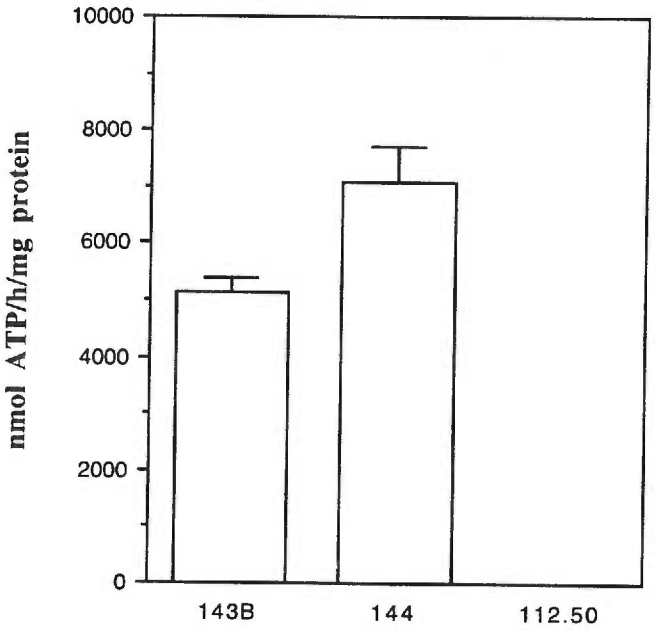
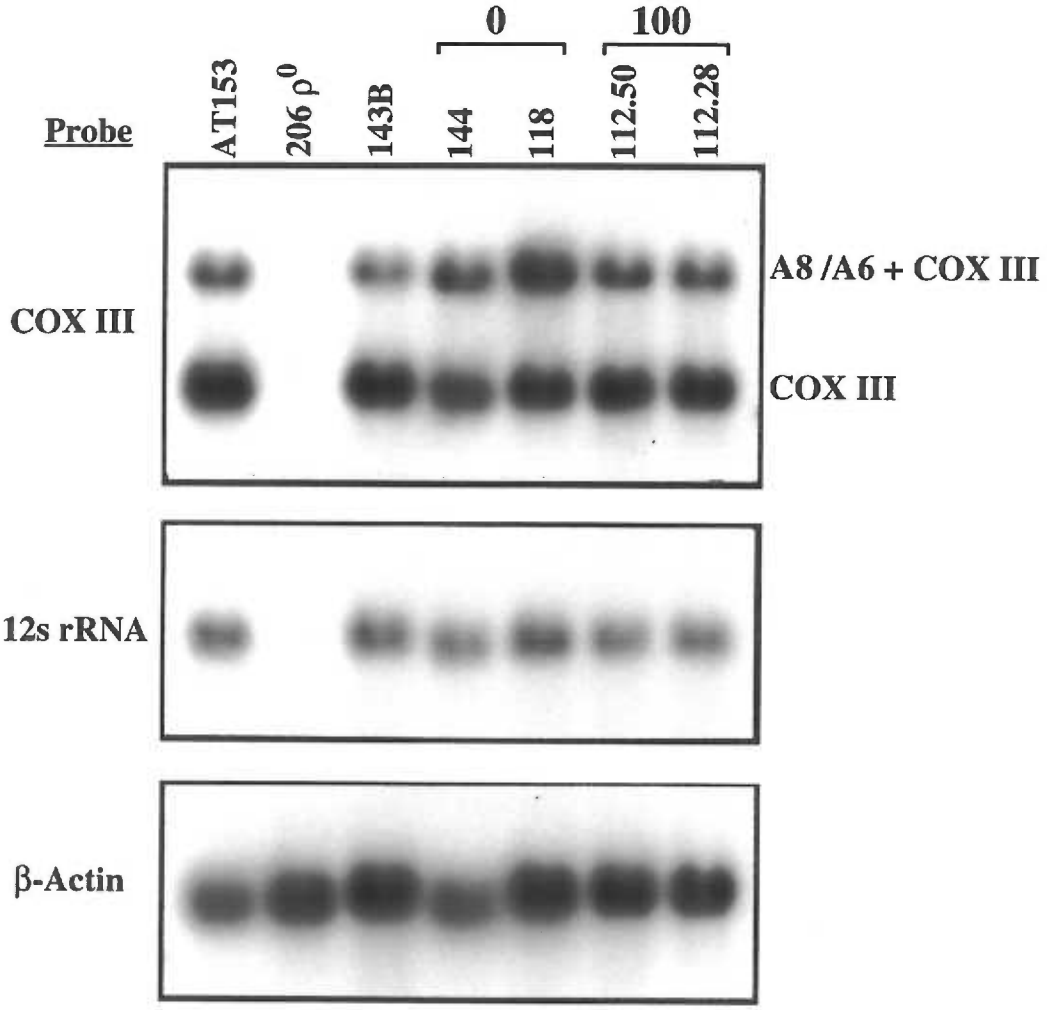


Figure 3.3. ATP synthesis in transmitochondrial cell lines. ATP synthesis was measured in wild-type cell line 143B (parental cell line of 143B206), wild-type transmitochondrial cell line 51.144 (0% mutation) and 100% mutant cell line 51.112.50. The rate of ATP synthesis was determined indirectly in isolated mitochondria by measuring the incorporation of P^{32} into glucose-6-phosphate following phosphorylation of ADP by inorganic phosphate. Error bars represent 1 standard error.

Figure 3.4. Northern Analysis. Total RNA was isolated from wild-type (0% mutation) and 100% mutant cell lines and two other wild-type cell lines, AT153 and 143B. The probes used are indicated on the left of the autoradiogram. The mRNA species representing the partially processed A8/A6 + COX III transcript and the fully processed COX III transcript are indicated on the right.


0
100
Probe
AT153
206 ρ0
143B
144
118
112.50
112.28
COX III
A8 /A6 + COX III
COX III
12s rRNA
β-Actin

analyses. Cell line 143B is the parental cell line of ρ^0 cell line 143B206. AT153 is a transmitochondrial derivative of the ρ^0 cell line 143B206 and has been shown by previous work to have a rate of O_2 consumption similar to 143B.

The COX III probe detected a band of approximately 1 kb, representing the COX III transcript, and a higher molecular weight band of 1.7 kb in both mutant and wild-type cell lines. The size of the higher molecular weight band was consistent with a partially processed mRNA species encompassing A8/A6 + COX III genes. Hybridization with an A8/A6 probe confirmed the identity of this 1.7 kb band and in addition, detected the fully processed 840 bp A8/A6 transcript (data not shown). The 1.7 kb mRNA species was not detected by other probes. The COX II probe detected a single band of 1 kb, the COX II mRNA transcript, in wild-type and mutant cell lines (data not shown). The 12S rRNA probe also identified a single band of 1.2 kb representing the 12S rRNA in wild-type and mutant cell lines. No probes specific for mtDNA regions detected any bands in ρ^0 cell line 143B/206.

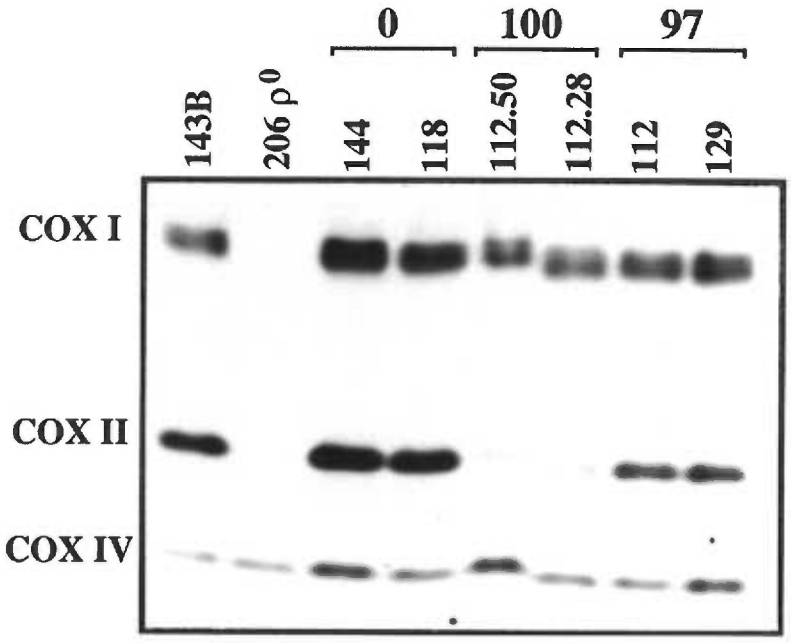
Hybridization signals were quantitated using phosphor-imaging analysis and the relative steady-state levels of mitochondrial transcripts in each cell line were normalized to nuclear encoded β-actin and 12S rRNA. The ratios are relative levels and do not reflect the absolute numbers of each species. The relative levels of COX III mRNA, the sum of processed and partially processed mRNA species normalized to β-actin in the 100% mutant cell lines (mean = 0.15 $\pm$ 0.01 [1SD]) were similar to wild-type cell lines, 52.144 and 51.118 (mean = 0.18 $\pm$ 0.02), and wild-type cell lines, 143B and AT153 (mean = 0.16 $\pm$ 0.08). The relative levels of COX II mRNA normalized to β–actin were not significantly different between 100% mutant cell lines (mean = 0.63 $\pm$ 0.03 [1SD]), and wild-type cell lines 52.144, 51.118, and AT153 (143B could not be quantitated) [mean = 0.612 $\pm$ 0.11]. These results indicated that the relative amount of COX II and COX III mRNA species was unchanged in mutant cells compared to wild-type. When the ratios of COX III mRNA, the sum of processed and partially processed mRNA species, normalized to 12S rRNA were

examined, no differences were observed in relative levels among 100% mutant cell lines (mean = 0.90 ± 0.57 [1SD]), wild-type cell lines, 52.144 and 51.118 (mean = 0.81 ± 0.45) and wild-type cell lines, 143B and AT153 (mean = 0.84 ± 0.16). The relative levels of COX II mRNA normalized to 12S rRNA in 100% mutant cell lines were similar to those of wild-type cell lines.

Steady-State Levels Of COX Polypeptides. Steady-state levels of mtDNA encoded COX I and COX II polypeptides and nuclear DNA encoded COX IV were investigated by western blotting analysis of mitochondria isolated from two wild-type cell lines (52.144 and 51.118), two 100% mutant cell lines (51.112.50 and 51.112.28) and two 97% mutant cell lines (51.129, 51.112). Mitochondria isolated from cell line 143B and ρ^0 cell line 143B206 were included in these analyses. The steady-state levels of COX III polypeptide could not be directly determined because an antiserum specific for human COX III was not available. As shown in Fig. 3.5, the steady-state levels of both COX I and COX II polypeptides were reduced in mutant cell lines compared to wild-type cell lines and 143B. A greater reduction in the steady-state levels of COX II was observed in the 100% mutant cell lines. The lower levels of mtDNA encoded COX I and COX II polypeptides may reflect a decreased stability of these subunits in mutant cell lines. Steady-state levels of nuclear encoded subunit COX IV in mutant cell lines were similar to the wild-type cell line 143B and ρ^0 cell line 143B/206.

Translation And Stability Of COX III Polypeptide. To examine mitochondrial translation, cells were labeled with [^{35}S]-methionine in the presence of a cytoplasmic protein synthesis inhibitor such as emetine or cycloheximide. Mitochondrial translation products were analyzed by electrophoresis of cell lysates or isolated mitochondria through tricine SDS-PAGE. Although emetine and cycloheximide are the most common cytoplasmic protein synthesis inhibitors for this type of analysis, both have drawbacks

Figure 3.5. Western Analysis. Immunoblot of the steady-state levels of mtDNA encoded COX I and COX II polypeptides and nuclear DNA-encoded COX IV in two wild-type cell lines (0% mutation), two 97% mutant cell lines and two 100% mutant cell lines. Immunoblotting was performed according to standard techniques. Bound monoclonal antibodies were visualized with alkaline phosphatase conjugated goat anti-mouse IgG, followed by incubation with CDP star substrate [Tropix] for chemiluminescent detection.


0
100
97
143B
206 ρ0
144
118
112.50
112.28
112
129
COX I
COX II
COX IV

when examining mitochondrial protein synthesis. Emetine has been shown to be an irreversible inhibitor of cytoplasmic protein synthesis and results in cell death in HeLa cell cultures (Grollman, 1966; Costantino and Attardi, 1977). An alternative to emetine, cycloheximide has been reported to be a reversible, but incomplete inhibitor of cytoplasmic protein synthesis with no effect on cell growth (Grollman, 1966; Costantino and Attardi, 1977). The growth properties of wild-type and 100% mutant cell line were examined after a one hour exposure to these drugs. Similar results were obtained for both cell lines; representative growth curves are shown in Fig. 3.6. Both emetine and cycloheximide had adverse effects on cell growth. Limited cell growth occurred after exposure to emetine. Exposure to cycloheximide resulted in slower rates of growth compared to untreated cells with less than two population doublings over a 120h period compared to approximately 6 population doublings in untreated cells over the same period.

Cell lines were labeled in the presence of emetine or cycloheximide to compare the efficiency of labeling in the presence of either drug and the level of inhibition of cytoplasmic protein synthesis. Levels of [^{35}S]-methionine incorporation into mitochondrial translation products were higher in cells labeled in the presence of cycloheximide compared to emetine, but levels of cytoplasmic protein synthesis also were higher in cycloheximide labelings which created difficulties for quantitation of labeled mtDNA encoded translation products. Incomplete inhibition of cytoplasmic protein synthesis by cycloheximide was shown by multiple bands of varying molecular weights in the electrophoresed extracts of the 143B206 cells (ρ^0 cell line). These translation products were not present in emetine labelings (data not shown). Based on these results, emetine was the cytoplasmic protein synthesis inhibitor routinely used in labeling experiments for analysis of the COX III mutation.

Mitochondrial protein synthesis was examined in wild-type and mutant cell lines by labeling cells with [^{35}S]-methionine in the presence of emetine for a period of 1.5 - 2h (pulse). Translation products were analyzed by tricine SDS-PAGE of isolated

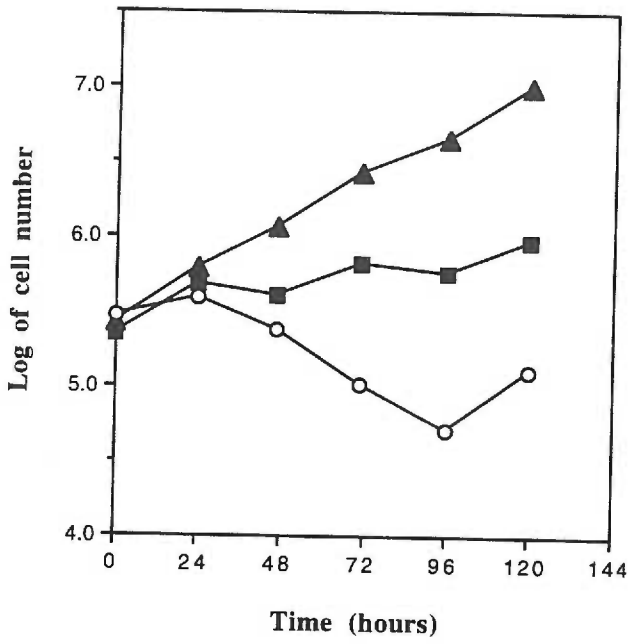


Figure 3.6 Growth properties of transmitochondrial cells after exposure to emetine and cycloheximide. The growth curves of transmitochondrial cell line 51.144 were determined by plating multiple 10cm petri dishes with a constant number of cells. Cells were incubated for 30 min. in either DMEM containing 100ug/ml emetine (open circles), DMEM containing 100ug/ml cycloheximide (black squares) or DMEM alone (black triangles). After treatment, cells were washed twice with DMEM and incubated at 37^{O}C. At various time intervals, individual plates were trypsinized and counted.

mitochondria. The COX III polypeptide was synthesized in mutant cell lines containing either 97% or 100% of the mutation but displayed a slightly greater electrophoretic mobility than that of wild-type cell lines (Fig. 3.7 and 3.8, pulse lanes). To compare relative levels [^{35}S]-methionine incorporation into translated polypeptides in different cell lines, levels were normalized to mtDNA-encoded A6. The A6 polypeptide is an abundant polypeptide which is fairly stable, well resolved by tricine SDS-PAGE and has relatively high levels of [^{35}S]-methionine incorporation. When the levels of labeled A6 polypeptide were examined in wild-type and mutant cell lines, levels of [^{35}S]-methionine incorporation were similar in all cell lines after a one hour labeling (ratio of levels in mutant cell lines/ levels in wild-type = 1.3). The stability of the labeled A6 polypeptide also was examined by labeling the cells for 1-2h and then "chasing" cells in emetine-free medium containing an excess of unlabeled methionine. Levels of labeled A6 polypeptides remaining after a 17h chase period were similar to initial incorporation levels in both wild-type and mutant cell lines (Fig. 3.7 and 3.8; ratio of levels in mutant cell lines/ levels in wild-type = 1.2), confirming that A6 polypeptide is a stable polypeptide with a low rate of turnover, and would be appropriate for normalizing levels of labeled mitochondrial translation products for comparison of different cell lines. The levels of [^{35}S]-methionine incorporation in two other mitochondrial translations products, Cyt *b*, a component of complex III, and ND2, a subunit of complex I were similar in wild-type and mutant cell lines after a 1h-2h labeling. The stability of these polypeptides was examined by pulse-chase experiments and levels of labeled polypeptides remaining after a 15h chase were found to be similar in both wild-type and mutant cell lines.

In contrast, levels of [^{35}S]-methionine incorporation into COX polypeptides after a 1.5 - 2h labeling were reduced by 25-50% in 100% mutant cell lines as compared to wild-type cell lines (Fig. 3.8, pulse lanes). A smaller reduction in levels of labeled COX polypeptides (12%-34%) was observed in the 97% mutant cell line (Fig. 3.7, pulse lanes). The lower levels of [^{35}S]-methionine incorporation into COX translation products in

Figure 3.7. Analysis of mitochondrial translation products in wild-type and 97% mutant cell lines. Fluorogram of mitochondrial translation products from pulse and pulse-chase labelings after electrophoresis through 10% tricine SDS-PAGE (Schagger and von Jagow, 1987). Wild-type (52.144) and 97% mutant cell lines (51.129) were labeled for 1.5h in the presence of 250-500μCi [^{35}S]-methionine and 100μg/ml emetine. After labeling, cells were either harvested for mitochondrial isolation (pulse) or incubated with an excess of unlabeled methionine at 37^{o}C for 17h, prior to mitochondrial isolation. The same amount of mitochondrial fraction (50μg of protein) was loaded in each lane of the gel.

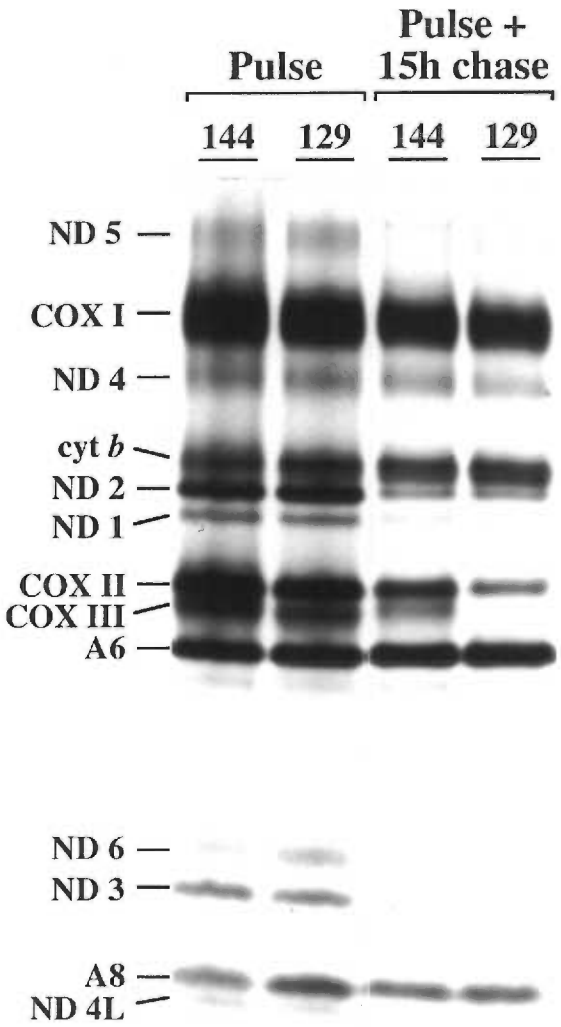
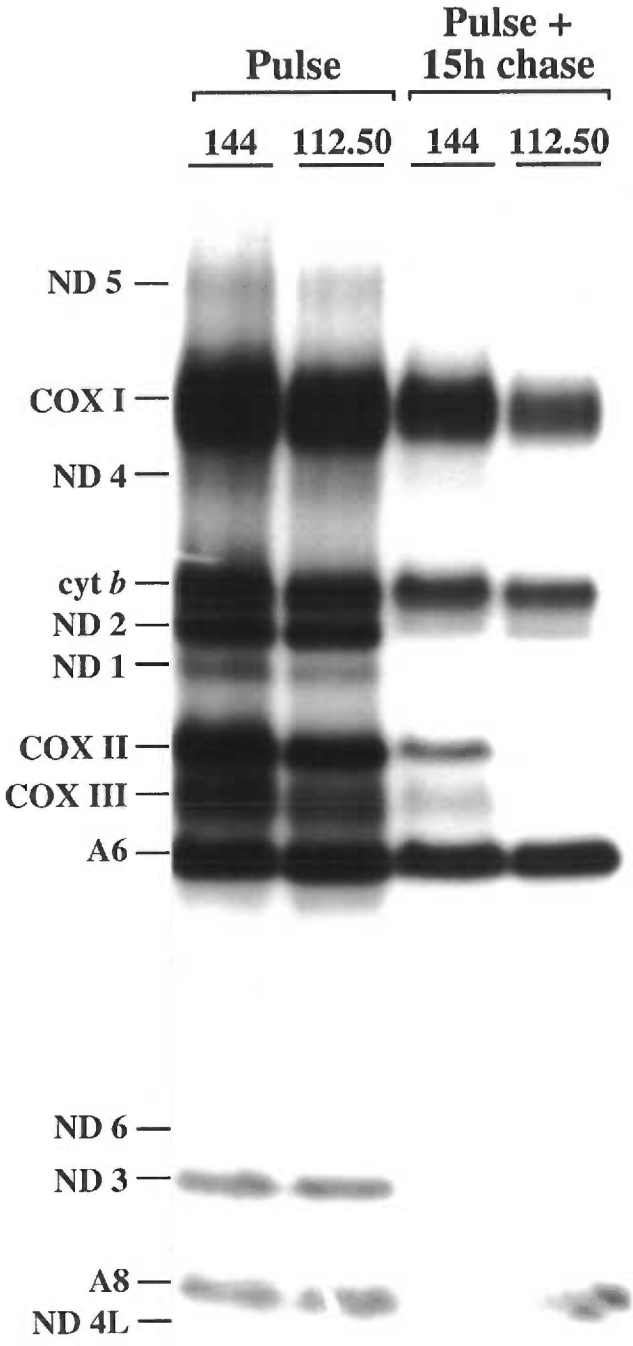

Pulse
Pulse +
15h chase
144
129
144
129
ND 5
COX I
ND 4
cyt b
ND 2
ND 1
COX II
COX III
A6
ND 6
ND 3
A8
ND 4L

Figure 3.8. Analysis of mitochondrial translation products in wild-type and 100% mutant cell lines. Fluorogram of mitochondrial translation products from pulse and pulse-chase labelings after electrophoresis through 10% tricine SDS-PAGE (Schagger and von Jagow, 1987). Wild-type (52.144) and 100% mutant cell lines (51.112.50) were labeled for 1.5h in the presence of 250-500μCi [^{35}S]-methionine and 100μg/ml emetine. After labeling, cells were either harvested for mitochondrial isolation (pulse) or incubated with an excess of unlabeled methionine at 37^{o}C for 17h, prior to mitochondrial isolation. The same amount of mitochondrial fraction (50μg of protein) was loaded in each lane of the gel.


Pulse
Pulse +
15h chase
144
112.50
144
112.50
ND 5
COX I
ND 4
cyt b
ND 2
ND 1
COX II
COX III
A6
ND 6
ND 3
A8
ND 4L

mutant cell lines may indicate reduced rates of synthesis of COX polypeptides. Alternatively, translated COX polypeptides in mutant cell lines may be unstable and degraded soon after translation resulting in lower levels even after a two hour pulse. To address these possibilities, both the rates of translation and the metabolic stability of COX polypeptides in mutant cell lines were examined.

To determine whether rates of translation were lower in mutant cell lines, levels of translated polypeptides in wild-type cell line (52.144) and 100% mutant cell line (51.112.50) were examined after short periods of labeling (15 min, 1h and 2h). At least two independent labelings were performed for each pulse time point. The results of these experiments are shown in Fig. 3.9 where the levels of [^{35}S]-methionine incorporation into translation products in the 100% mutant cell line are normalized to levels of the A6 polypeptide and expressed as a percentage of wild-type levels, for each pulse time. After a 15 min labeling, COX I, II and III polypeptides in the 100% mutant cell line had levels of [^{35}S]-methionine incorporation similar to wild-type, indicating comparable rates of synthesis of COX polypeptides in both mutant and wild-type cell lines. Reductions in the levels of [^{35}S]-methionine incorporation in COX II and COX III polypeptides were observed in the 100% mutant cell line compared to wild-type levels, only after the one and two hour labelings (56 - 62% of wild-type levels after a two hour pulse). Levels of [^{35}S]-methionine incorporation in COX I polypeptide were reduced, but to a lesser extent, in the 100% mutant cell line relative to wild-type levels after a two hour pulse (82% of wild-type levels). Levels of [^{35}S]-methionine incorporation into cyt*b*, ND 2 and ND 3 polypeptides in the 100% mutant cell line were similar to wild-type after a 15 min labeling (90-105% of wild-type levels), but lower levels were observed after the one and two hour labeling (80-94% of wild-type levels), Fig. 3.9b.

The stability of mtDNA-encoded COX polypeptides in wild-type cell line (52.144), 100% mutant cell line (51.112.50) and 97% mutant cell line (51.129) was investigated by pulse-chase labeling experiments. Cells were labeled with [^{35}S]-methionine for 1.5 - 2h in

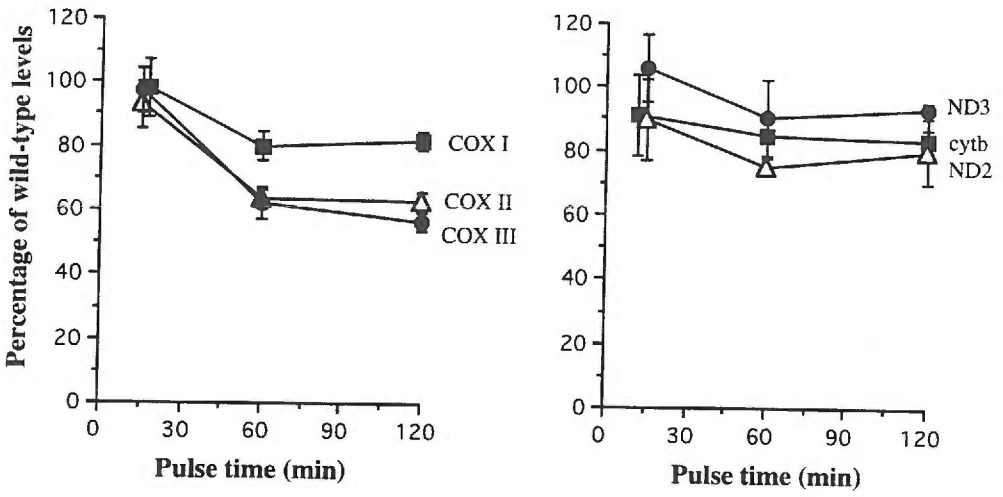


Figure 3.9. Analysis of mitochondrial translation products in the 100% mutant cell line after short pulse labelings. The levels of [^{35}S]-methionine incorporation in mtDNA-encoded COX polypeptides (**a**) and cyt b, ND 3 and ND 2 (**b**) in the 100% mutant cell line (cell line 51.112.50) after 15min, 1h and 2h pulse labelings. The levels [^{35}S]-methionine incorporation for each translation product in the 100% mutant cell line were normalized to A6 levels and expressed as a percentage of [^{35}S]-methionine incorporation levels in the wild-type cell line. At least two independent labelings were performed for each pulse time point; error bars represent 1 standard error. A reduction in levels of [^{35}S]-methionine incorporation in COX II and COX III polypeptides was observed in the 100% mutant cell line after 1h and 2h pulse labelings (a). Wild-type and 100% mutant cell lines were labeled for 15min, 1h and 2h in the presence of 500 μCi [^{35}S]-methionine and 100μg/ml emetine. Equal amounts of protein were electrophoresed through 10% tricine SDS-PAGE (Schagger and von Jagow, 1987), and translation products were quantitated by phosphorimaging.

the presence of emetine (pulse), and then incubated in emetine free medium with an excess of unlabeled methionine at 37°C for an additional 17h (pulse-chase). As shown in Fig. 3.7 and 3.8, the [^{35}S]-methionine-labeled COX III polypeptide was barely detectable in mutant cell lines after a 17h chase. Electrophoretic profiles of one representative pulse-chase labeling from wild-type and 100% mutant cell lines are shown in Fig. 3.10. The peak corresponding to the COX III polypeptide was absent from the electrophoretic profile of mutant cell lines. These data indicated that the stability of the COX III polypeptide in mutant cell lines was decreased compared to wild-type. Levels of [^{35}S]-methionine labeled COX I and II polypeptides remaining after a 17h chase also were lower in mutant cell lines compared to the wild-type cell line. This was in contrast to the levels of ND2, cyt *b* and A6 polypeptides which were similiar in mutant and wild-type cell lines after a 17h chase.

The kinetics of turnover of COX polypeptides was examined further in the wild-type (52.144) and 100% mutant cell line (51.112.50) by pulse-chase experiments with chase time points ranging between 1-22h. Two types of experiments were performed: cells were labeled either for 30 min. in the presence of emetine and chased over a time period of 1-8h in emetine-free medium containing an excess of unlabeled methionine or cells were labeled for 2h and chased over a time period of 2-22h. Labeled polypeptides were analyzed by tricine-SDS PAGE of isolated mitochondria or whole cell lysates and quantitated by phosphor-imaging analysis. Results from both chase experiments are shown in Fig. 3.11. Levels of labeled polypeptides remaining after each chase in wild-type and 100% mutant cell lines were expressed as a percentage of the initial [^{35}S]-methionine incorporation levels in wild-type cell line, relative to time. After a 30 min. or a 2h labeling period, COX II and COX III polypeptides in the 100% mutant cell line had lower levels of [^{35}S]-methionine incorporation (54 - 70% levels of wild-type) (Fig. 3.11b, 3.11d, time 0 on the graph). Since previous experiments with 15min. labeling periods (Fig. 3.9) had shown that wild-type and mutant cell lines had similar rates of protein synthesis, the lower levels of [^{35}S]-methionine incorporation observed during longer labeling periods are

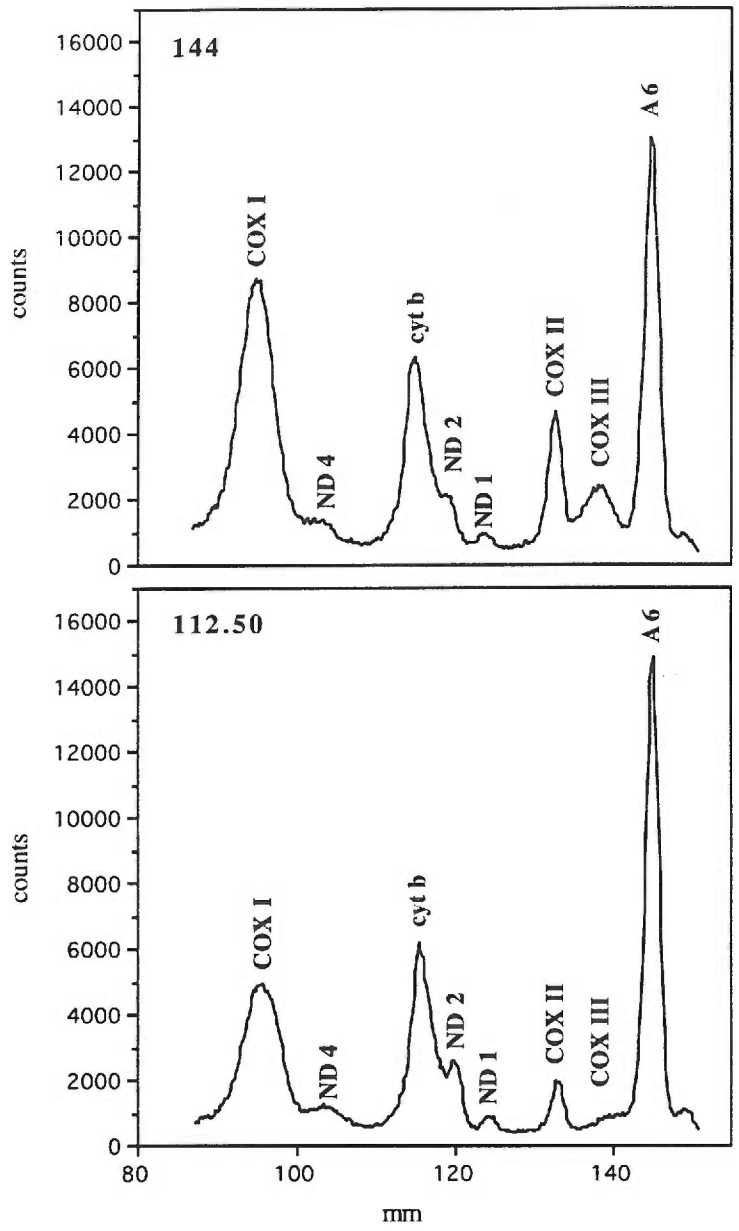
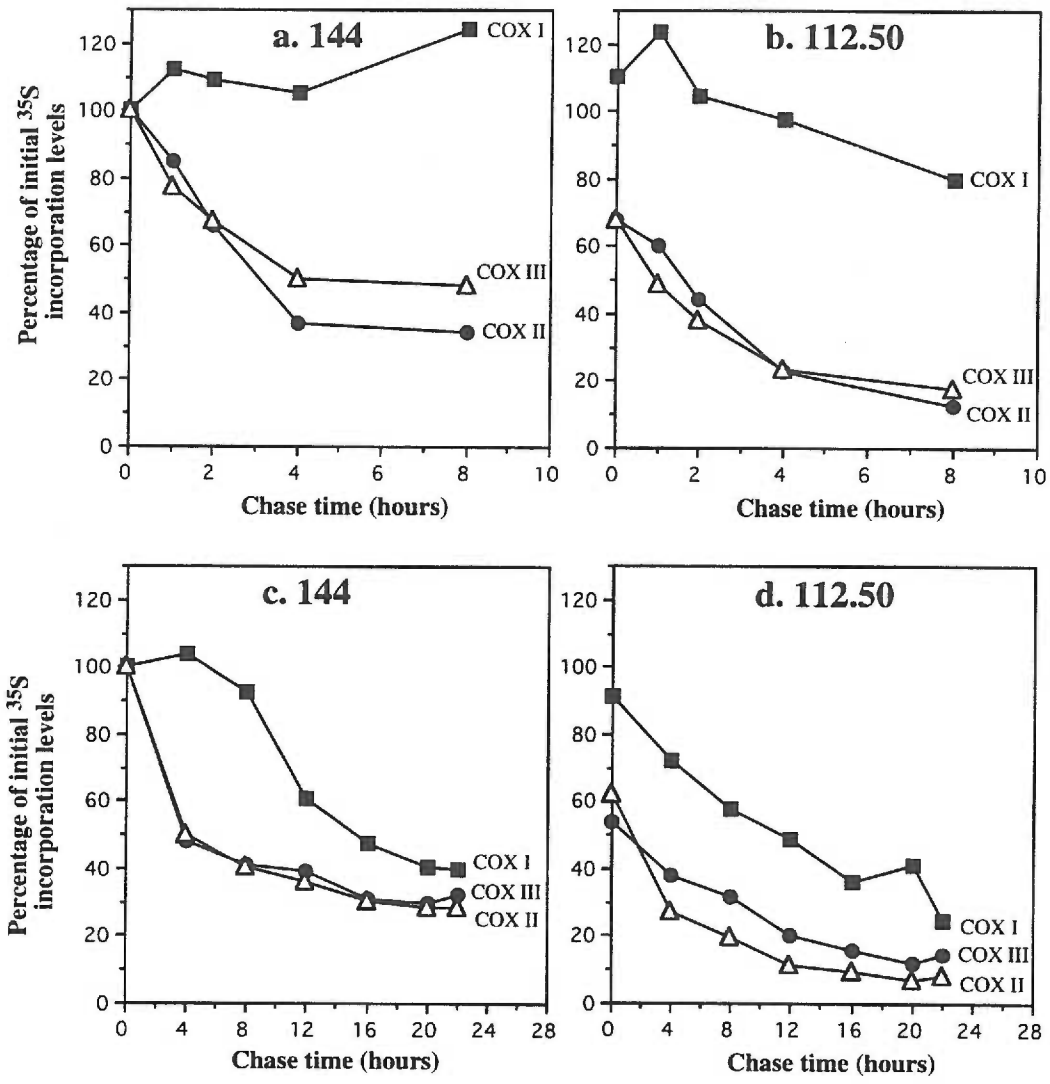


Figure 3.10. Electrophoretic profiles of mitochondrial translation products after pulse-chase labeling. Profiles of the pulse-chase labelings of the fluorogram shown in figure 3.8. The peak corresponding to the COX III polypeptide in the wild-type cell line (**a**) is absent in the profile of the 100% mutant cell line (**b**)

Figure 3.11. Turnover of mtDNA encoded COX polypeptides in wild-type and 100% mutant cell lines. Wild-type cell line 52.144 (**a**) and 100% mutant cell line 51.112.50 (**b**) were labeled with [^{35}S]-methionine in the presence of emetine for 30min., and then incubated in DMEM containing unlabeled methionine at 37^{o}C for 0-8h chase periods. After each chase period, cells were harvested for mitochondrial isolation and equal amounts of protein were electrophoresed through 10% tricine SDS-PAGE (Schagger and von Jagow, 1987) and translation products were quantitated by phosphor-imaging. Levels of labeled polypeptides remaining after each chase in both cell lines were expressed as a percentage of the initial [^{35}S]-methionine incorporation levels in the wild-type cell line. Wild-type cell line 52.144 (**c**) and 100% mutant cell line 51.112.50 (**d**) were labeled with [^{35}S]-methionine in the presence of emetine for 2h, and then incubated in DMEM containing unlabeled methionine at 37^{o}C for 0-22h chase periods. After each chase period, cells were lysed by the addition of 1% SDS and 10mM, Tris-HCl pH 7.4 directly to cells on petri plates. Electrophoresis and quantitation of labeled polypeptides were carried out as described above. Levels of labeled polypeptides remaining after each chase in both cell lines were expressed as a percentage of the initial [^{35}S]-methionine incorporation levels in the wild-type cell line


a. 144
b. 112.50
c. 144
d. 112.50
COX I
COX III
COX II
Percentage of initial 35S incorporation levels
Chase time (hours)
0
20
40
60
80
100
120
0
2
4
6
8
10
12
16
20
24
28

indicative of higher rates of turnover of the COX II and COX III polypeptides in the 100% mutant cell line.

The turnover of the COX II and COX III polypeptides during the chase period in both wild-type and 100% mutant cell lines was characterized by an initial rapid decrease in levels of labeled polypeptide during the first 4h of the chase period. As shown by the 1-8h chase experiment (fig 3.11a and b), levels of labeled COX II and COX III polypeptides were 50-60% lower in the wild-type cell lines and 80% lower in mutant cell lines after a 4h chase compared to the initial incorporation levels in the wild-type cell line. The wild-type cell line exhibited a lower rate of decrease in levels of labeled COX II and COX III polypeptides during 4-16h of the chase. No further degradation of COX II and COX III polypeptides were observed between 16-22h (Fig. 3.11a and c). A more rapid decrease in COX II and COX III polypeptide levels was observed in the 100% mutant cell line compared to the wild-type cell line between 4-22h (Fig. 3.11b and d). Labeled COX II and COX III polypeptides were not detectable in the 100% mutant cell line after a 16 hour chase.

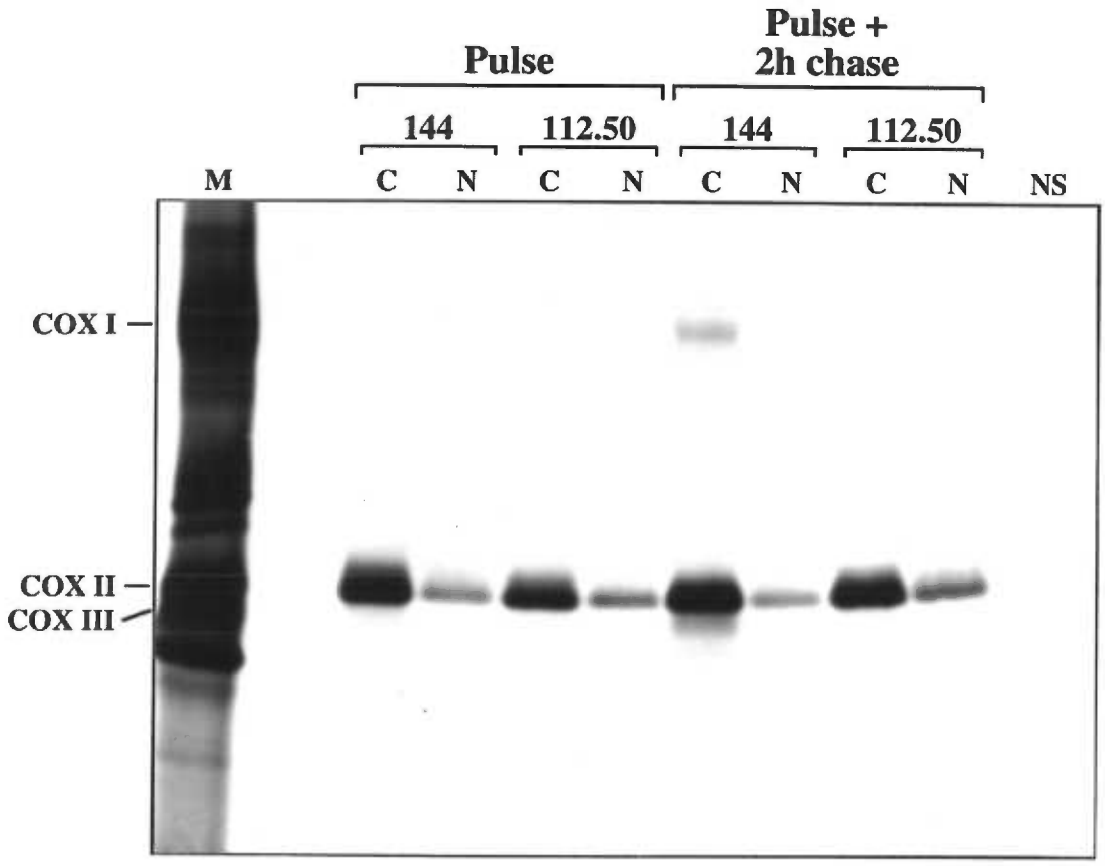
The turnover of COX I polypeptide in the wild-type cell line was characterized by a sharp decrease in levels of labeled polypeptide between 8-16h, followed by a much slower decline after a 16h; a 60% reduction in levels was observed after 22h chase (Fig. 3.11c). A more rapid decrease in the levels of labeled COX I polypeptide was observed in the 100% mutant cell line between 0-8h resulting in a 60-80% reduction after 8h (Fig. 3.11b and d). Later chase time points showed a steady decline in COX I polypeptide levels in the 100% mutant cell line.

Assembly Of COX. Immunoprecipitations were performed to investigate assembly of COX in the wild-type cell line (52.144), 100% mutant cell line (51.112.50), and 97% mutant cell line (51.129) using two antibodies directed against COX II. Wild-type and mutant cell lines were labeled in the presence of emetine for 1h, or labeled for 1h and

chased for 2h with excess unlabeled methionine. Mitochondria isolated by differential centrifugation from pulse and pulse-chased samples, were immunoprecipitated by two antisera directed against either the C-terminus or N-terminus of COX II. Antiserum to the C-terminus of COX II has been shown to immunoprecipitate the multisubunit enzyme complex in addition to the unincorporated COX II polypeptide (Mariottini *et al.*, 1986). Antiserum directed against the N-terminus of COX II immunoprecipitates the COX II polypeptide only when COX II is not incorporated into the holoenzyme. At least two sets of immunoprecipitations from independent pulse and pulse-chase labelings were performed for each cell line.

Representative immunoprecipitations of wild-type and 100% mutant cell lines are shown Fig. 3.12. The first four immunoprecipitations in Fig. 3.12 are those of mitochondria isolated from cells pulse labeled for 1h. In wild-type and 100% mutant cell lines, only the COX II polypeptide was detected in immunoprecipitations with the antiserum to either the C-terminus or N-terminus of COX II. These results suggested that after a one hour labeling, very little COX II polypeptide was assembled into the multisubunit enzyme complex in either cell line. The next four lanes of Fig. 3.12 are immunoprecipitations of mitochondria isolated from cells labeled for 1h and chased for 2h. In the wild-type cell line, in addition to the COX II polypeptide, COX I and COX III polypeptides were immunoprecipitated by the antiserum to the C-terminus of COX II (144, C lane), but only COX II was immunoprecipitated by the antiserum to the N-terminus of COX II (144, N lane). The co-precipitation of COX I and COX III polypeptides with COX II by the antiserum to the C-terminal of COX II, indicated that some of the labeled COX II peptide was assembled into the enzyme complex after a 2h chase, but a portion remained unassembled as shown by immunoprecipitations with the antiserum to the N-terminus of COX II. The next two lanes (112.50, N and C) of Fig. 3.12, are immunoprecipitations of mitochondria isolated from the 100% mutant cell line after a 1h pulse and a 2h chase. Only the COX II polypeptide was immunoprecipitated by antiserum to either the C or N-

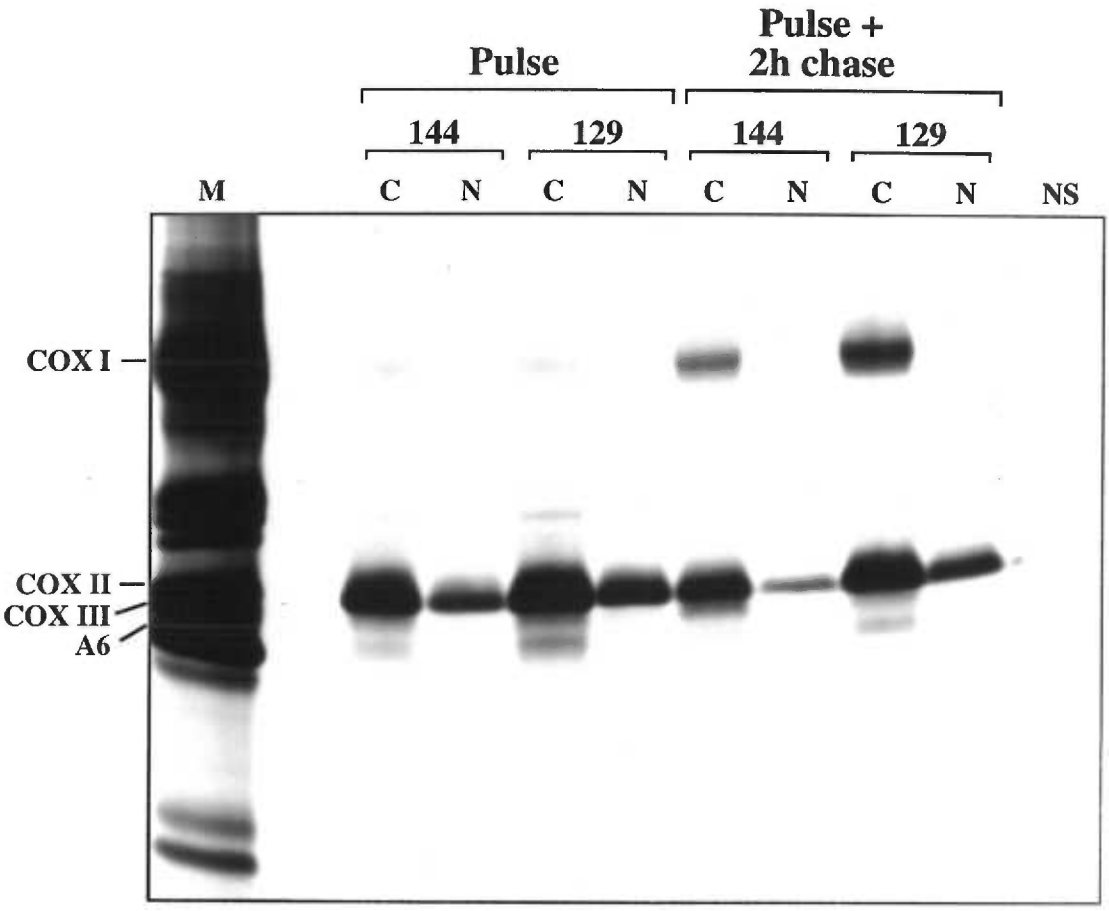
Figure 3.12. Assembly of mtDNA-encoded COX polypeptides in wild-type and 100% mutant cell lines. Immunoprecipitations of mitochondria isolated from wild-type cells (cell line 52.144) and 100% mutant cells (cell line 51.112.50) after a 1h pulse labeling (first four immunoprecipitations) and after a 1h pulse labeling and a 2h chase in DMEM containing excess unlabeled methionine (last four immunoprecipitations). Immunoprecipitations were performed using antiserum directed against the C-terminus of COX II (C) and antiserum directed against the N-terminus of COX II (N). Antiserum directed against the C-terminus of COX II has been shown to immunoprecipitate the multi-subunit enzyme complex and antiserum directed against the N-terminus of COX II only immunoprecipitates unincorporated COX II. The immunoprecipitates were analyzed by electrophoresis through 10% tricince SDS-PAGE (Schagger and von Jagow, 1987) and visualized by fluorography. NS = Immunoprecipitation performed with normal rabbit serum. M = pattern of mitochondrial translation products present in wild-type cell line after 1h pulse labeling and separated by 10% tricine SDS-PAGE.


Pulse
Pulse +
2h chase
144
112.50
144
112.50
M
C
N
C
N
C
N
C
N
NS
COX I
COX II
COX III

terminus of COX II, suggesting that no COX II polypeptide was assembled into the multisubunit enzyme complex in the 100% mutant cell line. No labeled polypeptides were detected when mitochondria were immunoprecipitated with normal rabbit serum (NS lane) or with a protein A-negative *Staphylococcus aureus* strain.

Results of immunoprecipitations with the wild-type cell line and the 97% mutant cell line are shown in Fig. 3.13. The first four immunoprecipitations in figure 3.13 are those of mitochondria isolated from cells pulse labeled for 1h. In wild-type and 97% mutant cell lines, the COX I, II and III polypeptides were immunoprecipitated by antiserum to the C-terminus of COX II (C lanes), but only COX II was immunoprecipitated by antiserum to the N-terminus of COX II (N lanes). The co-precipitation of COX I and COX III polypeptides with COX II by antiserum to the C-terminus of COX II indicated that some of the labeled COX II peptide was assembled into the enzyme complex in both wild-type and mutant cell lines after a 1h pulse. However, a portion remained unassembled as shown by the immunoprecipitations with antiserum directed to the N-terminus of COX II . The next four lanes of Fig. 3.13 are immunoprecipitations of mitochondria isolated from cells pulse-labeled for 1h and chased for 2h. The relative amounts of COX I and COX III labeled polypeptides immunoprecipitated by antiserum to the C-terminus of COX II in the wild-type cell line were greater after a 2h chase period compared to the amounts precipitated after a 1h pulse. These results suggested that a greater amount of labeled COX II polypeptide was assembled into the enzyme complex after a 2h chase in the wild-type cell line. In the 97% mutant cell line, the relative amount of COX I labeled polypeptide immunoprecipitated by the C-terminal antiserum was also greater after a 2h chase compared to the amount precipitated after a 1h pulse. In contrast, the relative amount of COX III labeled polypeptide immunoprecipitated after the 2h chase was similar or slightly reduced compared to the amount precipitated after a one hour pulse. These data suggested that after a 2h chase, a greater portion of labeled COX II polypeptide was assembled with COX I polypeptide, but that the COX III polypeptide may not always be a part of the multisubunit

Figure 3.13. Assembly of mtDNA-encoded COX polypeptides in wild-type and 97% mutant cell lines. Immunoprecipitations of mitochondria isolated from wild-type cells (cell line 52.144) and 97% mutant cells (cell line 51.129) after a 1h pulse labeling (first four immunoprecipitations) and after a 1h pulse labeling and a 2h chase in DMEM containing excess unlabeled methionine (last four immunoprecipitations). Immunoprecipitations were performed using antiserum directed against the C-terminus of COX II (C) and antiserum directed against the N-terminus of COX II (N). Antiserum directed against the C-terminus of COX II has been shown to immunoprecipitate the multi-subunit enzyme complex and antiserum directed against the N-terminus of COX II only immunoprecipitates unincorporated COX II. The immunoprecipitates were analyzed by electrophoresis through 10% tricince SDS-PAGE (Schagger and von Jagow, 1987) and visualized by fluorography. NS = immunoprecipitation performed with normal rabbit serum. M = pattern of mitochondrial translation products present in wild-type cell line after 1h pulse labeling and separated by 10% tricine SDS-PAGE .


Pulse
Pulse +
2h chase
144
129
144
129
M
C
N
C
N
C
N
C
N
NS
COX I
COX II
COX III
A6

complex in the 97% mutant cell line. Similar results have been observed with immunoprecipitations of mitochondria isolated from cells pulse labeled for 1h and chased for 12h (data not shown). No labeled polypeptides were detected when mitochondria were immunoprecipitated with normal rabbit serum (NS lane) or with a protein A-negative *Staphylococcus aureus* strain.

DISCUSSION

The 15bp deletion within the mtDNA encoded COX III gene is the first example of a mutation in a COX subunit (encoded by mtDNA or nuclear DNA) associated with isolated COX deficiency (see appendix 1, Keightley *et al.,* 1996). To investigate the consequences of the COX III mutation on COX function and structure, transmitochondrial cell lines were created by transferring mitochondria from the patient with the COX III mutation into a human cell line devoid of mtDNA. We obtained transmitochondrial cell lines containing 0%, 97% and 100% of the COX III mutation. The results presented here demonstrate a direct correlation between the presence of the 15bp deletion within the mtDNA-encoded COX III gene and a specific and severe deficiency in COX activity in transmitochondrial cell lines harboring this mutation.

One surprising finding of our studies was the inability to isolate any transmitochondrial cell lines with the COX III mutation from fusions of ρ^0 cell lines with muscle satellite cells. Previous analysis had shown high levels of mutation (93%) in skeletal muscle (Keightley *et al.*, 1996, see appendix 1). It is possible that myoblasts containing high levels of mutation were selected against in culture because of a growth disadvantage conferred by the mutation. Pools of myoblast clones and thirty individual myoblast clones were screened for the mutation after 3-4 weeks in culture. The mutation was not detected in any clones with a sensitivity that would detect levels of <0.1%. If high levels of the COX III mutation do confer a growth disadvantage, it is unclear why clones were not isolated with intermediate or low percentages of mutation, unless the

majority of the initial population of muscle satellite cells were either homoplasmic for wild-type mtDNA or mutated mtDNA. Alternatively, muscle satellite cells may have much lower levels of mutation compared to mature muscle in this patient. Others have reported difficulty establishing clonal cell lines with deletions of mtDNA and a point mutation in $tRNA^{(CUN)Leu}$ when muscle satellite cells were the source for the primary myoblast cultures (Moraes *et al.*, 1989; Fu *et al.*, 1996).

The most striking finding of our studies was the severity of the respiratory defect in transmitochondrial cell lines homoplasmic for the mutation. These cell lines had no detectable levels of COX activity and no detectable respiratory ATP synthesis. The rate of oxygen consumption was 5% of wild-type levels, similar to levels observed for the ρ^0 cell line 143B206, a human cell line lacking mtDNA. These cell lines have an absolute requirement for uridine and pyruvate, the same phenotype as the ρ^0 cell line 143B206. These growth properties indicate a complete absence of respiratory chain activity in cell lines with 100% mutated mtDNA. The uridine requirement is explained by the dependence of dihydroorotate dehydrogenase, an enzyme in the pyrimidine biosynthesis pathway, on intact respiratory chain activity (Gregoire *et al.*, 1984). Why cell lines completely deficient in respiratory function need pyruvate is less clear. Pyruvate supplementation may be required to drive the oxidation of excess cytoplasmic NADH coupled to the reduction of pyruvate to lactate (King and Attardi, 1996a).

The respiratory deficiency was less severe in cell lines harboring 97% mutated mtDNA (Fig. 3.2). COX activity in these cell lines was 6% of the levels in wild-type cell lines and the rate of oxygen consumption was 20% of the rates in wild-type cell lines. These cell lines grew normally in the absence of uridine, indicating that 3% wild-type mtDNA in the 97% cell lines was sufficient to restore some respiratory function. These data suggest that the COX III mutation behaves in a recessive manner. The markedly higher rates of oxygen consumption in 97% mutant cell lines compared to those of the 100%

mutant cell lines is consistent with other studies which show that COX may not be the rate limiting step in the respiratory chain (Taylor *et al.*, 1994; Davey and Clark, 1996)

To understand the mechanisms of COX deficiency associated with the COX III mutation, our studies focused on examining the consequences of this mutation for the function and stability of the COX III polypeptide within the enzyme complex. The mutation had little or no effect on the steady state levels of COX III mRNA transcripts in transmitochondrial cell lines with 97% and 100% mutated mtDNA (Fig. 3.4). To examine translation and stability of the COX III polypeptide in mutant cell lines, mitochondrial translation products were analyzed by labeling cells with [^{35}S]-methionine in the presence of emetine, a cytoplasmic protein synthesis inhibitor. Short 15min labeling periods demonstrated that the COX III polypeptide was translated in mutant cell lines at rates comparable to wild-type. However, no labeled COX III polypeptide could be detected in mutant cell lines 17h after labeling (Fig. 3.7 and 3.8). The stability of the COX III polypeptide thus, was reduced in the mutant cell lines. The increased turnover of the COX III polypeptide in the 100% mutant cell lines was evident even during short metabolic labeling periods of 1h and 2h, levels of labeled COX III polypeptide being 56% of wild-type levels after the 2h labeling period (Fig. 3.9). The metabolic labeling periods of 15min were performed to confirm that the lower levels of labeled COX III polypeptide observed in longer labeling periods were the result of increased turnover of the newly synthesized COX III polypeptide and not decreased translation. Presumably, the increased turnover of COX III polypeptide resulted in severely reduced steady-state levels of this polypeptide.

Analysis of the crystal structure of bovine COX revealed that the COX III polypeptide is composed of seven transmembrane segments. The 15bp deletion in the COX III gene results in the loss of 5 amino acids (PheAlaGlyPhePhe) from the third transmembrane domain of the polypeptide. The loss of 5 amino acids in the third transmembrane (15% of the total amino acids in this transmembrane region) could disrupt stability of the polypeptide. There is some evidence that mutations in transmembrane

domains can destabilize membrane proteins. A point mutation in the transmembrane domain of the proto oncogene p185 resulted in more than a four fold decrease in half-life of the p185 polypeptide (Stern *et al.*, 1988). The loss of five amino acids in the third transmembrane domain could also disrupt interactions of COX III with other subunits in the enzyme complex. The third transmembrane domain of COX III is one of two transmembrane domains which contact subunit COX I in the enzyme complex (Fig 1.3c, see chapter 1) (Tsukihara *et al.,* 1996). The loss of 5 amino acids in this transmembrane domain may prevent the association of COX III with COX I within the enzyme complex, presumably leading to decreased stability of the COX III polypeptide.

Immunoprecipitation experiments were performed to determine if assembly of COX would occur in the presence of the mutant COX III polypeptide. There was no evidence of assembly of COX in 100% mutant cell lines (Fig. 3.12). These studies indicated that the COX III polypeptide in 100% mutant cell lines was not incorporated into the enzyme complex thus preventing the assembly of the mtDNA encoded COX I and COX II.

The functional role of COX III within the enzyme complex has been debated. It has been suggested that COX III may modulate proton pumping activity or serve a structural and/or regulatory role in the complex (Brunori *et al.*, 1987; Haltia *et al.*, 1989). COX I forms the center of the enzyme complex with COX II associated at one side and COX III bound to the other (Fig. 1.3a-c, see chapter 1) (Tsukihara *et al.,* 1996). The immunoprecipitation experiments with the 100% mutant cell line suggested that COX III may be required to stabilize the interaction between COX I and COX II polypeptides.

The immunoprecipitation experiments with the 97% mutant cell line revealed that COX I, COX II and COX III polypeptides were assembled in the 97% mutant cell line (Fig. 3.13). In both wild-type and 97% mutant cell lines, similar amounts of COX I and COX II polypeptides were immunoprecipitated after a 2h chase with antiserum directed against COX II. The amounts of COX I polypeptide which co-precipitated with the COX II were higher than would be expected in a cell line with 97% levels of the COX III

mutation and suggested that a high proportion of COX I and COX II polypeptides were assembled in the 97% mutant cell lines. The relative amounts of the COX III polypeptide immunoprecipitated after a 2h chase, however, were lower in 97% mutant cells compared to levels in the wild-type. These results suggested that the COX III polypeptide was not always a part of the assembled COX I and COX II complex. The association of the COX I and COX II polypeptides in the 97% mutant cell line did not appear to be functional since COX activity in these cell lines was 5% the level of wild-type cell lines. Further studies are needed to examine the assembly COX I and COX II polypeptides in the 97% cell line and to determine if COX III serves a "catalytic" role in the enzyme complex by promoting the assembly of COX I and COX II polypeptides in the enzyme complex.

The steady state levels of mtDNA-encoded COX I and COX II polypeptides were reduced in the both the 97% and 100% mutant cell lines, as shown by western blotting analysis (Fig. 3.5). The lower steady-state levels in the 100% mutant cell lines were probably a direct consequence of these polypeptides not being assembled into the enzyme complex. COX I and COX II polypeptides also were shown to have decreased stability by metabolic labeling experiments in 100% mutant cell lines. These data indicated that COX I and COX II polypeptides which are not assembled into the enzyme complex are degraded. In the 97% mutant cell line, there were smaller reductions in the steady state levels of COX I and COX II polypeptides. These observations were consistent with the immunoprecipitation experiments which indicated that COX I and COX II polypeptides were at least transiently assembled.

In yeast, several ATP-dependent proteases have been identified which are responsible for the degradation of unassembled subunits of inner mitochondrial membrane proteins. Two proteases located in the mitochondrial inner membrane, Afg3/Yta10-12 complex and Yme101p, have been shown to be responsible for degradation of unassembled mtDNA-encoded COX subunits (Pajic *et al.*, 1994; Guelin *et al.*, 1996; Nakai *et al.*, 1994; Pearce and Sherman, 1995). Interestingly, these proteases are necessary for

normal respiratory function and may serve both a proteolytic role and a chaperone-assembly role in the membrane (Arlt *et al.*, 1996). The degradation of unassembled subunits of the respiratory chain enzyme complexes may be important for maintaining the stoichiometric assembly of the multi-subunit enzyme complexes (Hare 1990; Rep and Grivell,1996).

In this study, a proportion of the newly synthesized COX I, COX II and COX III translation products were unstable in wild-type cell lines. A reduction of approximately 40-60% in the levels of mtDNA encoded COX polypeptides was observed after a 12h chase in wild-type cell lines. The turnover of COX subunits after 12h was characterized by a much slower decrease in polypeptide levels, suggesting that this proportion of translation products was assembled into the enzyme complex. MtDNA-encoded COX polypeptides may be synthesized at higher levels than those which are assembled into the complex, leading to degradation of excess subunits. There is some evidence that mtDNA encoded COX subunits and other respiratory chain components are produced in non-stoichiometric levels compared to the levels of the nuclear encoded subunits. A subset of mitochondrial translation products was found to be unstable in HeLa cells and rat hepatoma cells after labeling in the presence of a cytoplasmic protein synthesis inhibitor, suggesting that only a certain proportion of newly synthesized mitochondrial translation products were assembled with nuclear subunits (Costantino and Attardi, 1977; Hall and Hare, 1990). Other studies specifically examining levels of mtDNA encoded COX subunits have shown that mRNA transcripts and translated polypeptides of mtDNA encoded COX subunits may be produced in excess compared to nuclear subunits (Van den Bogert *et al.*, 1993; Spelbrink *et al.*, 1994). The analysis of cell lines harboring 70% levels of a mtDNA deletion encompassing regions between the COX II gene and ND 5 genes, revealed that a 50% reduction in the steady state levels of COX II polypeptide had no effect on the concentration of COX or on COX activity (Spelbrink *et al.*, 1994).

With the creation of transmitochondrial cell lines harboring the COX III deletion, it has been possible to examine the cellular and molecular consequences of the 15bp deletion in the mtDNA encoded COX III gene. These studies may lead to a better understanding of the etiology and the pathogenesis of COX deficiency as well as the function of the COX III polypeptide in the enzyme complex.

CHAPTER 4

A POLYMORPHISM ELIMINATING THE STOP CODON OF ATP SYNTHETASE SUBUNIT 6 GENE DOES NOT IMPAIR STABILITY OR FUNCTION OF MITOCHONDRIAL DNA-ENCODED ATP SYNTHETASE SUBUNIT 6.

ATP synthetase, also termed F_1F_0-ATPase, catalyzes the formation of ATP from ADP and P. ATP synthetase is composed of two domains: F_1, the hydrophilic catalytic domain which protrudes into the mitochondrial matrix, and the F_0 complex which is embedded within the inner mitochondrial membrane. ATP synthetase in mammalian mitochondria is composed of 16 subunits (Walker and Collinson, 1994). The majority of the subunits are encoded by the nuclear genome, but two components of the F_0 domain (ATP synthetase subunit 6 and 8) are encoded by the mtDNA .

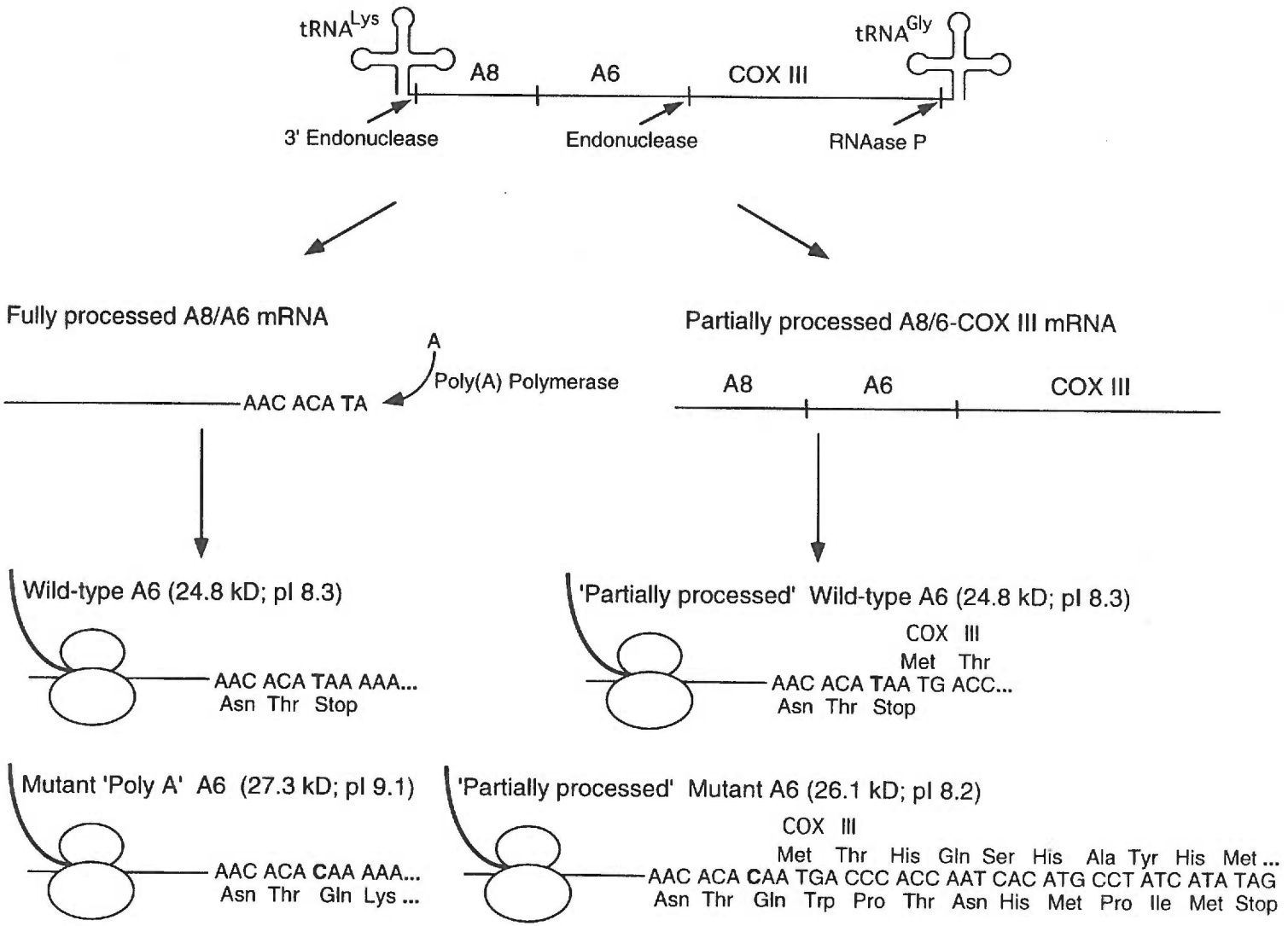
Two mutations within the mtDNA-encoded ATP synthetase 6 (A6) gene, T8993C and T8993G, have been associated with neuromuscular diseases. These mutations can result in maternally inherited Leigh syndrome (MILS) or neuropathy, ataxia and retinitis pigmentosa, NARP (Holt *et. al.*, 1990; Tatuch *et. al.*, 1992). The T8993C and T8993G mutations change a highly conserved amino acid ($Leu^{156} \rightarrow$ Pro or $Leu^{156} \rightarrow$ Arg). High levels of mutation resulted in decreased rates of ATP synthesis and a reduction in fully assembled F_1F_0 ATP synthetase in cultured cell lines (Tatuch and Robinson, 1993; Trounce *et. al.*, 1994; Davidson and King, 1997).

We previously reported a T9205C mutation in the A6 gene in the mtDNA of a patient with recurrent myoglobinuria and isolated COX deficiency associated with a 15 bp deletion in the mtDNA-encoded subunit III of cytochrome c oxidase (COX) (Keightley *et. al*, 1996). The T9205C, an OCHRE mutation, changed the A6 stop codon to glutamine and was present in the mtDNA of both the patient and her unaffected mother. In the mtDNA genome, the A6 gene terminates after the first 2 bp of the A6 stop codon and immediately

precedes the COX III gene, without intervening nucleotides. The mtDNA genome is transcribed as a polycistronic message which is then processed into individual mRNA species. The genes for ATP synthetase 8 (A8) and A6 are encoded by overlapping sequences and are encompassed within the same mRNA transcript. The A8/A6 + COX III mRNA polycistronic transcript is usually processed between the A6 and COX III genes, but both fully processed and partially processed transcripts have been documented in transmitochondrial cell lines (chapter 3, Masucci *et al.*, 1995). In cells lacking this mutation, the complete stop codon is formed by either polyadenylation of the processed A8/A6 mRNA transcript or by the first nucleotide in the ATG initiation codon of the COX III gene of the partially processed A8/A6 + COX III transcript (Fig. 4.1). In cells with the T9205C mutation, translation of either the processed or partially processed mRNA transcript would result in an aberrant form of A6. Translation of the A8/A6 + COX III partially processed transcript would result in a full length A6 polypeptide with 10 additional amino acids at the carboxyl terminus. Translation of the processed A8/A6 transcript also would produce an aberrant polypeptide. Because approximately 55 adenosines are added post-transcriptionally to mitochondrial transcripts (Perlman *et al.*, 1973), translation of this mRNA species would result in approximately seventeen lysines at the carboxyl terminus. Both aberrant polypeptides have a predicted molecular weight larger than wild-type A6 (26 Kd or 27 Kd) and the polypeptide with the lysine tail would have a isoelectric point that is dramatically different than that of the wild-type A6 polypeptide.

To determine the consequences of this mutation for translation of the A6 polypeptide and ATP synthetase activity, molecular analyses of transmitochondrial cell lines with the A6 mutation were performed.

Figure 4.1 Schematic diagram of RNA processing and translation of the modified A6 mRNAs. Transcription of the mtDNA occurs as a large polycistronic transcript that is processed to the individual RNA species. Shown is a portion of this transcript encompassing the A6 gene region. Processing normally occurs at the A6 -- COX III gene boundary. Poly adenylation of the A8/A6 mRNA creates the A6 TAA stop codon. In the partially processed A8/A6 transcript, a TAA stop codon is also present. The T9205C mutation creates a glutamine codon in both the fully processed and partially processed transcripts. Translation of the fully processed and poly adenylated mutated transcript would result in approximately 17 lysines following glutamine. Translation of the partially processed transcript would continue for 10 amino acids before encountering a stop codon.


tRNA Lys
tRNA Gly
A8
A6
COX III
3' Endonuclease
Endonuclease
RNAase P
Fully processed A8/A6 mRNA
A
Poly(A) Polymerase
AAC ACA TA
Partially processed A8/6-COX III mRNA
A8
A6
COX III
Wild-type A6 (24.8 kD; pI 8.3)
AAC ACA TAA AAA...
Asn Thr Stop
'Partially processed' Wild-type A6 (24.8 kD; pI 8.3)
COX III
Met Thr
AAC ACA TAA TG ACC...
Asn Thr Stop
Mutant 'Poly A' A6 (27.3 kD; pI 9.1)
AAC ACA CAA AAA...
Asn Thr Gln Lys ...
'Partially processed' Mutant A6 (26.1 kD; pI 8.2)
COX III
Met Thr His Gln Ser His Ala Tyr His Met ...
AAC ACA CAA TGA CCC ACC AAT CAC ATG CCT ATC ATA TAG
Asn Thr Gln Trp Pro Thr Asn His Met Pro Ile Met Stop

MATERIALS AND METHODS

Genomic DNA isolation and direct sequencing of mtDNA. Total DNA was isolated from exponentially growing cell lines 143B and AT153 according to established protocols (Davis *et al.*, 1986). PCR fragments of 1.4 -3.0 kb, generated from genomic DNA, were purified using Qiaex gel extraction resin (Qiagen) and sequenced directly using a combination of automated sequencing (Applied Biosystems at Columbia University Sequencing Facility and Oregon Health Sciences University Sequencing Facility) and cycle sequencing (New England Biolabs) with ^{32}P labeled primer. Sequencing reactions were electrophoresed through 6% polyacrylamide 8 M urea gels, dried and exposed to X-ray film for 24-48 hours. The A6 and A8 genes were sequenced in both cell lines; the sequence boundaries were nt 8365-9220

ATP synthesis assay. Mitochondria were prepared according to Millis and Pious (1973) after scraping monolayer cultures from five 15 cm culture dishes (approximately 5 x 10^6 cells) into 30 ml of an isolation buffer consisting of 0.27M mannitol, 0.1mM EDTA, 0.05% bovine serum albumin (BSA) and 10mM Tris-HCl pH7.3. After a brief treatment with protease (protease XIV, Sigma), cells were disrupted by four passes in a teflon/glass homogenizer, and the mitochondrial fraction was then isolated by differential centrifugation. The ability of isolated mitochondria to synthesize ATP during oxidative phosphorylation was assayed using succinate as a substrate. ATP synthesis was assayed indirectly by measuring the incorporation of ^{32}P into glucose-6-phosphate following phosphorylation of ADP by inorganic phosphate in the presence of the myokinase inhibitor adenosine pentaphosphate (Tuena de Gomez-Puyou *et al.*, 1984). Freshly isolated mitochondria (100-200 µg protein) were incubated in 0.3 ml of 30 mM Tris-acetate pH 7.4, 10 mM $MgCl_2$, 10 mM phosphate-tris (0.5µCi ^{32}Pi orthophosphate NEN), 6 mM substrate (succinate), 20 mM glucose, 20 U of hexokinase, 1 mM ADP and 50 µM

adenosine pentaphosphate in a shaking water bath at 30°C. If necessary as a control, 1µl of 5mg/ml of the F_0 inhibitor oligomycin was added. The reaction was terminated after 35min by adding 30% trichloroacetic acid to a final concentration of 10%. The reaction mixture was then centrifuged, and a 300µl aliquot was removed, to which was sequentially added 1ml H_2O, 1ml 3.3% ammonium molybdate in 3.75N H_2SO_4, and 200µl of acetone. Unincorporated phosphate was removed by extracting 6 times with 1ml of benzene:isobutanol mixture (50:50, vol:vol, H_2O saturated), after vortexing vigorously for 45 seconds each time. The amount of radioactivity present in an aliquot of the extracted reaction mix, determined by scintillation counting, was taken to represent incorporation of radioactive phosphate.

RNA Isolation and Northern Analysis. Total RNA was isolated from exponentially growing cell lines from approximately 1.5 x 10^7 cells using the commercial RNA isolation reagent, RNAzol B (Tel-test Inc.). RNA was resuspended in RNAase free H_2O for cDNA synthesis or formazol (Tel-test Inc.), a commercial brand of purified formamide and was quantitated by 260/280 UV absorbance.

Total RNA (10 - 15µg) was electrophoresed through a 1% agarose gel containing 0.66M formaldehyde, transferred to Zeta probe (Biorad) using blotting conditions as described by Fourney *et al.* (1988) and hybridized according to manufacturer's suggested directions. The following probes were labeled by random priming (Feinberg and Vogelstein, 1983) following manufacturer's directions (Random prime labeling kit, Boehringer Mannheim Co): pHFBA-1, a plasmid containing the human cytoplasmic β-actin gene (Gunning *et al.*, 1983); a PCR amplified fragment corresponding to nt 9268-9645 of mtDNA encoded COX III gene; a PCR amplified fragment corresponding to nt 7640-8051 of mtDNA encoded COX II gene. Strand specific probes for the following were obtained by extension of the universal M13 primer according to the method of Sucov *et al.* (1987): mp9.M9 (mtDNA 1-739) which hybridizes to 12S rRNA; a Pst digest of OP-

5 (mtDNA 8592-9020) which hybridizes to A8/A6. RNA hybridization signals were quantitated with Biorad phosphor-imager (Molecular Imager) and visualized by autoradiography.

Analysis Of Mitochondrial Protein Synthesis. For pulse labelings, 6-10 10 cm plates of exponentially growing cells were incubated for 60-120 minutes in methionine free DMEM containing 250μCi ^{35}S (> 1,000 Ci/mmol) labeled methionine, 5% dialyzed FBS, and 100μg emetine/ml, according to previously described methods (Chomyn, 1996b). For pulse-chase labelings, 8-15 10 cm plates of exponentially growing cells were labeled with 250-500μCi ^{35}S (> 1,000 Ci/mmol) methionine in the presence 100μg emetine per ml for 30-120 minutes. After labeling, cells were washed twice with DMEM containing 100μg emetine per ml and 2mM unlabeled methionine, washed once with DMEM containing 2mM unlabeled methionine and then incubated in DMEM containing 2mM unlabeled methionine and 5% dialyzed FBS at 37°C. At each chase time point, cells were lysed by addition of 1% SDS, 10mM Tris-HCl, pH 7.4 directly to the cells (1 10cm plate) or the cells were trypsinized for mitochondrial isolation (3-4 10cm plates). Labeled polypeptides from isolated mitochondria were analyzed by 10% tricine SDS-PAGE (Schagger and von Jagow, 1987), subjected to fluorography (NEN Intensify, Dupont) on Kodax film or were analyzed with Biorad phosphor-imager (Molecular Imager). The assignment of mtDNA-encoded translation products was based on similarity to those described by Chomyn and Lai (1990). Translation products were quantitated using Molecular Analyst software (Molecular Imager, Biorad). Each translation product was quantitated by drawing a box around the band and calculating the volume of the box (representing the mean pixel density multiplied by the area of the box), substracting background (volume of a box of equal area) and then dividing by the volume of the A6 polypeptide (to compare levels of translation products in different cell lines).

2-Dimensional Electrophoresis. Five 10 cm plates of exponentially growing cells were incubated for 60-120 minutes in methionine free DMEM containing 500μCi ^{35}S (> 1,000 Ci/mmol) labeled methionine, 5% dialyzed FBS, and 100μg emetine per ml, according to previously described methods (Chomyn, 1996b). A mitochondrial fraction was isolated by differential centrifugation and resuspended in 0.3% SDS and 10 mM Tris pH 7.4 containing five types of protease inhibitors; PMSF 174μg/ml, leupeptin 10μg/ml, pepstatin A 3.4μg/ml, E-64 3.6μg/ml, benzamidine HCl 56μg/ml. Two dimensional electrophoresis adapted for the resolution of basic proteins was performed according to the method of O'Farrell (O'Farrell *et al.*, 1977) by Kendrick Labs (Madison, WI). Non-equilibrium pH gradient electrophoresis (NEPHGE) using 1.5% pH 3.5-10 and 0.25% pH 9-11 ampholines (LKB Instruments, Baltimore, MD) was performed at 150v for 12h. After equilibration for 15 min. in SDS sample buffer (10% glycerol, 50mM dithiothreitol, 2.3% SDS and 0.0625 M Tris, pH 6.8) the tube gel was sealed to the top of a 4% acrylamide spacer gel on the top of a 10% acrylamide peptide slab gel (Schagger and von Jagow, 1987) (0.75 mm thick). SDS slab gel electrophoresis was started at 15 mamp/gel for the first four hours and then carried out overnight at 12 mamp/gel. The slab gel electrophoresis was stopped after the bromophenol blue dye front had run off. After electrophoresis, the slab gel was fixed in 50% methanol 10% acetic acid for 1h, and subjected to fluorography (NEN Intensify, Dupont) on Kodax Biomax MS film.

RESULTS

All transmitochondrial cell lines with the A6 mutation were derived from the patient with the COX III mutation. The A6 mutation appeared to be homoplasmic in cell lines with both 0% and 100% of the COX III mutation. There was no evidence of heteroplasmy of the A6 mutation by sequencing or RFLP analysis of PCR fragments (data not shown). The T9205C A6 mutation was not detected in mtDNA isolated from leukocytes of 50 controls.

To examine the consequences of the A6 mutation for enzymatic function, cell lines with only the A6 mutation (52.144 and 51.118), cell lines with the A6 and COX III mutation (51.112.50 and 51.112.28) and cell lines without the A6 mutation (143B and AT153) were chosen for molecular studies. Cell line 143B is the parental cell line of ρ^0 cell line 143B206. AT153 is a transmitochondrial derivative of the ρ^0 cell line 143B206 and has been shown by previous work to have a rate of O_2 consumption similar to 143B. Genes for A8 and A6 were sequenced in 143B and AT153 cell lines and no coding differences from the Cambridge sequence were identified in either of these genes (Anderson *et al.*, 1981).

ATP Synthesis. ATP synthesis was examined in cell lines with only the A6 mutation (52.144), with both the A6 mutation and the COX III mutation (51.112.50), and also in two wild-type cell lines without the A6 mutation (143B and AT153), Fig. 4.2. The ability of isolated mitochondria to synthesize ATP by oxidative phosphorylation was assayed using succinate as a substrate. ATP synthesis was assayed indirectly by measuring the incorporation of ^{32}P into glucose-6-phosphate following phosphorylation of ADP by inorganic phosphate in the presence of the myokinase inhibitor adenosine pentaphosphate (Tuena de Gomez-Puyou *et al.*, 1984). The rates of ATP synthesis in cell line 52.144 (7060 ± 660 [1 SE] nmol ATP/hr/mg protein) were comparable to the rates in the two cell lines without the A6 mutation (av.= 7240 ± 1200 nmol ATP/hr/mg protein). In contrast, cell line 51.112.50, with both the A6 and COX III mutation, had severely reduced rates of ATP synthesis (7.84 ± 1.10 nmol ATP/hr/mg protein), approximately 0.1% the rate of the other cell lines. These results indicated that the T9205C mutation in the A6 gene did not affect the rates of ATP synthesis in cell line 52.144, and therefore was probably not contributing to the severely reduced rates of ATP synthesis observed in cell line 51.112.50, containing both the A6 and the COX III mutations.

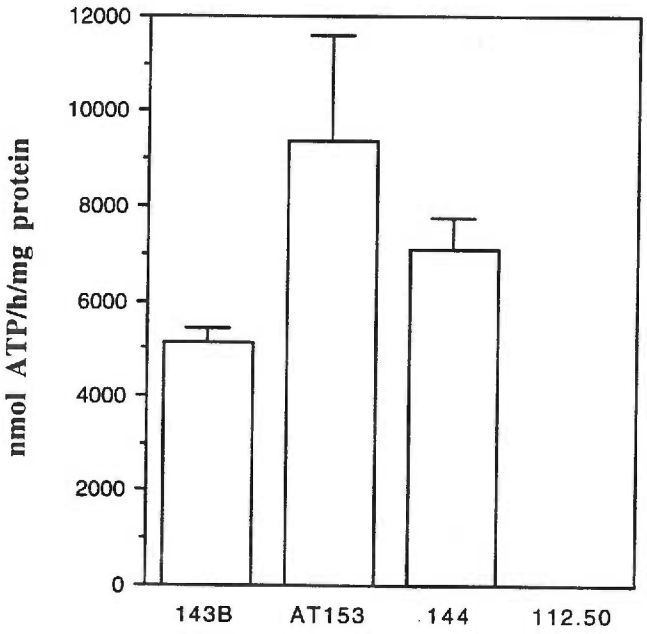


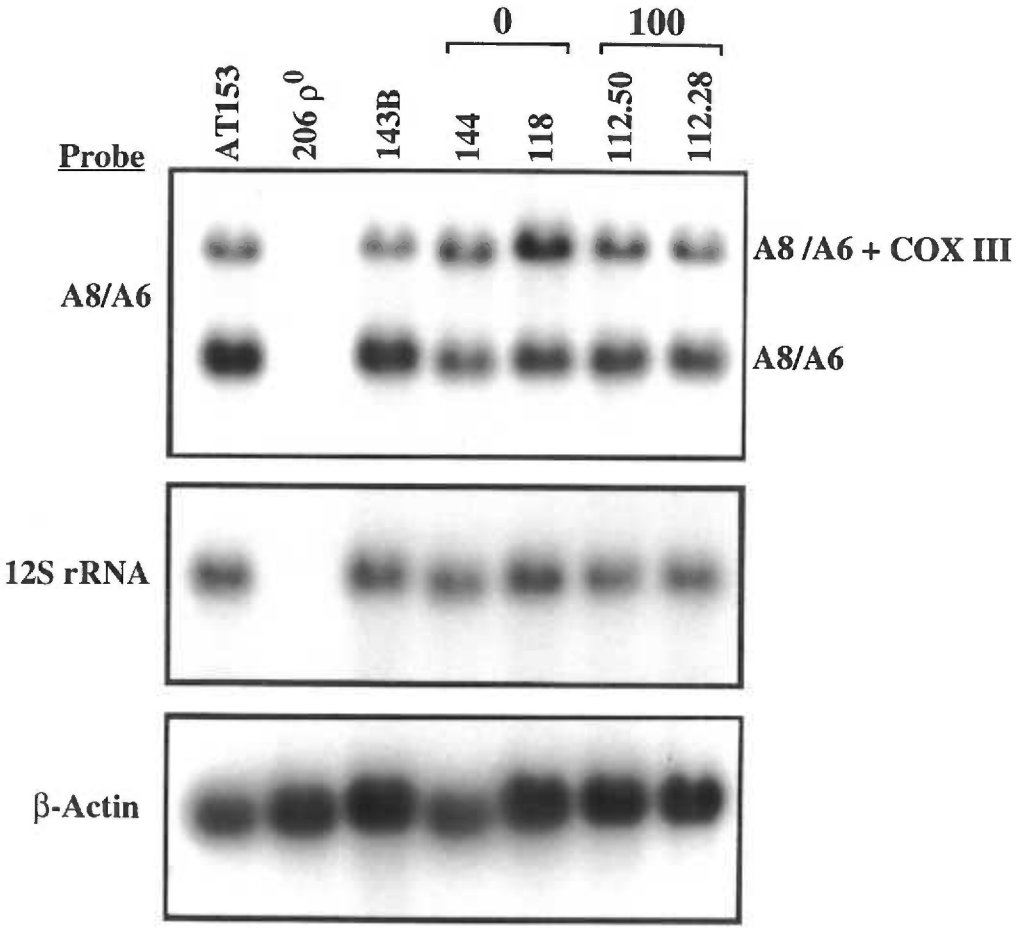
Figure 4.2. ATP synthesis in transmitochondrial cell lines. ATP synthesis was measured in two wild-type cell lines (143B and AT153) the cell line with the T9205C mutation (51.144) and the cell line with the T9205C mutation and homoplasmic levels of the COX III mutation (51.112.50). The rate of ATP synthesis was determined indirectly in isolated mitochondria by measuring the incorporation of P^{32} into glucose-6-phosphate following phosphorylation of ADP by inorganic phosphate. Error bars represent 1 standard error.

Northern Analysis. To determine if the T9205C A6 mutation affected steady-state levels or processing of RNA transcripts, steady-state levels of A8/A6 mRNA were investigated by northern analysis. Total RNA was isolated from exponentially growing cells and examined by northern analysis (Fig. 4.3). The RNA was hybridized with five probes which identify five mtDNA-encoded genes (COX III, A6, A8, COX II and 12s rRNA) and one nuclear encoded gene (β-actin). Two cell lines with the A6 mutation alone (52.144, 51.118), two cell lines with both the A6 mutation and the COX III mutation (51.112.50 and 51.112.28), and two cell lines without the A6 mutation (143B and AT153) and the ρ^0 cell line 143B206 were analyzed.

The A8/A6 probe detected a band of approximately 1 kb representing the A8/A6 transcript and a more slowly migrating band of 1.7 kb in both mutant and wild-type cell lines. The size of the 1.7 kb species was consistent with a partially processed mRNA species encompassing A8/A6 + COX III genes. Two mRNA species were detected with the COX III probe, a 780 bp COX III transcript and a transcript of approximately 1.7 kb, representing the partially processed A8/A6 + COX III transcript (chapter 3, Fig. 3.4). This 1.7 kb mRNA species was not detected by the other probes. The COX II probe detected a transcript of approximately 1 kb, the COX II mRNA, in wild-type and mutant cell lines (data not shown). The 12S rRNA probe identified a band of approximately 1.2 kb representing the 12S rRNA in wild-type and mutant cell lines. None of the probes specific for mtDNA genes detected any bands in ρ^0 cell line 143B206.

The relative steady-state levels of mitochondrial transcripts in each cell line were normalized to nuclear encoded β-actin to control for equal loading of RNA. Blots were quantitated using phosphor-imaging analysis. Levels of A8/A6 mRNA were calculated as the sum of processed and partially processed mRNA species normalized to β-actin. The relative levels of A8/A6 mRNA (sum of processed and partially processed) in four cell lines with the A6 mutation (mean = 8.6 $\pm$ 0.28 [1 SD]) were similar to levels for two cell lines without the A6 mutation (mean = 9.2 $\pm$ 0.45). However, cell lines with the A6 mutation

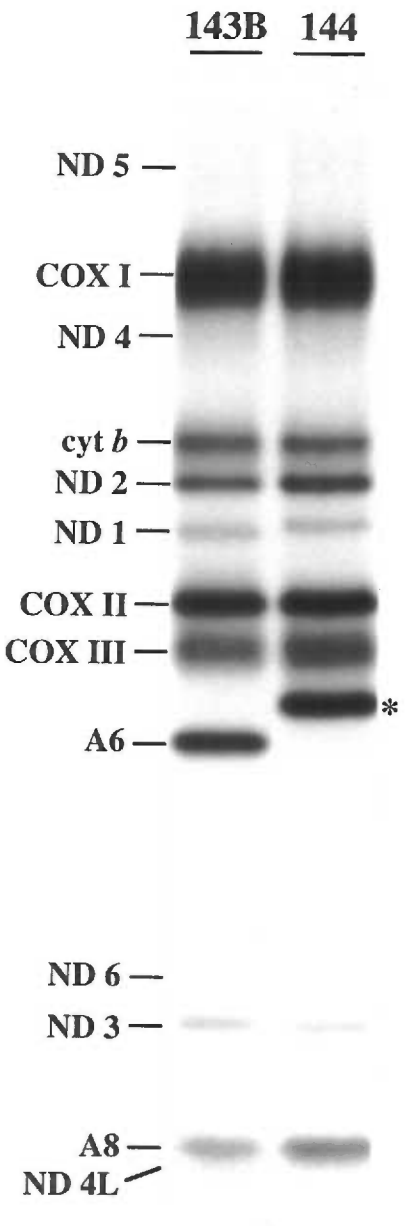
Figure 4.3. Northern Analysis. Total RNA was isolated from two wild-type cell lines (143B and AT153) the two cell lines with the T9205C mutation (51.144 and 51.118) and two cell lines with the T9205C mutation and homoplasmic levels of the COX III mutation (51.112.50 and 51.112.28). The probes used are indicated on the left of the autoradiogram. The mRNA species representing the partially processed A8/A6 + COX III transcript and the fully processed A8/A6 transcript are indicated on the right.


0
100
AT153
206 ρ0
143B
144
118
112.50
112.28
Probe
A8/A6
A8 /A6 + COX III
A8/A6
12S rRNA
β-Actin

(52.144, 51.118, 51.112.50 and 51.112.28) had a higher proportion of partially processed transcripts compared to the cell lines 143B and AT153. Levels of partially processed A8/A6 + COX III transcripts expressed as a percentage of total amount of transcript (processed + partially processed) were approximately 20% higher in cell lines with the A6 mutation (mean = 0.47 ± 0.01 [1SD]) compared to cell lines without the A6 mutation (mean = 0.27 ± 0.07). The results were similar when levels of partially processed A8/A6 + COX III transcript and processed COX III transcripts were quantitated. Levels of other mtDNA transcripts were not markedly different among cell lines.

Translation of A6 polypeptide. Mitochondrial protein synthesis was examined in cell lines with the A6 mutation by labeling cells with [^{35}S]-methionine in the presence of emetine, a cytoplasmic protein synthesis inhibitor, for a period of 1 hour. Translation products were analyzed by tricine-SDS PAGE of isolated mitochondria and quantitated by phosphor-imaging analysis. A representative labeling is shown in Fig. 4.4. The A6 polypeptide in cell lines with the T9205C mutation displayed a slower electrophoretic mobility on tricine-SDS PAGE compared to the A6 polypeptide in cell lines 143B and AT153. This slower electrophoretic mobility of the A6 polypeptide could be consistent with the molecular weight of either aberrant A6 polypeptides shown in Fig. 4.1. When levels of [^{35}S]-methionine incorporation were quantitated, the levels of labeled A6 polypeptides in cell lines with the mutation were similar to cell line 143B, (ratio of levels of A6 in 144/ levels in 143B = 1.1), indicating all cell lines had similar rates of translation of A6 polypeptide. The stability of the labeled A6 polypeptide was examined in cell lines with the A6 mutation (52.144 and 51.112.50) by labeling the cells for 1-2 hours in the presence of emetine and then incubating cells in emetine-free medium containing an excess of cold methionine for a chase period up to 17 hours. Levels of labeled A6 polypeptides with the T9205C mutation after 17h chase period (78%) were not significantly different from the initial incorporation levels.

Figure 4.4. Analysis of mitochondrial translation products in cell lines 143B and 52.144. Fluorogram of mitochondrial translation products from pulse labelings after electrophoresis through 10% tricine SDS-PAGE (Schagger and von Jagow, 1987). The aberrant A6 polypeptide in cell line 52.144 is indicated by an asterisk. Wild-type cell line (143B) and the cell line with the T9205C mutation (51.144) were labeled for 1h in the presence of 500μCi [^{35}S]-methionine and 100μg/ml emetine. After labeling, cells were harvested for mitochondrial isolation and separated through 10% tricine SDS-PAGE. The same amount of mitochondrial fraction (50μg of protein) was loaded in each lane of the gel.


143B
144
ND 5
COX I
ND 4
cyt b
ND 2
ND 1
COX II
COX III
*
A6
ND 6
ND 3
A8
ND 4L

2-Dimensional Electrophoresis. Because of the presence of both partially processed and processed A8/A6 mRNA transcripts, two types of aberrant A6 polypeptides could be present in cell lines with the A6 mutation (Fig. 4.1). An A6 polypeptide with an extra 10 amino acids, out of frame, of the COX III gene would be synthesized from the partially processed A8/A6 + COX III transcript. The estimated molecular weight of the A6[COX III] polypeptide would be 26kD, 1kD larger than the wild-type A6 polypeptide, but the predicted isoelectric point of the A6[COX III] polypeptide (pI=8.2) would not be very different from that of the wild-type (pI=8.3). Translation of the processed and polyadenylated A8/A6 mRNA would produce an A6 polypeptide with a poly-lysine tail A6[Lys]. This polypeptide would have a predicted molecular weight of 27kD. However, its isoelectric point, 9.1, would be markedly different from that of the wild-type A6 polypeptide. To determine if one or both types of aberrant A6 polypeptides were translated in cell lines with the A6 mutation, labeled mitochondrial translation products from cell line 52.144 (A6 mutation) and 143B were analyzed by non-equilibrium pH gradient gel electrophoresis (NEPHGE) of isolated mitochondria (O'Farrell *et al.*, 1977). NEPHGE is a 2-dimensional electrophorestic procedure which has been reported to be more effective than conventional isoelectric focusing in resolution of basic and hydrophobic proteins. In this type of analysis, both basic and acidic proteins can be separated according to isoelectric points, but because of shorter electrophoresis times, proteins typically don't reach equilibrium (O'Farrell *et al.*, 1977). The cell lines were labeled with [^{35}S]-methionine in the presence of emetine, a cytoplasmic protein synthesis inhibitor, for a period of 1 hour. Mitochondrial translation products were separated in the first dimension by NEPHGE and in the second dimension, according to molecular weight by tricine SDS-PAGE. The predicted separation of mitochondrial translation products is shown by Fig. 4.5. Two dimensional analysis of cell line 143B, cell line 52.144 (T9205C mutation) and an equal mix of mitochondria isolated from both cell lines is shown in Figs. 4.6, 4.7 and 4.8. To aid identification, total mitochondrial translation products were electrophoresed in the

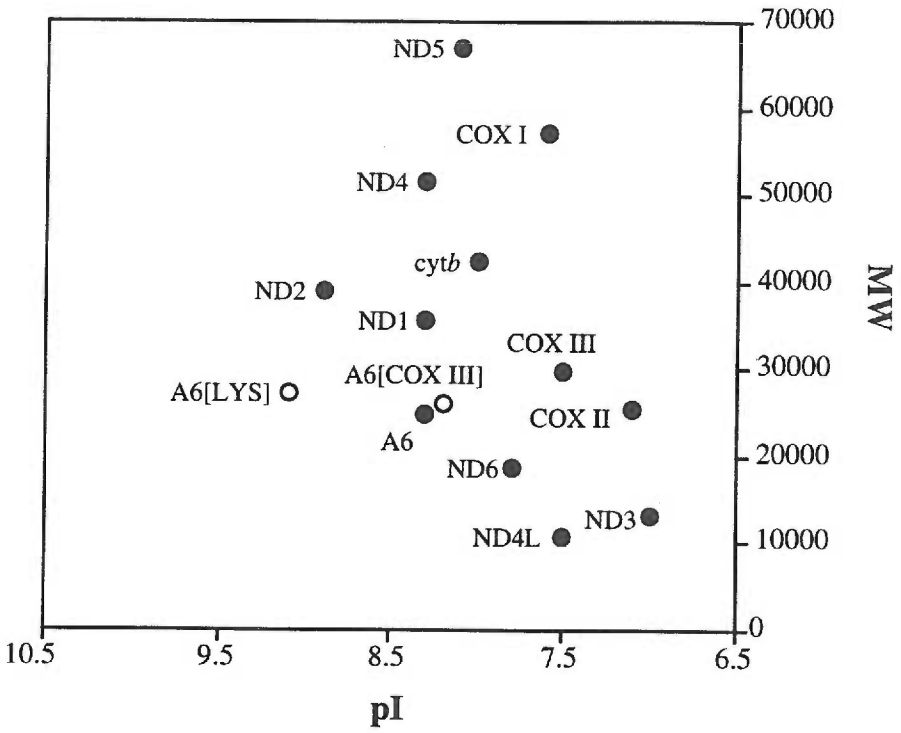
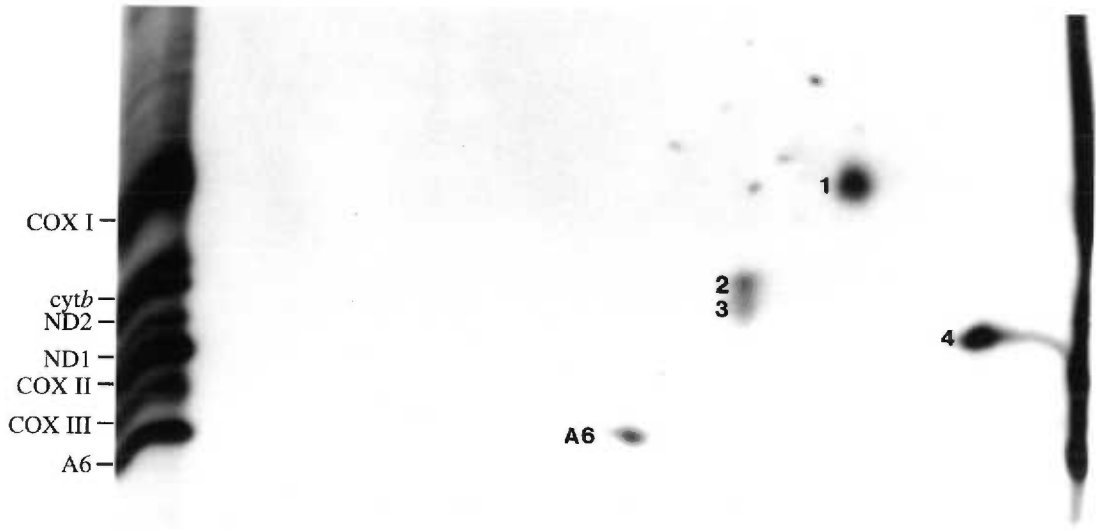


Figure 4.5. Predicted separation of mitochondrial translation products by two-dimensional electrophoresis. Predicted separation of mitochondrial translation products (black circles) and the putative aberrant A6 polypeptides (open circles) translated from either processed mRNA transcripts (A6[Lys]) or partially processed mRNA transcripts (A6[COX III]) according to molecular weight and pI.

Figure 4.6. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) of mtDNA-encoded translation products in cell line 143B. The upper panel shows the separation of isolated mitochondria of cell line 143B by NEPHGE after a 1h pulse labeling in the presence of 500µCi [^{35}S]-methionine and 100µg/ml emetine. The identification of five species representing polypeptides COX I, cyt b, ND 1, COX II and A6 was based on the predicted separation of polypeptides by molecular weight and pI (shown in the bottom panel) and the migration of total mitochondrial translation products electrophoresed only in the second dimension, shown on the left of the fluorogram.


COX I
cytb
ND2
ND1
COX II
COX III
A6
1
2
3
4
A6

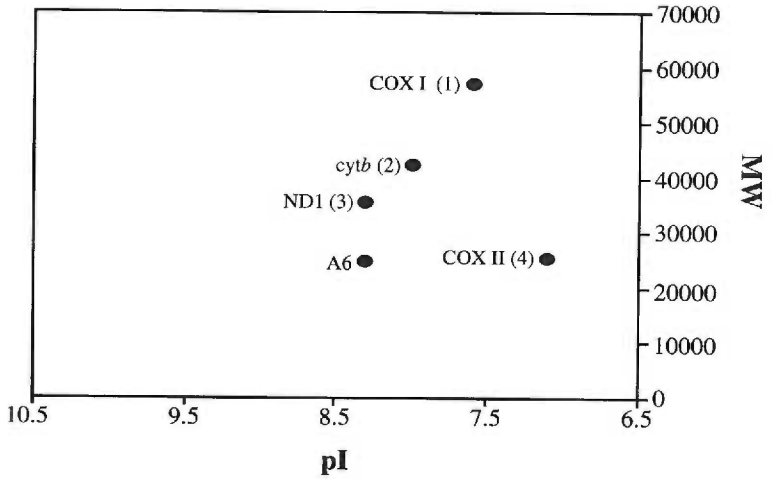
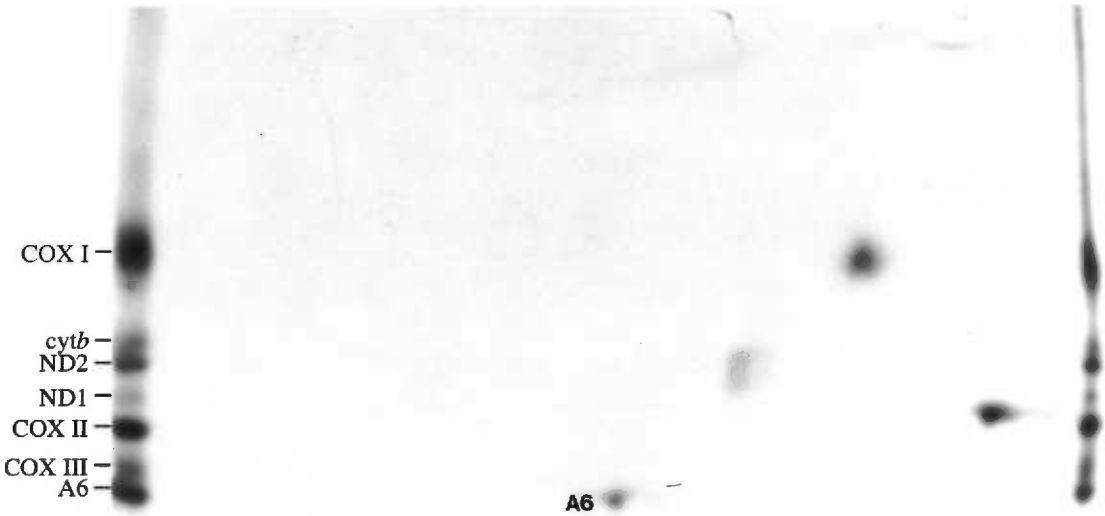

COX I (1)
cytb (2)
ND1 (3)
A6
COX II (4)
70000
60000
50000
40000
30000
20000
10000
0
MW
10.5
9.5
8.5
7.5
6.5
pI

Figure 4.7. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) of mtDNA-encoded translation products in cell line 52.144. The upper panel shows the separation of isolated mitochondria of cell line 52.144 by NEPHGE after a 1h pulse labeling in the presence of 500μCi [^{35}S]-methionine and 100μg/ml emetine. The identification of five species representing polypeptides COX I, cyt b, ND 1, COX II and A6 was based on the predicted separation of polypeptides by molecular weight and pI (shown in the bottom panel) and the migration of total mitochondrial translation products electrophoresed only in the second dimension, shown on the left of the fluorogram.


COX I
cytb
ND2
ND1
COX II
COX III
A6
A6

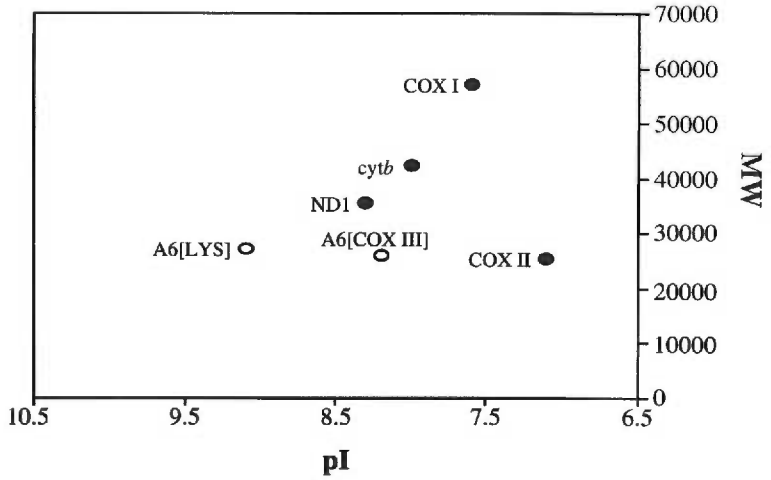
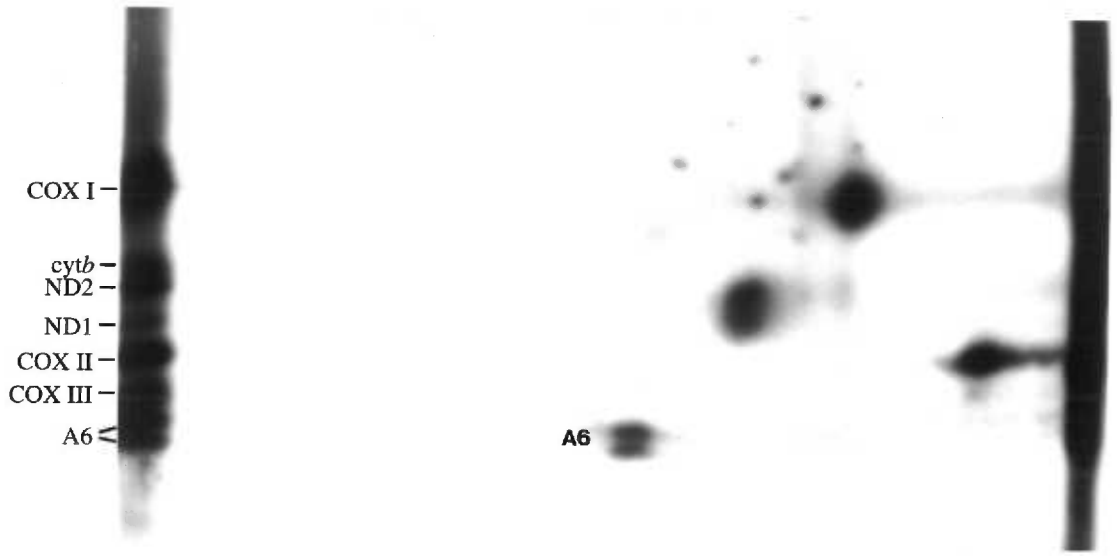
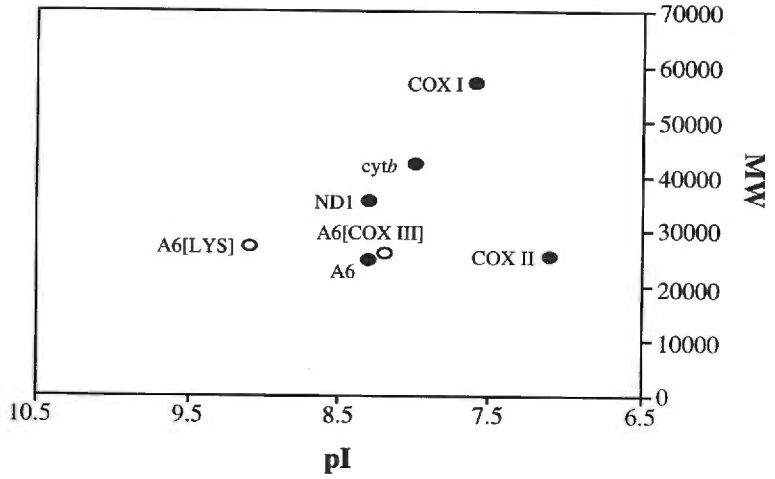

70000
60000
50000
40000
30000
20000
10000
0
MW
COX I
cytb
ND1
A6[LYS]
A6[COX III]
COX II
10.5
9.5
8.5
7.5
6.5
pI

Figure 4.8. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) of mtDNA-encoded translation products in cell lines 143B and 52.144. The upper panel shows the separation of an equal mix of isolated mitochondria from cell lines 52.144 and 143B by NEPHGE after a 1h pulse labeling in the presence of 500μCi [^{35}S]-methionine and 100μg/ml emetine. The predicted separation of mitochondrial translation products (black circles) and the two aberrant A6 polypeptides (open circles) is shown in the lower panel. Two species representing A6 polypeptides in cell line 52.144 and cell line 143B were identified on NEPHGE gels. The migration of both polypeptides was similar in the pH gradient, but the A6 polypeptide with the T9205C mutation had a slower electrophoretic mobility.


COX I
cytb
ND2
ND1
COX II
COX III
A6
A6


70000
60000
50000
40000
30000
20000
10000
0
MW
COX I
cytb
ND1
A6[COX III]
A6[LYS]
A6
COX II
10.5
9.5
8.5
7.5
6.5
pI

second dimension of each gel. Eight species representing polypeptides COX I, cyt *b*, ND 2, COX II, A6, ND 5, ND 4 and COX III were identified based on gel migration corresponding to their calculated pI and molecular weight (ND 5, ND 4 and COX III were identified after longer exposures). Other mitochondrial translation products could not be detected. As shown by Figs. 4.6 and 4.7, the migration of A6 polypeptide in cell line 52.144 (T9205C mutation) was similar to that of the A6 polypeptide in the 143B cell line. When two dimensional analysis of the mixture of mitochondria isolated from both cell lines was performed (Fig. 4.8), two spots were identified corresponding to the wild-type A6 and A6 polypeptide with the mutation. Migration of both polypeptides was similar in the pH gradient, but the A6 polypeptide with the mutation had a slower electrophoretic mobility in the tricine SDS- PAGE dimension. These results suggest that the A6 polypeptide found in the cell lines with the T9205C mutation was derived from the translation of the partially processed A8/A6 + COX III transcript. There was no evidence, even after longer exposures, of a species corresponding to the A6 polypeptide with the poly lysine tail.

DISCUSSION

The results presented here have shown that an aberrant A6 polypeptide is synthesized in cell lines with a T9205C mutation in the mtDNA-encoded A6 gene. The T9205C mutation, which changed the A6 stop codon to a glutamine codon, resulted in the translation of an A6 polypeptide which had a higher molecular weight than the wild-type A6 polypeptide. This aberrant A6 polypeptide appeared to be derived from the translation of a partially processed mRNA transcript encompassing A8/A6 and COX III coding sequences. Translation of this mRNA species was predicted to result in an extra ten amino acids at the carboxyl terminus of the full length A6 polypeptide. Neither the stability of the A6 polypeptide nor the rate of ATP synthesis in cell lines was affected by the T9205C mutation.

The T9205C A6 mutation originally was identified in a patient with isolated COX deficiency associated with a 15bp deletion in the COX III gene (Keightley *et al.*, 1996). This A6 mutation appeared to be homoplasmic in both the patient and her unaffected mother and was not present in leukocyte mtDNA of 50 controls. It was of interest to study the biochemical phenotype of this mutation to determine if the effects of the COX III mutation were exacerbated by the presence of the A6 mutation. It is possible that the mother of the patient, although unaffected, could have impaired ATP synthesis but no clinical phenotype. All transmitochondrial cell lines derived from the patient (0% and 100% COX III mutation) appeared to be homoplasmic for the T9205C mutation. This study confirms that the T9205C mutation did not contribute to the defect in ATP synthesis and overall respiratory function in transmitochondrial cell lines with the COX III mutation. Interestingly, a brief report has just described the identification of a homoplasmic 2bp deletion at nt 9204-9205 in an infant with COX deficiency and chronic lactic acidosis (Seneca *et al.*, 1996). The 2bp deletion resulted in the loss of the A6 stop codon. The authors predicted that the loss of the A6 stop codon would disrupt mRNA processing and produce a full length A6 + COX III fusion protein.

The tRNA genes are thought to serve as processing signals in the mitochondrial polycistronic messages (Ojala *et al.*, 1981). The junction between A8/A6 and the COX III genes is one of the few regions of the mtDNA genome which has no intervening tRNA genes, but is a target for mRNA processing. It is unclear what the processing signals are for the A8/A6 + COX III mRNA transcript. Although this study did not directly investigate whether the T9205C mutation affected the specificity of mRNA processing at this cleavage site, both fully processed A8/A6 mRNA transcripts and partially processed A8/A6 + COX III transcripts were detected in two wild-type cell lines and four cell lines with the T9205C mutation by northern analysis. The relative steady-state levels of the partially processed transcript ranged from 27% (of total transcript) in the two wild-type cell lines to 48% (of total transcript) in four cell lines with the T9205C mutation. One other study has

documented the presence of fully processed and partially processed transcripts in transmitochondrial cell lines (Masucci *et al.*, 1995). Up to 70% of steady state levels were present as the partially processed A8/A6 + COX III mRNA transcripts. Processing of the A8/A6 + COX III transcript normally may be inefficient in cells, but there are no phenotypic consequences of partially processed A8/A6 + COX transcripts. Transcription of either mRNA transcript would result in wild-type A6 polypeptide because the termination codon is completed either by polyadenylation (the processed transcript) or by the first nucleotide of the initiation codon of the COX III gene (the partially processed transcript).

In cells with the T9205C mutation, translation of either the processed or partially processed mRNA transcript would result in an aberrant form of A6 polypeptide. Both aberrant polypeptides have a predicted molecular weight larger than wild-type A6 (26 kD or 27 kD) and the polypeptide with the lysine tail would have an isoelectric point that is dramatically different than that of the wild-type A6 polypeptide. Analysis of mitochondrial protein synthesis revealed that the A6 polypeptide in cell lines with the T9205C mutation displayed a slower electrophoretic mobility on SDS-tricine gels compared to the wild-type A6 polypeptide which was consistent with the molecular weight of either aberrant A6 polypeptides. To determine if both types of aberrant A6 polypeptides were translated in cell lines with the A6 mutation, labeled mitochondrial translation products were analyzed by two-dimensional electrophoresis. Our studies have indicated that the predominant translation product in cell lines with this mutation was the A6 polypeptide derived from the partially processed A8/A6 + COX III mRNA transcript which results in a full length A6 polypeptide with ten additional amino acids at the carboxyl terminus. There was no evidence of a translation product derived from the processed A8/A6 mRNA transcript. Because of polyadenylation, translation of the processed A8/A6 mRNA transcript would extend the reading frame by approximately 17 lysines, but this polypeptide would have no termination codon. There is considerable evidence that the termination codon may be

important for cytoplasmic mRNA stability (Jacobson and Peltz, 1996). Both premature nonsense mutations and mutations resulting in the loss of the termination codon have been shown to accelerate the decay of mRNA (Weiss and Liebhaber, 1994; Jacobson and Peltz, 1996). In cell lines with the T9205C mutation, steady-state levels of A6 mRNA (processed + unprocessed) were similar to wild-type cell lines, but the proportion of processed A8/A6 mRNA transcript was lower. This may reflect reduced mRNA stability due to the absence of a termination codon in the processed A8/A6 mRNA transcript. Alternatively, translation of the A6 polypeptide with the lysine tail may occur, but the polypeptide may be rapidly degraded due to the effect of the considerable charge difference.

Although the T9205C mutation resulted in translation of an aberrant A6 polypeptide, rates of ATP synthesis in cell lines with the T9205C mutation were similar to wild-type cell lines. Thus, the additional ten amino acids at the carboxyl terminus of the A6 translation product had no detectable phenotypic consequences for ATP synthesis in these cell lines. The A6 polypeptide is a component of the membrane-embedded F_0 domain of F_1F_0 ATP synthetase. The F_0 domain serves as a proton pore which allows the movement of protons back into the matrix (Senior, 1988; Cox *et al.*, 1992). Hydropathy plots of the a-subunit in *E. coli* (homologous to the A6 polypeptide in the mammalian enzyme) have indicated that the a-subunit may have 4-7 transmembrane domains, but good structural models are still lacking (Senior, 1990; Cox *et al.*, 1992). Membrane spanning regions located near the C-terminus of the a-subunit share the strongest homology with the mammalian A6 polypeptide and are essential for ATP synthetase activity (Walker *et al.*, 1984; Cain and Simoni, 1986; Eya *et al.*, 1990; Vik *et al.* 1991; Hartzog and Cain, 1993). Our studies demonstrate that ten additional amino acids on the carboxyl terminus of the A6 polypeptide do not affect ATP synthesis. This may indicate that the C-terminus is not embedded in the complex.

CHAPTER 5

SUMMARY AND CONCLUSIONS

COX deficiencies are disorders of the respiratory chain usually associated with neuromuscular diseases. COX deficiency may present either in combination with other respiratory enzyme deficiencies or as an isolated deficiency (French *et al.*, 1972; Wallace *et al.*, 1988; Holt *et al.*, 1989; DiMauro *et al.*, 1990; Zeviani *et al.*, 1991). Isolated COX deficiency is poorly characterized at the molecular level. No mutations have been identified in any case of COX deficiency or Leigh syndrome associated with COX deficiency, even though in some patients many or all of the COX structural genes have been sequenced (Merante *et al.*, 1993; Adams *et al.*, 1997).

The focus of this thesis was to investigate the molecular basis for COX deficiency. Chapter 2 describes the molecular analyses of five patients with a tissue specific expression of COX deficiency. The clinical presentations of all five patients were characterized predominantly by a myopathy and/or cardiomyopathy associated with isolated COX deficiency (2 patients) or combined complex I and COX deficiency (3 patients). The tissue specific subunits of COX or the mtDNA-encoded subunits of COX were the most probable candidates for molecular defects because of the tissue specific expression of COX deficiency in these patients. No mutations were detected in the liver and heart isoforms of COX subunits VIa and VIIa, and no differences were found in mRNA expression of these subunits or in the expression of the mitochondrial encoded COX genes (COX I, COX II and COX III) in patients and controls. The mtDNA encoded COX genes were sequenced in one patient with a well characterized tissue specific form of isolated COX deficiency and no mutations were identified.

My results suggested that defects within genes other than COX structural genes may be involved in the pathogenesis of COX deficiency. COX biogenesis is controlled by

numerous factors which regulate the expression of COX subunits, import the nuclear encoded subunits into the mitochondrion, and coordinate the assembly of the multi-subunit enzyme complex. Molecular defects in any one of these processes could result in COX deficiency. Complementation studies have indicated that Leigh disease associated with COX deficiency is the result of a nuclear defect and a single disease locus may be responsible for the majority of cases (Tiranti *et al.*, 1995; Munaro *et al.*, 1997). Identifying the molecular defects causing the tissue specific forms of COX deficiency is more difficult because the expression of the defect is limited to one or a few tissues. Determining how the tissue specific isoforms are regulated in different tissues, may lead to a better understanding of the tissue specific expression of these diseases.

The first documentation of any mutation (encoded by mtDNA or nuclear DNA) associated with COX deficiency was described by our laboratory (Keightley *et. al*, 1996, see appendix 1). A 15 bp, in frame, deletion was identified in the COX III gene, encoding subunit III of cytochrome *c* oxidase in a patient with recurrent episodes of myoglobinuria and isolated COX deficiency. The second part of this thesis (chapter 3) describes the biochemical and the molecular characterization of transmitochondrial cell lines harboring the 15bp deletion in the COX III gene. To investigate the consequences of the COX III mutation on COX function and structure, transmitochondrial cell lines were created by transferring mitochondria from the patient with the COX III mutation into a human cell line devoid of mtDNA. We obtained transmitochondrial cell lines containing 0%, 97% and 100% of the COX III mutation. The creation of cell lines homoplasmic for both wild-type and mutated DNA provided a powerful system for evaluation of the COX III mutation in the presence of a neutral nuclear background. Because all cell lines are derived from the same patient material, there should be only one difference (the mutation) between mtDNA genomes (Davidson and King, 1997).

My results demonstrated a direct correlation between the presence of the 15bp deletion within the mtDNA-encoded COX III gene and a specific and severe deficiency in

COX activity in transmitochondrial cell lines harboring this mutation. Cell lines with 100% mutated mtDNA had no detectable COX activity and no detectable respiratory ATP synthesis. The rate of oxygen consumption, a measurement of entire respiratory chain activity was 5% of wild-type levels and was similar to levels previously measured for ρ^0 cell line 143B206. These cell lines have an absolute requirement for uridine and pyruvate, similar to the growth requirements of ρ^0 cell line 143B206. The respiratory deficiency was less severe in cell lines harboring 97% mutated mtDNA. These cell lines had a 94% reduction in COX activity and an 80% reduction in the rate of oxygen consumption. The 3% wild-type mtDNA in these cell lines was sufficient to restore some COX activity and respiratory function, suggesting that the COX III mutation behaves in a recessive manner.

My studies focused on examining the consequences of this mutation for function and stability of the COX III polypeptide within the enzyme complex. Analysis of mitochondrial protein synthesis revealed that the COX III polypeptide was translated in mutant cell lines at rates comparable to wild-type, but there was a higher turnover of the COX III polypeptide in the mutant cell lines. The 15bp deletion in the COX III gene results in the loss of 5 amino acids (PheAlaGlyPhePhe) from the conserved third transmembrane domain of the polypeptide. The loss of these five amino acids in this region may cause a reduction in the stability of the COX III polypeptide or may prevent the association of COX III with COX I within the enzyme complex, leading to decreased stability of the COX III polypeptide.

Immunoprecipitation experiments were performed to determine the consequences of an unstable mutant COX III polypeptide for assembly of COX catalytic subunits COX I and COX II. There was no evidence of assembly of these COX subunits in the 100% mutant cell lines. Taken together, these data indicated that homoplasmic levels of the COX III deletion resulted in a complete loss of COX activity due to the absence of assembled COX in these cell lines.

Similar immunoprecipitation experiments with the 97% mutant cell line revealed that a relatively high percentage of COX I and COX II polypeptides were assembled in this cell line. The relative amounts of the COX III polypeptide immunoprecipitated were much lower in the 97% mutant cells which suggested that the COX III polypeptide was not always a part of the assembled COX I and COX II complex. In the these cell lines, the assembly of COX I and COX II polypeptides appeared to be promoted by the small percentage of COX III polypeptide in a non-stoichiometric manner. However, most of the assembled COX I and COX II polypeptides in the 97% mutant cell line appeared not to be functional, because COX activity in these cell lines was 6% of the level in wild-type cell lines.

These experiments posed interesting questions concerning the role of COX III in the enzyme complex. Does the COX III polypeptide have a "catalytic" role within the complex by promoting the assembly of COX I and COX II in addition to serving a structural role? Does the COX I and COX II interaction in the 97% cell line represent a stable assembly intermediate? Are the COX nuclear subunits assembled in the enzyme complex in the 97% mutant cell lines? One approach towards addressing some of these questions would be to examine the assembly of the entire enzyme complex in the 97% mutant cell lines. Immunoprecipitations could be performed using antiserum directed against mtDNA encoded COX II and nuclear encoded COX IV of cells labeled in the presence of [^{35}S]-methionine, but in the absence of a cytoplasmic protein synthesis inhibitor. These experiments could indicate whether some of the nuclear subunits are assembled in the holoenzyme in the 97% mutant cell line. Additional immunoprecipitations of pulse labeled polypeptides with longer chase time periods of 12h and 24h, could indicate if COX I and COX II polypeptides are stably or transiently assembled in the 97% mutant cell lines.

One of our long-term goals with the creation of transmitochondrial cell lines with the COX III deletion was to develop a better understanding of the etiology and the

pathogenesis of COX deficiency in this patient. The patient had high levels of mutation in her skeletal muscle (93%) and a severe COX deficiency, COX activity being 14% of normal controls. This reduction in COX activity, however did not appear to limit the energy production in the patient's muscle under normal circumstances (Keightley *et al.*, 1996, see appendix 1). These findings suggest that COX is not the rate limiting step in the respiratory chain and that an earlier step may exert a higher control strength (Taylor *et al.*, 1994; Davey and Clark, 1996). In vitro studies of tRNA mutations A3243G associated with MELAS and A8344G associated with MERRF, revealed that high levels of mutation (> 90%) were required for expression of respiratory deficiencies, and the respiratory defect was expressed in a threshold-dependent manner (Chomyn *et al.*, 1992, Boulet *et al.*, 1992, Yoneda *et al.*, 1994). The COX III mutation also may have a sharp threshold of expression, in which respiratory chain function and ATP synthesis are impaired only in the presence of high levels of the COX III mutation. This is supported by the previous analysis of single muscle fibers from this patient which showed COX deficiency only at high levels of mutation (Keightley *et al.*, 1996, see appendix 1). Creating transmitochondrial cell lines with intermediate levels of mutation would provide an ideal system for examining threshold levels of the COX III mutation for COX activity and overall respiratory function.

Chapter 4 describes the characterization of a T9205C mutation in the mtDNA encoded A6 gene which was identified in the patient with the COX III deletion. The T9205C changed the A6 stop codon to a codon for glutamine and was present in the mtDNA of both the patient and her unaffected mother. It was of interest to study the biochemical phenotype of this mutation to determine if the effects of the COX III mutation were exacerbated by the presence of the A6 mutation. My studies confirmed that the T9205C mutation did not contribute to the defect in ATP synthesis and overall respiratory function in the transmitochondrial cell lines with the COX III mutation.

The mtDNA genome is transcribed as a polycistronic message which then is processed into individual mRNA species. The A6 gene terminates with the first 2 bp of the A6 stop codon and immediately precedes the COX III gene, without intervening nucleotides. The A8/A6 + COX III mRNA polycistronic transcript is usually processed further between the A6 and COX III genes. Although this study did not directly investigate whether the T9205C mutation affected the specificity of mRNA processing at this cleavage site, both fully processed A8/A6 mRNA transcripts and partially processed A8/A6 + COX III transcripts were detected in two wild-type cell lines and four cell lines with the T9205C mutation. The relative steady-state levels of the partially processed transcript ranged between 25% - 55% of total transcript in the wild-type cell lines and the cell lines with the T9205C mutation. One other study has documented the presence of fully processed and partially processed A8/A6 transcripts in transmitochondrial cell lines (Massucci *et al.*, 1995).

In cells with the T9205C mutation, translation of either the processed or partially processed mRNA transcript would result in an aberrant form of A6 polypeptide. Translation of the A8/A6 + COX III partially processed transcript would result in a full length A6 polypeptide with 10 additional amino acids at the carboxyl terminus from the out of frame read through into COX III. Translation of the processed A8/A6 mRNA transcript would extend the A6 protein by approximately 17 lysines, since the reading frame has been extended by polyadenylation. Both aberrant polypeptides have a predicted molecular weight larger than wild-type A6 (26 kD or 27 kD) and the polypeptide with the lysine tail would have a isoelectric point that is dramatically different than that of the wild-type A6 polypeptide. Analysis of mitochondrial protein synthesis revealed that the A6 polypeptide in cell lines with the T9205C mutation displayed a slower electrophoretic mobility on SDS-tricine gels compared to the wild-type A6 polypeptide which was consistent with the molecular weight of either aberrant A6 polypeptides. To determine if one or both types of aberrant A6 polypeptides were translated in cell lines with the A6 mutation, labeled

mitochondrial translation products were analyzed by two-dimensional electrophoresis. These studies indicated that the predominant translation product in cell lines with this mutation was the A6 polypeptide derived from the partially processed A8/A6 + COX III mRNA transcript. There was no evidence of a translation product derived from the processed A8/A6 mRNA transcript. It is possible that the A6 polypeptide with the lysine tail is translated in cell lines with the T9205C mutation, but this aberrant polypeptide may be unstable and degraded due to the considerable charge difference at the carboxyl terminus. Future experiments involving two-dimensional analysis of cells labeled for short periods of time in the presence of ^{35}S[lysine] or ^{14}C[lysine] may help determine if the A6 polypeptide with the lysine tail is synthesized in these cell lines.

My thesis work has examined the cellular and molecular consequences of two mutations in mtDNA encoded COX III and A6 genes. These studies have increased our understanding of the etiology and the pathogenesis of COX deficiency in this patient as well as the function of the COX III polypeptide in the enzyme complex. These transmitochondrial cell lines will be important for future studies examining the biogenesis and regulation of COX.

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A microdeletion in cytochrome c oxidase (COX) subunit III associated with COX deficiency and recurrent myoglobinuria

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We have identified a 15-bp microdeletion in a highly conserved region of the mitochondrially encoded gene for cytochrome c oxidase (COX) subunit III in a patient with severe isolated COX deficiency and recurrent myoglobinuria. The mutant mitochondrial DNA (mtDNA) comprised 92% of the mtDNA in muscle and 0.7% in leukocytes. Immunoblots and immunocytochemistry suggested a lack of assembly or instability of the complex. Microdissected muscle fibres revealed significantly higher proportions of mutant mtDNA in COX-negative than in COX-positive fibres. This represents the first case of isolated COX deficiency to be defined at the molecular level.

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The mitochondrial myopathies and encephalomyopathies comprise a wide and emerging spectrum of diseases[1,2]. Mitochondrial DNA (mtDNA) is a circular, double-stranded molecule of 16,569 nucleotide (nt) pairs that comprise two rRNA, 22 tRNA and 13 structural genes encoding subunits of respiratory complexes I, III, IV (cytochrome c oxidase; COX) and V (ATP synthase). Several well defined syndromes are associated with a variety of mutations of mtDNA. Disorders of mtDNA can be classified as a) large rearrangements (deletions and/or duplications), b) tRNA mutations, c) rRNA mutations or d) missense mutations in the protein coding genes[3,4]. The major rearrangements of mtDNA are frequently associated with progressive external ophthalmoplegia (PEO), Pearson marrow-pancreas syndrome or the multisystem disorder known as Kearns-Sayre syndrome[5]. Transfer RNA mutations have been described in isolated myopathy and/or cardiomyopathy and in several multisystem diseases, such as MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) or MERRF (myoclonus epilepsy and ragged red fibres), frequently associated with a point mutation in the $tRNA^{Leu(UUR)}$ or $tRNA^{Lys}$ gene, respectively[6,7]. Several missense mutations are associated with Leber's hereditary optic neuropathy (LHON)[8] and one, in the ATPase 6 gene, presents as a multisystem mitochondrial disease with variable expression[9]. Although the major rearrangements are usually sporadic, most disorders of mtDNA are maternally inherited, reflecting the maternal origin of this genome[10]. Mutant mtDNA molecules usually co-exist with wild-type mtDNA, a phenomenon known as heteroplasmy, with proportions that can vary widely in different tissues, presumably accounting for some of the clinical variability and apparent tissue specificity of these disorders. Proliferation of mitochondria containing mutant mtDNAs gives rise to the morphological hallmark of these diseases, the ragged red fibre (RRF), usually associated with deletion/duplication[11] or tRNA mutations[12], but not with the missense mutations[8,9]. Although a generalized defect in mitochondrial protein synthesis has been demonstrated in both tRNA mutations[13–16] and deletions[17], the individual respiratory chain activities have frequently shown poor correlation with the specific molecular defect.

Cytochrome c oxidase, the terminal component of the mitochondrial respiratory chain, transfers electrons from reduced cytochrome c to molecular oxygen and conserves the energy as an electrochemical proton gradient across the inner membrane[18]. COX deficiency[1,2,19,20] has been associated with a variety of syndromes including fatal infantile myopathy with or without renal Fanconi syndrome, subacute necrotizing encephalomyelopathy (Leigh syndrome)[21], myopathy with predominant cardiomyopathy[22] and benign reversible COX deficiency[23]. Most of these disorders appear to be autosomal recessive, but no molecular abnormality has been identified. COX deficiency has also been found in disorders of mtDNA, particularly those with mutations of tRNA genes or large deletions which include tRNA genes. In such cases, it is frequently associated with other respiratory chain deficiencies, reflecting a generalized reduction of mitochondrial protein synthesis.

In disorders of mtDNA, the myopathy can manifest

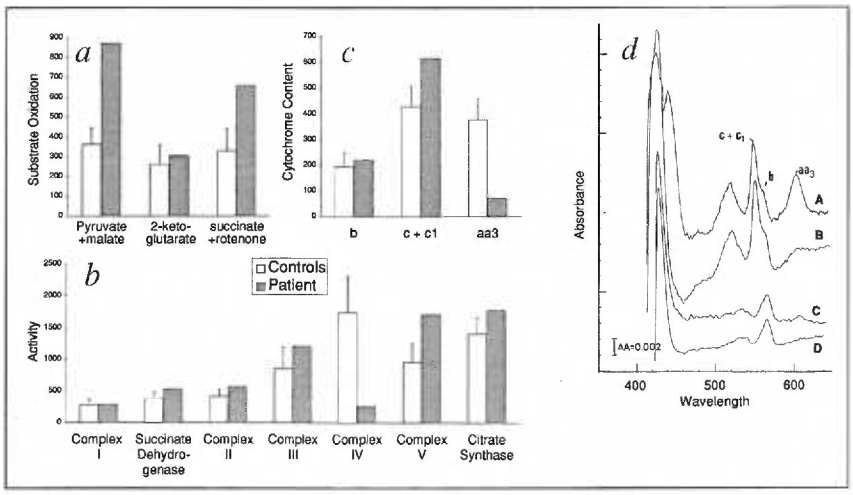

Fig. 1 Characterization of COX deficiency. Mitochondria isolated from skeletal muscle were used to assess respiratory chain function. *a*, Substrate oxidation (nmol ferricyanide reduced/min per mg protein). Error bars, 1 SD; $n = 5$. *b*, Individual respiratory chain activities and citrate synthase expressed as nmol/min per mg protein with the exception of complex III and complex IV (expressed as apparent first order rate constant ×10/min per mg protein). Error bars, 1 SD; $n = 20$. Complex IV (COX) activity was >2 SD below the mean control value. *c*, *d*, Reduced-oxidized spectra of cytochromes (pmol/mg protein; controls, $n = 12$) following reduction with succinate in the presence of cyanide (cytochromes aa_3 and $c + c_1$; curves A and B) or succinate in the presence of antimycin A (cytochrome b; curves C and D). Curves A and C represent a control; curves B and D, the patient.

as muscle weakness, hypotonia, fatigue or exercise intolerance, but rarely with myoglobinuria. We now report a patient with recurrent myoglobinuria, mitochondrial myopathy, a severe and specific deficiency of COX activity in muscle mitochondria and a novel mutation in the gene for COX subunit III (*COIII*). Preliminary reports have been published[24,25].

Clinical description

Two episodes of muscle cramps and myoglobinuria, the first precipitated by a viral illness, the second by prolonged exercise, prompted investigation in a previously normal 15-year-old white female. Both episodes were associated with decreased caloric intake. Two further episodes have since occurred during which creatine kinase (CK) levels rose to 17,000–44,000 I.U. (normal < 170 I.U.). No history of myalgia, seizures or encephalopathy, although mildly reduced endurance was reported. Family history was negative for neuromuscular disease.

Bicycle ergometry[26] at 16 yr showed a normal anaerobic threshold but high initial respiratory quotient (RQ) of 0.90, without excessive end-exercise RQ (1.24), and below normal maximal oxygen uptake (27 ml/kg/min; predicted value 37 ml/kg/min). Magnetic resonance spectroscopy of resting muscle[27] showed a Pi/PCr ratio of 0.081 (normal 0.075–0.155) and pH 7.06 (normal 7.03–7.11). On exercise, she showed mild acidification and normal bioenergetic efficiency. Other clinical and laboratory evaluations were normal (see Methods).

Muscle histochemistry showed type 1 fibre predominance, many RRF, which stained heavily for succinate dehydrogenase (SDH) activity, and a high proportion (64%) of COX negative fibres (see later). On EM, scattered areas revealed increased numbers of mitochondria, some enlarged and irregular, with disordered cristae (see Methods).

Characterization of COX deficiency

We first measured the oxidation of several substrates in freshly isolated skeletal muscle mitochondria. These measurements were coupled to the reduction of ferricyanide, which accepts electrons from reduced cytochrome c, and were therefore not dependent on COX activity. The oxidation of pyruvate + malate, 2-ketoglutarate, and succinate was normal or slightly elevated (Fig. 1*a*), ruling out a severe deficiency of respiratory complexes I, II or III. Indeed, COX activity at 14% of normal was the only respiratory chain activity to be deficient (Fig. 1*b*). Cytochrome aa_3 was also markedly reduced whereas cytochromes b and $c + c_1$ were both above the mean control value (Figs 1*c* and *d*). These data confirm the severe COX deficiency seen on muscle histochemistry.

Since there are 13 subunits in mammalian COX, three of which are encoded on mtDNA (subunits I, II and III), the subunit composition of COX was analysed by western blot analysis and by immunocytochemistry. The immunoblot of COX (Fig. 2) showed a severe deficiency of subunits II, Vb, VIb and VIc in muscle mitochondria from the patient, with a milder deficiency of subunits IV and Va. When the same blot in Fig. 2*a* was re-probed with antibody to core 2 of complex III, elevated levels were seen in the patient, consistent with the higher than average complex III activity. Immunoblotting of complex I (not shown) was normal.

The cellular distribution of the COX deficiency was examined in serial sections of skeletal muscle (Fig. 3).

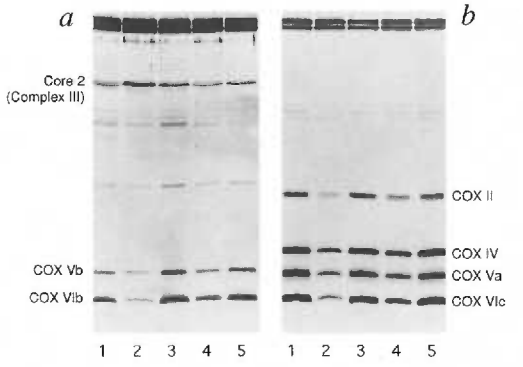

Fig. 2 Immunoblot of skeletal muscle mitochondria demonstrating deficiency of multiple subunits of COX in the patient. Lanes 1, 3 and 5, controls; lane 2, patient; lane 4, patient with ocular myopathy and mtDNA deletion. Mitochondrial proteins (100 μg) were separated by SDS 10–18% PAGE, blotted onto nitrocellulose and incubated with either *a*, a mixture of monoclonal antibodies to COX subunits Vb and VIb followed by a polyclonal antibody to core protein 2 of complex III or *b*, a mixture of monoclonal antibodies to COX subunits IV, Va and VIc followed by a polyclonal antibody to the C-terminal peptide of COX subunit II.

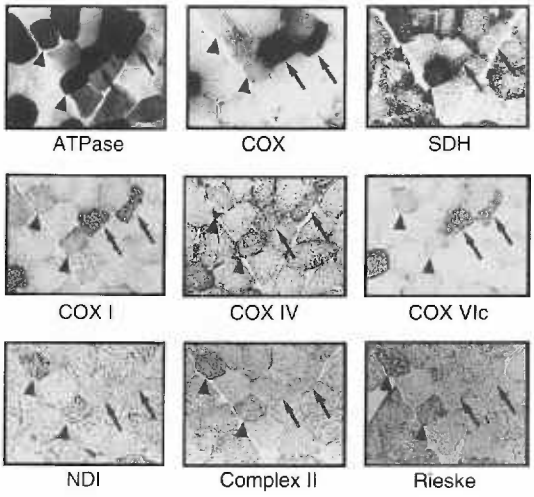

Fig. 3 Histochemistry and immunocytochemistry of skeletal muscle from the patient. Serial sections were stained for SDH, COX and ATPase (dark fibres are type 1, intermediate fibres type 2B and light fibres type 2A), and immunostained with monoclonal antibodies to COX subunits I, IV and VIc and polyclonal antibodies to ND1 of complex I, holo complex II and the Rieske protein of complex III. Two COX-negative RRF are indicated by arrowheads and two COX-positive fibres by arrows; all are type I.

Many RRF were present, (approximately 12% of the total), manifesting high SDH activity. The RRF were all COX-negative and were of both fibre types, indicating that the mosaic distribution of COX deficiency was not related to fibre type. COX-positive fibres showed strong reactivity with antibodies to COX subunits I and VIc whereas COX-negative fibres had little or no reactivity with these antibodies, indicating that the deficiencies of specific COX subunits correlated with the COX activity in individual fibres. COX-negative fibres stained positively, although less intensely than normal, with antibodies to COX subunit IV, confirming the less severe deficiency of subunit IV previously seen on immunoblotting (Fig. 2). Immunodetection of subunit I of complex I (ND1), holo complex II and the Rieske protein of complex III was good in all fibres and, in fact, high in RRF, consistent with the normal activities of complexes I, II and III and the immunoblot experiments. All of the above data indicated a severe and specific deficiency of COX in the patient's muscle, and the mosaic pattern of COX activity suggested a primary abnormality of one of the mitochondrial-encoded subunits of this enzyme.

Identification of a mutation in the *COIII* gene

Initially, we ruled out the presence of a large deletion of mtDNA in muscle by Southern analysis. Cycle sequencing of the $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ genes was also normal, ruling out the common mutations associated with MELAS and MERRF syndromes, respectively. However, PCR amplification and digestion of a portion of *COIII* identified a 658-bp *Msp*I fragment spanning nt 9293–9950 (numbers are from ref. 28), which migrated slightly faster than its normal counterpart. Further restriction analysis, followed by cloning and sequencing, revealed a 15-bp deletion in *COIII* (Fig. 4*a*, *b*). This in-frame deletion removes five amino acids from the third hydrophobic domain of COX subunit III and includes two highly conserved phenylalanine residues[29] (Fig. 4*c*). To determine if this deletion was heteroplasmic, and to quantitate the percentage of mutant mtDNA, we designed a PCR assay which generated an 85-bp (wild type) or 70-bp (mutant) fragment spanning the deletion breakpoints; we quantitated the products following non-denaturing polyacrylamide gel electrophoresis. The mutant mtDNA comprised 92% of total mtDNA in the patient's skeletal muscle and approximately 0.7% in her leukocytes (Fig. 5). It was not detectable in cultured skin fibroblasts but was present in low amounts (approximately 0.5%) in EBV-transformed lymphocytes (data not shown). The mutation was not detectable in her mother's leukocytes, in muscle from 30 individuals with a variety of muscle disorders, or in leukocytes from 71 controls. The possibility of heterogeneity at the deletion breakpoints was ruled out by denaturing PAGE of the radioactive PCR product, which revealed only the 70-bp (mutant) and 85-bp (wild type) bands.

To rule out the possibility of another mutation cosegregating with the 15-bp deletion, the genes for *COI*, *COII* and *COIII* were sequenced. No differences from the "Cambridge" sequence[28] were detected in either

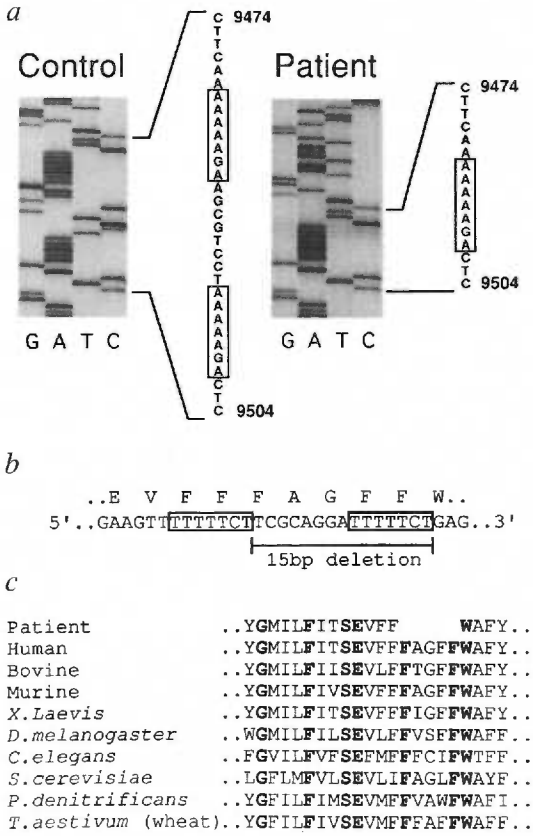

Fig. 4 Characterization of 15-bp deletion in *COIII*. *a*, Autoradiograph of DNA sequencing gel showing control (patient's mother) and mutant (patient) H-strand sequence in region of deletion. The sequence reads 5' to 3' from bottom to top and includes nt 9474–9504 (Cambridge sequence)[28]. Two 7-bp direct repeats are boxed. *b*, L-strand sequence, reading 5' to 3' from nt 9474, with deduced amino acid sequence (one letter code) showing deletion in the patient. *c*, Alignment of amino acid sequences in the third hydrophobic domain of COX subunit III, showing high degree of conservation (bold letters). Deletion in patient is indicated by gap.

the *COI* or *COII* gene. A T→C transition was identified at nt 9836 in mtDNA from the patient's muscle, but not in her mother's leukocytes. This transition within *COIII* does not change the resulting amino acid and is therefore, presumably, a neutral polymorphism.

Two other differences from the "Cambridge" sequence were identified: a G→A transition at nt 8387 within the ATPase 8 gene, resulting in substitution of a methionine for valine; and a T→C transition at nt 9205 in the ATPase 6 gene which changes the stop codon to a glutamine. Both of these changes appeared to be homoplasmic in the patient's muscle and in her mother's leukocytes, and therefore, presumably, are not related to the disease phenotype.

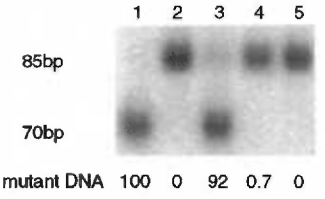

Fig. 5 Quantitation of mutant and wild-type mtDNA in affected (muscle) and unaffected (leukocytes) tissues. MtDNA was amplified by PCR, labelled with ^{32}P-dCTP in the last cycle, separated on a 12% polyacrylamide gel and quantitated by phosphorimaging. Lanes 1, mutant clone; 2, control leukocytes; 3, patient's muscle; 4, patient's leukocytes; 5, mother's leukocytes.

Correlation of mutant genotype with phenotype

To establish that the 15-bp deletion in *COIII* was responsible for the phenotype of COX deficiency, we determined the proportion of normal and mutant mtDNA in microdissected single muscle fibres, utilizing quantitative PCR and gel electrophoresis. COX-positive fibres contained low to moderately high proportions of mutant mtDNA (25.2 ± 25.1%) (Fig. 6). In contrast, COX-negative non-ragged red fibres contained very high proportions (97.4 ± 1.4%) while COX-negative RRF had even higher proportions (98.6 ± 0.8%). The difference between COX-negative and COX-positive fibres was highly significant ($P < 0.001$) and established that this microdeletion was responsible for the deficient COX activity.

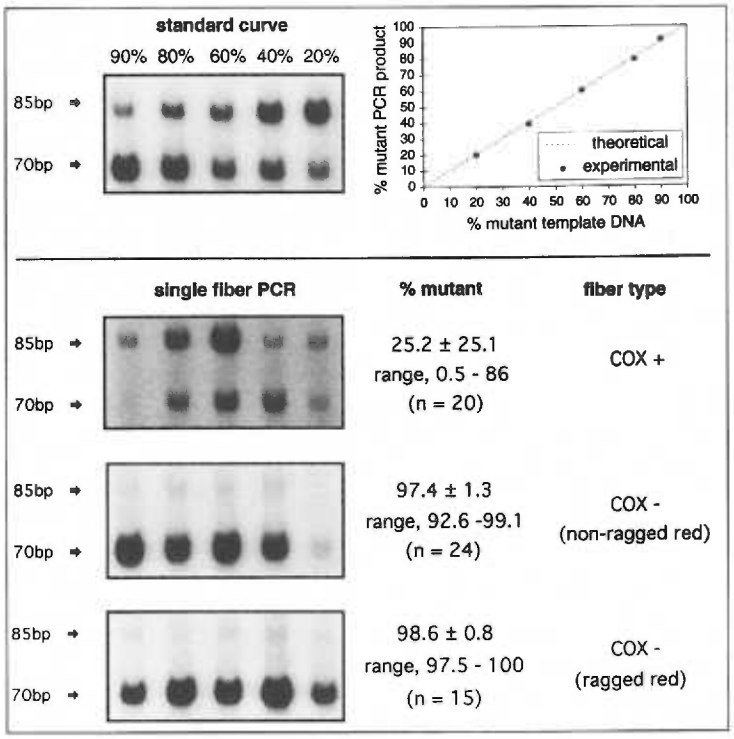

Fig. 6 Correlation of genotype (% mutant *COIII*) with phenotype (COX activity) in single microdissected muscle fibres. Fibres from COX-stained tissue sections were amplified by PCR, with incorporation of ^{32}P-dCTP, separated on a 12% polyacrylamide gel and quantitated by phosphorimaging. The top panels show a standard curve obtained from pure plasmid mixes containing 20, 40, 60, 80 and 90% mutant DNA. The percentage (mean ±SD with range below) of mutant DNA in COX positive (normal phenotype), COX negative non-ragged red (mutant phenotype) and COX negative ragged red (mutant phenotype) muscle fibres is shown.

Discussion

The patient in our study was shown to have a mitochondrial myopathy, based on the presence of structurally abnormal mitochondria, RRF with high SDH activity and, in particular, the mosaic pattern of COX-positive and -negative muscle fibres. Similar findings have been seen in patients with large deletions of mtDNA[11,30] or point mutations of tRNA genes[12,16,31,32] frequently resulting in low activities of one or more of the respiratory chain complexes containing mitochondrially encoded subunits (complexes I, III, IV and V). However, several lines of evidence suggested that our patient had a primary abnormality of one of the three mitochondrially encoded subunits of COX. First, the deficiency of COX was severe, as manifest by the high percentage of COX-negative fibres (64%), low COX activity in muscle mitochondria (14% of normal), and low levels of cytochrome aa_3 (19% of normal). Second, the COX deficiency was specific, all other respiratory chain activities being normal or high. This was consistent with the normal levels of cytochromes b and $c+c_1$, and the normal or high oxidation of all substrates which, when coupled to ferricyanide reduction, does not depend on COX activity.

Immunoblot analyses and immunocytochemistry confirmed the specificity of the COX deficiency. In particular, normal immunoreactivity of the mitochondrially encoded subunit 1 of complex I (ND1) distinguished our patient from those with a generalized defect of mitochondrial protein synthesis, secondary to mutations or deletions of tRNA genes[11,12,16]. Furthermore, the profile of complex I subunits was normal, the Rieske protein was highly immunoreactive, and we observed a slightly elevated level of core 2 of complex III, both consistent with the higher than average complex III activity. In contrast, all detectable subunits of COX were reduced.

A 15-bp microdeletion in *COIII* fulfills the criteria for the pathogenic mutation in this patient in the following respects. 1) The mutation is heteroplasmic and present in high proportion in the affected tissue (muscle), but not in other tissues. 2) It has not been identified in control subjects. 3) No other mutations that are likely to be pathogenic were detected in the entire coding sequences of *COI*, *COII* or *COIII*. 4) There is a genotypic to phenotypic correlation in individual muscle fibres, COX-negative fibres having a higher proportion of mutant mtDNA than COX-positive fibres. 5) The mutation causes the loss of five amino acids from a highly conserved transmembrane segment of the polypeptide[29], and would be predicted to affect the function, assembly or stability of COX.

Only a few specific mutations of the COX genes have been described. These include: a termination codon mutation, which also occurred in controls[33], and a heteroplas-

mic mutation (T6681C)[34], both in *COI*; a mutation in *COII* in 2 sibs who also carried a $tRNA^{Asp}$ mutation which is more likely to be pathogenic[35]; two missense mutations in *COIII* (G9438A or G9804A), each occurring in several LHON families[36]; and a heteroplasmic mutation (T9957C) in *COIII* in a patient with MELAS[37]. None of these reports had any documentation of COX deficiency or evidence that the COX mutation was of major pathogenic significance. Thus, our patient represents the first documentation of a primary abnormality in any of the mitochondrially encoded subunits, or indeed of any subunit of human COX, associated with COX deficiency and manifesting as a mitochondrial myopathy.

The large deletions of mtDNA seen in PEO or Kearns-Sayre syndrome are almost always flanked by direct repeats, suggesting that they have arisen by a mechanism of homologous recombination or slipped mispairing during replication[38,39]. The occurrence of 7-bp direct repeats at the boundaries of the deletion in our patient suggests that a similar mechanism may have generated the microdeletion described here. The high percentage of mutant DNA in muscle, compared to leukocytes, suggests that the 15-bp deletion may have undergone negative selection in rapidly dividing tissues. In Kearns-Sayre syndrome, the percentage of deleted mtDNA appears to increase over time, suggesting that the smaller molecule may have a replicative advantage[40]. This is less likely to be so in our patient, because of the small size of the deletion, and the mechanism leading to the high percentage of mutant mtDNA in muscle remains unclear. However, detection of the mutation in leukocytes, although at very low levels, indicates that it originated either during oogenesis in her mother or during embryogenesis, before differentiation of the haematopoietic and muscle precursor germ lines.

Mammalian COX is made up of 13 subunits, the three largest corresponding to the three subunits of the bacterial enzyme that perform the catalytic and proton pumping activities of the complex. Although subunit III does not contain any of the redox active metal centres, recent studies of mutants of *P. denitrificans* suggest that it may play a role in energy conversion[41] as well as assembly of the complex[42]. In our patient, the 15-bp deletion would be expected to affect stability, function and/or assembly of the *COIII* polypeptide rather than translation. When mitochondria containing the *COIII* deletion were transferred to rho^0 cells (cells depleted of mtDNA), the rate of translation was comparable to wild-type cells[43], indicating that translation of COX subunit III was not impaired. Further studies are being conducted to determine if stability or assembly of the complex is compromised by the deletion. These data are consistent with the western blot analysis, which showed moderate to severe deficiency of all detectable subunits of COX. Markedly reduced levels of subunits I and VIc were also apparent by immunocytochemistry in COX-negative fibres, the combined data indicating that most unassembled subunits of COX were rapidly degraded, whether of mitochondrial or nuclear origin. The less severe reduction of subunit IV suggests that it is less susceptible to degradation. Normal immunoreactivity of subunit IV in COX-negative fibres of patients with large deletions of mtDNA[44] or mutations of tRNA genes[16] had previously suggested that unassembled subunits of COX may not be rapidly degraded. Our data indicate subunit IV may not be a sensitive indicator of such an effect.

Recurrent episodes of muscle cramps and myoglobinuria are frequently associated with disorders of glycogen or glucose metabolism, or carnitine palmityltransferase deficiency[45], none of which our patient has. Episodic myoglobinuria is rare in patients with mitochondrial myopathy[46–49]. Our findings suggest that respiratory chain defects should be considered in the differential diagnosis of recurrent myoglobinuria.

In view of the high percentage of mutant mtDNA in our patient's muscle, and the severity of the COX deficiency, it is surprising that she does not manifest a more severe phenotype. This may be explained in part by the observation that the morphological abnormalities (RRF) and biochemical phenotype (COX-negative fibres) occurred only at very high proportions of mutant mtDNA (>92% and >97%, respectively), and the fact that the mutant phenotype could be "rescued" by a relatively small percentage (8–14%) of normal mtDNA, as seen in COX-positive fibres. This phenomenon often occurs in single muscle fibres or in cultured cells from patients with one of several mitochondrial tRNA mutations[12–16]. Our data also indicate that reduction of COX activity to 14% of normal does not limit energy production in the patient's muscle, under normal circumstances, consistent with the ^{31}P-magnetic resonance spectroscopy, which showed normal bioenergetic function both at rest and with exercise, although this was not continued to exhaustion. Interestingly, she did manifest slightly low maximum oxygen consumption following bicycle ergometry, suggesting some deficiency of energy production. Our data also imply that COX activity may be present in normal muscle in considerable excess and that an earlier step of the respiratory chain exerts a higher control strength[50] on the overall activity. This is supported by studies in rat muscle mitochondria, treated with cyanide, in which respiration remained nearly maximal until COX activity was reduced by about 80%[51].

The association of RRF with a mutation in *COIII* contravenes the previous proposal that mitochondrial proliferation is a consequence of a generalized defect of mitochondrial protein synthesis, as seen in patients with mutations of tRNA genes or large deletions of mtDNA which include tRNA genes[11,12] but not in patients with mutations of protein-encoding genes such as *ND1, ND4* or cytochrome b (associated with LHON)[8] or ATPase 6 (associated with a multisystem disease)[9]. Our data suggest that a severe defect in the function of a specific protein of the respiratory chain may be sufficient to trigger this proliferative response. Although, in all of the above cases, the mitochondrial proliferation could reflect a severe defect of COX activity *per se,* it seems more likely that it results from a severe impairment of respiratory chain function, regardless of the cause. The identification and detailed analyses of other patients with unique abnormalities of mtDNA will help to answer these questions and eventually clarify the mechanisms of pathogenesis of mitochondrial diseases.

Methods

Patient. Developmental and medical history were unremarkable. At 15 yr, neurological and general physical examination (ht 63"; wt 112 lb) were normal and school grades were above average. Between episodes of myoglobinuria, described in the

text, CBC, routine blood chemistry, CK, resting blood lactate, plasma carnitine and urine organic acids were normal. Brainstem auditory evoked responses, electrocardiogram, ophthalmological examination and electroretinography, and forearm ischaemic lactate test were also normal. The patient's mother (44 yr) and four siblings (14–23 yr) are healthy.

Apart from the typical findings of a mitochondrial myopathy described earlier, the muscle biopsy at 16 yr showed normal levels of glycogen, phosphorylase a and b, phosphofructokinase, phosphoglycerate kinase, phosphoglycerate mutase and lactate dehydrogenase, and intermediate levels of myoadenylate deaminase (14 μmol/min per g; normal 29–35 μmol/min per g). Carnitine palmityltransferase activity was slightly elevated (125 μmol/min per g; normal 76 ± 16 μmol/min per g).

Measurement of substrate oxidation, respiratory chain activities and cytochrome content. Control muscle was obtained, with permission, from individuals who died of trauma, patients having orthopedic surgery or patients undergoing diagnostic muscle biopsies in whom there was no evidence of mitochondrial dysfunction. Mitochondria were isolated from fresh skeletal muscle as described[52]. Substrate oxidations, coupled to ferricyanide reduction, were recorded at 420 nm on a Beckman DU-7 spectrophotometer as described[53]. Measurements were made of respiratory chain activities and citrate synthase[49] and ATPase[54]. Reduced-oxidized spectra were obtained as described[55], following reduction by 10 mM succinate in the presence of either 1 mM KCN (cytochromes aa_3 and $c + c_1$) or antimycin A (6.7 μg/ml) (cytochrome b).

Immunoblot analysis of COX subunits. This was performed as described[49]. Bound monoclonal antibodies were visualized with biotinylated horse anti-mouse IgG, and polyclonal antibodies with biotinylated protein A, followed by avidin-linked alkaline phosphatase reaction with NBT/BCIP colour development.

Histochemistry and immunocytochemistry. Frozen muscle biopsy sections (6–8 μm) were processed as described[56]. In fixed frozen muscle sections, the COX II antibodies only stained mitochondria, and in western blots of mitochondrial preparations they recognized only subunit II of COX[57].

Polyclonal and monoclonal antibodies. Polyclonal antibodies against the C-terminal peptides of COX subunit II and ND1 of complex I were kindly provided by R.F. Doolittle, University of California, San Diego, CA. Polyclonal antibodies against holo complex II and the Rieske protein of complex III were kindly provided by B. Ackrell, University of California, San Francisco, CA, and L. Yu, Oklahoma State University, OK, respectively. Polyclonal antibodies specific for core 2 of complex III and mouse monoclonal antibodies against subunits I, IV and VIc of COX were generously provided by R. Capaldi, University of Oregon, OR.

Sequencing of cloned PCR product. Genomic DNA was isolated from skeletal muscle by standard protocols and amplified by PCR to generate a 1,998-bp PCR fragment spanning nt 7955–9952 (ref. 58). A *BanII* restriction fragment encompassing nt 9265–9643 was cloned into the phagemid vector Bluescript II (Stratagene) and sequenced in both directions (Sequenase, USB).

Amplification and direct sequencing of mtDNA. PCR fragments of 1.4–3.0 kb, generated from genomic DNA, were purified using Qiaex gel extraction resin (Qiagen) and sequenced directly using a combination of automated sequencing (Applied Biosystems, Columbia University Sequencing Facility) and cycle sequencing (New England Biolabs) with ^{32}P labelled primer. Sequencing reactions were electrophoresed through 6% polyacrylamide 8 M urea gels, dried and exposed to X-ray film for 24–48 h. A total of 3,800 bp of mtDNA was sequenced with primers spaced approximately 300 bp apart. The boundries of sequence regions were nt 3150–3332, 5903–8390 and 8700–10060.

Quantitation of mutant and wild-type PCR products. DNA was isolated from skeletal muscle as described above and from leukocytes, EBV-transformed lymphocytes and fibroblasts using Puregene (Gentra Systems Inc.). MtDNA was amplified by PCR using 10 pmoles of forward and reverse primer (nt 9440–9463 and nt 9525–9502) in a 20 μl reaction volume containing 10 mM TrisCl, pH 8.3, 50 mM KCl, 1.5 mM $MgSO_4$, 160 μM dNTPs and modified *Taq* polymerase. The reactions were cycled 30 times for 1 min at 94 °C, 30 s at 60 °C and 30 s at 72 °C followed by a 10 min extension step. Last cycle labelling was performed with addition of 0.2 μCi of [α^{32}P]dCTP. Templates for the standard curve consisted of purified plasmid clones containing mutant and normal DNA. PCR reactions were electrophoresed through a non-denaturing 10% polyacrylamide gel, dried and quantitated using the Molecular Dynamics phosphorimaging system. Analysis was performed using the software IPLab Gel-1.5 g (Signal Analytics Corp.), subtracting local background prior to quantitation. Genomic DNA from controls was amplified by PCR as described but with omission of the last hot step cycle. PCR fragments were electrophoresed and visualized by ethiduim bromide staining.

Homogeneity of the deletion breakpoints. To verify the homogeneity of the deletion breakpoints, the forward primer was labelled with [γ^{32}P]ATP (New England Nuclear), using T4 polynucleotide kinase, and subjected to PCR under the conditions described above. Denaturing (50% urea) polyacrylamide gel electrophoresis of the resulting products showed only the mutant 70-bp and normal 85-bp bands (data not shown).

Analysis of microdissected muscle fibres. Individually microdissected muscle fibres from 40 μm COX stained sections of patient muscle were transferred to PCR tubes containing 20 μl H_2O, and stored at –20 °C. Just prior to amplification, the fibres were dried under vacuum, resuspended in 10 μl H_2O, heated to 96 °C for 20 min, chilled on ice for 20 min, and centrifuged for 1 min at 12,000 rpm. Plasmid mixes for the standard curve were diluted to provide approximately 5×10^{-5} fmoles of DNA, which we estimated to be in the range of the muscle fibres. PCR was performed as described above except that 2.67 μCi of [α^{32}P]dCTP was included throughout 25 cycles and cold dNTPs (30 μM dCTP and 160 μM dATP, dTTP and dGTP) were withheld on top of a wax cap for 'hotstart PCR'. The experimental curve matched the theoretical within experimental limits (Fig. 6).

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