Genetic defects in patients with
mitochondrial encephalomyopathies

Cover: Extreme darkfield imaging. Dynamic view of mitochondria. Left panel shows a healthy COS-7 cell with the mitochondria very elongated and extending far into the cell periphery. Right panel shows a stressed cell with the mitochondria, showing up pinkish and densely packed around the nucleus after labelling with mitotracker (Photography: Katharina Draegestein and Niels Galjart, Department of Cell Biology and Genetics, Erasmus MC, Rotterdam).

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Genetic defects in patients with mitochondrial encephalomyopathies

Genetische afwijkingen bij patiënten met mitochondriële encephalomyopathieën

Proefschrift

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Voor mijn patiënten en hun families

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List of abbreviations

cDNA	Copy DNA (deoxyribonucleic acid)
CNS	Central nervous system
COX	Cytochrome c oxidase (complex IV)
CPEO	Chronic progressive external ophthalmoplegia
CSF	Cerebrospinal fluid
Cyt b_L	Large cytochrome *b*
Cyt b_S	Small cytochrome *b*
DGGE	Denaturing gradient gel electrophoresis
D-HPLC	Denaturing high performance liquid chromatography
ETC	Electron transport chain
HMSN	Hereditary motor and sensory neuropathy
KSS	Kearns-Sayre syndrome
LHON	Leber's hereditary optic neuropathy
MC	Mitochondrial cytopathy
MDS	mtDNA depletion syndrome
MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged red fibres
MIDD	Maternally inherited diabetes and deafness
MiMyCa	Maternally inherited mitochondrial myopathy and cardiomyopathy
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
mtDNA	Mitochondrial deoxyribonucleic acid
mtETC	Mitochondrial electron transport chain
NARP	Neuropathy, ataxia, retinitis pigmentosa
np	Nucleotide pair
OXPHOS	Oxidative phosphorylation
PEO	Progressive external ophthalmoplegia
PGL	Hereditary paraganglioma
Pi	Inorganic orthophosphate
RRF	Ragged red fibre
SSCP	Single stranded conformation analysis

CHAPTER 1

Introduction

Living cells require the continuous input of free energy for many biochemical processes, such as the activation of metabolites, biosynthesis of macromolecules, motion, active transport of ions and molecules, repair processes, heat generation and signal transduction and amplification. The adenosine triphosphate (ATP)-adenosine diphosphate (ADP) cycle is the main mode of energy exchange in biological systems. ATP molecules exported out of the mitochondria provide the energy for the biological work. The energy is made available by the hydrolysis of ATP into ADP and inorganic phosphate (P_i), or into AMP and pyrophosphate (PP_i), the latter molecules are converted into ADP and P_i by the action of adenylate kinase and pyrophosphatase, respectively. Cells maintain a high level of ATP by oxidation of substrates like glucose and fatty acids. In oxygen consuming (aerobic) cells most of the oxidation energy is transferred in the form of reducing equivalents (H^+ or electrons) by NADH and FADH to the mitochondrial electron transport chain or respiratory chain. In this chain the energy is converted into an electrochemical potential ($\Delta\mu_H$) across the inner mitochondrial membrane and the electrons are ultimately donated to oxygen with the formation of water. The electrochemical energy is then converted to chemical energy again by driving the conden-

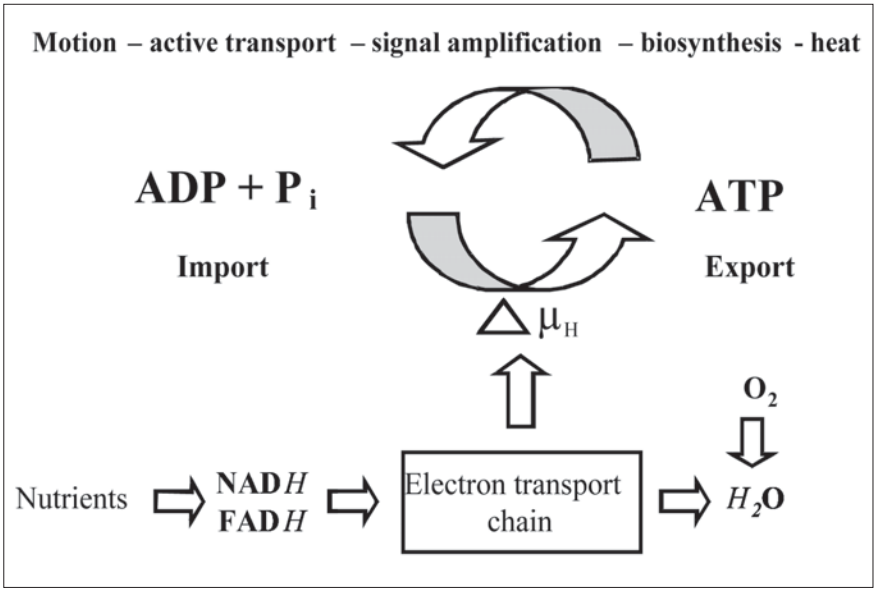


Fig. 1. The bare essentials of aerobic energy metabolism. The critical transfer of hydrogen to oxygen to give water is indicated in italics. See text above for further explanation.

sation of ADP and P_i to ATP. For this latter reaction to occur the ADP and P_i have to be imported from the cytosol into the mitochondria. This whole process of energy transduction is named oxidative phosphorylation (see Fig. 1).

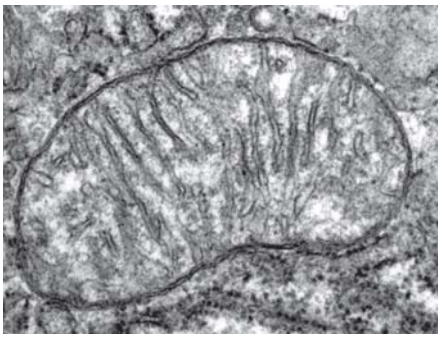
Strong selective pressure has resulted in versatile systems to meet the highly variable energy requirements under various internal and external conditions. It is therefore not surprising that energy metabolism appears to be a highly specialized, regulated and directed cell characteristic, in which multiple complex enzyme systems cooperate. Many of the basic components of energy metabolism have been studied in great detail, but there remains still much to be learnt, in particular how the energy metabolism is regulated. The biochemical complexity of energy metabolism has also a complex genetic counterpart. The proteins constituting the electron transport chain are coded for by genes in the nuclear genome as well as by genes in the mitochondrial genome. In this thesis methods are described to analyse the mitochondrial genes and some of the nuclear genes involved in oxidative phosphorylation. Selected patients with neuromuscular disorders were screened for mutations in these genes to find the genetic cause for their disease.

STRUCTURE AND FUNCTION OF MITOCHONDRIA

It was thought previously that biological tissues consume oxygen uniformly throughout the cell. At the time of World War II, studies of isolated mitochondria demonstrated that this was not the case and that most oxygen was taken up by the mitochondria. Mitochondria are about 3 μm long and 0.5-1 μm in diameter and are surrounded by a double membrane structure (see Fig. 2). The mitochondrial shape may vary from these classic bean-shaped organelles to extended and dynamic reticular networks in specialised cells. All mitochondria contain mtDNA coding for a limited number of mtRNAs and proteins essential for formation of a functional mitochondrion. In most species mitochondrial DNA is circular (Nosek et al., 1998) and multiple copies are found per mitochondrion. The part of the mitochondrion located between the two membranes is called the intermembrane space and the region surrounded by the inner membrane, the matrix (Yaffe, 1999).

Mitochondria are a prominent cell characteristic and cells may contain hundreds to thousands of these organelles occupying up to 30% of the cell volume. Hundreds of mitochondrial proteins that are involved in numerous

biosynthetic and degradative reactions fundamental to cell function have been discovered over the years. These activities depend on a distinctive mitochondrial structure, with the different proteins localized in transmembrane position, at the inner or outer face of the inner or outer membrane, or in the aqueous compartments.



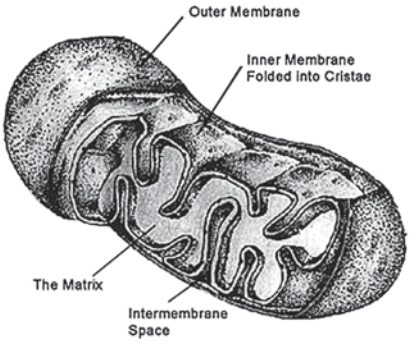


Fig. 2A (left). Electron micrograph of a typical mitochondrion. Human lymph node, neck region (magnification 32,000 x). (By courtesy of Dr. J.M. Kros, Josephine Nefkens Institute, Erasmus MC, Rotterdam, the Netherlands). See text.
Fig. 2B (right). Schematic drawing of the structure of a mitochondrion.

The mitochondrial inner membrane

The energy transducing function of mitochondria is reflected by their most outstanding feature, which is the large amount of internal membrane. This inner membrane is heterogeneous in structure. An inner boundary membrane region where at discrete places contact is made with the outer membrane can be distinguished from the extensive invaginations, called cristae. Cristae are more prevalent in mitochondria in tissues that consume much oxygen, like heart muscle and are structurally and functionally the more complex part of the mitochondrion. The inner membrane has an unusually high protein to phospholipid ratio; 75% of the dry weight is protein. Most of the phospholipid in the inner membrane is the hydrophobic cardiolipin. In addition to its role in the maintenance of membrane fluidity and osmotic stability, cardiolipin interacts strongly with membrane proteins modulating their activity. Most of the inner membrane proteins are involved in oxidative phosphorylation, the process in which electrons are passed along a series of three carrier molecules (the electron transport chain) to oxygen. These electrons are generated from NADH (reduced nicotinamide adenine dinucleotide) by the first complex and to a lesser extent from FADH (reduced

flavin adenine nucleotide) via coenzyme Q. The electrons are produced by oxidation of nutrients such as glucose and fatty acids and are ultimately transferred to molecular oxygen. The electron transport chain consists of four respiratory enzyme complexes in the mitochondrial inner membrane. The passage of electrons between three of these complexes converts energy using a proton gradient across the membrane. The potential energy of the protein gradient is then used by ATP synthase to make ATP from ADP (adenosine 5'-diphosphate) and phosphate. So five different protein complexes, each consisting of 4 to 46 polypeptides, can be distinguished in this oxidative phosphorylation pathway. They catalyse the series of redox steps

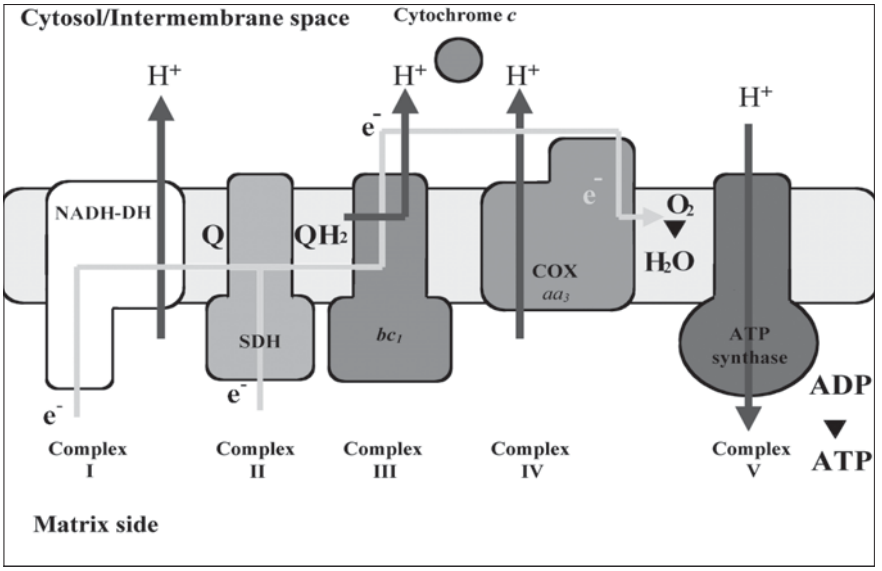


Fig. 3. Schematic view of the oxidative phosphorylation. The complete process by which ADP, P_i and oxidative substrates, are imported into the mitochondria and oxidized via the electron transport chain by oxygen to produce and export ATP, is called oxidative phosphorylation. Electrons (e^-) are fed in the chain (light grey vector) via NADH-dehydrogenase (Complex I) and succinate dehydrogenase (Complex II). Complex I, cytochrome bc_1 (Complex III) and cytochrome oxydase (Complex IV) pump protons across the membrane (dark gray arrows). Ubiquinone (Q) and cytochrome c *(circle) are the lipid soluble and aqueous soluble electron carriers, respectively, in the electron transport chain. The generated proton gradient is used by ATP synthase (Complex V) to make ATP. Redrawn from Saraste (Saraste, 1999).*

from NADH to coenzyme Q (complex I), succinate to coenzyme Q (complex II), coenzyme Q to cytochrome *c* (complex III) and cytochrome *c* to oxygen (complex IV). At the same time potential energy is generated by an outward proton flux by the complexes I, III and IV (see Fig. 3). The potential energy is converted to chemical energy by the fifth complex of the oxidative phosphorylation. Complex V catalyses the formation of ATP and is driven by incoming protons. This proton cycle as a mechanism for energy conservation was postulated by Mitchell (Mitchell, 1961) in the early sixties. This is the final step in the process in which the oxidation of substrate is coupled to the phosphorylation of ADP to form ATP. ATP is then exported in exchange for ADP by the adenine nucleotide translocator.

Most, but not all, mitochondrial functions are directly related to the ATP production, such as the activation of fatty acids, the carnitine system for the import of activated long-chain fatty acids, the import of other fuel substrates and their conversion to substrates for the dehydrogenases, the specific calcium transport protein that imports Ca^{++} to stimulate several mitochondrial 'key' enzymes, the import of other cations to create the suitable matrix composition, and the synthesis of metabolic intermediates, cofactors and holo-enzymes. Mitochondria also catalyse steps in the synthesis of phospholipids, heme, urea, glucose, steroid hormones among others.

The mitochondrial outer membrane

The mitochondrial outer membrane is the frontier between the mitochondrion and the rest of the cell, and molecular activities at this boundary largely determine mitochondrial behaviour. Mitochondria are a highly dynamic structure in the cell and show large variations in structure and distribution in cells. Mitochondria are frequently found as extended reticular networks which are not-randomly distributed. Recently, a number of proteins involved in movement, fusion or fission of mitochondria has been identified and some of them are localized to the outer membrane. Three of such proteins, all belonging to the kinesin superfamily of motor proteins, are involved in the intimate interaction of mitochondria and microtubules. Microtubules are formed by the aggregation of tubulin, and disruption of this aggregation results in aberrant distribution of the mitochondria in the cells, indicating a prominent role of microtubules in the positioning of the mitochondria. Other outer membrane proteins have been shown to be involved in transmission of mitochondria (budding yeast) or in mitochondrial fusion. The "fuzzy onions" protein on the mitochondrial outer membrane was demonstrated to be involved in the fusion of mitochondria in developing sperm cells of Drosophila and is the first identified component of a larger protein complex which connects the inner and outer membrane.

Mutations in the GTPase *mitofusin 2* gene (The D. *melanogaster* homologue is called mitochondrial assembly regulatory factor) cause Charcot-Marie-Tooth neuropathy type 2A. This enzyme is localized to the mitochondrial outer membrane and regulates the mitochondrial network by fusion of mitochondria (Chen et al., 2003; Zuchner et al., 2004).

The outer membrane appears to play a central role in a newly recognized function of mitochondria, namely as integrator of the cell death machinery. Apoptosis or programmed cell death is a property of all metazoans and is essential for life. Apoptosis is a mechanism for elimination of unwanted or supernumerary cells in developmental processes and cellular homeostasis. Mitochondrial apoptosis is initiated by pore forming proteins and efflux of cytochrome *c*. Opening of the mitochondrial permeability transition pore (mtPTP) has been considered as the irreversible step in mitochondrial apoptosis. The voltage dependent anion channel, the adenine nucleotide translocator and other ancillary proteins are thought to come together at the mitochondrial inner and outer membrane contact point to create the mtPTP. Cytochrome *c* is then completely released into the cytoplasm and initiates an apoptotic pathway consisting essentially of effector molecules (caspases), adaptor molecules (Apaf-1) and regulatory molecules [pro- and anti-apoptotic Bcl-2 family members, including inhibitors of apoptosis (IAPs)]. The proteolytic activity of caspases causes proteolysis, DNA condensation and fragmentation and membrane blebbing. In addition to cytochrome *c*, other proteins are released from the intermembrane space by apoptotic stimulation, such as apoptosis inducing factor which is translocated to the nucleus where it induces large scale chromatin fragmentation. It also results in the dissipation of the mitochondrial transmembrane potential and exposure of phosphatidylserine on the cell membrane. Mitochondria appear therefore the integrator of cellular pathways leading to the destruction of their host and their own demise. This latter observation and all the other mitochondrial proteins essential for cellular function brings me to the question discussed in the next paragraph of how the mitochondria containing eukaryotic cell originated.

Evolution of mitochondria

The evolution of mitochondria and the significance of DNA retained in the organelle is still poorly understood. Although the function of the mitochondrial DNA – namely coding for a number of RNAs and proteins essential for the formation of a functional mitochondrion – is similar in all organisms, the amount of mitochondrial DNA and gene content varies

widely. The smallest mitochondrial DNA known is that from the human malaria parasite Plasmodium *falciparum* of less than 6,000 base pairs and coding for three genes and the largest mtDNA known is that from the plant Arabidopsis *thalania* of 366,900 basepairs and coding for (only) 32 genes. The mitochondrial DNA from the protozoan Reclinomonas *americana* is the most gene-rich to date, harbouring 97 genes and all sequenced mitochondrial genomes contain a subset of these 97 genes.

Until recently it was generally accepted that the eukaryotic cell is the product of an endosymbiosis between a primitive eukaryotic cell and a bacterium (Margulies, 1981) and that during evolution most of the bacterial genome was transferred to the host genome forming together the nuclear genome. The sequencing of numerous microbial genomes and mitochondrial genomes from protists (mostly unicellular eukaryotes) yielded data that largely supported this endosymbiont hypothesis and indicated that all mitochondria relate to a single eubacterial ancestor shared with a subgroup of the α-Protobacteria. The genome of such an α-Protobacterium, Rickettsia, an obligate intracellular parasite, containing 834 genes is phylogenetically most close to mitochondrial genomes sequenced to date. In particular the genes that are involved in energy production are conserved. The majority of these genes was found to be positioned in the nuclear DNA. Andersson et al. (1998) hypothesized that in evolution there was an off-loading of these genes from the early mitochondrion to the nucleus. The mitochondrion became more dependent on the host cell since most of its genes were incorporated in the host nucleus, and they could survive within the nucleated cell only.

However, from largescale genome sequencing efforts it became clear that the nuclear genome is a chimera from two bacterial ancestors. Most genes involved in biosynthesis of low molecular weight compounds, energy metabolism and intermediary metabolism (the so-called operational genes) are from eubacterial origin. Eubacteria are the more common bacteria such as E. *coli* and are distinct from Archaebacteria, the latter which can be found at places with extreme conditions such as in hot water springs and at deep sea level. Most nuclear genes involved in replication, transcription and translation (the so-called informational genes) appear to be from archaebacterial origin. Interestingly, also eubacterial genes with no apparent mitochondrial function can be found in current day nuclear genomes. Also, there exist eukaryotes without mitochondria, but who carry nevertheless "typical mitochondrial" genes in their genomes.

Following the discovery of hydrogenosomes, a cell organelle containing mitochondrial proteins, which produce H_2 as a byproduct of ATP synthesis, a new concept of the origin of mitochondria has been postulated which

is not based on a symbiosis based on oxidative phosphorylation, but a symbiosis based on hydrogen metabolism. The "host" is now supposed to have been a hydrogen requiring Archaebacterium and the "metabolic symbiont" a hydrogen producing eubacterial species. Subsequently the eubacterial genome made a major contribution to the nucleus. The remnants of the eubacterial genome then developed into mitochondrial DNA as we know it today. This "hydrogen hypothesis" may cause a drastic revision of the opinion that mitochondria are the direct descendants of a bacterial endosymbiont that became established at an early stage in a nucleus-containing (but amitochondriate) host cell. Thus the origin of mitochondria is placed very close to, if not coincident with, the origin of the eukaryotic cell itself. Instead of the two-step scenario with a step involving an amitochondriate eukaryote intermediate and in a later evolutionary step the acquisition of the mitochondrion, this hypothesis is based upon the simultaneous creation of the eukaryote plus mitochondria (Gray et al., 1999).

A conspicuous aspect of mitochondrial DNA is the maternal inheritance (Kroon et al., 1978). This uniparental inheritance cannot be ascribed to the fact that in mammals the females are the homogametic sex as in birds, in which the males are the homogametic sex and where the mitochondrial DNA is inherited also maternally (Kuroiwa et al., 2002). A likely evolutionary explanation for the uniparental transmission is that it prevents the spread of deleterious mutations in the mitochondrial DNA. As there is only limited DNA repair mechanism in mitochondria, a deletion in the mitochondrial DNA may give it a replication advantage, eventually replacing all mitochondrial DNA in the population. Uniparental transmission limits the spread of the mutated mtDNA to only one parental line, thus protecting the population as a whole from extinction.

What actually the selective advantage is for eukaryotic cells to maintain the coding DNA in the mitochondria is still unknown. A number of hypotheses have been put forward, such as that some proteins of the oxidative phosphorylation are too hydrophobic to pass the mitochondrial membranes, or that some of the mitochondrial proteins might be toxic when exposed to the cytoplasm, or that the alternate codon use by the mitochondrial DNA precludes nuclear expression. Finally, it might be that we are looking at an on-going process of transfer of mitochondrial genes to the nucleus and that at the "evolutionary end" all mitochondrial genes will be found in the nucleus. The first three hypotheses have been found to be untenable, while the last hypothesis is difficult to disprove. Recently, a new selection mechanism was proposed for the maintenance of coding potential in mitochondria (and in chloroplasts, the other chromosomal DNA containing cell organelles in photosynthetic organisms). That selection is

operational can be deduced from observation that the genes retained in mitochondria (and plastids) function preferentially in oxidative phosphorylation (and photosynthesis). It is suggested that these genes code for proteins that by their localization in the electron transport chain might be very vulnerable for oxidative damage. It is well known that due to the large potential difference generated from the transfer of hydrogen bound to carbon to hydrogen bound to oxygen (see Fig. 1) the electron transport chain might be short circuited and reactive oxygen species are liberated leading to cellular damage. This would account for the fact that most (but not all) hydrogenosomes do not contain chromosomal DNA anymore. Also hydrogenosomes produce ATP, but by substrate level phosphorylation instead of electron transport across membranes relaxing the functional constraints for the organellar genome.

Demonstration of biochemical and genetic defects in mitochondrial energy metabolism

In view of the central role of the mitochondria in energy conversion and intermediary metabolism it seemed impossible until the seventies that there could be any diseases found in which mitochondrial defects were the primary causative factors. Let alone that one could imagine that a defect in the OXPHOS causing a disruption of the ATP synthesis could be compatible with life. The knowledge about OXPHOS defects gained in the last decades, changed this view gradually and showed, amongst others, that not only many OXPHOS defects can be compatible with (diseased) life but showed also the great adaptability of mitochondria.

To state the level of knowledge, the following phrase, meant as a concluding remark spoken by Martin at the first symposium about mitochondria and muscular diseases held in Rotterdam in 1980, is illustrative: *'Clinical or morphological criteria do not allow adequate identification and classification of myopathies with abnormal mitochondria. As is the case for lysosomal disorders, a logical classification depends upon the recognition of specific enzymatic deficiencies. Of course, the same remark applies to the encephalopathies with mitochondrial abnormalities. Despite the tremendous amount of biochemical research being carried out, it appears difficult to determine whether some low enzymatic activities are really primary or secondary to other metabolic disturbances in many of the conditions discussed here. Combined clinical, morphological and biochemical investigations must be pursued.' (Martin, 1981)*

Around 1960 the first report indicating a pathogenic role of mitochon-

dria appeared. It concerned a patient with hypermetabolism in which large numbers of mitochondria were found in skeletal muscle. These accumulations of abnormal mitochondria stain patchy red by the modified Gomori trichrome staining of muscle slice (these so named "ragged red fibres" are now considered as the histological hallmark of mitochondrial myopathies). The patient was a 35-year-old woman who presented with severe hypermetabolism likely due to a defective feedback mechanism by which the ATP/ADP ratio controls the rate of oxidative phosphorylation (Ernster et al., 1959; Luft et al., 1962). Also in the same decade the first patients with morphological abnormal mitochondria and decreased oxidative phosphorylation were described, due to the better possibilities to study mitochondrial muscle pathology by histochemical, electron-microscopic and biochemical techniques. From that time onwards the spectrum of phenotypes of diseases associated with OXPHOS widened from mainly myopathic with or without ragged red fibres (Engel and Cunningham, 1963) to encephalomyopatic, also often associated with ragged red fibres. There is no consensus about the word or phrase that describes best the diseases connected to structural and/or functional abnormalities of the mitochondria. Mitochondrial cytopathy, mitochondrial diseases, mitochondrial encephalopathy, mitochondrial myopathy, mitochondriopathy, myopathy with abnormal mitochondria (last term suggested by Bethlem (Bethlem, 1980)) are all used to label the condition.

As there was a natural hesitance to accept defects in the respiration to be compatible with life, this was even more so with the possibility of defects in the mitochondrial DNA. The "small but beautiful" genome (Borst and Grivell, 1981) was considered the ultimate concise information carrier, not allowing for any functional variation. However, in 1988 the first mtDNA mutations (Holt et al., 1988; Lestienne and Ponsot, 1988; Wallace et al., 1988) in patients suffering from mitochondrial encephalomyopathies were described. In Leber's Hereditary Ophthalmic Neuropathy (LHON), a maternally inherited blindness, a missense mutation was found in a mtDNA gene coding for a complex I subunit and in Progressive External Ophthalmoplegia (PEO) large deletions encompassing multiple genes were found in muscle mtDNA.

Many more pathogenic mutations and different rearrangements (also duplications and insertions) were identified. Specific syndromes became associated with mtDNA mutations like Kearns-Sayre syndrome (KSS), Pearson syndrome, neuropathy, ataxia, retinitis pigmentosa syndrome (NARP), mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), myoclonus epilepsy and ragged red fibres (MERRF), maternally inherited diabetes and deafness (MIDD) and a

maternally inherited disorder with adult-onset myopathy and cardiomyopathy (MIMyCa) (see Table 1, Chapter 12, General Discussion). It soon became clear that in addition to the typical mitochondrial syndromes mutated mtDNA might also be involved in more common disease like Parkinson(-ism) (Rana et al., 2000; Korlipara and Schapira, 2002) and Alzheimer disease (Hutchin and Cortopassi, 1995; Bonilla et al., 1999), and in the process of aging (Chomyn and Attardi, 2003; Ross and Short, 2004). Not all mitochondrial DNA diseases are maternally inherited, for example for a mother carrying a single large deletion mutation in her mtDNA is the estimated recurrence risk about 4% (Chinnery et al., 2004) and multiple mtDNA deletions or depletion of mtDNA show a clear Mendelian inheritance pattern. Defects in nuclear genes have been found for these disorders, as well as in a number of nuclear encoded genes for subunits of the oxidative phosphorylation complexes (see for a extensive description chapter 12). Nowadays more than 100 pathogenic mutations are known in the mtDNA (Servidei, 2004).

From the analysis of mutated mtDNA in various tissues (mitotic segregation) and the inheritance patterns (meiotic segregation) the following aspects of mitochondrial genetics were highlighted:

- Cells typically contain hundreds of mitochondria and thus thousands of mitochondrial genomes. Mutations can arise in a mtDNA population, thus more than one genotype can exist in a cell, tissue or individual. This coexistence of mutated and normal DNA is called heteroplasmy.
- If a mutation is pathogenic, the proportion of mutated molecules will determine the severity of the defect, but not necessarily in a linear fashion. Because different cell types may have different energy requirements, the genotypic threshold for a phenotypic effect may vary (Attardi et al., 1995; Chinnery et al., 1997).
- Replication and inheritance of mtDNA is a stochastic process, thus the mutational load during the patient's life may change and the inheritance of a heteroplasmic mtDNA mutation may increase or decrease in successive generations (Weber et al., 1997; Chinnery et al., 1998; Liu et al., 1998; Morgan-Hughes and Hanna, 1999).

Research strategy

The patient group described in this thesis were selected on the basis of one or more defects in complexes I-V or by a mutation in mtDNA or nuclear DNA. When we started our investigations in 1993 eight maternally inherit-

ed mitochondrial diseases with distinct clinical phenotypes [LHON (Wallace et al., 1988), MERRF (Rosing et al., 1985; Shoffner et al., 1990), MELAS (Pavlakis et al., 1984; Goto et al., 1990; Goto et al., 1991), MIDD (van den Ouweland et al., 1992), PEO (Ciacci et al., 1992), MiMyCa (Zeviani et al., 1991), NARP (Holt et al., 1990; de Vries et al., 1993), Leigh's disease (Tatuch et al., 1992)] were associated with point mutations in mitochondrial mtDNA. Another quantitative important group of mitochondrial DNA mutations were the large single deletions. These occur in isolated patients and are sporadic in nature although patients with maternal transmission have been reported (Poulton et al., 1991; Bernes et al., 1993; Shanske et al., 2002). The large single deletions were found to be associated with PEO (DiMauro et al., 1988; Holt et al., 1988), KSS (Lestienne and Ponsot, 1988; Zeviani et al., 1988) and Pearson syndrome (Rotig et al., 1991). The mtDNA mutations appeared often to be associated with the disease phenotypes but it became sometimes likely that a nuclear factor attributed to the phenotype (Zeviani et al., 1997b; Poulton et al., 1998; Khogali et al., 2001) or even became the primary factor in the case of multiple mtDNA deletions (Zeviani et al., 1989; Spelbrink et al., 2001).

AIM AND OUTLINE OF THE THESIS

At the time this project was started in about 30% of the patients with a clinical diagnosis of a possible mitochondrial myopathy a biochemical defect in the oxidative phosphorylation could be demonstrated. In 6% of the patients a mutation in the mtDNA could be detected, mostly single mtDNA deletions and the MELAS mutation. Our experience was not much different from other diagnostic centres worldwide (Munnich et al., 1996; Chinnery and Turnbull, 1997; Zeviani et al., 1997a; DiMauro et al., 1999; Christodoulou, 2000; Larsson and Oldfors, 2001). So most patients suspected to suffer from an OXPHOS disease remained without a diagnosis. In case of the biochemically defined patients it was not possible to predict if the biochemical defect found in a particular patient was primary or secondary. Our aim was to develop a more comprehensive DNA diagnosis protocol for mitochondrial diseases. This would not only lead to a better understanding of the diseases, which is badly needed for possible treatment, but also to improve the genetic counselling of the families struck by these frequently early-lethal diseases. In this thesis we have focussed on the OXPHOS structural subunit genes although we realised that many other genes are necessary for the OXPHOS subunits proteins to be able to function properly. Like the genes responsible for the proteins to become

imported (TOM/TIM mitochondrial protein translocation machinery), folded, assembled and maintained. More than 20 of these genes and diseases have been detected (see Table 3-6 in Chapter 12).

In Chapters 2-7, we describe the methods developed for a more simple screening procedure for mtDNA mutations. Some of the consequences of the applied methods for patient screening were elaborated on in Chapters 3, 5, 6 and 7. These mtDNA screening methods made it possible to select a group of patients in which mtDNA involvement appeared to be highly unlikely.

In part two we studied the involvement of nuclear genes encoding OXPHOS proteins in the patients. From biochemical studies we know that isolated complex I and IV deficiencies are the most frequent findings. We choose to investigate the genes for complex I. Complex I consists of 7 mitochondrial-encoded subunits and of 39 (Carroll et al., 2003) nuclear-encoded subunits. Immunochemical analysis revealed heterogeneity of complex I deficiency with disproportionate loss of one or several subunits (Ichiki et al., 1988; Morgan-Hughes et al., 1988; Schapira et al., 1988; Robinson et al., 1990; Slipetz et al., 1991). The pronounced decrease of the 24 kDa Fe-S subunit of complex I found in patients by Schapira and Morgan-Hughes (Morgan-Hughes et al., 1988; Schapira et al., 1988), prompted us to search for a genetic defect in this subunit first (Chapter 9). Another subunit which was supposed to have an important function within complex I is the 51 kDa subunit (Walker, 1992). This was the next subunit to characterize (Chapter 10). The 24- and 51-kDa subunits form together with the 10-kDa subunit a group within the complex I enzyme complex called the flavoproteins. Although the function of the 10-kDa subunit was not clear we also characterized this gene in the human (Chapter 8) enabling a comprehensive mutation screening of all the genes coding for the flavoproteins. The elucidation of the gene structures was facilitated by the work of Walker and co-workers who made available the cDNA sequences of the 32 subunits of complex I of the bovine heart (Walker, 1992; Walker et al., 1992). In Chapter 11 we used the knowledge acquired by the gene characterizations to develop a search tool to detect mutations at the genomic level in the three genes encoding for the flavoprotein fraction proteins of complex I in complex I deficient patients, in whom mutation in mtDNA is unlikely [part I]. Finally I suggest in the discussion (Chapter 12) strategies for nowadays screening of patients with mitochondrial encephalomyopathies and describe some future developments in mitochondrial diagnostics.

CHAPTER 2

A PCR test for progressive external ophthalmoplegia and Kearns-Sayre syndrome on DNA from blood samples

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Abstract

Progressive external ophthalmoplegia (PEO) and Kearns-Sayre syndrome (KSS) are caused by deletions in mitochondrial DNA. Identification of these deletions is important for diagnosis, prognosis and genetic counselling. As yet, the most frequently used test is Southern blot analysis of DNA isolated from a muscle biopsy. Here, we describe a sensitive PCR-based test for the identification of these deletions in DNA isolated from blood. The main advantage is that in the majority of cases a muscle biopsy is no longer necessary for the molecular diagnosis of PEO and KSS.

INTRODUCTION

A variety of diseases, in which clinical symptoms point to dysfunction of tissues with high energy demand, are caused by defects in mitochondrial energy metabolism. Mutations in mitochondrial DNA (mtDNA) are associated with many of these disorders and can be classified as deletions, duplications/insertions or point mutations (Shoffner and Wallace, 1990). The identification of mutations in mtDNA is important, both for diagnosis and prognosis as well as for genetic counselling. In this respect, it is important to mention two distinct features of mtDNA: First, each cell contains hundreds of mitochondria and the phenotypic expression of mtDNA mutations depends on the ratio between normal and mutated mitochondrial DNA (heteroplasmy). Secondly, mtDNA and mtDNA point mutations are inherited maternally, except for large single deletions and duplications, which are *de novo* events with low recurrence risk (Holt et al., 1989; Shoffner et al., 1989).

Single deletions of mtDNA were identified in PEO (Moraes et al., 1989), KSS (Zeviani et al., 1988), Pearson syndrome (de Vries et al., 1992a), diabetes mellitus and deafness (Ballinger et al., 1992), and in migraine and stroke (Bresolin et al., 1991). These deletions were easily detectable in DNA of muscle biopsies, using conventional Southern blot analysis with the complete mtDNA being used as a probe (Holt et al., 1988; Holt et al., 1989). Unfortunately, using this test, the same defect was, in most cases, not detectable in DNA from peripheral blood cells (Holt et al., 1989; Larsson et al., 1992). Either the sensitivity of the test was too low or the mutation was not (or barely) present in peripheral blood cells (Holt et al., 1989; Moraes et al., 1989). Based on characterised deletions in mtDNA from muscle, PCR tests specific for these deletions have been reported (Johns et al., 1989). One or two primer sets were necessary to amplify a fragment bridging the mtDNA deletion (Poulton et al., 1991). Because deletion breakpoints are highly variable among patients with the same disease, this test cannot be used for routine diagnostic applications.

Recently, thermostable DNA polymerases have been described that allow the *in vitro* amplification of DNA fragments of up to 40 kb (Barnes, 1994; Cheng et al., 1994a). These polymerases have been used to amplify the complete mitochondrial genome of 16.5 kb (Cheng et al., 1994b). In a case of Pearson syndrome with a high percentage of deleted mtDNA in blood, the deletion could directly be shown after 28 PCR cycles on blood DNA (Kreuder et al., 1995). The same PCR test was used for detecting large deletions in skeletal muscle DNA from PEO patients (Yamamoto et al., 1996). Using a similar approach, we tested the blood DNA of two PEO

patients, two Kearns-Sayre patients and one Pearson syndrome patient for deletions and compared our results with the Southern blot analysis of blood and muscle DNA.

PATIENTS AND METHODS

Patients

The five selected patients (A-E) were derived from a collection of several hundred patients with possible mitochondrial malfunctioning. These patients were routinely investigated for biochemical and molecular defects (Table 1). One patient with Pearson syndrome (E) had a clinical phenotype consisting of aplastic anaemia with failure to thrive and persistent diarrhoea (de Vries et al., 1992a). Two patients with PEO (A and B) were studied, one of whom (Patient A) showed a PEO (plus) syndrome with muscle weakness. Patients C and D, with KSS, showed the typical phenotype with progressive external ophthalmoplegia, retinitis pigmentosa, myopathy, cardiac conduction defect and an elevated cerebro-spinal fluid (CSF) protein level.

Table 1. Southern blot- and PCR analysis of mtDNA from PEO, KSS and Pearson patients.[a]

Patient	Sex	Diagnosis	Blood			Muscle
			PCR[b] I	II	Southern blot	Southern blot
A	F	PEO Plus	-	-	-	6 (70%)
B	M	PEO	10	-	10 (nd)	nd
C	F	KSS	4	4	4 (60%)	4 (60%)
D	M	KSS	8	8	8 (30%)	8 (60%)
E	F	Pearson	6	6	6 (91%)	6 (82%)

nd = not done

a The estimated sizes of the deletions found (in kb) and the percentage of the deleted mtDNA are indicated in brackets.

b The size of the deletion in blood DNA found with primer sets I and II, respectively. For the position of the primers, see Fig. 1.

- = no signal

Methods

Mitochondrial DNA was isolated from muscle biopsies (de Vries et al., 1992b) and blood (Miller et al., 1988). Southern blot analysis was performed according to standard procedures (Sambrook et al., 1989). For the

analysis of mtDNA, 2 μg muscle DNA or 10 μg blood DNA were digested with *Pvu*II. For quantification of heteroplasmy, the autoradiograph was scanned on a Hewlett Packard ScanJet IIc and the percentage of mutated DNA was determined using ImageQuant software (Molecular Dynamics).

Two primer sets were developed (Fig. 1) and several enzymes and enzyme mixtures [Taq-extender (Stratagene, Cambridge, UK), RTth DNA polymerase, Pwo polymerase (Expand™ Long Template PCR System, Boehringer, Mannheim, Germany) and TaKaRa Ex Taq (Takara biomedicals, Japan)] were used to amplify total mitochondrial DNA, isolated from blood and muscle tissue. The PCR was performed on 500 ng DNA according to the protocols supplied by the manufacturers. PCR products were separated on an 0.8% agarose gel and stained with ethidium bromide.

Restriction digestion of PCR-amplified mtDNA was performed with *Dra*I, *Bcl*I, *Sca*I and *Acc*I. Junction fragments of deleted PCR products were generated by conventional PCR amplification using *Taq*I polymerase.

Results and discussion

The complete mtDNA (Anderson et al., 1981) could routinely be amplified. Primer set 1 yielded a fragment of 16 kb, primer set 2, a fragment of 16.2 kb. We obtained the best results with TaKaRa Ex Taq (TaKaRa). We used two primer sets to exclude amplification failures, because of a deletion of one or both of the priming sites. The position of the two primer sets was such that they could not be removed in a single deletion event, as this deletion would have to include either the D-loop region, which controls both transcription and replication, or almost the entire mtDNA (Fig. 1). Blood DNA of the 5 patients was tested by PCR analysis, using both primer sets (Fig. 2). In 4 cases deletions were visible. The size of the deletions ranged from 4 to 10 kb (Table 1). The deletion of 10 kb in patient B, with PEO, was only detectable with primer set I, which illustrated the need for applying two primer sets at different locations (Fig. 1). Using Southern blot analysis, we showed that the deletion included the cytochrome *b* gene (data not shown). Only in patient A no deletion could be detected with our long distance PCR system. The sensitivity of the PCR test was determined by mixing blood DNA of Pearson patient E (91% deleted mtDNA) with increasing amounts of control DNA, containing only the intact mtDNA. The detection limit was between 0.5 and 1% deleted DNA (data not shown).

The results from the PCR test were compared with those of the Southern

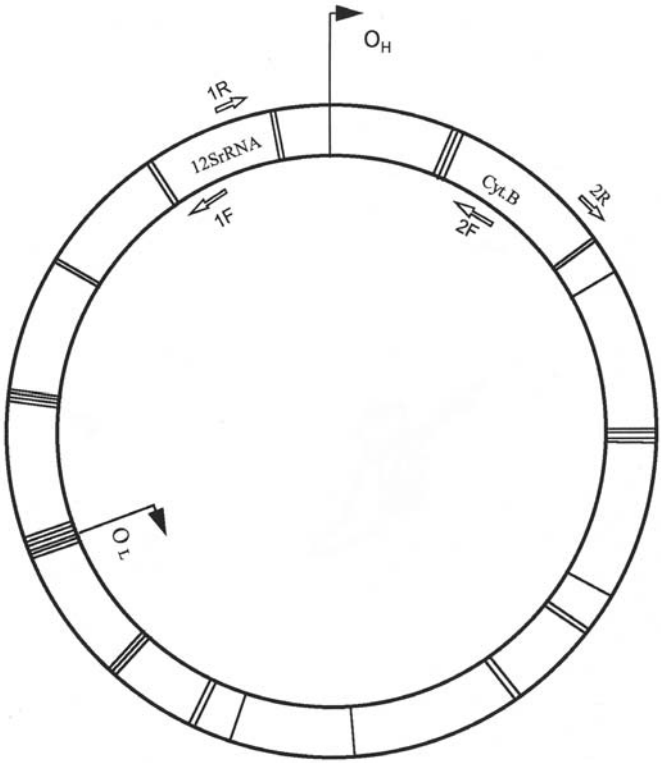

*Fig. 1. Map of the circular human mitochondrial genome. The structural genes and the position of the primers used in the long PCR are shown. Primer set I positioned in the 12*S *ribosomal (*12SrRNA*) gene:* 1F*=nucleotide position (np)1330-1355*,* 1R*=np778-753; Primer set II situated in the cytochrome* b *(*Cyt. B*) gene:* 2F*=np15149-15174,* 2R*=np14841-14816.* O_H, O_L*: origin of replication of the heavy (H) or light (L) strand (arrows indicate the direction of duplication or transcription).*
**Numbering of nucleotides are according to the Cambridge human mtDNA sequence (Anderson et al., 1981).*

blot analysis of muscle DNA samples of 4 patients and blood DNA samples of all 5 patients (Table 1). In patient E with Pearson syndrome, the deletion of 6 kb was also detected in several other tissues, as has been reported before (de Vries et al., 1992a). The sizes of the deletions were identical to the PCR analysis. Deletions were detectable in muscle DNA of all patients, including patient A. The heteroplasmy in muscle DNA varied from 60 to 82% deleted mtDNA (Table 1). In contrast to earlier reports (Holt et al., 1989; Johns et al., 1989; Larsson et al., 1992; Zupanc et al.,

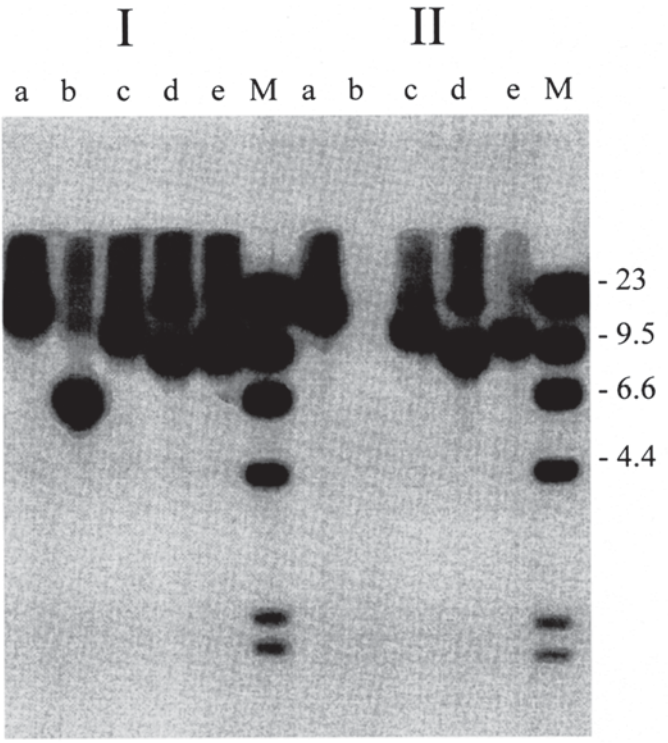

Fig. 2. Ethidium bromide staining of the long PCR products of blood DNA with primer set **I** *(situated in the 12*S *ribosomal gene) and primer set* **II** *(situated in the cytochrome* b *gene). Lanes:* **a***=PEO patient, (A);* **b***=PEO patient, (B);* **c***=KSS patient (C);* **d***=KSS patient (D);* **e***= Pearson patient (E);* **M***=Molecular weight marker (Lambda-DNA cut by* Hin*dIII).*

1991), the deletions in patients B, C and D were also detectable in blood DNA, using Southern blot analysis. This was probably caused by the high amount of deleted mtDNA in the blood samples of our group of patients. Similar to the negative PCR result, no deletion could be detected by Southern blot analysis of blood DNA from patient A. The deletion in muscle DNA of patient A was characterized by digestion of the PCR product with *Dra*I, *Bcl*I, *Sca*I and *Acc*I. The breakpoints were located between nucleotide positions 8591 and 9745 and between nucleotide positions 15254 and 16008. Two primers at nucleotide position 9437-9457 (forward) and nucleotide position 16050-16069 (reversed) were used to generate a junction fragment. This specific junction fragment was detectable in blood DNA by PCR analysis (data not shown). Our result indicated that PCR

analysis of the entire mtDNA was less sensitive than a specific PCR in which competition of the wild type mtDNA could be excluded. However, amplification of the entire mtDNA is a generally applicable strategy, which is not the case for the amplification of junction fragments and, therefore, preferable for diagnostic questions.

We conclude that a high percentage of diagnostic questions regarding mtDNA deletion syndromes can be answered by performing a simple PCR test on blood DNA, thereby saving the patient a muscle biopsy.

Acknowledgement

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CHAPTER 3

A mitochondrial $tRNA^{Val}$ gene mutation (G1642A) in a patient with mitochondrial myopathy, lactic acidosis and stroke-like episodes

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Abstract

We studied a patient with the diagnosis of mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) for mitochondrial DNA mutations in muscle. Established MELAS mutations were excluded. Mitochondrial DNA was further analysed for mutations in the 22 tRNA genes by single-strand conformation polymorphism (SSCP) analysis; a $tRNA^{Val}$ mutation (G1642A) was found. The structure of the altered tRNA, the heteroplasmy and the absence of the mutation in the mother and in 100 control subjects suggests that the $tRNA^{Val}$ mutation is associated with the MELAS syndrome.

Introduction

Mutations in mitochondrial DNA (mtDNA) are associated with a number of diseases, in which clinical symptoms reflect a primary defect in tissues with high oxidative demand. These mutations involve either large-scale deletions and rearrangements or point mutations. In general, single partial deletions of mtDNA are found in isolated patients, in contrast to point mutations in mtDNA, which are usually found to be associated with familial disease (Larsson and Clayton, 1995). About half of the point mutations described were detected in tRNA genes (Wallace et al., 1993) and are associated with a number of maternally transmitted disorders, like mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibres, and myopathy-cardiomyopathy (Larsson and Clayton, 1995). Patients with single tRNA point mutations often show biochemical evidence of respiratory chain dysfunction with decreased activity of complex I and IV (Wallace, 1992).

In a patient with the MELAS phenotype, clinical, biochemical, and morphologic data pointed very strongly to a mitochondrial disease. Having excluded the known MELAS mutations in the $tRNA^{Leu(UUR)}$ gene, we set out to screen the other mitochondrial tRNAs. One pathogenic $tRNA^{Val}$ mutation was identified in the MELAS patient.

Case report

The patient was the first son of unrelated healthy parents. He had one healthy brother. The medical history of his mother's brother and sister and his mother's grandmother were unremarkable. His psychomotor development was normal until the age of 7 years, when behavioural changes were noted after a viral infection. Neurologic examination demonstrated bradyphrenia and a left-sided hemianopsia. Metabolic investigations revealed elevated levels of lactic acid in venous blood ranging between 2.2 and 4.0 mmol/l. MRI of the brain showed infarction of the right occipital cortex not corresponding to the territory of larger arteries. Angiography showed occlusion of cortical branches of the right posterior cerebral artery, for which no cause could be found on further investigations. A muscle specimen was obtained by an open biopsy. It showed apart from an excess of lipids only minor changes. There were no ragged red fibres or cytochrome *c* oxidase-negative fibres. Some small arteries showed an intense reaction for succinate dehydrogenase. Six months later he developed partial complex epilepsy and cortical blindness. The seizures were

easily controlled by carbamazepine. MRI now showed similar infarcts of the left occipital cortex. Several times he had a transient right-sided hemiparesis. At the age of 9, he developed severe loss of initiative and global aphasia progressing to mutism. Repeated neurologic examination revealed tunnel vision but no signs of either retinitis pigmentosa or deafness. CT now also showed bilateral vaguely demarcated hypodense areas in the basal part of the frontal lobes, extending into the frontal operculum and Sylvian fissure on both sides. At the end of his life, the boy also suffered from severe dementia and aphasia. He died at the age of 10 years from pneumonia in a severely regressive state. No autopsy was obtained.

Biochemical investigations of skeletal muscle homogenate showed a decrease in complex I to 39% of the average control subjects: complex I (μmol NADH to decyl-Q_2): control subjects (n=12), average 5.24 (range, 4.14 to 8.85); patient, average 2.04 (39%); citrate synthetase (μmol); control subjects (n=10), average 16 (range, 8.6 to 36.1), patient, 30.3 (189%). The activities of the other respiratory chain complexes and of pyruvate dehydrogenase complex were in the normal range. Total carnitine was decreased to 50% of the average control subject.

METHODS

DNA was isolated from blood and muscle samples. Thirteen primer pairs flanking the tRNA loci were synthesized to amplify all 22 tRNA genes (de Coo et al., unpublished data). Polymerase chain reaction (PCR) fragments were separated on a 0.5×mutation detection enhancement (MDE) gel (AT Biochem, Malvern, PA, USA), and SSCP analysis was performed at two different electrophoresis conditions, 6 hours at 40 W and 16 hours at 6 W both at 4°C in a 0.6×TBE buffer. The PCR fragments for which a band shift was observed were purified from agarose gels by the freeze-squeeze method, cycle sequenced, and analysed on an ABI 373 automated sequencer (ABI, Foster City, CA, USA). Mutations altering a restriction enzyme site were tested by digesting the PCR fragment with the appropriate restriction enzyme (New England Biolabs Inc., Beverly, MA, USA). The bands were resolved on an agarose gel and stained with ethidium bromide. For quantification of heteroplasmy, the tRNA genes were amplified and a γ-^{32}P-labeled reversed primer was added before the last extension reaction of the PCR. Products were separated on a 6% polyacrylamide/bisacrylamide gel. The autoradiograph was scanned on a Hewlett Packard ScanJet IIc and the percentage of mutated DNA was determined using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

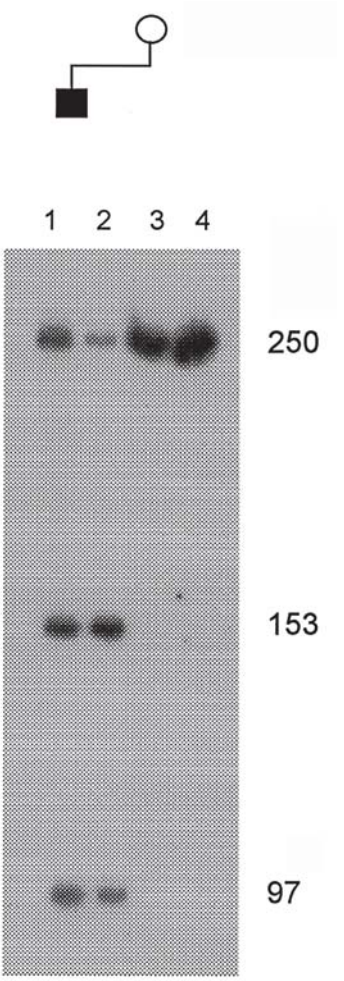

Fig. 1. Autoradiogram of a Mbo*II-digested 250-bp mtDNA PCR fragment encompassing the G1642A tRNA*^{Val} *mutation site. The mutation induces a* Mbo*II restriction site.* Mbo*II fragments are 153 and 97 bp. Lane* **1***, Patient, G1642A mutation (blood); lane* **2***, patient, G1642A mutation (muscle); lane* **3***, mother of the patient, wild type (blood); lane* **4***, control subject, wild type (blood).*

Results

Screening for gross rearrangements of the mtDNA and the point mutations in tRNA$^{Leu(UUR)}$ (positions 3243, 3252, 3271, 3291), tRNA^{Cys} (position 5814), *COX III* gene (position 9957) and the *ATPase 6* gene (position 8993) in mitochondrial DNA was negative in the MELAS patient. The 22 mitochondrial tRNA genes of the patient were amplified in 13 PCR fragments and screened for mutations by SSCP analysis and direct sequencing. In the patient, one PCR fragment showed a mobility shift. Sequence analysis revealed a (G→A) substitution at nucleotide position 1642 in the tRNA gene for valine. The mutation created an *Mbo*II restriction site that facilitated further analysis. Heteroplasmy for the G1642A tRNA^{Val} mutation was 60% in DNA from blood and 80% in DNA from muscle of the patient (Fig. 1). The mutation was neither found in blood DNA from the unaffected mother nor in a group of 100 control subjects.

Discussion

The morphological data in our patient with succinate dehydrogenase-reactive blood vessels were characteristic for MELAS. The lack of ragged-red

fibres in the muscle biopsy material is not unusual in MELAS patients (Melberg et al., 1996), especially in children. An additional finding was the occlusion of branches of the right cerebral posterior artery, detected by angiography. This occlusion explained only part of the symptomatology and MRI findings in our patient. Abnormal MRI or CT findings in MELAS patients in general have been explained by abnormal cellular

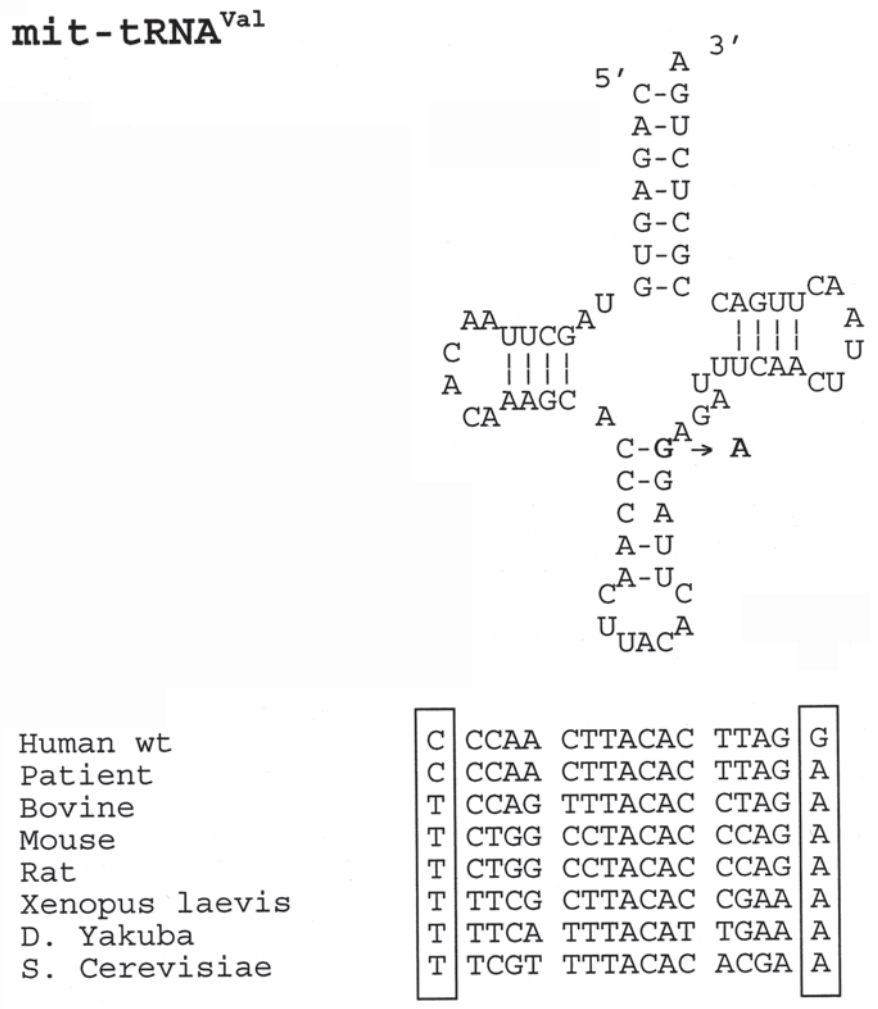

Fig. 2. Secondary (or cloverleaf) structure and evolutionary conservation of the tRNA^{Val}. The mutated nucleotide is indicated. The pathogenic mutation in the tRNA^{Val} gene is located in the anticodon stem, which is predicted to become one basepair shorter due to the mutation.

metabolism and by the proliferation of abnormal mitochondria in the smooth muscle and endothelial cells of the small arteries, arterioles of the brain and the pia mater (Fujii et al., 1991; Mizukami et al., 1992). The MRI pathology and the symptomatology found in our MELAS patient indicated that the symptoms of MELAS have to be explained by a combination of abnormal cellular metabolism, a mitochondrial capillary angiopathy (with or without cardiomyopathy), and sometimes an involvement of the larger vessels. Recently, a patient with a clinical MELAS syndrome and an occlusion of the left posterior cerebral artery was described (Ito et al., 1995), but no molecular studies were reported.

The mutation in the $tRNA^{Val}$ gene, the second mutation ever reported in this tRNA gene, is likely pathogenic for the following reasons: the mutation was heteroplasmic in different tissues of the patient, the G1642A $tRNA^{Val}$ mutation is absent or very rare in the normal population, the absence of the mutation in the healthy mother, or the $tRNA^{Val}$ G1642A alters a nucleotide that is located in a conserved anticodon stem (Fig. 2). The mutation was expected to disrupt the structure of the anticodon stem. It was hypothesized that the $tRNA^{Val}$ mutation, which was situated between the 12*S* and 16*S* ribosomal RNAs (rRNA), might hamper the cleavage from the polycistronic messenger by a nuclear-encoded RNase P-like endonuclease (Doersen et al., 1985). However, Northern blot analysis of mRNA from blood of the patient did not show any abnormalities in the splicing pattern (data not shown). This is the second patient reported with the MELAS phenotype and a heteroplasmic $tRNA^{Val}$ G1642A mutation. This mutation was originally described by Taylor et al. (1996).

To our knowledge our patient is the first reported with a clinical phenotype of MELAS syndrome and the involvement of large cortical artery branches and a unique mtDNA mutation. A causative relation between this $tRNA^{Val}$ mutation and the involvement of both the small arteries and the large cerebral vessels is hard to prove. However, in patients with stroke-like episodes, it should be worthwhile to test not only for the classic $tRNA^{Leu(UUR)}$ MELAS mutations but also for $tRNA^{Val}$ gene mutations.

Acknowledgements

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CHAPTER 4

A 4-base pair deletion in mitochondrial cytochrome *b* gene associated with Parkinsonism/MELAS overlap syndrome

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Abstract

Five patients with a diminished activity of complex III of the mitochondrial respiratory chain have been screened for mutations in the mitochondrial cytochrome b *(cyt* b*) gene. In 1 patient, a young boy with an akinetic rigid syndrome and a mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), a novel 4-base pair deletion was identified. This mutation in this highly conserved gene is considered to be pathogenic since it is a heteroplasmic frame shift mutation predicted to lead to a truncated protein.*

Introduction

The oxidative phosphorylation system comprises five multipolypeptide enzyme complexes, which produce most of the adenosine triphosphate required for normal cellular function. Complex III (ubiquinol: ferricytochrome *c* oxidoreductase EC 1.10.2.2) catalyses electron transfer between coenzyme Q and cytochrome *c*, thereby translocating protons across the mitochondrial inner membrane. Complex III is composed of 11 subunits, one of which, (cytochrome *b* [cyt *b*]) is encoded by the mitochondrial DNA (mtDNA). The cyt *b* gene is located at nucleotide position 14747 to 15887 of the mtDNA and is flanked by the transfer RNA (tRNA) genes for glutamic acid and threonine (Anderson et al., 1981). It codes for a protein of 380 amino acids.

Mutations in mtDNA genes can impair adenosine triphosphate production by the oxidative phosphorylation system. A group of 5 patients with isolated diminished activity of complex III (Mourmans et al., 1997) was analysed for mutations in the cyt *b* gene by direct sequence analysis. We detected a heteroplasmic frame shift mutation in 1 patient. The particular phenotype and biopsy findings associated with the mutation are discussed.

Patients en methods

Patients

Patient A to E have been described (Mourmans et al., 1997). All patients showed lactic acidosis and defective complex III activity in muscle. In patient E, we identified a likely pathogenic mutation in the cyt *b* gene. This 20 year-old patient is the only child of healthy nonconsanguineous parents. At 6 years of age, difficulties with fine motor coordination and concentration were noticed. From the age of 14.5 years, he started to complain of fatigue and yawning, and he showed periods with regressive behaviour, echolalia, anxiety, social isolation, and absent-mindedness. Examination revealed a lack of facial expression, reduced blinking frequency, and a hypophonic monotonous voice. Eye fundoscopy and vision were normal. He walked with a flexed body posture, reduced arm swing, and mild shuffling gait. He showed a resting tremor of the hands. The muscle tone was increased, particularly on the right side. He had a stimulus-responsive myoclonus. Cerebellar signs were absent, and sensory deficits could not be tested adequately. Muscle strength was normal, but he was easily fatigued. Arm and leg reflexes were low, with extensor plantar response reactions. Blood pressure and heart rate showed normal responses to orthostatic test-

ing. Results from standard laboratory investigations (van Erven et al., 1989) were normal. Lactic acid levels were increased in blood and cerebrospinal fluid: 2,969 μmol/l (normal, 400-2,100 μmol/l) and 9,750 μmol/l (normal, 500-1,900 μmol/l), respectively. Magnetic resonance imaging showed diffuse atrophy of both cerebral and cerebellar hemispheres. The basal ganglia were not involved.

Electroencephalographic results demonstrated an irregular slow background pattern with sporadic generalized paroxysmal epileptic discharges. Cardiological investigations revealed a Wolff-Parkinson-White syndrome and a mild hypertrophic left ventricle. Electroneurography and the pattern-reversal visual evoked responses were within the normal limits. Brainstem auditory responses (interpeak latency I-III, 2.56 msec) and somatosensory evoked responses (N13-N20, 7.2 msec) were borderline delayed.

At the age of 16 years, a routine surgical quadriceps muscle biopsy showed normal percentages of type I, IIA, and IIB fibres, but type IIC fibres were increased (7 vs 1-2% in controls). The diameters were smaller than normal.

Table 1. Biochemical analysis of the respiratory chain of the skeletal muscle of Patient E.

	Patient	Control range	(n)
Oxidation rate[a]			
[1-^{14}C]pyruvate + malate	2.20	3.61-7.48	(15)
[1-^{14}C]pyruvate + carnitine	4.16	2.84-8.24	(15)
[U-^{14}C]malate +acetylcarnitine + malonate	2.72	3.43-7.30	(15)
[1,4-^{14}C]succinate + acetylcarnitine	0.16	2.54-6.39	(15)
Adenosine triphosphate + phosphocreatine production rate[a]			
Pyruvate + malate	16.5	42.1-81.2	(15)
Activity[b]			
NADH:Q_1 oxidoreductase	0.096	0.044-0.265	(30)
Succinate:cytochrome *c* oxidoreductase	0.26	0.30-0.97	(39)
Succinate dehydrogenase	0.34	0.067-0.177	(11)
Decylubiquinol:cytochrome *c* oxidoreductase	0.42	2.85-6.61	(21)
Cytochrome *c* oxidase	1.81	0.81-3.13	(21)
Citrate synthase	75.0	48-162	(43)

a nmol.hr^{-1}/mU^{-1}citrate synthase.

b mU/mU^{-1}citrate synthase, except for the citrate synthase activity, which is given in mU/mg^{-1} of protein.

One ragged red fibre was observed and type I fibres showed variable cytochrome *c* oxidase staining. Succinate dehydrogenase staining was unremarkable. Electron microscopy showed aggregates of subsarcolemmal mitochondria but did not reveal clear structural abnormalities. Biochemical studies on supernatant prepared from fresh muscle tissue (Mourmans et al., 1997) revealed an isolated severely diminished activity of complex III (decylubiquinol:cytochrome *c* oxidoreductase ratio, Table 1).

From the age of 15 years, his spontaneous motor performances were characterized by an akinetic rigid syndrome. Between the age of 14 and 19 years, he suffered from epileptic seizures presenting with short episodes of disturbed consciousness. These attacks were partially controlled with antiepileptic drugs. At the age of 19 years, a status epilepticus occurred. Six months later, he suffered from two other prolonged tonic-clonic seizure attacks. After the last attack, a further loss of initiative and a cortical blindness were noted. Magnetic resonance imaging of the brain performed after these attacks showed diffuse atrophy and; in addition, lesions consistent with cerebral infarcts in the left and right parieto-occipital region were seen.

Molecular Genetic Investigations

DNA extraction and Southern blot analysis were performed according to standard procedures (Sambrook et al., 1989). The most common mutations associated with mitochondrial encephalomyopathy were analysed (de Coo et al., 1998). In Patient E, all 22 tRNA genes were tested by single-stranded conformational polymorphism (SSCP) analysis (de Coo et al., 1998).

The complete cyt *b* gene was amplified using the polymerase chain reaction (PCR) with the forward (f) primer 1f (np 14574-14595) and the reverse (r) primer 7r (np 16050-16069). Small PCR products were generated using nested primers purified on ultralow gelling temperature agarose (Sigma Chemicals, Zwijndrecht, the Netherlands) and analysed by direct cycle sequence analysis on an ABI 373 automated sequencer (ABI, Foster City, CA, USA). The amount of mutated mtDNA was determined by adding [γ-^{32}P]deoxycytidine triphosphate in the last of 30 PCR cycles. After electrophoresis on a 6% denaturing polyacrylamide gel, the autoradiograph was scanned on a Hewlett Packard ScanJet IIc and the relative percentages were determined with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

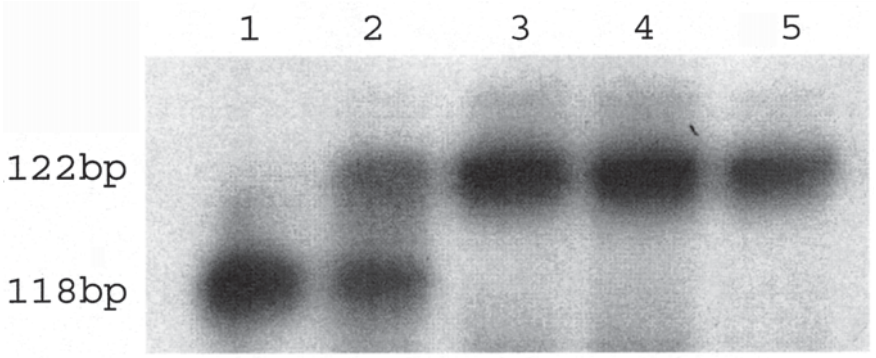

Fig. 1. Quantitation of mutant and wild-type mitochondrial DNA (mtDNA) in tissues from Patient E (lane **1***, skeletal muscle; lane* **2***, blood) and in different tissues from his mother (lane* **3***, blood; lane* **4***, hair follicles; lane* **5***, oral mucosa). The mtDNA was amplified by polymerase chain reaction labelled with* ^{32}*P-dCTP in the last cycle and separated on a 6% polyacrylamide gel.*

RESULTS

No gross rearrangements or any of the point mutations in the mtDNA associated with the mitochondrial encephalopathy with ragged red fibres, MELAS, or NARP/Leigh syndromes were found in our group of patients. SSCP analysis of all tRNA genes did not reveal any heteroplasmic mutations in Patient E. Sequence analysis of the cyt *b* gene revealed two synonymous polymorphisms: a homoplasmic 15452C→A polymorphism (Brown et al., 1992) in patient B and a homoplasmic 15693T→C polymorphism (Howell et al., 1995) in patients D and E. A heteroplasmic deletion of 4 base pairs (bp) (14787**del4**: cccctaata aaattaa**tt aa**ccactca [nucleotide numbering according to Anderson et al. (1981)]) in patient E was identified. This microdeletion was absent in 20 controls. In blood, hair follicles, oral mucosa, and fibroblasts, about 60% of the mtDNA carried the 4-bp deletion, although more than 95% of the mtDNA in muscle was mutated (Fig. 1). The 4-bp deletion causes a frame shift from codon 13 onward in the cyt *b* gene and is predicted to result in a nonsense stretch of amino acids terminating at an early stop at codon 50. To assess whether this 4-bp deletion was a *de novo* mutation, we tested the clinically unaffected mother of the proband. No cyt *b* deletion was found in her blood, oral mucosa, or hair follicles by PCR with primers 2f and 12r (data not

shown). No other maternal relatives were available for further investigations.

Discussion

A predominant complex III deficiency has been reported for only 30 patients (Morgan-Hughes, 1994; Marin-Garcia et al., 1996). In 1 of the 5 patients we screened, a complex III deficiency could be related to a mtDNA mutation. In contrast to the other 4 patients (phenotype dominated by failure to thrive, hypotony and myopathy), Patient E showed signs of parkinsonism and MELAS, symptoms that are associated with mutations in the mitochondrial tRNA genes. For this reason, we analysed the mitochondrial tRNAs but did not find any heteroplasmic mutations. As mutations in the cyt *b* gene were excluded, a mutation in 1 of the other 10 nuclear-encoded subunits of complex III might well be responsible for the disease found in the phenotypically different other patients. The 4-bp pathogenic deletion is the first frame shift mutation in the cyt *b* gene described. In the normal mtDNA sequence these four nucleotides are part of a doublet and the deletion may be caused by slippage of the replication machinery. Homoplasmic cyt *b* mutations are found in patients with Leber hereditary optic neuropathy (Johns and Neufeld, 1991), probably being only a polymorphism (Oostra et al., 1994), whereas heteroplasmic missense mutations are associated with exercise intolerance (Dumoulin et al., 1996), or with a progressive cardiomyopathy (Bouzidi et al., 1996; Dumoulin et al., 1996).

The main presenting clinical feature of Patient E is a juvenile akinetic rigid syndrome (parkinsonism). Although incidentally mentioned (van Erven et al., 1989), this movement disorder is rare as an initial symptom of a mitochondrial encephalomyopathy. Dystonia, myoclonus, and chorea are more frequently described as first manifestations (Nelson et al., 1995; Thyagarajan et al., 1995; de Vries et al., 1996). Parkinsonism has been observed in families with Leigh syndrome or as one of the symptoms of another type of mitochondrial encephalomyopathy (van Erven et al., 1989). Idiopathic Parkinson's disease has been associated with a decline in activities of complex I, II and IV but not with a selective deficiency of complex III. Parkinson's disease patients also do not have a lactic acidosis or become acidotic on exercise (Hattori et al., 1992). In a period of about 5 years, the akinetic rigid syndrome in our patient was complicated into an encephalopathy with seizures and stroke-like events. Apart from the onset as an akinetic rigid syndrome, the clinical symptoms together with the

morphological results of a muscle biopsy specimen in this patient resemble classic MELAS (Mariotti et al., 1995). Therefore, the clinical picture can be considered that of an overlap syndrome between juvenile parkinsonism and MELAS syndrome.

The high percentage of mutated cyt *b* gene in skeletal muscle can explain the strongly diminished complex III activity in this tissue. The clinical phenotype of MELAS in relation to a defective complex III activity is uncommon and is associated with a deficiency of complex I or complex IV activity in the majority of cases (Mariotti et al., 1995). At the DNA level, it is interesting that a drastic mutation in a complex III subunit gene eventually can have a clinical (MELAS) manifestation similar to that of a tRNA mutation (de Coo et al., 1998). Our data extend the genetic basis of MELAS further.

CHAPTER 5

Isolated case of mental retardation and ataxia due to a *de novo* mitochondrial T8993G mutation

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To the Editor:

Neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) and subacute necrotizing encephalomyelopathy (Leigh disease) are both associated with an alteration of nucleotide 8993 in the mitochondrial *ATPase 6* gene. In NARP, the T-to-G transversion at that position changes leucine into arginine (Holt et al., 1990). In Leigh syndrome, the same mutation can be found (Tatuch et al., 1992), as can a T-to-C transition (de Vries et al., 1993), which changes this leucine into proline. Clinical manifestations occur for NARP, when ~60-90% mutated mtDNA is present. In case of Leigh, these percentages usually exceed 95% (Tatuch et al., 1994). It is known that this mutation can segregate very rapidly within pedigrees (Santorelli et al., 1993; Tulinius et al., 1995). Here we report on a sporadic case with mental retardation and ataxia without retinitis pigmentosa in which the T8993G mutation was found.

The patient was born after an uncomplicated pregnancy, with a perinatal asphyxia. The neonatal period was unremarkable. Because of complaints about exercise intolerance and slow recovery after viral infections, she was medically investigated at the age of 5 years. Clinical examination determined her to be moderately mentally retarded. She showed a cerebellar ataxia with a normal muscle tone and extensor plantar responses. No signs of hearing or vision impairment were noted, and retinitis pigmentosa was excluded by careful ophthalmological investigations. Tests for cardiac

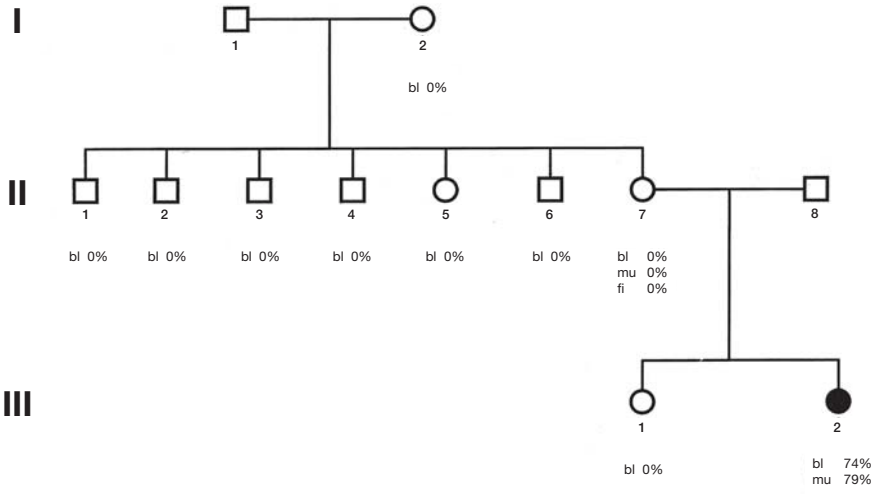

Fig. 1. Pedigree of the T8893G-positive patient with mental retardation and ataxia. The percentages given below the pedigree symbols are the percentages mutated mtDNA found in blood (bl), fibroblasts (fi), and muscle (mu).

functions, magnetic resonance imaging, electroencephalography, electromyography, and nerve-conduction studies showed no abnormalities. Biochemical investigation revealed an elevated lactic acid level in blood and cerebrospinal fluid. A muscle biopsy revealed no morphological and histochemical abnormalities. The clinical findings are consonant with the spectrum of symptoms in Leigh disease (Tatuch et al., 1992). None of the members of the family shown in figure 1 showed evidence of a neurological disorder, on clinical examination.

DNA isolated from the muscle sample of the patient was analysed for mutations in the mtDNA. Deletions and the point mutations at nucleotide positions 3243, 3271, 8344, and 8356 were excluded. In order to detect the NARP/Leigh mutations, a 633-bp DNA fragment containing nt 8993 was amplified and digested with *Hpa*II for the T-to-G or the T-to-C mutation and with *Ava*I for the T-to-G mutation (de Vries et al., 1993). The PCR fragment could be digested with *Ava*I and *Hpa*II (data not shown), indicating the T8993G transversion. A radioactive PCR was performed, and the signal intensities of the bands after *Ava*I digestion were compared, to determine the amount of mutated mtDNA. Radioactive [γ-^{32}P]dCTP was added before the last elongation of the PCR reaction, to ensure that the intensity ratio of the *Ava*I fragments was a true reflection of the *in vivo* heteroplasmy. The patient's DNA from peripheral blood cells and muscle showed 74% and 79% mutated DNA, respectively. The same test was performed on DNA samples of the mother, sister, and maternal relatives of the patient. For the mother, peripheral white blood cells, fibroblasts, and a muscle sample were tested. However, even after prolonged autoradiographic exposure, no mutated mtDNA could be observed in any of the maternal tissues (data not shown). The sensitivity of the radioactive PCR test, estimated by dilution of the patient sample with a normal sample, was estimated to be >0.1%. Supportive evidence for the absence of the mutation in the mother can be derived from her healthy sibs and daughter, who were also negative for the T8993G mutation. To our knowledge, it has never been reported that the muscle tissue was negative in patients in whom a mtDNA mutation was found. The occurrence of a *de novo* mutation is the most likely explanation for our observations.

The spontaneous occurrence of mutated mtDNA in this patient is similar as the occurrence of deleted mtDNA seen in most patients with Pearson syndrome who also have a negative family history. Santorelli et al. (1993) and Tulinius et al. (1995) have also reported evidence for new mutations, but in these cases the asymptomatic mothers were carrier of appreciable amounts of mutated mtDNA. In cows, it has been observed that the mitochondrial genome can be replaced completely by a nucleotide-sequence

variant within a single generation (Koehler et al., 1991). Very recently, it has been shown that low levels of deleted mtDNA do occur in oocytes, which are comparable to levels seen in tissue specimens of older subjects (Chen et al., 1995).

Since point mutations do arise on aging, it is not unlikely that point mutations occur in oocyte mtDNA also. Attardi and coworkers have put forward the hypothesis that the mitochondrion is the unit of segregation (Yoneda et al., 1992). It might very well be that in our case the mutation has arisen before or during the mitochondrial expansion in the oocyte. In the stochastic purification following the dilution of the mtDNA down to one copy per organelle (Ashley et al., 1989), one mitochondrion with only mutated mtDNA may have survived. In the developing organism, the mutated mtDNA may have expanded because of the feedback mechanism of the functionally compromised mitochondria. One may wonder, then, whether this should be a rare event. Recent *in vitro* experiments with various mtDNA point mutations in mtDNA-less cells indicate that, in culture, the shift to either wild type or mutant may be dependent on the nuclear background (Yoneda et al., 1992; Chomyn et al., 1994; Mariotti et al., 1994; Dunbar et al., 1995). Obviously, identification of such nuclear-encoded, possibly tissue-specific, modifier genes will be needed to understand the genesis and frequency of mtDNA disorders.

Acknowledgements

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CHAPTER 6

Mutation analysis of the entire mitochondrial genome using denaturing high performance liquid chromatography

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

Abstract

In patients with mitochondrial disease a continuously increasing number of mitochondrial DNA (mtDNA) mutations and polymorphisms have been identified. Most pathogenic mtDNA mutations are heteroplasmic, resulting in heteroduplexes after PCR amplification of mtDNA. To detect these heteroduplexes, we used the technique of denaturing high performance liquid chromatography (DHPLC). The complete mitochondrial genome was amplified in 13 fragments of 1-2 kb, digested in fragments of 90-600 bp and resolved at their optimal melting temperature. The sensitivity of the DHPLC system was high with a lowest detection of 0.5% for the A8344G mutation. The muscle mtDNA from six patients with mitochondrial disease was screened and three mutations were identified. The first patient with a limb-girdle-type myopathy carried an A3302G substitution in the tRNA$^{Leu(UUR)}$ gene (70% heteroplasmy), the second patient with mitochondrial myopathy and cardiomyopathy carried a T3271C mutation in the tRNA $^{Leu(UUR)}$ gene (80% heteroplasmy) and the third patient with Leigh syndrome carried a T9176C mutation in the ATPase6 *gene (93% heteroplasmy). We conclude that DHPLC analysis is a sensitive and specific method to detect heteroplasmic mtDNA mutations. The entire automatic procedure can be completed within 2 days and can also be applied to exclude mtDNA involvement, providing a basis for subsequent investigation of nuclear genes.*

INTRODUCTION

Energy production in cells by the process of oxidative phosphorylation (OXPHOS) is one of the important functions of mitochondria. Part of the proteins of the OXPHOS complex is encoded by the mitochondrial DNA (mtDNA). This is a maternally inherited 16,569 bp long, circular double-stranded molecule with 37 genes, encoding OXPHOS subunits, two rRNA genes and 22 tRNA genes. Mutations in the mtDNA as well as mutations in nuclear genes can cause a clinical phenotype of a mitochondrial disorder. In the past 12 years numerous mtDNA mutations have been described in patients with mitochondrial disease (Servidei, 2000). These mutations were inherited maternally, or originated *de novo.* Except for the mtDNA mutations causing Leber hereditary optic neuropathy, most other pathogenic mutations are in general heteroplasmic (i.e. a mixture of wild-type and mutant mtDNA in the same tissue or cell) and the mtDNA mutations become clinically manifest when the percentage of a mutation increases above a tissue-specific threshold (DiMauro and Moraes, 1993). Non-pathogenic polymorphisms are normally homoplasmic (Zeviani and Antozzi, 1997).

Many mtDNA disorders show a wide spectrum of clinical manifestations and variation in the mode of onset, course and progression of the disease (Rose, 1998). This is seen between families, but also within families. Maternal relatives can inherit different percentages of mutant mtDNAs and can present with different clinical manifestations (DiMauro and Moraes, 1993). Patients with a mitochondrial disorder are usually screened for the presence of deletions and specific mtDNA mutations in blood or muscle, based on these symptoms. The protocols are becoming increasingly complex, as the number of mutations in mtDNA accumulate and the specificity of the symptoms lags behind. Furthermore, only known mutations are detected with these protocols and unknown mutations remain unresolved. As an alternative the entire mitochondrial genome can be screened, but these approaches remain laborious [denaturing gradient gel electrophoresis (DGGE) analysis], lack sufficient sensitivity (sequence analysis) or do not sufficiently discriminate between hetero- and homoplasmic variants [single stranded conformation polymorphism (SSCP) analysis]. Therefore, we applied the relatively new and automatable denaturing high performance liquid chromatography (DHPLC) technology (Underhill et al., 1997; Liu et al., 1998; O'Donovan et al., 1998) which specifically identifies heteroduplexes that result from heteroplasmic mutations. When known mutations were readily detected even at low levels of heteroplasmy, we developed a protocol to screen the mtDNA for mutations

by DHPLC in a rapid and sensitive way. A group of six patients with mitochondrial disease was screened for mutations as a test case and three mutations were identified.

Materials and methods

Patients

Five patients with 4.5, 9, 18, 55 and 76% of the A3243G mutation, respectively, and three patients with 0.5, 7 and 19% of the A8344G substitution, respectively, were used to test the sensitivity and specificity of the DHPLC system. Genomic DNA was isolated from blood and (when available) muscle according to the protocol described by Mullenbach et al. (1989). Heteroplasmy was determined, as described below, using PCR primers encompassing the mutations (Table 1). Another patient carried a 4 bp deletion in the cytochrome *b* gene (60% heteroplasmy). The mtDNA of six patients with mitochondrial (encephalo-)myopathies, lactic acidosis, OXPHOS deficiencies and ragged red fibres was screened.

Table 1. Primers used to amplify genes containing the A3243G, A8344G, A3302G, T3271C and T9176C mutations.

Mutation	Position	Length (bp)	Sequence forward primer	Sequence reverse primer
A3243G	3189-3383	195	5'-CAACTTAGTATTATACCCACAC-3'	5'-TTTCGTTCGGTAAGCATTAT-3'
A8344G	8211-8410	200	5'-TCG CCTAGAATTAATTCCC-3'	5'-GGGGGTAATTATGGTGGGCC-3'
A3302G	3130-3383	254	5'-AGGACAAGAGAAATAAGGCC-3'	5'-TTTCGTTCGGTAAGCATTAT-3'
T3271C	3130-3301	172	5'-AGGACAAGAGAAATAAGGCC-3'	5'-TAAGAAGAGGAATTGAACCTCTGACCTTAA-3'
T9176C	9035-9203	169	5'-TCATGCACCTAATTGGAAGCG-3'	5'-GTGTTGTCGTGCAGGTACCAGCTTACT-3'

Mismatches are underlined.

DHPLC analysis of specific mutations

Primers (Gibco BRL, Life Technologies, Cleveland, OH, USA) used for the amplification of fragments containing the A3243G mutation [in $tRNA^{Leu(UUR)}$] and the A8344G mutation (in $tRNA^{Lys}$) are displayed in Table 1. Reactions were performed in a 50 µl vol using 66 ng genomic DNA as template, 8.33 µM dNTP each (Pharmacia Biotech, Buckinghamshire, UK), 14 pmol of the forward and reverse primer, 1 U *Taq* DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) and OptiTaq buffer B (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.4) for $tRNA^{Leu(UUR)}$ and OptiTaq buffer G (50 mM NaCl instead of 50 mM KCl) for $tRNA^{Lys}$. Using the GeneAmp® PCR system 9700 (PE Applied Biosystems), PCR conditions for $tRNA^{Leu(UUR)}$ were as follows: first, one cycle of 94°C for 5 min, followed by 32 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 45 s,

and finally, one cycle of 72°C for 7 min followed by cooling to 4°C. PCR conditions for $tRNA^{Lys}$ were similar, except for the annealing temperature of 54°C and the number of cycles, which was 35. PCR products were tested by gel electrophoresis on a 2% agarose gel stained with ethidium bromide.

DHPLC analysis was performed on an automated DHPLC instrument (Transgenomic Inc., San Jose, CA, USA). The stationary phase consisted of a DNA Sep® column, which binds DNA during analysis. The mobile phase consisted of two eluents (pH 7.0). Buffer A contained triethylammonium acetate (TEAA), which interacts with the negatively charged phosphate groups on the DNA as well as with the surface of the column (http://www.transgenomic.com/Pages/Applicationnotes.shtml#101). Buffer B contained TEAA with 25% of the denaturing agent acetonitrile. Fragments were eluted with a linear acetonitrile gradient of 2% per min at a flow rate of 0.9 ml/min. Increasing the concentration of acetonitrile at a fixed temperature will denature the fragments. Temperatures for successful resolution of heteroduplexes were both calculated by the DHPLC Melt program (http://insertion.stanford.edu/cgi-bin/melt.pl) and experimentally determined for the fragments containing the A3243G and A8344G mutation. For this latter purpose samples were analysed at increasing column temperatures, until a significant decrease in retention time occurred.

DHPLC analysis of the complete mitochondrial genome

For DHPLC analysis of the mtDNA in small fragments, appropriate restriction sites were selected (Clone Manager 4.0 program, Scientific & Educational software) to yield fragments between 90 and 600 bp. These fragments were generated from longer PCR fragments using the primers shown in Table 2 (Gibco BRL, Life Technologies). Reactions were performed in a 50 µl vol using 330 ng genomic DNA as template, 8.33 µM dNTP each (Pharmacia Biotech), 14 pmol each primer, 2 U *Taq* DNA polymerase (PE Applied Biosystems) and OptiTaq buffer B. Fragment 2 was amplified using 14 pmol of the forward and 28 pmol of the reverse primer. Using the GeneAmp® PCR system 9700 (PE Applied Biosystems), PCR conditions were as follows: first, one cycle of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 57°C for fragments 1-10 and 67°C for fragments 11-13 for 1 min, 72°C for 2 min, and finally, one cycle of 72°C for 7 min followed by cooling to 4°C. PCR results were tested by gel electrophoresis on a 1% agarose gel stained with ethidium bromide.

Fragments were denatured after PCR at 95°C for 10 min, reannealed at 65°C for 10 min and slowly (1°C/s) cooled to 4°C to form heteroduplexes. PCR fragments were then cleaved in smaller fragments varying in size

Table 2. Primer sequences used to amplify the mtDNA in 13 fragments of 1-2 kb in length.

Fragment	Position	Length (bp)	Sequence forward primer	Sequence reverse primer
1	656-2490	1835	5'-TGGTCCTAGCCTTTCTATTAGC-3'	5'-GGGTAAGATTTGCCGAGTTCC-3'
2	2433-4224	1792	5'-CAGGCATGCTCATAAGGAAAGG-3'	5'-GGAGACATATCATATAAGTAATGC-3'
3	4152-5735	1584	5'-CGACCAACTCATACACCTCC-3'	5'-GAGAAGTAGATTGAAGCCAG-3'
4	5470-6908	1439	5'-CGCTACTCCTACCTATCTCC-3'	5'-AGATCATTTCATATTGCTTCCGT-3'
5	6789-8000	1212	5'-GGAATAGACGTAGACACACGAG-3'	5'-CAACGTCAAGGAGTCGCAGGT-3'
6	7699-8738	1040	5'-CCTGTATGCCCTTTTCCTAAC-3'	5'-ATAAGAGATCAGGTTCGTCCTT-3'
7	8577-10407	1831	5'-ACCCGCCGCAGTACTGATCAT-3'	5'-CCAATTCGGTTCAGTCTAATCC-3'
8	10233-11249	1017	5'-GCTATTACCTTCTTATTATTTGATC-3'	5'-GTGCGATGAGTAGGGGAAGG-3'
9	10866-12420	1554	5'-TCATCCCTCTACTATTTTTTAACC-3'	5'-TTTGTTAGGGTTAACGAGGG-3
10	12175-13708	1534	5'-TGACAACAGAGGCTTACGACC-3'	5'-CCAGGCGTTTAATGGGGTTTAGT-3'
11	13354-14458	1105	5'-TTTATGTGCTCCGGGTCCATCAT-3'	5'-GATGGCTATTGAGGAGTATCCT-3'
12	14399-15593	1195	5'-ACACTCACCAAGACCTCAACC-3'	5'-ATCGGAGAATTGTGTAGGCGAAT-3'
13	15498-711	1783	5'-GCGACCCAGACAATTATACCCT-3'	5'-AACGGGGATGCTTGCATGTGT-3'

Table 3. Enzymes used for restriction digestion (in a volume of 50 µl) and DHPLC analysis temperatures.

Fragment	Enzyme	Restriction site	Enzyme used (U)	Incubation temperature (°C)	Fragments after restriction	Temperatures for DHPLC analysis (°C)
1	*Bfa*I	C↓TAG	10	37	132 187 260 365 401 484	55, 57, 58, 59, 60
2	*Nla*III	CATG↓	10	37	120 295 382 460 527	55, 58, 59, 60, 61
3	*Hpa*II	C↓CGG	5	37	135 396 493 560	55, 56, 58, 59, 60
4	*Hae*III	GG↓CC	5	37	123 190 233 369 524	55, 57, 59, 60, 61
5	*Hae*III	GG↓CC	5	37	113 170 240 300 389	55, 57, 58, 59, 60
6	*Alu*I	AG↓CT	10	37	229 377 434	54, 57, 58, 59, 60
7	*Taq*I	T↓CGA	5	65	159 227 270 308 381 486	50, 55, 58, 59, 60
8	*Hin*fI	G↓ANTC	15	37	141 205 278 393	53, 56, 57, 58, 60
9	*Hph*I	GGTGA$(N)_8$↓	7.5	37	284 330 413 528	55, 57, 58, 59, 60
10	*Sfa*NI	GCATC$(N)_5$↓	0.8	37	292 347 410 485	55, 57, 58, 59, 60
11	*Hae*III	GG↓CC	5	37	500 593	57, 58, 59, 60, 61
12	*Dpn*II	GA↓TC	5	37	191 235 297 472	55, 57, 59, 60, 61
13	*Alu*I	AG↓CT	10	37	90 130 218 280 482 548	53, 57, 58, 59, 60

from 90 to 560 bp by the enzymes shown in Table 3. For each reaction ~300 ng of PCR product and 0.8-15 U of the appropriate enzyme (Roche Diagnostics, Mannheim, Germany and New England Biolabs, Beverly, MA, USA) were used in a volume of 50 µl, according to the conditions given by the manufacturer, leaving out the dithiothreitol, which may be harmful to the DNA Sep® column. Digestion results were tested by gel electrophoresis on a 3% agarose gel stained with ethidium bromide.

The optimal conditions for DHPLC analysis of fragments created by digestion were calculated for each individual fragment using the DHPLC Melt program (http://insertion.stanford.edu/cgi-bin/melt.pl) and the WAVEmaker Utility Software (Transgenomic Inc.). The fragments were first analysed under non-denaturing conditions (50°C) to test for the presence of aspecific fragments and the resolution. Each vial of 100 µl diges-

tion product is sufficient for analysis at five temperatures using 10-20 µl per injection. The injection volume can be adjusted to the signal of the product on agarose.

Sequence analysis

PCR fragments were purified with the QIA quick PCR purification kit protocol (Qiagen, Valencia, CA, USA) and cycle sequenced in a volume of 20.0 µl using 8.0 µl BigDye Terminator Ready Reaction Kit (PE Applied Biosystems), 3.2 pmo1 of the forward or reverse primer and 6.0 µl of PCR product. Using a GeneAmp® PCR system 9700 (PE Applied Biosystems), cycle sequence conditions were as follows: 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min, and finally cooling to 4°C. Then, DNA was precipitated by mixing 20 µl of the product with 74 µl 70% ethanol and 0.5 mM $MgCl_2$, incubated at room temperature for 15 min and centrifuged at 10,000 *g* for 15 min. Samples were loaded on a denaturing Long Ranger gel (Pharmacia Biotech), on the ABI Prism 377 automatic sequencer/genetic analyser and analysed with the Sequence® 2.1 analysis software (PE Applied Biosystems).

Determination of heteroplasmy

The percentage heteroplasmy of the A3243G, A8344G, A3302G, T3271C and T9176C mutations was determined by analysing fluorescently labelled PCR products on a 4% poly-acrylamide gel under non-denaturing conditions. Primers (Gibco BRL, Life Technologies) used for the amplification of fragments containing these five mutations are displayed in Table 1. PCR amplification, as described for the A3243G mutation, was followed by a single last cycle, using fluorescent dUTPs (dTTP:fdUTP, 250:1). The PCR fragment with the A3243G mutation was digested with *Apa*I (New England Biolabs), yielding two fragments in the presence of the mutation (140 and 60 bp) and one fragment in the wild-type (200 bp). The PCR fragment with the A8344G mutation was digested with *Bgl*I (New England Biolabs), yielding two fragments in the presence of the mutation (133 and 42 bp) and one fragment in the wild-type (175 bp). The PCR fragment containing the A3302G mutation was digested with *Dde*I (New England Biolabs), yielding three fragments (106, 85 and 63 bp) in the patient and two fragments (191 and 63 bp) in the wild-type. The PCR fragment containing the T3271C mutation was generated with a mismatch primer, creating an A*fl*II site (New England Biolabs), yielding two fragments in the patient (26 and 146 bp) and one fragment (171 bp) in the wild-type. The PCR fragment containing the T9176C mutation was also generated with a mismatch primer, creating a *Bst*XI site (New England Biolabs), yielding

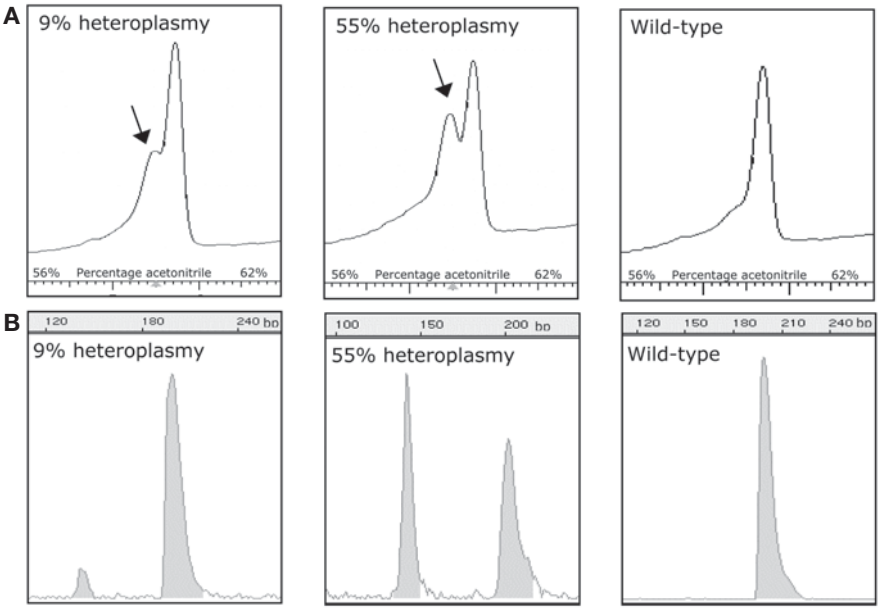
two fragments (148 and 21 bp) in the patient and one fragment (169 bp) in the wild-type. Digestion products were then analysed on an ABI Prism 377 automatic genetic analyser with the Genescan® 2.1 analysis software (PE Applied Biosystems). The ratio of the mutation peaks versus wild-type was determined. In case not all peaks could be quantified, a correction was made for the size of the fragments and the related incorporation of fdUTP.

Results

The sensitivity of DHPLC analysis

DHPLC analysis was performed on a variety of percentages of the A3243G and A8344G mutations in PCR fragments of 200 bp (Fig. 1). In the absence of heteroplasmic mutations, the chromatogram shows only a single homoduplex peak, whereas a heteroplasmic mutation leads to two peaks, the first one representing the heteroduplexes and the second one the homoduplexes. In the case of a low percentage of heteroplasmy, heteroduplexes are represented as a 'shoulder' in the peak. The A3243G mutation was detected in samples with heteroplasmy ranging from 4.5 to 76% (Fig. 1B). Figure 1A shows 9 and 55% mutated mtDNA compared to 0% het-

Fig. 1.



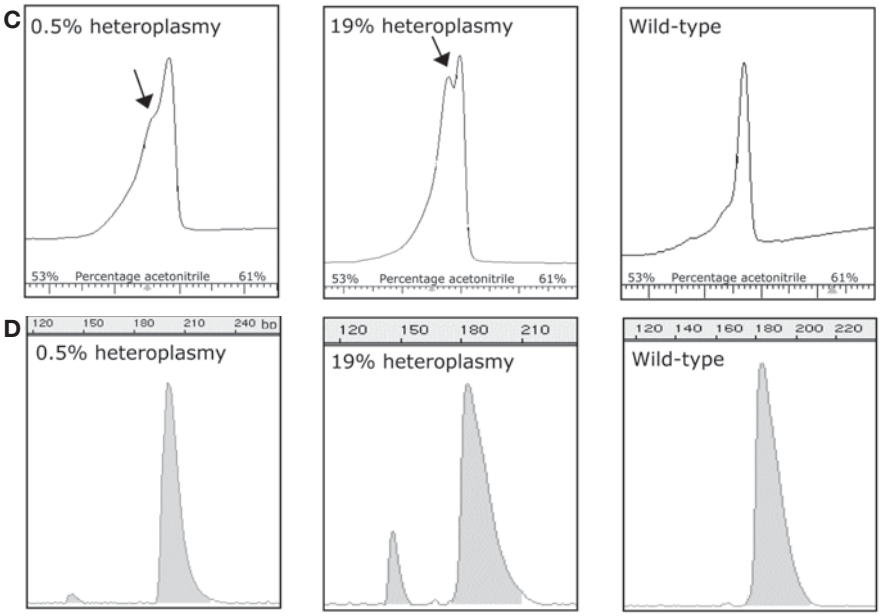


*Fig. 1. DHPLC analysis of two known mutations with different amounts of mutated mtDNA. (***A** *and* **C***) DHPLC elution peaks of characterized percentages of the A3243G and A8344G mutation (***B** *and* **D***) using increasing amounts of acetonitrile (x-axis). The y-axis represents the intensity of the peaks. The arrow indicates the heteroduplexes. (***B** *and* **D***) The same samples using last-cycle PCR followed by restriction digestion to determine the exact percentage of the mutation. On the x-axis the length of the fragments after digestion is displayed.*

eroplasmy (wild-type). The A8344G mutation was also detected in samples with 0.5, 7 and 19% heteroplasmy (Fig. 1D). Figure 1C shows 0.5 and 19% mutated mtDNA compared to 0% heteroplasmy. The sensitivity of the method is thus high for the examples given.

Next we amplified the entire mtDNA in 13 fragments (numbered 1-13, primers in Table 2). PCR products were optimised and restriction enzymes were chosen to generate fragments in the DHPLC size range without overlap (Table 3). Figure 2 shows chromatograms of the digestion products of fragments 4 and 9 under non-denaturing conditions. Fragment 4 yields *Hae*III fragments of 123, 190, 233, 369 and 524 bp in length and fragment 9 *Hph*I fragments of 284, 330, 413 and 528 bp. The resolution was good and no additional fragments were observed.

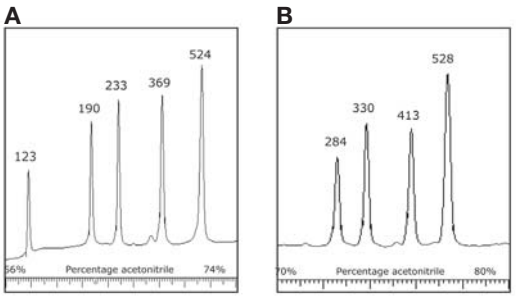


Fig. 2. DHPLC analysis of fragments 4 and 9 after digestion with Hae*III and* Hph*I respectively. Fragment 4 is cleaved into fragments of 123, 190, 233, 369 and 524 bp (***A***) and fragment 9 into fragments of 284, 330, 413 and 528 bp (***B***). Intensities of the elution peaks are an indication of the size of the fragment. In case of ambiguity, additional digestion reactions were performed to determine which fragment each of the peaks represents. Fragments 4 and 9 are representative for all other fragments.*

Table 4. Polymorphisms creating (+) or deleting (-) restriction sites of the enzymes used.

Polymorphism	Gene	Restriction site	Result	No. of patients (6 in total)
A2706G	16S rRNA	+	295 bp → 146 + 149 bp	4
G6260A	COX I	-	233 + 123 bp → 356 bp	2
C8958A	ATPase 6	-	381 + 486 bp → 867 bp	1
T11299C	ND4	+	528 → 139 + 389 bp	1
A15607G	Cyt b	+	280 → 170 + 110 bp	2

Because polymorphisms in the mtDNA can occur within the restriction sites of the enzymes, sometimes less and sometimes more fragments are observed. Five such polymorphisms were identified (Table 4). At higher temperatures the number of fragments decreased, depending on the melting temperature of the individual fragments. It should be noted that separation of the fragments is based on melting characteristics and that it is not always the largest fragment which is finally last eluted. Additional digestion reactions were performed in case of ambiguity to determine which fragment each of the peaks represents. Figure 3A displays the A3243G mutation with 55% heteroplasmy detected in peak 2 (460 bp) of fragment 2. Figure 3C shows the A8344G mutation with 7% heteroplasmy at 57°C. Figure 3E shows the heteroplasmic l4787delTTAA mutation (60%) in the cytochrome *b* gene, present in peak 4 (472 bp) of fragment 12 at 57°C. The corresponding wild-type fragments are shown in Figure 3B, 3D and 3F, respectively. All mutations were readily detected in the digestion products, although the patterns can be quite complex because

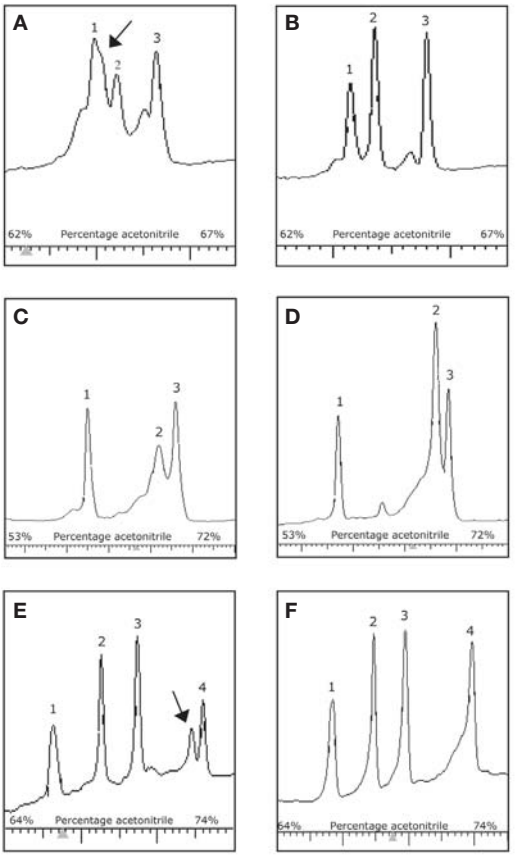


*Fig. 3. DHPLC analysis of known mutations in digestion products compared with wild-types. The A3243G mutation (55% heteroplasmy) in tRNA$^{Leu(UUR)}$ (***A***) is compared with the wild-type fragment (***B***) representing the 382 (peak 1), 460 (peak 2) and 527 bp (peak 3) digestion products of fragment 2 at 58°C. The mutation is present in peak 2, which decreases in intensity. The arrow indicates the heteroduplexes merging into peak 1. The A8344G mutation (7% heteroplasmy) in tRNA^{Lys} (***C***) is compared with the wild-type fragment 6 (***D***), representing the 229 (peak 1), 434 (peak 2) and 377 bp (peak 3) products of fragment 6 at 57°C. The mutation is present in peak 2, which decreases in intensity and broadens, whereas the size of peaks 1 and 3 remains the same. The 14787delTTAA (60% heteroplasmy) in cytochrome b (***E***) is compared with the wild-type fragment 12 (***F***), showing four homoduplex peaks at 57°C representing the 191 (peak 1), 235 (peak 2), 297 (peak 3) and 472 bp (peak 4) products. The mutation is present in peak 4. The arrow indicates the heteroduplexes.*

heteroduplexes of one fragment can overlap with homoduplexes of another fragment. Any alteration, which is either the presence of a heteroduplex or the relative decrease in intensity of the homoduplexes, is an indication for a mutation.

DHPLC analysis of patients with unknown mutations

The muscle mtDNA of six patients with evident mitochondrial disease were screened by DHPLC. One patient with a limb-girdle-type myopathy

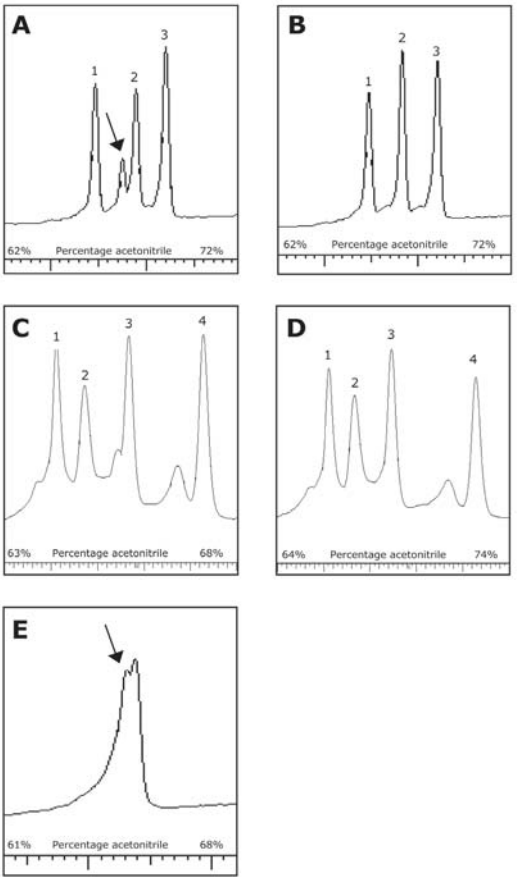


*Fig. 4. DHPLC analysis of fragments 2 and 7 of three patients with unknown mutations. Fragment 2 of patient 1 (***A***) was compared with the wild-type fragment 2 (***B***), showing three homoduplex peaks at 55°C representing the 382 (peak 1), 460 (peak 2) and 527 bp (peak 3) products. Patient 1 showed heteroduplexes in peak 2 (460 bp) as indicated by the arrow. Sequence analysis revealed the presence of an A3302G substitution. Fragment 2 of patient 2 showed a shoulder in peak 3 (460 bp) (***C***), as indicated by the arrow, compared to the wild-type at 58°C (***D***). The four peaks represent the 295 (peak 1), 382 (peak 2), 460 (peak 3) and 527 bp (peak 4) fragments. In these two samples the polymorphic restriction site, seen in the other patients, was absent, showing the fragment of 295 bp as the fourth peak. Sequence analysis revealed the presence of a T3271C substitution. Patient 3 showed heteroduplexes in the 867 bp digestion product of fragment 7 (***E***) as indicated by the arrow. No similar wild-type fragment was available because of a* Taq*I polymorphism. Sequence analysis revealed the presence of a T9176G substitution.*

showed heteroduplexes in the fragment containing the $tRNA^{Leu(UUR)}$ gene (Fig. 4A). Sequence analysis revealed a heteroplasmic A3302G substitution. Heteroplasmy was determined to be ~70% in muscle. A second patient also showed a heteroduplex in the $tRNA^{Leu(UUR)}$ gene (Fig. 4C), which by sequence analysis turned out to be the T3271C mutation. The percentage of the mutation was 80% in muscle from this patient with mitochondrial myopathy and cardiomyopathy. The pattern is different

from the previous patient because of an *Nla*III polymorphism. A third patient with signs of Leigh syndrome showed heteroduplexes in the largest individual fragment of 867 bp (Fig. 4E). Sequence analysis revealed a heteroplasmic T9176C substitution in the *ATPase6* gene. The percentage of the mutation in muscle was ~93%. No comparable wild-type pattern is available because of a polymorphism in a *Taq*I site in this patient. The other three patients did not carry a heteroplasmic mutation in the mtDNA.

Discussion

PCR reactions were optimised to amplify the entire mtDNA in 13 overlapping fragments using two different PCR conditions. Restriction digestion conditions were adjusted for each individual fragment. Testing the digestion products on agarose revealed five different polymorphisms (Wallace et al., 1988; Howell et al., 1995; Rieder et al., 1998). These changes in restriction digestion sites are important to know prior to the analysis, because the melting temperatures from these new fragments may differ from the original fragments. All fragments showed good resolution and no aspecific products were detectable. Digestion of fragment 9 yielded products that eluted after 10 min. It is possible to start at a higher acetonitrile concentration to decrease the time required for analysis. However, if the analysis time is too short, the risk of fragments eluting into the first injection peak exists. The optimal temperatures for DHPLC analysis and acetonitrile gradients of all individual fragments can be determined experimentally or using the DHPLC Melt program (http://insertion.stanford.edu/cgi-bin/melt.pl). The predicted temperatures did not differ by more than 1-2°C from the experimentally determined temperatures. When fragments are being analysed at 1°C below and above the recommended temperature, this 1-2°C difference is within the range of reliable detection of heteroduplexes. For each fragment of 1-2 kb, five temperatures are recommended for analysis, ranging from 50 to 60°C. Digestion products should be first analysed for wild-type fragments at each individual temperature to determine the normal melting behaviour. When screening for unknown mutations it is important to be able to discriminate between peaks, which appear as a consequence of the melting process and peaks caused by the presence of heteroduplexes.

Samples with 4.5, 9, 18, 55 and 76% of the A3243G mutation and 0.5, 7 and 19% of the A8344G mutation were used to test the sensitivity and accuracy of the DHPLC. All heteroplasmy percentages were detectable, making DHPLC analysis a very sensitive technology. Mutations were re-

presented as two peaks and mutations with low percentages, such as 0.5% heteroplasmy, as a 'shoulder' in the peak. Apart from the heteroduplex peak, which under some conditions can overlap with (melting) homoduplexes, the reduction in intensity of a peak in comparison with the other peaks is a strong indicator for the presence of a mutation in that specific fragment. Transgenomic™ describes the resolution of hetero- and homoduplexes to be optimal, when all four hetero- and homoduplex species are fully resolved (http://www.transgenomic.com/Pages/Applicationnotes.shtml#101). In the DHPLC analysis performed here, only two species were consistently visible. This was also reported by others using DHPLC as a mutation detection system (Liu et al., 1998; O'Donovan et al., 1998; Giordano et al., 1999). It has also been described that different mutations show different profiles (O'Donovan et al., 1998). The stability of heteroduplexes and the degree of partial denaturation surrounding it varies, depending on the nature of the mutation and the flanking base pairs. This may explain why some mutations show a better resolution of hetero- and homoduplexes than other mutations (O'Donovan et al., 1998).

DHPLC has been described as a very sensitive method in mutation screening of nuclear genes. Sensitivity and specificity of this method have been evaluated in a blind analysis performed on exon H of the *factor IX* gene and exon 16 of the *neurofibromatosis type 1* gene. In this analysis 55/55 (100%) individuals carrying 48 unique mutations were correctly identified, as were 55/55 individuals with wild-type alleles (Wagner et al., 1999). DHPLC has also been used to analyse sequence variation in the *BRCA2* gene, identifying 82 sequence variants (Giordano et al., 1999). In another study 37 out of 40 (92.5%) PCR products containing defined sequence variation were identified and no alterations among 196 PCR products containing homozygous normal sequence were indicated (Liu et al., 1998). The few exceptions encountered may relate to the extreme G-C content of the tested fragments. This high sensitivity indicates that the absence of heteroduplexes after DHPLC analysis of the mtDNA most likely excludes the presence of heteroplasmic mutations in the mtDNA, making the involvement of such a mtDNA mutation as the cause for the disease unlikely. This provides a strong basis for further investigation of nuclear genes. This is also an important issue for counselling as mtDNA is transmitted maternally, whereas nuclear gene defects are predominantly autosomal recessive.

The entire mitochondrial genome of six patients showing clinical signs of a mitochondrial disorder was screened with DHPLC. In one patient, the A3302G mutation in $tRNA^{Leu(UUR)}$ was found with ~70% heteroplasmy in muscle. This mutation involves the aminoacyl stem of $tRNA^{Leu(UUR)}$ and has

been described before, associated with abnormal mitochondrial RNA processing (Bindoff et al., 1993). A second patient carried the T3271C mutation in the anticodon stem of the $tRNA^{Leu(UUR)}$ gene, which was previously reported in MELAS patients (Goto et al., 1991). In a third patient, with signs of Leigh syndrome, the T9176C mutation in the *ATPase6* gene was found with ~93% heteroplasmy in muscle. This mutation was first described in two patients with familial bilateral striatal necrosis and later described in patients with Leigh disease (Thyagarajan et al., 1995; Campos et al., 1997; Dionisi-Vici et al., 1998). A high percentage of this mutation in blood and muscle is associated with poor prognosis (Thyagarajan et al., 1995; Campos et al., 1997). We feel that the power of the method has convincingly been demonstrated to rapidly identify mutations in the mtDNA.

Methods to screen part of or the complete mtDNA sequence for mutations have been reported previously (Fauser and Wissinger, 1997; Wong and Senadheera, 1997). Most of these methods have been based on SSCP analysis, sequence analysis and DGGE (Suomalainen et al., 1992; Barros et al., 1997; van Orsouw et al., 1998; Levin et al., 1999). DHPLC is superior to the first two, because these methods do not discriminate between pathogenic mutations and polymorphisms and lack sufficient sensitivity to detect low percentages of mutations. A two-dimensional DGGE method has a similar sensitivity and detects heteroduplexes, but can be less easily automated (van Orsouw et al., 1998). The protocol described here can be automated and can be completed for a single patient within 2 days.

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CHAPTER 7

Transmission and prenatal diagnosis of the T9176C mitochondrial DNA mutation

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Abstract

Background

Leigh syndrome is a progressive neurodegenerative disorder. Here we describe a family with 3 affected children with a T9176C mutation in the ATPase 6 *gene of mtDNA (OMIM 516060). Unaffected maternal relatives were tested for carriership and one of these was pregnant of her first child. The possibility of prenatal diagnosis was evaluated. The main problem in case of the T9176C mutation was the lack of data on genotype-phenotype correlations and on variation in tissues and in time. Therefore multiple tissues of affected and unaffected carriers were analysed. Eventually prenatal diagnosis was offered and carried out twice during the pregnancy on cultured and uncultured chorionic villi and amniotic fluid cells.*

Methods

The OXPHOS complexes were analysed in patients III-2 and III-3. The T9176C mutation was tested on DNA from several tissues. The fragment containing the mutation was PCR-amplified and labelled with fluorescent nucleotides in the last round. Digestion with a mutation-specific enzyme, followed by electrophoresis on a sequencing gel, allowed a reliable, quantitative determination of the mutation percentage.

Results

In muscle of patient III-2 and III-3, the activity of pyruvate dehydrogenase was increased and that of complex V decreased. The pregnant woman had a mutation load of 55% in blood. Literature data and our own results suggested that prenatal diagnosis might be reliable, although the number of data was too limited to make this significant. The result of the prenatal test was a mutation percentage just below the assumed threshold of expression (90%). The couple decided to continue the pregnancy and an apparently healthy child was born with an associated unclear prognosis.

Conclusions

This is the first prenatal diagnosis for a carrier with the T9176C mutation. Prenatal diagnosis for this mutation is technically reliable but prognostic predictions are not straightforward.

Key words

Leigh syndrome, mtDNA, prenatal diagnosis, preimplantation genetic diagnosis.

INTRODUCTION

Leigh syndrome (OMIM 256000) or subacute necrotizing encephalomyelopathy is a progressive neurodegenerative disorder with a poor prognosis and most of the patients die within a few years after age of onset (Rahman et al., 1996). Symptoms, occurring in early infancy or childhood, are psychomotor developmental regression, optic atrophy, ophthalmoparesis, nystagmus, ataxia, dystonia, failure to thrive and respiratory abnormalities. Characteristics are bilateral necrotic lesions on MRI in basal ganglia and brainstem and lactic acidosis. Leigh syndrome is caused by functional or molecular defects in the enzyme complexes involved in the mitochondrial energy production, including pyruvate dehydrogenase and OXPHOS complexes I, II, IV and V (DiMauro and Tanji, 1997; Dahl, 1998; Tanji et al., 2001). The inheritance of Leigh syndrome is either autosomal recessive, autosomal dominant, X-linked or maternal, and defects in nuclear genes or mitochondrial DNA (mtDNA) can cause this disease.

In this paper, we describe a family with Leigh disease caused by a mutation in the *ATPase 6* gene (OMIM 516060) at position 9176 of the mtDNA (Thyagarajan et al., 1995; Campos et al., 1997; Dionisi-Vici et al., 1998; Makino et al., 1998; Makino et al., 2000; Wilson et al., 2000; Akagi et al., 2002). The severe clinical manifestations of this disease occur at mutation percentages above 90% (Akagi et al., 2002). The mutation is maternally transmitted, but the percentage of this mutation may vary widely between oocytes, making the transmission of the associated Leigh disease fairly unpredictable (Hauswirth and Laipis, 1982; Howell et al., 2000). Mitochondria go through a genetic bottleneck in oogenesis or early embryogenesis, which involves both a reduction in number of mitochondria and of mtDNA molecules to only a very few, that subsequently repopulate the oocyte. For point mutations in the mtDNA three criteria have been proposed to allow reliable prenatal diagnosis (Poulton and Marchington, 2000; Poulton and Turnbull, 2000).

(I) A close correlation between the proportion of mutant: wild-type mtDNA (mutant load) and disease severity.
(II) A uniform distribution of mutant mtDNA in all tissues.
(III) No change in mutant load over time.

For most mtDNA mutations this information is lacking. Only a few mutations meet these criteria like the T8993G/C (associated with another form of Leigh syndrome) and A8344G (associated with MERRF syndrome i.e. myoclonic epilepsy and ragged red fibres) mutation, but not the common A3243G (associated with the MELAS syndrome, i.e. mitochondrial encephalopathy with lactic acidosis and stroke-like episodes). Here we

describe the segregation of the T9176C mutation in a family with Leigh syndrome and the development and implementation of a prenatal test for this mutation.

PATIENTS AND METHODS

Patients

Case histories of a Dutch family with the T9176C mtDNA mutation

The index patient (III-2, Fig. 1) first presented at the age of 21 months, with a sub-acute onset of cerebellar ataxia and speech retardation. The pregnancy was complicated by toxicosis but delivery and development during the first year were unremarkable. She walked at 12 months. At the age of 3 years, after a period with fever, she had an attack with loss of tone of her right arm and leg that lasted for three days. Clinical examination showed a further increased cerebellar ataxia with signs of dystonic posturing of the right arm and leg. She was a happy child and was apparently not impeded by the handicap. She spoke a few words only but was able to express herself with gestures. At 4 years and 9 months of age she was admitted in the hospital because of the suspicion for a metabolic derange-

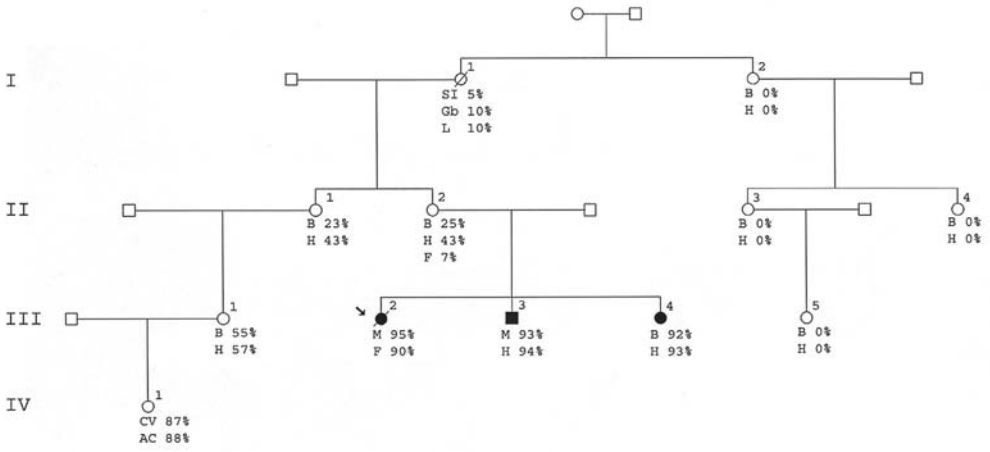


Fig. 1. Segregation of the mtDNA T9176C mutation in a large Dutch family. The percentage of the mutation is indicated below each individual. Solid symbols indicate affected and open symbols unaffected individuals. B: Blood, M: Muscle, F: Fibroblasts, H: Hair roots, Gb: Gall bladder, SI: Small intestine, CV: Uncultured Chorion villi, L: Liver, AC: Uncultured Amniotic fluid cells.

ment, after four weeks of stomach ache, dyspnoea, and increasing fatigue and weakness. A sick girl was seen with irregular breathing and deep set eyes. Apart from the cerebellar ataxia there were pyramidal tract signs with very easily elicited reflexes and extensor toe signs on both feet. Magnetic resonance imaging and computerized tomography of the brain showed extensive vaguely demarcated hypodensities in the cerebellar hemispheres, pons and mesencephalon. Supratentorially there were hypodense lesions in the caudate nuclei, the internal capsule and the basal ganglia. Peripheral and central CSF spaces were enlarged. Lesions could be compatible with infarctions in parts of the basilar and the internal carotid artery region. The clinical phenotype combined with lactic acidosis and CSF involvement made a mitochondrial encephalopathy or Leigh-like syndrome likely.

The child's condition worsened and in the following two weeks time she developed severe apnoeic and hypoventilation spells and became ventilator dependent. She died from progressive brainstem dysfunction. She underwent a muscle biopsy a few hours before death but there has been no post mortem examination.

The younger brother (III-3) and sister (III-4) of the index patient (III-2) developed a cerebellar ataxia with an onset at age 2 years. Their language development was slightly delayed. Both received, from age 4 and 2 respectively, daily vitamin B complex containing 100 mg vitamin B1, 2, 3, 5, 6, 50 mg vitamin E and 3 dd 330 mg carnitine. From the age of 3, after viral illnesses, attacks of one to several days occurred with loss of tone resulting in paraparesis losing ambulation, sighing, dyspnoea and dystonic movements. At the age of 4 the diagnosis mitochondrial encephalomyopathy of the boy was confirmed by determination of the T9176C mutation in DNA from muscle and blood (van den Bosch et al., 2000). Subsequently the DNA analysis in the other family members took place. The boy (III-3) was still ambulant at age 8, with a cerebellar ataxia, generalized mild dystonic movement disorder, a discrete pyramidal syndrome and speaks only a few words. His total IQ is 55. The EEG showed a slight delay of the background pattern. The cerebellar hemispheres showed in the cortical and subcortical regions on MRI extensive abnormalities in the flair and T2 spin echo. A quadriceps muscle biopsy showed at electron microscopy an increase of lipid droplets and enlarged mitochondria. At the age of 6, the girl (III-4) had milder problems than her brother. She speaks in short, poorly articulated sentences. Her EEG showed a slight delay in background pattern. The MRI showed very discrete abnormalities in the cerebellar hemispheres. The mother of the index patient (II-2) has periodically stomach ache but no neurological abnormalities. None of the unaffected family members showed movement disorders or other neurological or psy-

chiatric abnormalities. All numbered patients (Fig. 1) were clinically examined except the grand mother of the index patient (I-1). She died from a gastrointestinal tumour.

Methods

Muscle biochemistry

Muscle biopsies were obtained from the index patient (quadriceps) and brother under generalized anaesthesia.

Frozen muscle specimens were weighed and homogenised in 0.25 M sucrose, 10 mM HEPES and 1 mM EDTA, pH 7.1. The enzyme activities were assayed at 37ºC, unless indicated otherwise.

Complex I, NADH-coenzyme Q reductase, was measured as the rotonone sensitive oxidation of NADH with decylubiquinone as electron acceptor (de Vries et al., 1996). Complex II + III, succinate cytochrome *c* reductase, was measured as the antimycin sensitive reduction of cytochrome *c* in the presence of appropriate inhibitors at 25ºC (Scholte et al., 1995). Complex II, succinate-coenzyme Q reductase, was determined as the theonyl trifluoroacetone (2 mM) sensitive reduction of 2.6-dichlorophenolindophenol by succinate in the presence of decylubiquinone (Trounce et al., 1996). Complex III, ubiquinol cytochrome *c* reductase, was assayed as the antimycin sensitive reduction of cytochrome *c* by decylubiquinol at 25ºC (DiMauro and Tanji, 1997), in the presence of lauryl maltoside. Complex IV, cytochrome *c* oxidase, was measured according to Cooperstein and Lazarow (1951) at 25ºC, or with the detergent (Mayr and Sperl, 2000) Tween-20, at 37ºC. Complex V, ATP synthase, was determined as oligomycin sensitive uncoupler stimulated Mg-ATPase by assay of ADP with pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase and NADH (Rustin et al., 1994). This reaction was started by sonication (Mayr and Sperl, 2000). Pyruvate dehydrogenase was assayed as in Arts et al. (1987) and citrate synthase according to Srere (1969). The other methods were as in Scholte et al. (1995).

DNA extraction

DNA was extracted from blood, cultured fibroblast cells, hair roots, muscle tissue, chorionic villi cells (CVS) and amniotic fluid cells. DNA extraction from blood was done as described previously (Miller et al., 1988). DNA extraction from hair roots was done using the Qiagen RNA/DNA minikit (Qiagen GmbH, Hilden, Germany). For the DNA extraction of the other samples the standard phenol/chloroform method was used (Sambrook et al., 1989).

PCR

The mtDNA was amplified using the polymerase chain reaction (PCR) with primers, corresponding to positions 09035-09055 of the L-strand and positions 09203-09177 of the H-strand. The latter primer contains a mismatch at location 09184-09186 to create a restriction site for the restriction enzyme *Bst*XI in case of the T9176C mutation. The PCR was performed using 4 mM for each dNTP (Amersham Pharmacia Biotech AB, Uppsala, Sweden, 25 mM each), 15 mM $MgCl_2$, 0.5 M NaCl, 0.1 M Tris-HCl, 50 ng per primer, 1 U *Taq* DNA polymerase (Life Technologies, Breda, the Netherlands) in a 25 µl volume. PCR conditions were 94ºC for 5 min, followed by 32 cycles of 92ºC for 1 min, 52ºC for 1 min and 72ºC for 45 sec and a final step of 7 min at 72ºC (9600 Thermocycler, Applied Biosystems, Foster City, USA). In the last cycle 50 fmol R6G labeled dUTP (Applied Biosystems, Foster City, USA) was added. The PCR product was purified with the Qiagen PCR purification kit (Qiagen GmbH, Hilden, Germany), digested with *Bst*XI (Boehringer-Mannheim, Bayern, Germany) and separated by electrophoresis on a 4% non denaturing polyacrylamide gel at 40ºC (ABI 377 Applied Biosystems, Foster City, USA). The wild-type fragment has a length of 169 bp and the mutated fragment is cleaved in fragments of 148 and 21 bp. The mutation percentage is determined by calculating the ratio between the surface of the mutant peak (148 bp) and the total area of the mutant and normal peak (169 bp). The calculated mutation percentage is multiplied by 1.06 to correct for lower number of fluorescently labelled dUTPs that can be incorporated in the mutant peak. The experimental variation calculated for this method is 3%.

Prenatal Diagnosis

Data from literature and of the family were used to determine if this mutation was suitable for prenatal diagnosis.

Chorionic villi cells were obtained after 12 weeks of gestation and amniotic fluid after 17 weeks of gestation. Both samples were analysed immediately and after a culture period of two weeks.

Results

Clinical Biochemistry

In the index patient (III-2) blood lactate was 3.2 mmol/l (normal <1.8 mmol/l) and pyruvate was 85 µmol/l (normal <160 µmol/l). Lactate 4.6 mmol/l (normal range 0.9-2.8 mmol/l) was also elevated in cerebrospinal fluid (CSF). CSF pyruvate was 80 µmol/l. A muscle biopsy of the quadriceps muscle of the index patient (III-2) appeared normal. Histochemical staining of cytochrome *c* oxidase and succinate dehydrogenase yielded

normal results. Electronmicroscopy showed some irregularly formed fibrils with thickening of the Z-bands. One concentric laminated body was seen possibly derived from mitochondria. A muscle biopsy from patient III-3 also showed a normal morphology by routine histology and histochemistry. Electronmicroscopy showed enlarged intermyofibrillar mitochondria and increased amount of lipid droplets. One small aggregate was seen of elongated subsarcolemmal mitochondria. The brother (III-3) of the index patient showed a blood lactate of 2.2 mmol/l and a pyruvate of 140 µmol/l. CSF lactate was 3.2 mmol/l. Blood lactate in the sister (III-4) of the index patient was 2.3 mmol/l and pyruvate 150 µmol/l.

Muscle Biochemistry

The mitochondrial enzyme activities were strikingly abnormal with a severe decrease of complex V activity and an increase of pyruvate dehydrogenase activity. Complex V/CS is 0.10 and 0.08 (normal 0.40) and pyruvate dehydrogenase/CS is 0.10 and 0.09 (normal 0.03) for patient III-2 and III-3 respectively.

Mutation analysis and prenatal diagnosis

The identification of the T9176C mutation in muscle of the index patient was reported previously (van den Bosch et al., 2000). The internal variation of the method used to determine the mutation percentages is ±3%. The mutation percentage in muscle was about 95% and in fibroblasts 90%. The same mutation was identified in a symptomatic brother and sister. The brother has a mutation percentage of about 93% in muscle and 94 % in hair roots. The sister had a mutation percentage of about 92% in blood and 93% in hair roots (pedigree Fig. 1). A number of maternal relatives were tested showing either the absence or presence of the mutation (Fig. 1). Mutation percentages appeared to be more constant among tissues in patients with the higher mutation ranges (>90%). One of the female family members (III-1) was 6 weeks pregnant at the time of investigation. The mutation load in blood and hair roots was about 55% and 57%. She was at risk of having severely affected offspring, although the exact risk was unknown.

We investigated whether this mutation would meet the criteria to allow reliable prenatal diagnosis (Poulton and Turnbull, 2000). Both data from literature (Fig. 2) and of the family (Fig. 1) were used. No symptomatic patients have been reported with a mutation percentage below 90%, although the methods used vary in their precision. An estimate of the experimental variation is calculated, as it was not given in most references, and the number of patients is small (Fig. 2). Only one healthy individual had a percentage between 80% en 90% and two between 70% and 80%.

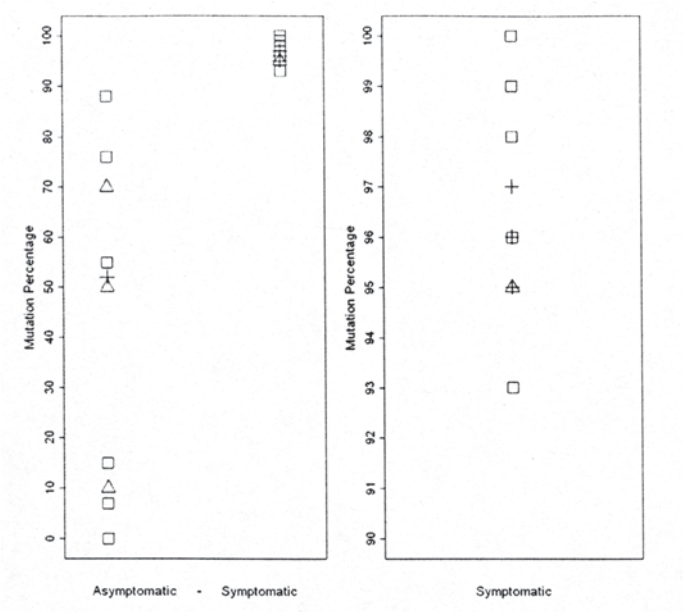
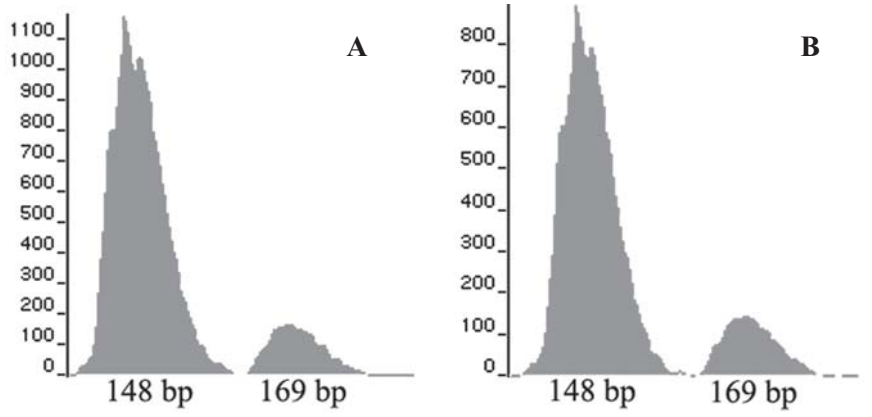


Fig. 2. Scatter plots of the literature data on the genotype-phenotype correlation of the mtDNA T9176C mutation and the detection methods used. Asymptomatic carriers show a variation in the mutation percentage of 0% to 88%, and symptomatic carriers show a variation in the mutation percentage of 93% to 100%.
□ *Method A: Last cycle [γ-^{32}P]dATP. Quantification by scanning in a Betascope 603 blot analyser (Thyagarajan et al., 1995; Campos et al., 1997; Wilson et al., 2000). Variance of the method is estimated at ±5% heteroplasmy.*
Δ Method B: Ethidium Bromide staining. Quantification using UV detection (Dionisi-Vici et al., 1998). Variance of the method is estimated at ±10% heteroplasmy.
+ Method C: Last cycle cold PCR/RFLP. Quantification on an image analyser FMBIO II (Makino et al., 1998; Makino et al., 2000). Variance of the method is estimated at ±5% heteroplasmy.

Obviously, data on the distribution of the mutation among different tissues was mainly limited to patients, all of whom had >90% mutation load in every tissue analysed. Since no unaffected mutation carrier with less than 90% mutation has been followed during a longer period no firm conclusion is possible on either time related changes in the mutation percentage or the onset of symptoms in carriers with sub critical mutation percent-

ages. Based on these limited data and on the data of the T9176G (Akagi et al., 2002; Carrozzo et al., 2001) (which has a somewhat more malignant disease course and also a lower mutation threshold for clinical expression than the T9176C, disease symptoms have been found with 70% mutation) and two other mutations in the *ATPase6* gene (T8993G/C) (which have been studied in much more detail and for which reliable prenatal diagnosis is possible (Harding et al., 1992; Bartley, 1996; Ferlin et al., 1997; White et al., 1999a; White et al., 1999b; Leshinsky-Silver et al., 2003), we decided to offer prenatal diagnosis. The prenatal sampling was undertaken with the strict understanding by the couple, written in an informed consent, that there could be considerable uncertainty in the interpretation of the results given the scarcity of data available. Somewhat arbitrary, a mutation percentage in fetal cells above 70% was considered a high risk of being affected, a mutant load between 50 and 70% would be inconclusive and a mutant load of less than 50% would mean a high chance of being unaffected.

To gather more information on tissue variation of the mutation and the time factor, we decided to analyse both a chorionic villus sample (12 weeks of gestation) and an amniotic fluid sample (17 weeks of gestation) directly and after a culturing period. The mutation percentage in chorionic villi was about 87% and after culturing 85% (Fig. 3A and 3B) and in amniotic fluid cells 88% and after culturing 86% (Fig. 3C and 3D). This difference is not statistically significant as the experimental variation was determined to be about 3%. Therefore the foetus was diagnosed as at risk of being severally affected, although the mutation percentage was just below the level of the other three affected children. Despite these results, uncertainties and associated risks the couple decided to continue the pregnancy. A healthy child was born at term after a normal pregnancy. The child did not show any



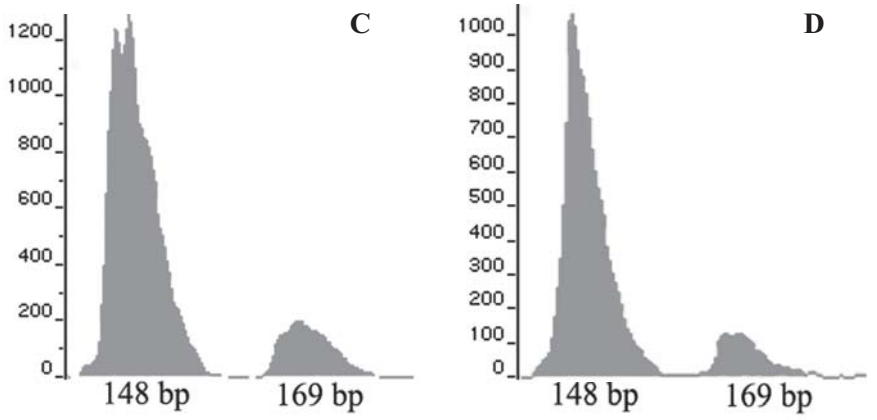


Fig. 3. Prenatal DNA-analysis for the T9176C mutation in the mtDNA. The right peak is the uncleaved PCR product and the left peak the cleaved PCR product, due to the presence of the mutation. The mutation percentage is calculated by dividing the surface of the cleaved peak (148 bp) with the surface of cleaved and uncleaved peaks (148 bp + 169 bp).
*(***A***) Uncultured Chorionic villi 87% mutation; (***B***) Cultured Chorionic villi 85% mutation; (***C***) Uncultured amniotic fluid cells 88% mutation; (***D***) Cultured amniotic fluid cells 86% mutation.*

abnormalities at the age of one year and for ethical reasons no further biochemical or genetic test will be performed as long as the child is healthy. It is important that the child remains under clinical control as the other affected children with a slightly higher mutation level did not show clinical signs of disease until the age of two.

Discussion

The segregation of point mutations in the mtDNA is not completely understood and the transmission of the mutation load is unpredictable for heteroplasmic mutations. The family reported in this paper, with 3 affected children with Leigh disease and the T9176C mutation, is a clear illustration of this. Maternal relatives of the patients either do not detectably carry the mutation or carry the mutation in varying percentages, they appear to be below the threshold of clinical expression, although in most cases no extensive neurological or biochemical examinations have been performed. However in such families to predict the recurrence risk, only DNA studies

can provide positive evidence for the carrier status of an individual. The exact estimate of the risk of having an affected child is not possible given the genetic bottleneck and limited number of data on this mutation (Thyagarajan et al., 1995; Campos et al., 1997; Dionisi-Vici et al., 1998; Makino et al., 2000; Wilson et al., 2000). It has been proposed that oocyte sampling and testing could be an acceptable approach to determine the mutation load in individual oocytes and estimate the recurrence risk (Poulton and Marchington, 2002; Thorburn, 2004).

Because of these uncertainties prenatal diagnosis, based on either DNA or enzyme tests of mtDNA mutations, is controversial. Until now seven prenatal tests were reported, for the T8993G and T8993C mutation (Harding et al., 1992; Bartley, 1996; Ferlin et al., 1997; White et al., 1999a; White et al., 1999b; Leshinsky-Silver et al., 2003). These mutations have well characterized genotype-phenotype correlations and effects of mutation load, based upon sufficient number of families tested. Recurrence risks have been calculated and a safe margin for the mutant load in case of prenatal diagnosis is established. Disease caused by the T8993C mutation is clinically less severe than the T8993G mutation. The probability of having severe symptoms of the T8993C and the T8993G mutations are low if mutation loads are below 80% and 60% respectively.

The differences in recurrence risks for point mutations in the mtDNA and the potential pitfalls prompted a statement by a group of researchers supported by the European NeuroMuscular Consortium concerning prenatal options for carriers of mtDNA mutations (Poulton and Marchington, 2000; Poulton and Turnbull, 2000). Only 3 mutations are known today with sufficient data available to judge these criteria properly. For the T8993G/C and A8344G mutation prenatal diagnosis can be reliably performed, but for the A3243G mutation this is not possible. For other mutations this is still unknown and the families involved can only be counseled in general terms. Whether prenatal diagnosis will be an option for these families will depend on the frequency of the mutation and the ethical discussions on acceptable risks.

The T9176C mutation has been described only a few times in the literature (Thyagarajan et al., 1995; Campos et al., 1997; Dionisi-Vici et al., 1998; Makino et al., 2000; Wilson et al., 2000). As the methods used are often not comparable and as usually no information is provided on detection level and experimental variation, it is difficult to draw general conclusions on the mutation threshold for clinical expression. Until now severe symptoms were only reported for patients with mutation percentages above 90% in various tissues. As the T9176C mutation is located in the same gene as the T8993G/C mutation and shows some resemblance in

clinical symptoms and progression of the disease, we considered this additional, though arguable, evidence that the T9176C mutation is also suitable for DNA-prenatal diagnosis. For the T9176C also a T9176G variant has been found with a somewhat more malignant progression similar to the T8993G mutation compared to the T8993C mutation (Carrozzo et al., 2000; Carrozzo et al., 2001; Akagi et al., 2002). Given the high chance of an affected foetus or a borderline result it is not advised to offer prenatal diagnosis to carriers with a mutation percentage of more than 50% of the T8993G/C mutation (Poulton and Turnbull, 2000). Because the woman in our study was already pregnant it was not possible to discuss alternatives. The parents were informed about the uncertainties and risks of borderline mutation percentages and an affected foetus and decided to continue with the prenatal diagnosis. The prenatal diagnosis revealed a percentage of 85-88% with a variation of 3%. Although there was a high likelihood that the child would be affected the parents decided to continue the pregnancy.

Assuming that the bottleneck occurs during oocyte development (Poulton et al., 1998), preimplantation genetic diagnosis (PGD) could be offered in case of mtDNA mutations as an alternative for conventional prenatal diagnosis (Thorburn and Dahl, 2001). For PGD, one or two blastomeres are removed from an 8-cellular embryo, obtained by IVF procedures and intracytoplasmic sperm injection (ICSI), and these blastomeres are tested for the specific mutation. Healthy embryos are transferred to the uterus. PGD is technically easier for mtDNA mutations than for nuclear genes, as the copy number is much higher. Protocols have been optimised at the single cell level in our lab (L.J. Jacobs et al., in preparation). It is however important to know that mutation percentages quantified in the biopsied cell are representative for the entire embryo. This seems to be the case in heteroplasmic mice (Dean et al., 2003). The same criteria apply for prenatal diagnosis as for PGD, but the main advantage of PGD is that no termination of pregnancy has to be considered in cases where the chances of affected offspring are high. That these chances can be very high has been demonstrated in the oocytes of a carrier of the T8993G/C mutation (50% in blood), who had 6 oocytes with more than 95% mutation and 1 oocyte with no mutation at all (Blok et al., 1997), yielding a chance of more than 85% for offspring being affected. PGD can prevent multiple terminations of pregnancy.

Acknowledgements

Prof. Dr. P.A. van Doorn and Prof. Dr. H.F.M. Busch performed routine histology, histochemistry and electronmicroscopy.

CHAPTER 8

Molecular cloning and characterization of the human mitochondrial NADH:ubiquinone oxidoreductase 10-kDa gene (*NDUFV3*)*

René F.M. de Coo, Paul Buddiger, Hubert J.M. Smeets and Bernard A. van Oost

Genomics, 1997, 45: 434-437

* The human 10-kDa subunit gene has been assigned the symbol *NDUFV3* by the Human Gene Mapping Workshop Nomenclature Committee.

Abstract

The human gene for the 10-kDa flavoprotein subunit of the mitochondrial NADH-ubiquinone oxidoreductase (Complex I) was completely cloned and sequenced. The so-called NDUFV3 *gene contains three exons, spanning 20 kb. The open reading frame contains a 34-codon import sequence and a 74-codon mature protein sequence. A database search revealed close homology to bovine and rat protein sequence but not to any other known protein. Northern blot analysis showed that the* NDUFV3 *gene is ubiquitously expressed. The* NDUFV3 *gene was assigned by FISH to a single location on chromosome 21q22.3 and might contribute to the Down syndrome phenotype.*

NADH:ubiquinone oxidoreductase (complex I; EC 1.6.5.3) is an inner mitochondrial membrane-bound multisubunit enzyme complex. Complex I consists of at least 41 subunits of which 7 are encoded by the mitochondrial genome. As one of the complexes of the mitochondrial respiratory chain, its function is the catalysis of the rotenone-sensitive oxidation of NADH and the reduction of ubiquinone. Using chaotropic agents complex I can be resolved in two hydrophilic fractions, the flavoprotein fraction and the iron-protein fraction, and a hydrophobic fraction. The flavoprotein fraction comprises the 51-, 24-, and 10-kDa subunits, which are all encoded by nuclear genes. This fraction plays a catalytic role in the oxidation of NADH as it is associated with flavoprotein and NAD binding. The tetranuclear Fe-S centre in the 51-kDa subunit and the binuclear Fe-S centre in the 24-kDa subunit are involved in electron transfer (Ohnishi et al., 1985). The function of the 10-kDa protein is unknown as yet, despite the fact that the primary structure of the bovine 10-kDa subunit is known (Skehel et al., 1991).

Specific or generalized deficiencies in subunits of complex I have been described in a subgroup of patients with a mitochondrial encephalomyopathy. In some cases mutations were detected in the mitochondrial DNA, but in most cases a defect in one of the nuclear coding genes was suspected (de Vries et al., 1994) although as yet it has not been found. To elucidate the possible pathological role for nuclear-encoded complex I genes, the human genes must be characterized first. We have chosen to begin with the functionally important flavoproteins. We reported earlier on the 24-

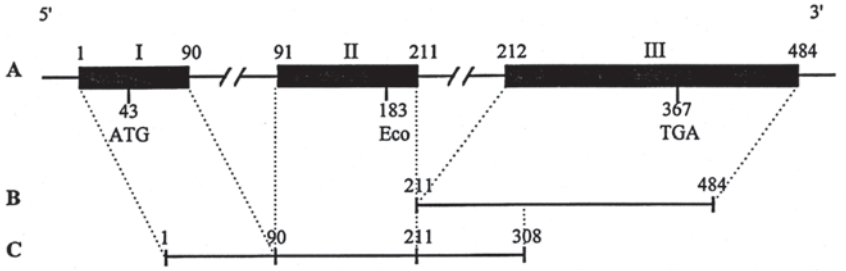


*Fig. 1. Structure of the human 10-kDa subunit gene (*NDUFV3*). (***A***) Genomic structure. Nucleotide 1 is the first nucleotide according to the cDNA (See Fig. 2). Exons I-III. The positions of the intron-exon boundaries are given. ATG, initiation codon; TGA, termination codon; Eco,* Eco*RI restriction site cuts at position 183. (***B***) Partial 10-kDa cDNA sequence derived from the kidney cDNA library. (***C***) Partial 10-kDa cDNA sequence derived from brain cDNA by a RACE procedure.*

kDa gene (de Coo et al., 1995), and we now report on the genomic structure, expression and localization of the human 10-kDa gene.

To isolate the 10-kDa cDNA a human renal λgt11 cDNA library was screened with a 10-kDa bovine cDNA probe (positions 135 to 361, EMBL/Genbank Accession No. X59048) (Skehel et al., 1991). After successive rounds of screening, we obtained two positive plaques, from which the same 1600-bp insert was isolated by PCR with T3 and T7 primers. The fragment was analysed by cycle sequencing and contained a hybrid cDNA sequence of which 273 bp coded for the carboxy-terminal region of the 10-kDa protein (Fig. 1B), and 1300 bp coded for an unknown sequence. To obtain the 5' end of the human 10-kDa cDNA, nested primers derived from the human cDNA sequence (see Fig. 2 for the positioning of the primers) were applied in a modified Rapid Amplification of cDNA ends (RACE) procedure (Apte and Siebert, 1993). Three fragments were obtained from

```
  1 GAAGCTGCTG TGGCCCTGCT TGGTGCGCCC GCTGTCACCG CCATGGCTGC CCCGTGTTTG
                                                  M  A   A  P  C  L
                                                                S  L

 61 CTGAGGAAAG GACGAGCCGG GGCGCTGAAG ACTATGCTCC AGGAAGCCCA GGTGTTTCGA
    L  R  K   G10 R  A   G  A  L  K   T  M  L   Q20 E  A   Q  V  F  R
          Q                             V      L          G

121 GGACTTGCTT CTACGGTTTC TTTGTCTGCG GAATCAGGGA AGAGTGAAAA GGGTCAGCCA
    G  L  A   S30 T  V   S  L  S  A   E  S  G   K40 S  E   K  G  Q  P
       V      P  A           →                      N           L

181 CAGAATTCCA AGAAGCAAAG TCCACCAAAA AAGCCAGCCC CA---GTGCC TGCTGAGCCG
    Q  N  S   K50 K  Q   S  P  P  K   K  P  A   P60    V   P  A  E  P
    P     P                                V   S  A  A      T

238 TTTGACAACA CTACCTACAA GAACCTGCAG CATCATGACT ACAGCACGTA CACCTTCTTA
    F  D  N   T  T70 Y   K  N  L  Q   H  H  D   Y  S80 T   Y  T  F  L

298 GACCTCAACC TCGAACTCTC AAAATTCAGG ATGCCTCAGC CCTCCTCAGG CCGGGAGTCA
    D  L  N   L  E90 L   S  K  F  R   M  P  Q   P  S100S   G  R  E  S
                D

358 CCTCGACACT GAGGGCCCTC GGTGTGAAGA TGAACCTTCC ACCGTCTTCA CTGCATCCTG
    P  R  H108

418 GAGTGCAAAA ATAAAATCCA CTCAAGAGTC ACAAGGCCCG CTGTGCATAA TCGGTTTCAC

478 TTTTACC
```

Fig. 2. Human 10-kDa cDNA sequence and comparison of the deduced protein sequences of the precursors of the human and bovine 10-kDa proteins. The human cDNA sequence is shown in the upper row and the deduced human protein sequence in the middle; where the bovine protein sequence differs is shown below the human protein sequence in the bottom row. The human cDNA sequence and protein sequence are numbered. (→) The N terminus of the mature protein. The ATG iniator codon, the TGA termination codon, and the polyadenylation signal are shown in boldface. The primers used for the nested PCR in the RACE procedure are shaded. The human sequence shows a one-codon deletion compared to the bovine sequence at position 60.

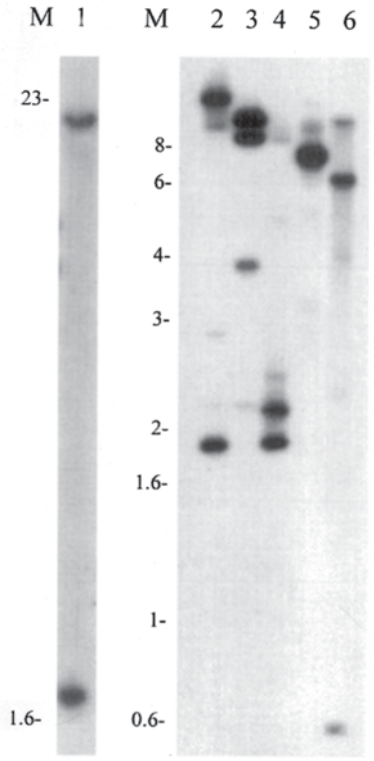


Fig. 3. Hybridization of Eco*RI-restricted genomic DNA with the human 10-kDa subunit cDNA as a probe. This probe contains the entire coding region. Lane* **1**, *human genomic DNA; lane* **2-6**, *cosmid clone C0564 restricted with, respectively* Eco*RI (lane* **2***),* Hin*dIII (lane* **3***),* Pst*I (lane* **4***),* Sst*I (lane* **5***) and PvuII (lane* **6***). The size marker used is the 1-kb ladder from Gibco BRL.*

a human brain cDNA library. The longest fragment of 308 bp was cloned in pCR-Script SK(+) and sequenced. From the overlapping renal cDNA and the brain cDNA fragments (273 and 308 bp, respectively), a full length 484-bp human cDNA was inferred (Fig. 1B and C).

The following procedures aided in clarifying the genomic structure and chromosomal assignment of the 10-kDa subunit gene. Southern blot analysis of an *Eco*RI digest of human genomic DNA with a nearly full length cDNA probe corresponding to nucleotides 1 to 399 revealed two bands of 20 and 1.8 kb (Fig. 3). The human cDNA harbours an *Eco*RI restriction site at position 183 (Fig. 1). The 1.8 kb genomic fragment is situated 5' of this *Eco*RI site of the gene and the 20 kb-genomic fragment downstream as a 3' cDNA probe (nucleotides 212-484) showed hybridization to the 20-kb *Eco*RI fragment only (data not shown). Based on the Southern blot analysis the length of the gene is between 8 and 20 kb (Fig. 3). Attempts to perform a long PCR spanning intron 2 to refine the length estimation failed. The length of intron 1 is less than 1.5 kb.

A Southern blot panel of human/hamster somatic cell hybrids (Geurts van Kessel et al., 1983) was analysed using the 10-kDa subunit cDNA as

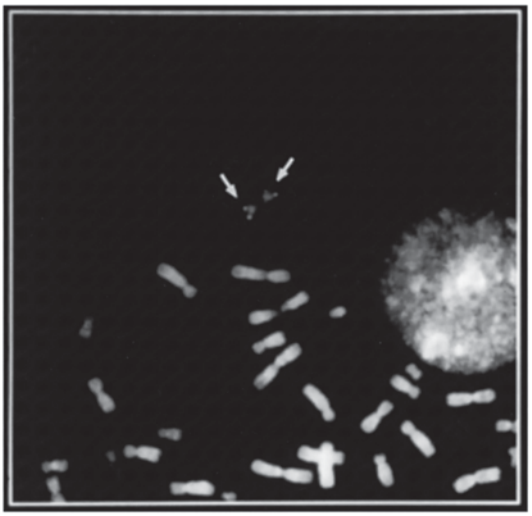


Fig. 4. Chromosome localization of NDUFV3 *by fluorescence* in situ *hybridization on chromosome 21. Arrows, paired signals present at 21q22.3.*

a probe. There was a 100% concordance for a chromosome 21 location (data not shown). Subsequently, filters containing a chromosome 21-specific cosmid library (Nizetic et al., 1991) were screened with the human cDNA probe (nt 1-399). Two clones (ICRFc103C0564 (10kdL) and the ICRFc103G087 (Lehrach, 1990)) were found to be positive. These two cosmid clones were virtually identical by restriction mapping. Fluorescence *in situ* hybridization was performed to map the C0564 cosmid more specifically. The hybridization signal appeared consistently on chromosome 21q22.3 (Fig. 4). No significant signals were seen on other chromosomes or on other parts of chromosome 21. The C0564 cosmid was used as a template in a cycle sequencing protocol with 10 different primers derived from the cDNA sequence. Analysis of the sequence obtained revealed the presence of three exons contiguous with the cDNA, varying in length between 90 and 273 bp and divided by two introns that follow the conventional intron splice donor and acceptor sequence rules. The in-frame ATG codon at position 43 is most likely the start codon, because the sequence upstream contains the consensus sequence for a eukaryotic translation initiation site, CCGCCATG (Kozak, 1984). Exon I codes for the first 16 amino acids, exon II for amino acids 17 to 56, and exon III for amino acids 57 to 108. The 5' untranslated region (UTR) is 42 nucleotides long and the 3' UTR 115 nucleotides. The poly(A) tail is located 52 nucleotides downstream from the polyadenylation signal (Fig. 2). Immediately following the 3' UTR an *Alu*-repeat sequence was detected. The exact size of the introns was not determined. In intron 2 we found four

copies of a 75-bp repeat (data not shown). The sequence is deposited with the EMBL Nucleotide Sequence Database under Accession Nos. X99726-X99728.

The 10-kDa subunit mRNA is expressed ubiquitously and shows alternative splicing. Northern blot analysis of human poly(A)$^+$ RNA from 16 different human tissues (Fig. 5) showed a single fragment of about 500 nucleotides in each lane. A relatively strong signal was seen in skeletal muscle and heart. RNA from testis showed a single slightly larger fragment. A reverse transcriptase (RT)-PCR from human fibroblast RNA and from human placenta RNA with primers 904f and 821r showed two cDNA fragments, one fragment of full length and one fragment lacking exon II, as was found by direct sequencing of the RT-PCR product. These fragments were not visible on the Northern blot (Fig. 1).

In our ongoing research to clone nuclear genes that encode for mitochondrial complex I proteins (de Coo et al., 1995), we have now characterized the human 10-kDa subunit gene. We describe here that there is only one 10-kDa gene in the human genome, which is ubiquitously expressed.

The human 10-kDa cDNA appeared to be quite similar to the bovine cDNA in a number of aspects. Both the human and bovine cDNA have an AATAAA polyadenylation signal, which is located, respectively, at 52 and 18 bases upstream of the poly(A) tail. The 5' regions of both cDNAs contain a preponderance of C and G residues, indicative for CpG islands associated with the 5' ends of the genes. The bovine coding region is 3 bases longer. Over the region of alignment, the nucleotide sequence differed in 55 of the 324 bases. Of these, 37 were within the mature protein coding region (aa 35-108), and 18 in the region encoding the import protein sequence (aa 1-34). This 83% identity with the bovine nucleotide sequence for the coding region is less than was found in other mitochondrial genes

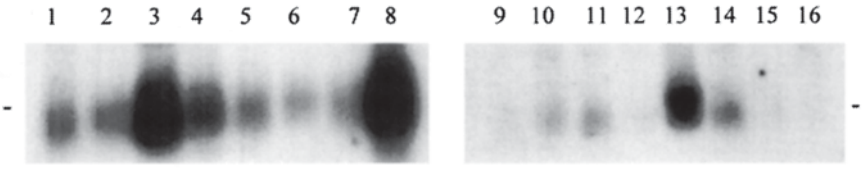


Fig. 5. Northern blot analysis. Hybridizations of the cDNA probe (nucleotides 1-399) from the NDUFV3 *gene to 2µg poly(A)$^+$ RNA from human tissues. Lanes* **1**, *pancreas;* **2**, *kidney;* **3**, *skeletal muscle;* **4**, *liver;* **5**, *lung;* **6**, *placenta;* **7**, *brain;* **8**, *heart;* **9**, *peripheral blood leucocyte;* **10**, *colon;* **11**, *small intestine;* **12**, *ovary;* **13**, *testis;* **14**, *prostate;* **15**, *thymus;* **16**, *spleen. RNA size marker band 0.5 kb are indicated at both sides.*

like the 24-kDa with 93% homology (de Coo et al., 1995). The 3' and the 5' UTR are completely divergent, including a 307-bp insertion in the bovine 3' UTR compared to the human 3' UTR.

The cDNA divergence corresponds with the protein divergence as the human and bovine protein sequences are 82% homologous. The mature bovine and human 10-kDa protein both have the same N-terminal sequence (S A E S) (Skehel et al., 1991). The 74 amino-acid mature protein sequence differs in 10 positions from the bovine protein with, as mentioned above, the bovine protein sequence being 1 amino acid longer. A further 9, mostly conservative, amino acid substitutions are present within the 34-amino-acid N-terminal presequence (Fig. 2).

As expected, the *NDUFV3* gene is ubiquitously expressed. The different RT-PCR products from the human fibroblast and human placental RNA suggest alternative splicing of the *NDUFV3* pre-mRNA, although this is not a major quantitative effect since only the full-length mRNA fragment is observed on the Northern blot. However, in testis tissue the major fragment appeared to be slightly longer than the 10-kDa mRNA in the other tissues. With a RT-PCR for the coding region and 32 nucleotides of the 3' UTR of the 10-kDa mRNA, no evidence for a length divergence was obtained. Further analysis of the 5' and 3' ends of the testis cDNA are warranted as a length difference may also be caused by a second polyadenylation signal situated more downstream.

The 10-kDa protein sequence is only moderately conserved between human and cow, compared to the highly homologous 24-kDa protein sequence (de Coo et al., 1995) and the 51 kDa subunit (de Coo et al., 1999). A search in the current databases did not reveal any proteins homologous to the 10-kDa subunit. The import presequence (Fig. 2) for the human protein is not extensively related in primary structure to other mitochondrial import sequences (Nicholson and Neupert, 1988). The mitochondrial presequences are in general quite divers, but have a net positive charge, and rarely contain acidic amino acids. These two latter features are present in the putative import sequence of the 10-kDa protein.

The 10-kDa protein is a protein with unknown function and structure and is present in the mammalian complex I only (Masui et al., 1991). Within complex I it is situated in close proximity to the 24- and 51-kDa subunits (Yamaguchi and Hatefi, 1993), suggesting a function in the electron transport. However, the mature protein sequence does not contain any cysteine residues so there is no evidence that it participates in the liganding of a 2Fe-2S centre necessary for the electron transfer, like the 24- and 51-kDa subunits. Probably it has a modifying or regulatory role in the electron transport. A defect in this subunit could thus be involved in mito-

chondrial encephalopathies, in particular in patients with a partial Complex I deficiency. Isolation and characterization of the *NDUFV3* gene allows us now to investigate these patients in detail.

The localization of the *NDUFV3* gene to a single locus at chromosome 21q22.3, may provide another clue for its function since Down syndrome has been postulated to be a contiguous gene syndrome (Korenberg et al., 1994). Prince et al. (1994) reported a mitochondrial enzyme defect in Down syndrome. This defect is present at an early stage in Down syndrome and might be related to the development of dementia. The 10-kDa subunit might contribute in a dosage-dependent manner to the phenotype of Down syndrome, similar as was suggested recently (Chen et al., 1995) for the mitochondrial ATP50 protein, which was located near the *AML1* gene at the border between 21q22.1 and 21q22.2.

Acknowledgements

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CHAPTER 9

Molecular cloning and characterization of the active human mitochondrial NADH:ubiquinone oxidoreductase 24-kDa gene (*NDUFV2*) and its pseudogene

René de Coo, Paul Buddiger, Hubert Smeets, Ad Geurts van Kessel, John Morgan-Hughes, Daniël Olde Weghuis, Joan Overhauser and Bernard van Oost

Genomics, 1995, 26: 461-466

Part of the results were presented at the chromosome 18 workshop held on July 19-20, 1993 in Doorwerth, The Netherlands: de Coo, I.F.M., Geurts van Kessel, A., Harding, A., Morgan-Hughes, J.A. and van Oost, B.A. (1994). Assignment of the 24-kDa subunit of complex I of the respiratory chain to chromosome 18. Cytogenet. Cell Genet. 65: 162. The human 24-kDa subunit gene has been assigned the symbol *NDUFV2* by the Human Gene Mapping Workshop Nomenclature Committee.

Abstract

*Two distinct loci for the 24-kDa subunit of the mitochondrial NADH:ubiquinone oxidoreductase (complex I of the respiratory chain) were detected in the human genome: a transcribed gene from chromosome 18 and an inactive locus on chromosome 19. Cosmid clones containing the functional gene (*NDUFV2*) and the pseudogene (*NDUFV2P1*) were isolated. The* NDUFV2 *gene spans approximately 20 kb and contains 8 exons. Refined mapping of both* NDUFV2 *genes by FISH resulted in an assignment of the* NDUFV2 *gene to 18p11.2-18p11.31 and of the* NDUFV2P1 *gene to 19q13.3-qter. The nucleotide sequence of the* NDUFV2P1 *pseudogene differs from the cDNA sequence by the lack of the methionine initiator codon, an additional 165 bp of the first intron sequence, and a 1-nucleotide deletion.*

INTRODUCTION

The mitochondrial NADH:ubiquinone oxidoreductase (complex I) (EC 1.6.5.3) of the respiratory chain is composed of at least 41 proteins. Seven of these are coded for by the mitochondrial genome, and 34 are of nuclear origin (Walker et al., 1992). Complex I catalyses the transfer of electrons from NADH to ubiquinone coupled to ADP phosphorylation. Together with the 51- and the 10-kDa subunit, the 24-kDa subunit constitutes the flavoprotein fraction of complex I (Galante and Hatefi, 1979). The 24-kDa subunit contains a Fe-S cluster and is as such involved in the electron transport (Ohnishi et al., 1985). The primary structure of the 24-kDa subunit is closely related to the first 188 amino acids of a component of a soluble NAD-dependent hydrogenase from the bacterium Alcaligenes *eutrophus* (Tran-Betcke et al., 1990).

As with mitochondrially encoded complex I genes, defects in nuclear-encoded complex I genes could result in a mitochondrial (encephalo-) myopathy. Complex I deficiency has been described in association with several mitochondrial myopathy syndromes, including (i) myopathy with exercise intolerance, (ii) (encephalo-) myopathy, or (iii) multisystem dysfunction (Morgan-Hughes et al., 1986). In blood, the mitochondrial dysfunction is reflected by lactic acidemia. Schapira et al. described one patient with a complex I deficiency and, on immunoblot, a partial deficiency of the 24-kDa subunit (Schapira et al., 1988). However, no direct relationship between a defect in a nuclear-encoded complex I subunit and disease has been demonstrated yet.

The important role played by the 24-kDa subunit in the catalytic function of complex I makes it a promising candidate gene for mutation analysis in patients with a complex I deficiency. Characterization of the 24-kDa subunit gene is a prerequisite for a comprehensive mutation analysis. In the present paper we describe the identification of the human 24-kDa subunit gene and its pseudogene.

MATERIALS EN METHODS

Southern blot analysis

Chromosomal DNA was isolated from peripheral blood lymphocytes (Miller et al., 1988). DNA (10 μg) was digested with *Eco*RI (Gibco BRL), resolved by agarose gel electrophoresis, and immobilized on GeneScreen Plus (DuPont NEN, Boston, MA, USA) by Southern blotting, according to standard procedures (Sambrook et al., 1989). The 24-kDa cDNA probe (see Fig. 4), which covers 813 of the 878 bp of the nearly full-length cDNA

(Pilkington and Walker, 1989; Toda et al., 1989), was ^{32}P-labeled by random primer synthesis (Feinberg and Vogelstein, 1983). Prehybridization and hybridization were performed at 65°C in 0.5 M sodium phosphate buffer (pH 7.2)/7% (w/v) sodium dodecyl sulfate/1 mM EDTA. Filters were washed three times for 5 min and once for 30 min at 65°C in 40 mM sodium phosphate (pH 7.2)/0.1% sodium dodecyl sulfate. For autoradiography, filters were exposed to Kodak X-ray films for up to 7 days at -70°C.

Northern blot analysis

Total RNA was isolated from $20x10^6$ cultured cells using RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX, USA) according to the instructions of the manufacturer. Multiple tissue Northern blots (Clontech Laboratories, Palo Alto, CA, USA) containing 2 μg of poly(A)$^+$ RNA from different human tissues were hybridized with the 24-kDa cDNA probe. The amount of poly(A)$^+$ RNA was normalized according to the β-actin expression.

Cell lines

cDNA was synthesized using RNA extracted from a chromosome 18-only specific hybrid cell line (Coriell Institute, Cell Repository No. GM12082) and using RNA isolated from a chromosome 19q-only hybrid (Schonk et al., 1989) using the Geneamp RNA PCR kit (Perkin Elmer Cetus). After reverse transcription, 10 μl of the cDNA preparation was used as a template in a 100-μl PCR reaction mixture containing the primers 750f and 853r (see Table 1). For the chromosomal localization of the 24-kDa cDNA gene, a panel of well-characterized human-rodent somatic cell hybrids was used (Geurts van Kessel et al., 1983). The chromosomal constitution of the hybrids was evaluated using reverse (R)-banding on air-dried spreads according to standard procedures. Of each cell line at least 16 metaphases were examined. The cells used for chromosome analysis and DNA extraction were always derived from the same culture batch.

In situ hybridisation

Two hundred nanograms of the biotinylated cosmid probe (derived from cosmid clones 188c and 189c, see Results) was preannealed in the presence of 10 μg Cot-1 DNA (Life Technologies) to compete out reiterated sequences. Then, the probe was hybridized to BrdU-incorporated high-resolution chromosomal preparations under a 18×18-mm coverslip for 16 h at 37°C and detected immunocytochemically as described (Suijkerbuijk et al., 1992). Counterstaining of the chromosomes was performed with DAPI

Table 1. Polymerase chain reaction primers used for amplification and cycle sequencing of the NDUFV2.

Primer	Position[a]	Exon	Sequences (5'-3')
41f	41-58	I	CCGCCATGTTCTTCTCCG
254f	254-274	IV	ATCCAGAAGGCCATAAAGCAG
412f	412-432	V	AATCGAAAGCCAGTTGGAAAG
523f	523-543	VI	GTTGGGGAGACTACACCTGAC
663f	663-683	VII	AAATTATTGATGAGCTCAAGG
750f	750-767	VIII	TGAACCACCCAAGGGACC
853r	853-833	VIII	CGTAGATTGGAAGTCCATATT
760r	760-740	VIII	TGGGTGGTTCAGTCAAAGAGG
693r	693-674	VII	GGTTTTGGGATTTTGCCAG
593r	593-573	VI	GCGTTCACACAGGCCCCTAAA
485r	485-465	V	CTGTCAGAGTTTCGAAGCATG
341r	341-821	IV	TTCATAGCAGAGATGGGCAAC
219r	219-199	III	TTCTGGTGTGAAATCAAATGG
165r	165-145	II	CACAAATAAAGCTCCTCCAGC
58r	58-40	I	GGAGAAGAACATGGCGGG
Intron primers		Intron	
5' f 1		5'	TAGGCCTGGGGTCGGGAG
1 f 1		1	TTAAGGCTATGATTTACTAAACTTTC
2 f 1		2	GTACAGTGTCATTCACACCTGAG

Note. f, forward primer; r, reverse primer; 5', 5' flanking region; 3', 3' flanking region.
a Nucleotide numbering according to cDNA sequence published by Toda et al. (1989).

(Sigma). Chromosomes were studied under a Zeiss Axiophot epifluorescence microscope equipped with appropriate filters for the visualization of FITC and DAPI, and digital images were recorded using a Photometrics high-performance CH 250/a cooled CCD camera.

Cosmid identifcation

The cDNA clone (see Fig. 4, nucleotides 41-853 of cDNA) was used to screen a chromosome 18 flow-sorted library (constructed by Peter de Jong, Lawrence Livermore Laboratory) and a human genomic cosmid library (made available by Martin Hofker, Leiden, the Netherlands). The Qiagen plasmid purification kit (Qiagen Inc., Chatsworth, CA, USA) based on the alkaline lysis method was used for the purification of cosmid DNA.

Sequence analysis

Two micrograms of cosmid DNA was subjected to direct cycle sequencing of both strands. Using the Taq Dye-Deoxy terminator cycle sequencing kit (Perkin Elmer/Applied Biosystems), we analysed the samples with an Applied Biosystems 373A DNA sequencer.

RESULTS

Southern blot analysis of the 24-kDa subunit genes

A human cDNA encoding the 24-kDa subunit of complex I (EMBL/GenBank Accession No. M22538) as published by Pilkington and Walker (1989) was isolated by PCR from total human mRNA. After reverse transcribing the RNA, an 813-bp fragment of the human cDNA (Fig. 4; primers 41f and 853r) was amplified and used as probe. Southern blot analysis of an *Eco*RI restriction digest of human genomic DNA revealed five bands (15, 8, 6.5, 4.5, and 1.6 kb; see Fig. 1). The difference in intensity of the bands suggested that there is more than one gene and/or pseudogene.

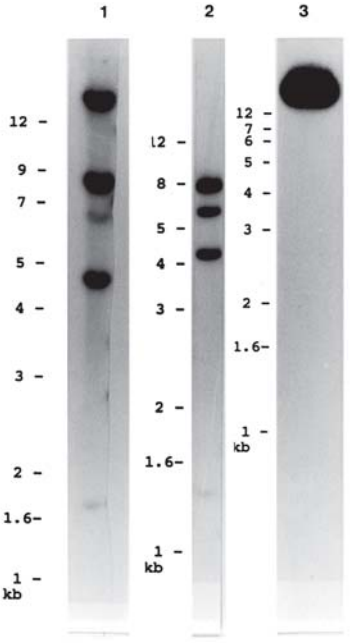

Fig. 1. Hybridization of Eco*RI-restricted genomic DNA with the human 24-kDa subunit cDNA as a probe. This probe contains the entire coding region and the 3' UTR. Lane* **1***, human genomic DNA; lane* **2***, cosmid clone 120H11; lane* **3***, cosmid clone 189c. The size marker used is the 1-kb ladder from Gibco BRL. The partial cDNA that is used as a probe causes the diminished intensities for the 6.5- and 1.6-kb bands in lanes* **1** *and* **2**.

Chromosomal assignment of the 24-kDa subunit genes

A Southern blot panel of human/hamster somatic cell hybrids were analysed using the 24-kDa subunit cDNA as a probe. The Southern blot hybridization pattern for the *Eco*RI digest of human DNA was clearly distinguishable from that for hamster DNA. The 15-kb *Eco*RI restriction fragment could be assigned to human chromosome 19. The other *Eco*RI restriction fragments of the 24-kDa subunit gene(s) were present only in somatic

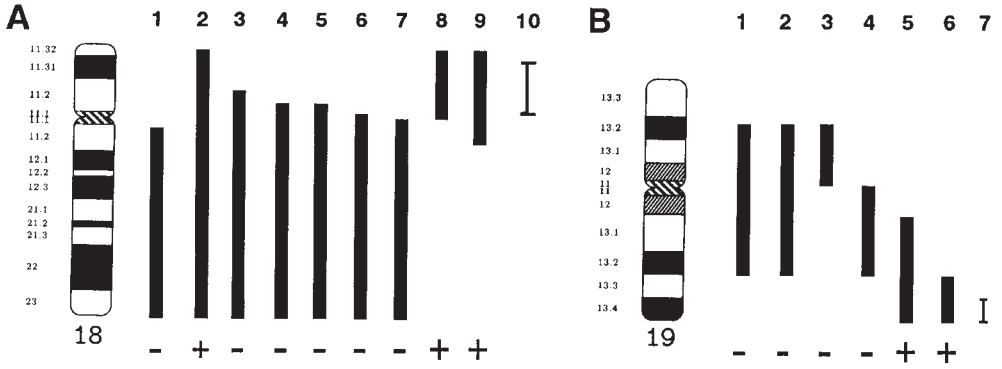

*Fig. 2. (***A***) Idiogram of chromosome 18 depicting the localization of the* NDUFV2 *gene (lanes* **1-9***) by PCR with primers 750 and 853 on somatic cell hybrids. Lane* **1***, truncated chromosome 18 (X;18)(p11.2;q11.2) (gift of B. de Leeuw); lane* **2***, HHW324; lane* **3***, JH407; lane* **4***, JH357; lane* **5***, HHW778; lane* **6***, JH252; lane* **7***, JH158; lane* **8***, JH341; lane* **9***, JH211, (lanes* **2-9** *see Overhauser et al., 1993). Lane* **10***, localization by FISH. (***B***) Idiogram of chromosome 19 depicting the localization of* NDUFV2P1 *determined by PCR with primers 750 and 853 on somatic cell hybrids. The human-specific bands are found in lanes* **5** *and* **6***. Lane numbers and corresponding hybrid numbers are as follows: lane* **1***, hybrid 908K1; lane* **2***, 908K1A; lane* **3***, 908K1A1; lane* **4***, 908K1B; lane* **5***, ORIM7-1; lane* **6***, GM89A9-C9-7 (Schonk et al., 1989); lane* **7***, localization by FISH.*

cell hybrids containing at least human chromosome 18 (data not shown).

Further refinement of the mapping of the loci of the 24-kDa subunit was achieved with somatic cell hybrids containing fragments from either chromosome 18 or chromosome 19 (Fig. 2). By doing so, the locus on chromosome 18 could be confined to 18p11.2-pter and the locus on chromosome 19 to 19q13.3-qter (Fig. 2).

Isolation of genomic clones for the 24-kDa subunit genes

Screening of a human cosmid library with the 24-kDa subunit cDNA probe yielded four cosmid clones. Two cosmids, 188c and 189c, were selected for further experiments. Both showed a PCR fragment of 104 bp with primer set 750f/853r. An *Eco*RI restriction map showed a single positive fragment of 2.5 kb for clone 188c and a strong positive fragment of 15 kb for clone 189c. Cosmid 189c was mapped to 19qter by FISH analysis (data not shown). Cosmid clone 188c, containing the 2.5-kb

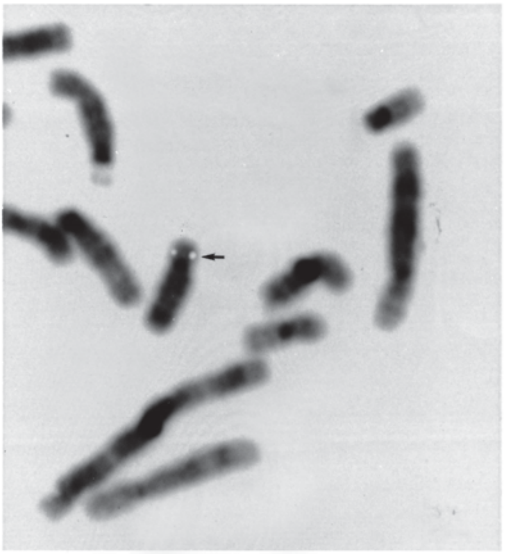

Fig. 3. Chromosome localization of NDUFV2 *by fluorescence* in situ *hybridization on chromosome 18. Arrow, paired signals are present at 18p11.2-p11.31.*

*Eco*RI restriction fragment, was mapped to chromosome 18p11.2-p11.31 by FISH (Fig. 3).

To avoid interference of the chromosome 19 locus with the isolation of additional chromosome 18 cosmids, a chromosome 18-specific cosmid library was screened. One cosmid, 120H-11, was analysed in detail. This cosmid apparently spans the whole gene, as it showed the same pattern of *Eco*RI restriction fragments as human genomic DNA on Southern blot analysis except the 15-kb band, which contains the pseudogene sequence (see below and Fig. 1).

The locus on chromosome 18 contains the whole open reading frame of the 24-kDa subunit gene

The 120H-11 clone was used as a template in a cycle sequencing protocol with primers derived from the cDNA sequence (Table 1). Six of seven forward primers yielded good sequence, and the intron-exon boundaries could be determined in all cases except exons 2 and 3 (Table 1). For exon 2 we determined the 5' boundary with a reverse primer and the 3' boundary was sequenced with the forward intron primer 1f. As exons 3 and 4 were separated by only 85 bp, sequencing with the reverse exon 4 primer 341r revealed the 3' end and the 5' end of exon 3 in one run. Based on this sequence information, reverse primers were designed to find the remaining intron-exon boundaries. The gene appeared to have eight exons (Fig. 4) and a maximal length of 20.6 kb (Fig. 1), as judged from the size of the

*Eco*RI restriction fragments of the cosmid clone (Fig. 1). The estimated intron lengths, found by PCR amplification with Taq Extender PCR Additive (Stratagene), varied between 0.85 and 6.5 kb (Table 2). This indicates a total exon-intron length of 18.2 kb.

The 24-kDa subunit gene is transcribed from the chromosome 18 locus only

Total mRNA was isolated from the somatic cell hybrids harbouring either a single chromosome 18 or a single chromosome 19. Reverse transcription followed by PCR for the 24-kDa subunit cDNA with primer pair 750f/853r revealed a PCR fragment of the predicted size for the chromosome 18 harbouring somatic cell hybrid only. The chromosome 19 cell hybrids ORIM7-1 and GM89A9-C9-7 (for chromosome content see Fig. 2) were negative, making transcription from this locus unlikely because a control RT-PCR for the myotonic dystrophy locus, using primers flanking the CTG repeat (Mahadevan et al., 1992), yielded the expected cDNA frag-

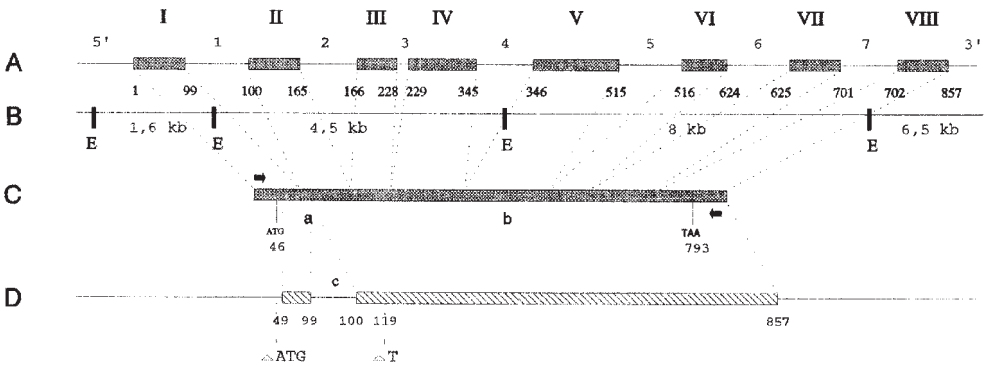

*Fig. 4. Structure of the human 24-kDa subunit gene (*NDUFV2*). (***A***) Genomic structure. Nucleotide 1 is the first nucleotide according to the cDNA as published by Toda et al. (1989). (***B***)* Eco*RI restriction map from cosmid clone 120H11. The sizes of* Eco*RI fragments harbouring* NDUFV2 *exons are given in kilobases. The position of the exon-intron boundaries is given. (***C***) The human cDNA with exon-exon boundaries. (***D***) The pseudogene* NDUFV2P1 *structure. Differences with the cDNA structure are shown. (→) primer 41f; (←) primer 853r; (▩) exon; (▨) pseudogene; (***a***) presequence; (***b***) mature protein sequence; (***c***) 165-bp intron;* **E**, Eco*RI restriction site;* **ΔT**, *deletion thymidine nucleotide;* **ΔATG**, *deletion of ATG nucleotides 46-48. The sequence of the pseudogene is deposited with the EMBL Nucleotide Sequence Database under Accession No. X84682.*

Table 2. Sequence of the exon-intron boundaries of the NDUFV2 *gene.*

Exon	Size (bp)	5' Splice donor		Intron	Size (kb)	3' Splice acceptor	
I	>99	CACTGG	*gt*aagg	1	3	aaattttag	GGAAGACAT
II	66	TTTGTG	*gt*aagt	2	1.5	gttgtgtag	CACAGAGAT
III	63	TATAAG	*gt*atgg	3	0.085	tatatacag	AGGATAGAG
IV	117	AACAAG	*gt*actg	4	2.7	atattttag	GTTGCAGAA
V	160	AGCTTG	*gt*aggg	5	2.0	tatttttag	GAATAAAGG
VI	109	TACTAT	*gt*gagt	6	1.5	tttttccag	GAGGATTTG
VII	77	GCCAAG	*gt*atgc	7	6.5	tcatttcag	GAGTGGACG
VIII	156						

Note. Uppercase bases are exon sequences. Lowercase letters indicate intron sequences. Splice acceptor and donor sites consensus sequences are shown in italics. For the complete exon sequence see Toda et al. (1989). The first base of the cDNA as isolated by Toda et al. has been numbered 1. Additional intronic sequence is deposited with the EMBL Nucleotide Sequence Database under Accession Nos. X84421-X84436.

ment. This negative experiment also showed that the 24-kDa subunit cDNA-specific primer pair employed did not yield a product for the coisolated mRNA from hamster origin.

The 24-kDa subunit mRNA is ubiquitously expressed

Northern blot analysis of human poly(A)$^+$ RNA from 16 different human tissues (Fig. 5) showed a single fragment of about 1000 nucleotides. A relatively strong signal is seen in heart and skeletal muscle.

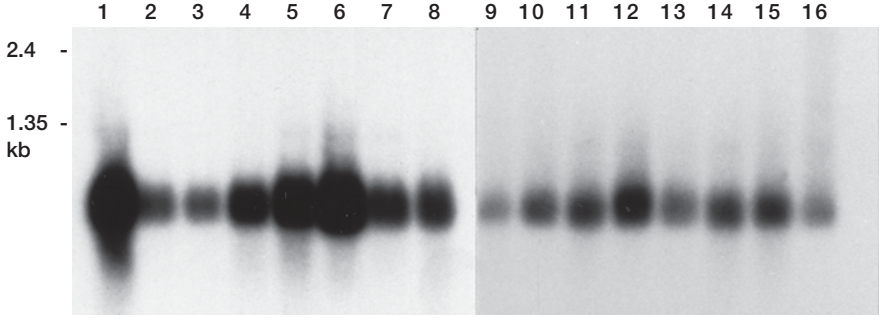

Fig. 5. Northern blot analysis. Hybridizations of the cDNA probe (nucleotides 41-853 of cDNA) from the NDUFV2 *gene to 2 µg poly(A)$^+$ RNA from human tissues. Lane* **1**, *heart;* **2**, *brain;* **3**, *placenta;* **4**, *lung;* **5**, *liver;* **6**, *skeletal muscle;* **7**, *kidney;* **8**, *pancreas;* **9**, *spleen;* **10**, *thymus;* **11**, *prostate;* **12**, *testis;* **13**, *ovary;* **14**, *small intestine;* **15**, *colon;* **16**, *peripheral blood leukocytes. RNA size marker bands are indicated to the left.*

The locus on chromosome 19 contains a 24-kDa subunit pseudogene

Automated cycle sequencing of cosmid clone 189c showed that the sequence of the *NDUFV2P1* gene was similar to the 24-kDa cDNA sequence with three differences. The pseudogene lacks the first 23 nucleotides, including the methionine initiator codon, and shows at position 119 a deletion of one thymidine nucleotide (Fig. 4). At position 99 there is a 165-bp insert, the sequence of which is identical to part of intron 1 of the expressed 24-kDa gene (data not shown).

DISCUSSION

As a first step toward identification of a nuclear gene responsible for a defect in complex I of the mitochondrial respiratory chain and understanding the pathogenetic mechanism in patients with a mitochondrial (encephalo-) myopathy, we have mapped the human 24-kDa subunit gene. We conclude that there is only one expressed 24-kDa gene, on chromosome 18, with a single transcript of 1 kb. Toda et al. (1989) speculated about differential splicing since they detected an extra band on a Northern blot from a HeLa cell line from brain. However, our Northern blot results showed only a single fragment in all tissues tested.

The eight exons vary in length from 63 to 160 bp and are divided by introns that all follow the conventional intron splice donor and acceptor sequence rules (Table 2). The length of the first exon was not determined exactly, but our data combined with sequence data from the literature (Pilkington and Walker, 1989; Toda et al., 1989) points to a transcription starting point between position -32 and +1 of the published sequence by Toda et al. This assumption is supported by our finding that with primer 5' fl (position -50 to -32) and primer 58r we could not get an amplified fragment from cDNA, while the same primer set used on cosmid clone 120H11 DNA yielded the expected 109-bp fragment (unpublished observations).

The open reading frame that codes for an import sequence of 32 amino acids and for 217 amino acids of the mature protein (Pilkington and Walker, 1989) starts at position 46 in exon 1 and ends at position 793 in exon 8. The cleavage site for the mature protein is at nucleotide position 140 in exon 2. It is very likely that the ATG triplet serves as the initiator codon since the sequence upstream is identical to that found as a consensus sequence for eukaryotic transcription initiation sites, namely CCGCCATG (Kozak, 1984).

The 24-kDa pseudogene on chromosome 19 has a sequence very similar to that of the human cDNA (Fig. 4). The differences with the chromosome 18 gene sequence make it unlikely that the *NDUFV2P1* locus produces a translation product similar to that from the *NDUFV2* locus. The structure of the pseudogene is anomalous in two respects. First, there is no 3'-terminal poly(A) tract, and second, part of the first intron of the expressed gene is present in the pseudogene. It is most likely that the pseudogene arose from an insertion of a copy of a partially processed 24-kDa gene transcript.

Detailed knowledge of the 24-kDa gene and the 24-kDa pseudogene will enable a comprehensive mutation screening of patients. In particular, one may look for those mutations that have inactivated the 24-kDa gene to generate the pseudogene. Gene conversion of adjacent active and inactive genes is a well-known mechanism to generate mutations. This mechanism may also be operational for unlinked genes. For example, it has been demonstrated for the Von Willebrand factor gene that acquisition of pseudogene mutations by the active gene on a different chromosome is a major source of pathological mutations (Eikenboom et al., 1994).

Isolation and characterization of the *NDUFV2* gene allows us now to investigate patients with a complex I deficiency and in particular those patients with a 24-kDa subunit deficiency (Morgan-Hughes et al., 1986).

Acknowledgements

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CHAPTER 10

The structure of the human *NDUFV1* gene encoding the 51-kDa subunit of mitochondrial complex I

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Mammalian Genome, 1999, 10: 49-53

Part of the results were presented at the annual meeting of the American Society of Human Genetics held on October 28 - November 1, 1997 in Baltimore, Maryland: P.A.L. Buddiger, W. Ruitenbeek, H.R. Scholte, B.A. van Oost, H.J.M. Smeets, I.F.M. de Coo. Molecular genetic analysis of complex I genes in patients with a deficiency of the respiratory chain. The American J. of Hum. Genet. (1997): 61; A305. The nucleotide sequence data reported in this paper have been submitted to EMBL and have been assigned the accession number Y16309 (Y17379-17383) and Y16310.

Abstract

*The genomic organization of the human 51-kDa subunit gene (*NDUFV1*) on human chromosome (chr) 11q13 was determined. The* NDUFV1 *gene consists of 10 exons. Exon 1 encodes for the 20-amino-acids-long import sequence and exon 1 through 10 codes for the 444-amino-acids-long mature protein. The protein sequence is highly conserved between human and bovine. Northern blotting analysis showed that the* NDUFV1 *gene expression varies widely among tissues and that in testis a unique mRNA species is present. In comparison with the other complex I flavoproteins, the expression of the 51-kDa gene in pancreatic tissue is high.*

INTRODUCTION

NADH:ubiquinone oxidoreductase (complex I) of the mitochondrial respiratory chain consists of at least 41 subunits, of which all but seven are nuclearly encoded. Complex I removes electrons from NADH and passes them on to the electron acceptor ubiquinone. This is the first step in the process of electron transfer through a number of intermediates to oxygen and drives the production of ATP (Walker, 1992). With chaotropic reagents complex I can be fragmented into three fractions (Ragan, 1987). Two hydrophilic fractions, the flavoprotein and the iron protein fraction, and one hydrophobic fraction. The flavoprotein fraction contains three subunits of 51-kDa, 24-kDa and 10-kDa, and the oxidation of NADH is mediated by this fraction. The 51-kDa is directly involved in the transfer of two electrons from NADH to ubiquinone with the simultaneous translocation of four electrons across the mitochondrial inner membrane (Deng et al., 1990). The 51-kDa protein probably contains binding sites for both the cofactor and for the oxidant of NADH, FMN (Krishnamoorthy and Hinkle, 1988; Patel et al., 1991), as well as a binding site for a tetranuclear iron-sulfur center (Ohnishi et al., 1985). The 51-kDa protein is an evolutionary conserved subunit since there is a strong amino acid sequence homology with part of the bacterial hydrogenase gene of Alcaligenes *eutrophus*. The gene for the α-subunit of the hydrogenase resides in the *hoxF* (α) part of the *hoxS* locus in Alcaligenes *eutrophus* (Tran-Betcke et al., 1990) and was shown to encode a fusion of closely related sequences from the 51- (Pilkington et al., 1991) and 24-kDa (Pilkington and Walker, 1989) subunits. The same relationships were reported in Neurospora *crassa* (Preis et al., 1991) and Paracoccus *denitrificans* (Xu et al., 1991).

In 1991 the bovine cDNA for the 51-kDa subunit was cloned by Patel and Pilkington. By serendipity, Spencer et al. (1992) localized the *NDUFV1* gene to be 30,000 bp downstream of the *GSTP1* gene on a cosmid originating from human chr 11. In 1993 Ali et al. sequenced the partial human 51-kDa subunit cDNA and used this cDNA to confirm the chromosomal localization on 11q13 by FISH analysis. We are screening for mutations in nuclear-encoded complex I genes of patients with mitochondrial (encephalo)myopathy and complex I deficiency. The genes encoding flavoprotein fraction subunits are plausible candidates. To screen for mutations at the genomic level, the gene structure of the different subunits has to be known. The gene structures of the 24-kDa subunit (de Coo et al., 1995) and the 10-kDa subunit (de Coo et al., 1997) have been reported previously by us. Here we complete the series by reporting the structure of the 51-kDa subunit gene (*NDUFV1*).

MATERIALS AND METHODS

cDNA library

One million clones from a large insert human kidney cDNA library in λGT11 (Clontech Laboratories, Palo Alto, CA, USA) was plated at 5×10^5 pfu/150 mm plate and grown for 6h. Plaques were transferred to Hybond N+, autoclaved and cross linked. Hybridizations were performed with a 1.55-kb full-length bovine 51-kDa cDNA clone (Patel et al., 1991) at 42°C, with a 50% formamide buffer, lacking Denhardt's recommended for short fragment probes. Filters were exposed for 3-4 days. Positive plaques were taken through second and third screens to obtain pure clones. The clones were subcloned into a pBluescript KS (+) vector (Stratagene, La Jolla, CA, USA) and checked for length after *Eco*RI digestion and resolving on a 0.8% agarose gel. Both strands of the insert were sequenced with PCR products amplified with T3 and T7 primers. The human brain cDNA 5' RACE-Ready kit (Clontech) was used with the nested reverse primers 1083r and 1080r to obtain the full-length cDNA. Products were analysed on a 0.8% agarose gel. The longest fragments were cloned with the pCR-Script™ SK(+) cloning kit (Stratagene). Adapted (higher melting temperatures) T3/T7 primers (Stratagene) were used to rescue the insert, followed by direct cycle sequencing.

Southern blot analysis

Chromosomal DNA was isolated from peripheral blood. DNA (10 μg) was digested with *Pvu*II, *Stu*I, *Sst*I, *Hin*dIII or *Eco*RI (Gibco, BRL, Breda, The Netherlands), resolved by agarose gel electrophoresis and immobilized on Gene Screen plus™ (Du Pont, NEN, Boston, Mass., USA) by Southern blotting, according to standard procedures (Sambrook et al., 1989). An almost full-length human 51-kDa cDNA probe was ^{32}P-labeled by random primer synthesis. Prehybridization and hybridization were performed at 65°C in 0.5 M sodium phosphate buffer (pH=7.2)/7% (wt/vol) sodium dodecyl sulphate/1mM EDTA. Filters were washed three times for 5 min at room temperature and once for 15 min at 65°C in 40 mM Na_2HPO_4/NaH_2PO_4 (pH=7.2)/0.1% SDS. The same procedure was also performed with 30 mM and with 15 mM sodium phosphate. For autoradiography, filters were exposed to Kodak X-ray films for up to 14 days at -70°C.

Sequence analysis

Two μg of genomic DNA was subjected to direct cycle sequencing of both strands. In Table 1 the oligonucleotides are shown that were used for

amplification and cycle sequencing with the Taq DyeDeoxy™ terminator cycle sequencing kit (Perkin Elmer/Applied-biosystems, Foster City, USA) of the *NDUFV1* gene. Samples were analysed with an Applied Biosystems 373A DNA sequencer. Pwo polymerase (Expand™ Long Template PCR System, Boehringer, Mannheim, Germany) was used to amplify long fragments.

Table 1. Oligonucleotides used for PCR and cycle sequencing of the NDUFV1 *Gene*

Primer[a]	Position[b]	Exon	Sequences (5'-3')
1128f	-21 - -1	intron 1	CCTGACCCTTTGTCTCCCTAG
1078f	456 - 476	IV	CGCTGCCTATATCTACATCCG
1112r	+45 - +22	intron 4	GCACACTGAACACCTTCTCATCTG
1081f	605 - 625	V	GGGCCTACATCTGTGGAGAGG
1083r	644 - 625	V	GACTCGATGAGCGCTGTCTC
1117f	865 - 885	VI	GAGGAGGAGATGTCTGTGCCC
1080r	885 - 865	VI	GGGCACAGACATCTCCTCCTC
1143f	934 - 955	VII	GACTACCTCCTTGCTGTGATCC
1150r	1163 - 1145	VIII	CACCGGTCACATGGGGTAC
1181f	1136 - 1154	VIII	GTGGCCAGTGTACCCCATG
1147r	1430 - 1410	X	TGGATAGACGCAGGACAGCAG

a f, forward primer; r, reverse primer.
b Nucleotide numbering according to cDNA sequence, Fig. 3.

Northern blot analysis

Multiple tissue Northern blots (Clontech Laboratories, Palo Alto, CA, USA) containing 2 μg of poly(A)$^+$ RNA from different human tissues were hybridized with the 51-kDa human cDNA probe. The amount of poly(A)$^+$ RNA was normalized according to the β-actin expression. For quantification of the different mRNA fragments, the autoradiograph was scanned on a Hewlett Packard ScanJet IIc and the relative percentages were determined with ImageQuant software (Molecular Dynamics, Sunny Vale, CA, USA).

RESULTS

cDNA library screening

A human kidney cDNA library was screened with a full-length bovine 51-kDa cDNA clone (Patel et al., 1991). Ten positive clones with insert sizes in the range of 800-1500 base pairs (bp) were obtained. The 1500 bp insert was further analysed by subcloning into a pBluescript KS(+) vector and by

subsequent sequencing of the insert on both strands. The sequence of the insert was found to be 90% homologous to the bovine 51-kDa sequence, confirming it to be the human equivalent. The human 51-kDa cDNA had a length of 1516 nucleotides coding for 464 amino acids. With a 5' RACE experiment using human brain cDNA as template, we were not able to extend further the sequence to the 5'-end.

Southern blot analysis of the 51-kDa subunit gene

Restriction digests of genomic DNA run on a 0.6% agarose gel were hybridized with a probe containing the almost full-length human cDNA (position 7-1475). One (*Pvu*II and *Hin*dIII) or two different (*Stu*I, *Sst*I and *Eco*RI) bands of similar intensity were present in the various digests (Fig. 1). After washing at different stringencies, the same number of bands was observed. This simple pattern pointed to a single gene with an estimated length of 7 kilobases (kb).

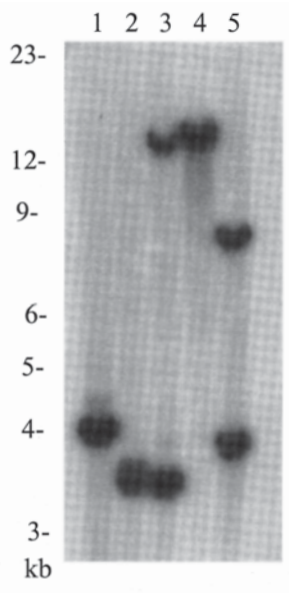

Fig. 1. Hybridization of the cDNA for the bovine 51-kDa protein with restriction digests of human genomic DNA. This probe contains the entire coding region. The human DNA was digested with the following restriction enzymes: **1**, Pvu*II;* **2**, Stu*I;* **3**, Sst*I;* **4**, Hin*dIII,* **5**, Eco*RI. The size marker used is the 1-kb ladder from Gibco BRL.*

Intron-exon organization and the restriction map of the **NDUFV1** *gene*

In order to elucidate the intron-exon organization of the 51-kDa gene, we generated a PCR fragment from genomic DNA using the 5' primer (1128f at nucleotide position -21 in intron 1) and the most 3' primer (1147R at nucleotide position 1478 of the coding sequence). As a long PCR fragment was anticipated, Pwo polymerase instead of *Taq* polymerase was added to

the PCR mixture. The resulting 4.7-kb PCR product was used as a template in a cycle sequencing protocol with nine different primers derived from the human cDNA sequence. Analysis of the sequence obtained revealed the presence of seven exons in addition to the first three exons described previously by Spencer (1992). Therefore, the *NDUFV1* gene comprises 10 exons varying in length between 82 and 213 bp and is separated by nine introns varying in length between 73 and 1428 bp that follow the conventional intron splice donor and acceptor sequence rules (Fig. 2 and 3A). The ATG codon at position 46 is most likely the translation start codon, because the sequence upstream contains the consensus sequence for a eukaryotic translation initiation site, (CC)RCCATG (Kozak, 1984). The ATG initiation codon was located in exon I, and the TAG stop codon and the poly(A) addition signal were located in exon X. The 5' untranslated region (UTR) and the 3' UTR were 45 nucleotides and 79 nucleotides long, respectively. The poly(A) tail is located 20 nucleotides downstream from the polyadenylation signal (Fig. 3A). The intron size was determined for introns 4 and 5 by estimation of the length of the PCR-fragments spanning those introns and for introns 6, 7, 8 and 9 by direct sequencing. New DNA sequence was deposited with the EMBL Nucleotide Sequence Database under Accession Nos.: Y16309 (=Y17379-Y17383) and Y16310.

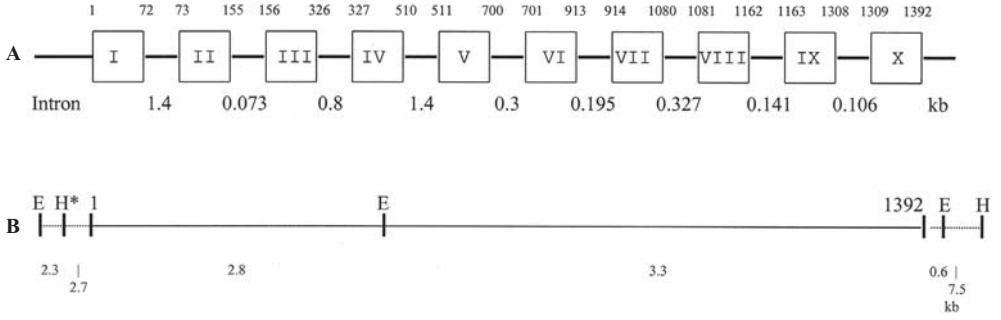

*Fig. 2. Structure of the human 51-kDa subunit gene (*NDUFV1*). (***A***) Genomic structure. Nucleotide 1 is the putative initiator codon ATG. Rectangles represent exons, which are numbered I-X. The numbers between rectangles represent intronic sizes (in kb). The position of the exon-intron boundaries is given in nucleotide number. (***B***) Restriction map of the* NDUFV1 *gene for (*H*)* Hin*dIII and (*E*)* Eco*RI. Distances in kilobases (kb) of the restriction sites up and downstream the gene are given.*
****** *This* Hin*dIII position is derived from Cowell (Cowell et al., 1988).*

Fig. 3A

```
                .         .         .         .
-45     TGCTATGAAGGTGACAGCGTGAGGTGACCCATCTGGCCCGCCGCGATGCT
                .         .         .         .         .
6       GGCAACACGGCGGCTGCTCGGCTGGTCGCTTCCCGCGCGGGTATCTGTGC
              .         .
56      GTTTCAGCGGCGACACGgtgaggcccagcctgggccacgggtgtttgggg
        cgggtgtcgcggccgcgctcaagttgcacgagcagtcctgggcgtagtgc
        tctgagagcctctcct .../Alu-sequence/...   cccctcctaa
        atagggagacccaagattctgtagcttctggcccaatccctcatggcccc
                                                        .
73      agagcactctgggcctcctgacccttgtctccctagACAGCACCCAAGA
                .         .         .         .         .
86      AAACCTCATTTGGCTCGCTGAAGGATGAAGACCGGATTTTCACCAACCTG
              .         .         .
136     TATGGCCGCCATGACTGGAGgtgagacagtgcccttagtgtgttgggtcc
                                                        .
156     cggagcaaggtgtccccttcattgccttccctattctgtccagGCTGAAA
                .         .         .         .         .
163     GGTTCCCTGAGTCGAGGTGACTGGTACAAGACAAAGGAGATCCTGCTGAA
                .         .         .         .         .
213     GGGGCCCGACTGGATCCTGGGCGAGATCAAGACATCGGGTTTGAGGGGCC
                .         .         .         .         .
263     GTGGAGGCGCTGGCTTCCCCACTGGCCTCAAGTGGAGCTTCATGAATAAG
              .
313     CCCTCAGATGGCAGgtgtgtgtggggtcggggcagatgtggctgtggg
        agagaccttgggggtggctggggtttccctgggcctttgggctctttcct
        tagaaacttagcatg       ...//... caactggatggagtaagatg
        ctgtcagagactcttgagctcctggtgagtgggtgcatgagttgaagacc
                                                        .
327     cagtcctgatggccctgtagcctgtctgacctgtgggcccctgcagGCCC
```

```
331     AAGTATCTGGTGGTGAACGCAGACGAGGGGGAGCCGGGCACCTGCAAGGA
                .         .         .         .         .
381     CCGGGAGATCTTACGCCATGATCCTCACAAGCTGCTGGAAGGCTGCCTGG
              .         .         .         .         .
431     TGGGGGGCCGGGCCATGGGCGCCCGCGCTGCCTATATCTACATCCGAGGG
              .         .         .
481     GAATTCTACAATGAGGCCTCCAATCTGCAGgtgggtagggagagatgtag
        acagatgagaaggtgttcagtgtgcactcacacacccctcacccagcaca
        gttgttctgaggtgttagtacctggtctgtcagtggttgaactg
        ...//...  ccaggagcattaggagcttcaccgtgggaggccttcaagg
        gcttcatgactcctgaagttataggctgactcctgggctggggtgggct
                                                .         .
511     gggaaactcacacctttgtcctgcagGTGGCCATCCGAGAGGCCTATGAG
              .         .       . .         .         .
535     GCAGGTCTGATTGGCAAGAATGCTTGTGGCTCTGGCTATGATTTTGACGT

              .         .         .         .         .
585     GTTTGTGGTGCGCGGGGCTGGGGCCTACATCTGTGGAGAGGAGACAGCGC
              .         .         .         .         .
635     TCATCGAGTCCATTGAGGGCAAGCAGGGCAAGCCCCGCCTGAAGCCCCCC
              .         .
685     TTCCCCGCAGACGTGGgtaaggcctggcgtaaccctgggtcagactgtgt
        cctgtgacacccgggatctggctaggctccctttggattgttcttaggga
        tttctgagtggcttcccggctggggccagatagagagtaggctggcaaca
        acacacaggctcccccaggggcgcctgctgtccctcc     ...//...
        ggggatattagagcaggggctctgccatgggtgcaggaccctgggacaca
        cctccctgccaggaaacttgccccacccccctagcagccaccagttctctt
                                        .         .         .
701     cccatttccctgaagGAGTGTTTGGCTGCCCCACAACTGTGGCCAACGTG
              .         .         .         .         .
```

736 GAGACAGTGGCAGTGTCCCCCACAATCTGCCGCCGTGGAGGTACCTGGTT

786 TGCTGGCTTTGGCAGAGAACGCAACTCAGGCACCAAACTATTCAACATCT

836 CTGGCCATGTCAACCACCCTTGCACTGTGGAGGAGGAGATGTCTGTGCCC

886 TTGAAAGAACTGATTGAGAAGCATGCTG*gt*aaggcctggggccagccagg
tggtgggggggtgcgcagtgggggcaggtgtccacaaagagagcctgggc
gggagggctcaggagacggggctgggtctaggggctgactaagggcctgg
gctcaggactaggcaggtgtgccggccccagccctgaccatgcatccctt

914 tggggaccgacttggggccccag*GGGGTGTCACGGGCGGCTGGGACAACC*

941 TCCTTGCTGTGATCCCTGGCGGCTCGTCTACCCCACTGATCCCCAAGTCT

991 GTGTGTGAGACGGTGCTGATGGACTTCGATGCGCTGGTGCAGGCACAGAC

1041 AGGCCTGGGCACAGCTGCGGTGATCGTCATGGACCGCTCG*gt*aagggttc
acacaccagccctggtccctgccctcctggttgctgtctccctccctggg
cctcccagaaaaccctcttgccagcactcaggtctcagttcctgcagcct
gagataaagcaaggtggaagaggagggaggaagtcttctctgaggagaat
accccggagtctggacagcacaggggacccagggaggctggaggaggcca
gaacgctgggtgggctgggaagagcttctggaactgggggagggtctgct
gcatgggggctgaggcccaggcttctgtctggccgtgggtgcctgctaat

1081 tgcccctcgtcaccc*ag*ACGGACATCGTGAAAGCCATCGCCCGCCTCATT

1114 GAGTTCTATAAGCACGAGAGCTGTGGCCAGTGTACCCCATGCCGTGAGG*g*
*t*gagcatcgggcaggttgggggcttgcttgctgtggcttcatttaacctc
ctccccaccacgtggcctgcagccctcaagcgccgccccacatcctggct

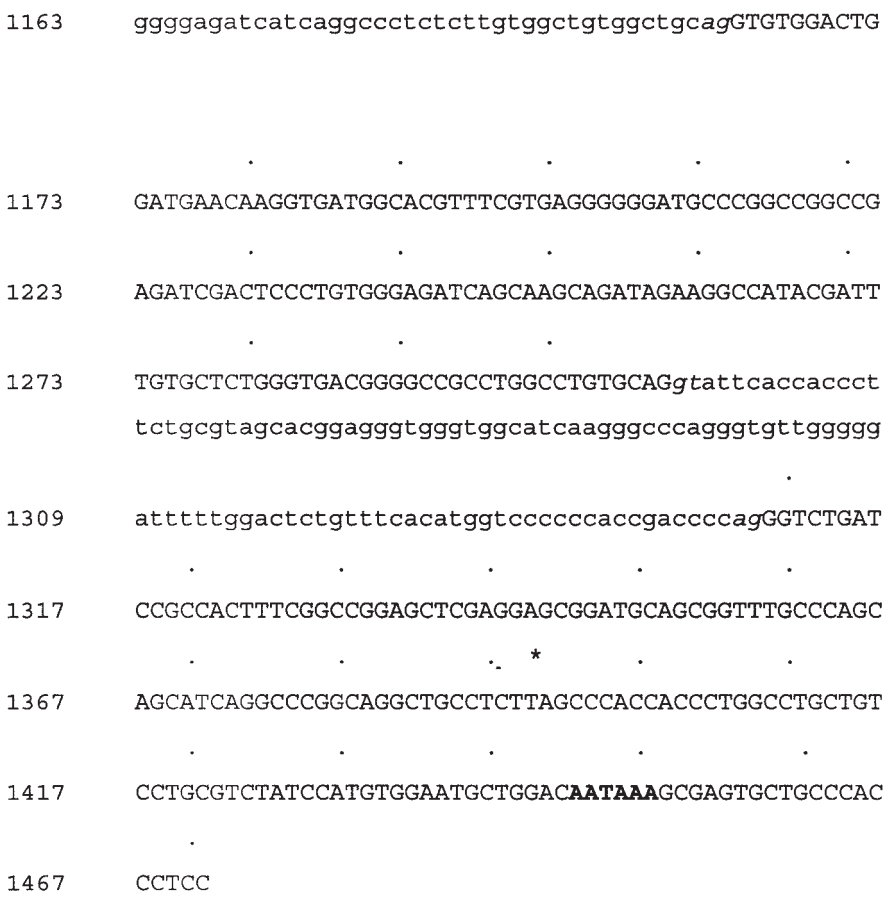

*Fig. 3. (**A**) Nucleotide sequence of the 51-kDa subunit of human NADH:ubiquinone oxidoreductase. The 6.3-kb genomic sequence encompasses exon I-X and the partial intronic sequences. The gene structure was deduced by comparison of this genomic sequence with the human cDNA sequence, generated by PCR from human fibroblast first-strand cDNA and the published cDNA sequences of the bovine 51-kDa subunit (Patel et al., 1991; Pilkington et al., 1991). The first three exons and two introns were published by Spencer et al. (1992) and the partial human cDNA sequence (nucleotide 304-960) by Ali et al. (1993). The numbers indicate the cDNA sequence. The putative initiator codon ATG is underlined, the TGA termination codon is shown by an asterisk, and the polyadenylation signal is shown in bold print. The* Eco*RI site is double underlined. Lowercase let-*

Fig. 3B

```
H  1    MLATRRLLGWSLPARVSVRFSGDTTAPKKTSFGSLKDEDRIFTNLYGRHD  50
B  1    ...A.....G.....................................     50
H  51   WRLKGSLSRGDWYKTKEILLKGPDWILGEIKTSGLRGRGGAGFPTGLKWS  100
B  51   .....AQ.....................V...................     100
H  101  FMNKPSDGRPKYLVVNADEGEPGTCKDREILRHDPHKLLEGCLVGGRAMG  150
B  101  ....................................V..........     150
H  151  ARAAYIYIRGEFYNEASNLQVAIREAYEAGLIGKNACGSGYDFDVFVVRG  200
B  151  .................................................     200
H  201  AGAYICGEETALIESIEGKQGKPRLKPPFPADVGVFGCPTTVANVETVAV  250
B  201  .................................................     250
H  251  SPTICRRGGTWFAGFGRERNSGTKLFNISGHVNHPCTVEEEMSVPLKELI  300
B  251  .........A.....................N.................     300
H  301  EKHAGFVTGGWDYLLAVIPGGSSTPLIPKSVCETVLMDFDALVQAQTGLG  350
B  301  ........................................I.......     350
H  351  TAAVIVMDRSTDIVKAIARLIEFYKHESCGQCTPCREGVDWMNKVMARFV  400
B  351  .................................................     400
H  401  RGDARPAEIDSLWEISKQIEGHTICALGDGAAWPVQGLIRHFRPELEERM  450
B  401  ............C.....................................     450
H  451  QRFAQQHQARQAAS                                      464
B  451  ............F                                       464
```

ters indicate intron sequences. Splice acceptor and donor sites consensus sequences are shown in italics. .../.../... only partial intronic sequence is given, additional intronic sequence is deposited with the EMBL Nucleotide Sequence Database under Accession Nos. Y16309 and Y16310. The complete NDUFV1 *gene sequence is deposited under No. AF053070 by Schuelke et al. (1998). (***B***) Alignment of the amino acid sequences (one letter code) of human (*H*) and bovine (*B*) 51-kDa subunits. The sequence of the bovine subunit is taken from Pilkington et al. (1991). The numbering for the protein sequences started at the first methionine codon. Dots indicate identical amino acids conserved in the two species.*

The 51-kDa subunit mRNA is ubiquitously expressed

Northern blot analysis of human poly(A)$^+$ RNA from 13 different human tissues showed a single fragment of about 1500 nucleotides (Fig. 4). For testis, a 2.4 kb and 3 kb fragment were also observed. In all lanes an additional double band at 4.5 kb showed up, probably representing mRNA aggregates. From a densitometric analysis of the autoradiograms it was estimated that the steady-state levels of the 51-kDa mRNA in pancreas is about 40-fold and in skeletal muscle about 20-fold higher than in brain RNA.

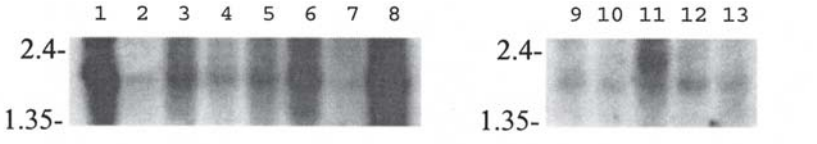

Fig. 4. Northern blot analysis. Hybridization of the human cDNA probe (nucleotides 337-930) from the NDUFV1 *gene to 2µg poly(A)$^+$ RNA from human tissues. Lane* **1***, heart;* **2***, brain;* **3***, placenta;* **4***, lung;* **5***, liver;* **6***, skeletal muscle;* **7***, kidney;* **8***, pancreas;* **9***, thymus;* **10***, prostate;* **11***, testis;* **12***, ovary;* **13***, small intestine. RNA size marker bands are indicated to the left.*

DISCUSSION

As part of our investigations towards identification of nuclear genes responsible for a defect in complex I of the respiratory chain in patients with a mitochondrial (encephalo-) myopathy, we here describe the structural organization for the 51-kDa subunit gene. We found that in the human genome there is a single 6.2-kb gene consisting of 10 exons and interspersed by 9 introns varying in length between 0.1 and 1.4 kb.

The Southern blot data for the various restriction enzymes are consistent with the presence of a single copy gene. The two *Eco*RI Southern blot fragments observed could be predicted on the basis of the *Eco*RI site found in exon 4 at nucleotide position 481 (Fig. 1). Our results are at variance with the Southern blot results reported by Patel et al., who used the bovine cDNA as a probe. An obvious explanation for this discrepancy is lacking, because at least three restriction site polymorphisms have to be invoked to this relatively short gene to reconcile both results.

Northern blot analysis revealed a single mRNA fragment in 13 different tissues. The single exception was testis where two larger fragments

were detected. Interestingly, multiple transcripts in testis were also observed for the 10-kDa flavoprotein complex I gene (de Coo et al., 1997), for the mouse cytochrome *c* gene (Hake and Hecht, 1993), and for the mitochondrial transcription factor A in human and mouse (Larsson et al., 1996; Larsson et al., 1997), but not for the 24-kDa flavoprotein complex I gene (de Coo et al., 1995). More detailed and comparative studies are needed to gain insight in the function of these alternative splice products.

The human 51-kDa cDNA was very similar in structure to the bovine cDNA, not only for the coding region, but also for the 5' and 3' UTRs. Both the human and bovine cDNA had an AATAAA polyadenylation signal, which was located, respectively, 20 and 13 bases upstream of the poly(A) tail. The 5' regions of both cDNAs contain a preponderance of C and G residues, which might indicate that CpG islands are associated with the 5' ends of the genes. The bovine coding region encompassed 464 amino acids also (Patel et al., 1991; Pilkington et al., 1991). The identity with the bovine nucleotide sequence for the coding region was 98%, which was similar to the values found in other mitochondrial genes, such as the 24-kDa with 93% homology (de Coo et al., 1995). The mature bovine and human 51-kDa protein both had the same N-terminal sequence. Two amino acid substitutions were present within the 20-amino acid N-terminal presequence, and the 444-amino acids mature protein sequence differed in 10 positions from the bovine protein sequence (Fig. 3B). While this paper was under review, Schuelke et al. (1998) reported that they had sequenced the 51-kDa and the intron-exon boundaries of the 51-kDa gene. Except for a silent 138 T>C transition, their published sequence is identical to ours.

Northern blotting analysis showed that the 51-kDa subunit is ubiquitously expressed. Compared with the 24- and 10-kDa subunit genes, the expression pattern is, apart for a high expression in pancreatic tissue, identical. It is firmly established that mitochondrial DNA (mtDNA, for example, $tRNA^{Leu(UUR)}$ 3243A→G mutation) mutations are associated with pancreatic exocrine dysfunction and or diabetes (Gerbitz et al., 1996; Gerbitz et al., 1995; van den Ouweland et al., 1992). Complete loss of 51-kDa protein is likely to be incompatible with life. A severe deficiency would probably cause multisystem disease, whereas a more moderate deficiency might impair insulin secretion, by perturbing energy metabolism. Alternatively, an allelic variant of the *NDUFV1* gene might exacerbate the effect of mtDNA mutations such as the $tRNA^{Leu(UUR)}$ 3243A→G mutation.

Acknowledgements

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CHAPTER 11

Classification of twenty patients with an isolated mitochondrial complex I deficiency and exclusion of mutations in the complex I flavoprotein genes

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Abstract

Complex I (NADH:ubiquinone oxidoreductase) is the largest protein in the mitochondrial respiratory chain and consists of 46 subunits, of which 7 are encoded by the mitochondrial DNA. Isolated Complex I deficiency is one of the most common causes of mitochondrial encephalomyopathies. We have studied twenty patients with an isolated complex I deficiency. The patients could be subdivided into three clinical categories. Six of the patients presented with a myopathic phenotype, in 12 patients encephalopathic features dominated the clinical symptoms and two patients had multiorgan failure. The onset of disease in the latter two groups was earlier and the disease showed a much more severe course. There was no clear correlation between the degree of diminished complex I activity in fresh muscle biopsies and the severity of symptoms. In the patients none of the common mitochondrial DNA mutations were found. The three flavoproteins of complex I were scrutinized at the genomic level by single stranded conformation analysis (SSCP) of all exons of NDUFV1, NDUFV2 *and* NDUFV3. *The observation of aberrant migrating SSCP bands was followed up by sequence analysis. Two polymorphisms but no pathogenic mutations were detected. This study confirms the heterogeneity of clinical and biochemical features in patients with a defective complex I, which is most likely mirrored by genetic heterogeneity.*

INTRODUCTION

Oxidative phosphorylation (OXPHOS) is the final common pathway of mitochondrial energy metabolism. The main function of the OXPHOS is the production of ATP. The OXPHOS is carried out in the inner mitochondrial membrane by the five enzyme complexes of the respiratory chain. Hydrogen, derived from the oxidation of organic acids, such as pyruvate and fatty acids, is oxidized with atomic oxygen. Oxygen consumption is coupled to ADP phosphorylation through the electrochemical gradient. NADH:ubiquinone oxidoreductase (complex I) plays an important role in this process by pumping protons while transferring electrons from NADH to coenzyme Q (Walker, 1992).

Complex I activity can be determined in cultured cells or muscle biopsies by polarographic analysis of the rate of oxygen utilization with NAD^+ -linked substrates, by decarboxylation of these [^{14}C] labelled substrates or by spectrophotometric analysis of the rotenone-sensitive NADH-CoQ reductase activity. Human complex I consists of 46 subunits, seven of which are coded for by the mitochondrial genome (Carroll et al., 2003; Murray et al., 2003). The presence of subunits of Complex I can be studied by 2D electrophoresis of OXPHOS complexes and by Western blotting analysis using specific antibodies e.g. (Bentlage et al., 1995a; Ugalde et al., 2004).

Defects of complex I have been identified in patients with a broad range of OXPHOS disease phenotypes, named mitochondrial encephalomyopathies (ME). The symptoms can range from myopathic complaints with exercise intolerance to either encephalopathy with seizures or dementia, or symptoms related to a multi-organ failure (Morgan-Hughes, 1994). But even within a subgroup there is considerable clinical, biochemical and genetic heterogeneity. Decreased complex I activities or absent individual complex I subunits (13, 20, 24 kDa, or 75 kDa) have been reported in patients with a ME (Moreadith et al., 1987; Ichiki et al., 1988; Morgan-Hughes et al., 1988; Schapira et al., 1988; Robinson et al., 1990; Slipetz et al., 1991; Bentlage et al., 1995b; Dionisi-Vici et al., 1997; Kim et al., 2001).

The most frequently encountered mutations associated with complex I deficiency are those found in the mitochondrial tRNA genes (Lebon et al., 2003). To date mutations in eleven nuclear encoded genes of the OXPHOS have been described, seven of them in complex I genes. The first mutation detected was in the flavoprotein subunit gene of succinate dehydrogenase (complex II, *SDH1*) (Bourgeron et al., 1995). Later, a 5 bp duplication in the gene encoding the 18 kDa subunit gene (*AQDQ*, *NDUFS4*) of complex

I (van den Heuvel et al., 1998) was found followed by a compound mutation in *NDUFS8* (Loeffen et al., 1998), three mutations in *NDUFV1* (Schuelke et al., 1999) and a missense mutation in *NDUFS7* (*PSST*) (Triepels et al., 1999). Other mutations have been found in *NDUFS1* (Bénit et al., 2001) and in *NDUFV2* (Bénit et al., 2003).

Here we have focused on the evolutionary highly conserved flavoprotein genes *NDUFV1*, *NDUFV2* and *NDUFV3*, encoding for the 51, 24 and the 10 kDa protein subunits of complex I, for their important role in electron transport, for the fact that four patients have been reported to have a defective 24 kDa protein subunit (Schapira et al., 1990) and for our observation that in a large group of patients with a mitochondrial encephalomyopathy (ME) and a defective complex I enzyme activity no mutations in the mtDNA were found (de Vries et al., 1994). Previously, we have described the genomic organization of the three FP genes facilitating such screening of patients at the genomic level (de Coo et al., 1995; de Coo et al., 1997; de Coo et al., 1999).

PATIENTS

Frozen muscle specimen from twenty, non-related, patients with a mitochondrial encephalomyopathy were selected from the patient tissue bank of two biochemical laboratories at the University Hospital of Nijmegen and that in Rotterdam, the Netherlands. The patients included in this study fulfilled the following criteria: 1. Decreased oxidation rates of NAD^+-linked substrates by a mitochondrial preparation from fresh unfrozen muscle as well as energy decreased phosphate production rates with these substrates (results not shown) (Scholte et al., 1995; de Vries et al., 1996). 2. Decreased complex I activity, in combination with normal activities of succinate:cytochrome *c* oxidoreductase (reflecting complex II + III activity), decylubiquinol:cytochrome *c* oxidoreductase if measured (reflecting complex III activity), cytochrome *c* oxidase (reflecting complex IV activity) and pyruvate dehydrogenase. To correct for a low mitochondrial content the ratio of complex I/complex IV was used. The threshold for inclusion was arbitrarily set at a level below 50% of the mean ratio found in controls.

Data from patient 3, 4, 6, 8 and 19 have been reported previously (Bernsen et al., 1991; Bernsen et al., 1993; Scholte et al., 1995; Loeffen et al., 1998; Rubio-Gozalbo et al., 1998).

MATERIALS/METHODS

A quadriceps muscle specimen was processed for histochemical and biochemical studies. The OXPHOS studies and the activities of the enzyme complexes of the respiratory chain were measured as described previously. Complex I was assayed as rotenone-sensitive NADH oxidase (NO) or as rotenone-sensitive NADH-CoQ_1 oxidoreductase (Q_1), complex IV as ferrocytochrome *c* oxidase (IV) (Fischer et al., 1986; Scholte et al., 1987). Enzyme activities were measured in 10 minutes, 600 *g* supernatants prepared from fresh muscle samples, or measured in supernatants or total homogenates from frozen muscle samples (Table 1).

Table 1. Biochemical and Clinical data.

Patient	Fresh muscle (Supernatant)		Sex	Diagnosis		Onset	Outcome	Family history	Reference
	Q_1/CS	IV/CS Units/Units	Q_1/COX						
control 2SD range	0.142 ± 0.06 (0.044-0.265) (n=37)	1.71 ± 0.68 (0.81-3.12) (n=68)	0.083 ± 0.048						
p1	0.0556	2.63	0.0211	M	Leigh	12 y		Unknown	
p2	0.0281	1.98	0.0142	M	Encephalo-pathy	Neonatal	Died	Sib, consan-guineous	
p3	0.0243	1.48	0.0164	M	Encephalo-pathy	Infancy	Died	Sib	(Rubio-Gozalbo et al., 1998)
p4	0.0229	1.59	0.0143	M	Myopathy neuropathy	Childhood		No	(Bernsen et al., 1991)
p5	0.0215	2.14	0.0100	F	Myopathy	Childhood		Mother affected	
p6	0.0201	1.96	0.0102	M	Leigh	Infancy	Died	No	(Loeffen et al., 1998)
p7	0.0180	1.69	0.0107	F	Encephalo-pathy	Neonatal	Died	No	
p8	0.0050	0.52	0.0098	F	Myopathy	Childhood		No	(Bernsen et al., 1993)
	Frozen muscle (10 min 600g supernatant) Q_1 mUnits/mg protein	IV	Q_1/IV						
control 2SD range	17.1 ± 4.8 (8.9-26.6) (n=16)	94 ± 51 (33-225) (n=18)	0.18 ± 0.11						
p9	1.8	120	0.015	M	Myopathy	Infancy		Sib	
p10	0.495	33	0.015	M	Failure to thrive	Neonatal		Sibs (2)	

Table 1 (continued) Biochemical and Clinical data.

Patient	Frozen muscle (homogenate)			Sex	Diagnosis	Onset	Outcome history	Family	Reference
	Q_1/CS	IV/CS Units/Units	Q_1/ IV						
control 2SD range	0.187 ± 0.081 (0.056-0.440) (n=23)	1.15 ± 0.39 (0.52-2.08) (n=20)	0.163 ± 0.089						
p11	0.009	1.98	0.005	F	Encephalo-pathy	Neonatal		No	
	Frozen muscle (homogenate) I Units/(min.g	IV *k*/(min.g)	I/IV Units/*k*						
Control mean range	3.66 (1.55-7.29) n=45	95 (45-161) n=49	0.040						
p12	2.27	138	0.0165	F	Leigh	9 y		No	
p13	1.42	75	0.0189	M	Encephalo-pathy myopathy	Infancy		Sib	
p14	1.20	73	0.0167	M	Myopathy	38 y		Unknown	
p15	0.76	195	0.0039	F	Myopathy	15 y		No	
p16	0.69	65	0.0106	F	Encephalo-pathy	16 y		Unknown	
p17	0.66	54	0.0122	M	Leigh	Childhood		Sib	
p18	0.40	43	0.0093	F	Encephalo-pathy myopathy	Infancy	Died	Sib	
p19	0.18	305	0.0006	M	MELAS	Infancy		Sib, consan-guineous	(Scholte et al., 1995)
p20	0.16	84	0.0019	M	Multi-organ failure	11 months	Died	No	

DNA-studies

Total genomic DNA was isolated from muscle samples. Mitochondrial DNA was screened for large deletions and common point mutations by routine procedures (de Coo et al., 1998). The coding regions of the nuclear *NDUFV1*, *NDUFV2* and *NDUFV3* genes were amplified with 10, 8 and 3 primer pairs, respectively, flanking the even so many exons. The primers were designed based on intronic sequences and, if possible, located outside the mRNA splicing region (A list of PCR primers and conditions for SSCP analysis of the *NDUFV1*, *NDUFV2* and *NDUFV3* gene can be requested by IFMdC). The PCR fragments were separated on a 0.5xMDE-gel (AT Biochem, Malvern, PA, USA) and SSCP analysis was performed at two different electrophoresis conditions: 6 h at 40 W and 16 h at 6 W both at 4ºC in a 0.6xTBE buffer as previously described (Lemmink et al., 1993).

The PCR fragments with a shifted band were purified from agarose gels by the freeze squeeze method, cycle sequenced and analysed on an ABI 373 automated sequencer (ABI, Foster City, CA, USA). Mutations altering a restriction enzyme site were tested by digesting the PCR fragment with the appropriate restriction enzyme (New England Biolabs Inc., MA, USA). The restriction fragments were resolved on an agarose gel and stained with ethidium bromide.

RESULTS

Patients

The group of twenty patients selected on biochemical criteria consisted of 12 males and 8 females (Table 1). Six of the patients suffered from a myopathic disease, 12 from an encephalopathic disease and 2 from a multisystemic disease. In the encephalopathic group four patients presented with Leigh syndrome and 1 patient showed a MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes) phenotype.

Biochemistry

Lactic acidemia was present in all patients except in patient 11. The results of the measurements are listed in Table 1 in decreasing order of activity of complex I.

DNA studies

Screening for gross rearrangements of the mtDNA and the point mutations in $tRNA^{Leu(UUR)}$ (positions: 3243, 3271), $tRNA^{Lys}$ (position: 8344) and the *ATPase 6* gene (position: 8993) were negative in all 20 patients tested. Mutations in all the mitochondrial tRNA genes were excluded in p3-8, 10 and 15 and mutations in the mitochondrial complex I genes were excluded in p3, 5-8 and 15. The results of the mutation screening in the three flavoprotein genes are listed in Table 2. With SSCP mutation analysis we could not detect any mutation or polymorphism in *NDUFV1* and *NDUFV3*. Patients 2, 7, 9 and 11 were heterozygous for a recently described ALA29VAL polymorphism in exon II of the *NDUFV2* gene (Hattori et al., 1998). Patient 9, 11 and 15 were heterozygous for a silent mutation (Val67Val) in exon III of the *NDUFV2* gene. Apparently, this is a novel polymorphism because it was also detected in 6 out of 24 control samples (25%). Finally, we found in the 3' region a base pair change at position 92 after the TAA stopcodon of the *NDUFV2* gene. This latter mutation was not followed up in controls.

Table 2. NDUFV2 *gene mutations in patients with a mitochondrial complex I deficiency.*

Patient	Sex	Gene	Primer pair detecting SSCP shift and mutation	Nucleotide position[a]	Type of mutation and Codon position[b]	Reference
p2	M	*NDUFV2*	Exon 2	C86T	Missense ALA29VAL (GCT>GTT)	(Hattori et al., 1998)
p7	F	*NDUFV2*	Exon 2	C86T	Missense ALA29VAL	(Hattori et al., 1998)
p9	M	*NDUFV2*	Exon 2	C86T	Missense ALA29VAL	(Hattori et al., 1998)
p20	M	*NDUFV2*	Exon 2	C86T	Missense ALA29VAL	(Hattori et al., 1998)
p9	M	*NDUFV2*	Exon 3	A201T	VAL67VAL (GTA>GTT)	
p11	F	*NDUFV2*	Exon 3	A201T	VAL67VAL	
p15	F	*NDUFV2*	Exon 3	A201T	VAL67VAL	
p11	F	*NDUFV2*	3'Extragenic region	C > G 92 bp after stopcodon TAA	-	

a Nucleotides are numbered with respect to the A of the first ATG of the ORF (de Coo et al., 1995; de Coo et al., 1997; de Coo et al., 1999) (GenBank accession No. *NDUFV1*: Y16309, Y16310; *NDUFV2*: M22538; *NDUFV3*: X59048).
b Codons are numbered with respect to the first ATG of the ORF.

DISCUSSION

In twenty patients with a complex I deficiency and an apparently normal mtDNA composition, we were not able to find an explanation for their pathology by mutation screening of the FP genes. With the combination of SSCP and sequence analysis we detected two polymorphisms in the *NDUFV2* gene. The ALA29VAL missense mutation has been associated in homozygous form with Parkinson disease (Hattori et al., 1998). We found only 4 heterozygous patients (20%). The VAL67VAL silent mutation is a novel polymorphism with a frequency in a control group of 6 out of 24. In p11 we detected a mutation in the 3' extragenic region.

From the more than 300 muscle specimens analysed, only twenty patients had an isolated complex I deficiency. In none of them a common mtDNA mutation has been found. By the applied criterion of an isolated complex I deficiency, a group of in total about a hundred patients was excluded. The excluded group consisted of patients with a progressive external ophthalmoplegia (PEO), a mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS), a myoclonic epilepsy with ragged red fibres (MERRF), a Lebers hereditary optic neuropathy (LHON), or patients with a deletion or a point mutation in the mtDNA. By this standard also a group of 11 out of 12 patients with the MELAS phenotype but without a mtDNA tRNA mutation was excluded because none of these latter patients had an isolated complex I defect lower than 50% below the mean ratio.

The only patient (p2) with a MELAS phenotype that we included was

from a consanguineous marriage. A family member from this MELAS patient, born from consanguineous parents too, was also known with MELAS. The two patient's families were linked by their paternal lineage, strongly implying a nuclear gene to be involved in the pathogenesis of this MELAS phenotype. The likelihood of a nuclear gene involvement and a possible autosomal recessive mode of inheritance in our patient group was further enhanced by the 2 families with consanguineous marriage and the high percentage of affected sibs (43%, 8 out of 17 patients with a known family history). For patient 5, with an affected mother, an involvement of the mitochondrial genome was made unlikely by excluding a mutation in the mtDNA genome by sequence analysis of the tRNA and ND-genes.

Based on heterogeneity in phenotype and biochemistry, Pitkänen et al. (1996) classified 12 complex I patients in five categories (type I through V). Following their classification our patient material could be split in 3 patients (p2, 7, 10) with a severe neonatal acidosis (type I), 6 patients (p1, 3, 6, 12, 17, 18) with a Leigh and two patients with a "Leigh-like" (p3, 18) syndrome (type II), none with cardiomyopathy and cataract (type III), one (p20) with a hepatopathy and tubulopathy (type IV) and eight patients (p4, 5, 8, 9, 11, 13, 14, 15) with mild symptoms and lactic acidosis (type V). Two patients could not be classified, p19 with the MELAS phenotype and p16 with epileptic seizures as an important symptom. As also seen in most other inborn errors, Pitkänen found that the earlier the onset of disease the more severe the course. The patients who died a few weeks to a few years after the onset of their disease belonged mainly to disease type I or II. In our group of complex I deficient patients we can confirm the findings of Pitkänen et al. (1996) in that there was little correlation between the decrease in complex I activity and the severity of clinical symptoms or with the classification according to Pitkänen.

An obvious explanation for our negative results is that we have searched only for mutations in three subunits of complex I. We did also not exclude in all cases the possibility that the complex I deficiency was due to a rare mutation in the mtDNA. This latter possibility was recently highlighted by Lebon et al. (2003). Five heteroplasmic mutations (20%) were detected by screening a group of 50 complex I patients for all mitochondrially encoded complex I subunits.

Besides the limited number of genes surveyed and the relative low number of patients screened, other explanations for our negative results are possible. The selective loss of individual subunits as described in literature (Bentlage et al., 1995a; Schapira et al., 1988) may be a secondary phenomenon or a biochemical artefact (assemblage, breakdown) pointing to the central role of some of the subunits (e.g. the 24kda subunit) (Almeida et

al., 1999). We were not able beforehand to get direct indications for a pathogenetic role of the flavoprotein genes in our patients. One of the reasons was that in many patients no fibroblasts or mRNA was available to analyse the gene expression. Only in p4, 5 and 19 we were able to look for abnormalities in mRNA expression level, but they were all within the control range (results not shown). Another cause could be the sensitivity of the applied SSCP mutation detection method, being restricted to about 90% (compared to sequence analysis). Mutation detection of the promotor region has not been performed, but abnormalities in that region were unlikely in p4, 5 and 19 in view of their normal mRNA expression. Finally it is highly likely, that the detected polymorphisms are real ones and not attributive mutations (as e.g. described by Hattori et al. 1998 for the *NDUFV2* gene ALA29VAL), since we did not detect any homozygous changes in our patients.

During and after our analysis of three Complex I genes at the genomic level, more than 15 publications have appeared in which screening at the cDNA level (varying between screening of a single gene or conserved genes [*NDUFV1*, *NDUFS8*, *NDUFS7*, *NDUFB6*, *NDUFA8*, *NDUFS1* and *NDUFS2* (Lebon et al., 2003)] to an extensive [most cDNAs of complex I (see Triepels et al., 2001)] of many different genes was described. In none of the publications screening of the *NDUFV3* genes has been reported. In three publications mutations in the *NDUFV1* gene were detected. Three mutations (one compound) were found in two different families from a group of twenty complex I deficient patients (Schuelke et al., 1999) (selected in a similar way to the present study), and 6 *NDUFV1* mutations were found in 3 out of 36 patients screened (Bénit et al., 2001). Beskow et al. reported negative findings for the *NDUFV1* gene in 8 patients. One homozygous mutation in the *NDUFV2* gene in three sibs of an consanguineous family were reported by Bénit et al. (2003). Theirs and our study represent about 86 complex I deficient patients in which to date in 5% (6 patients) a flavoprotein defect has been found. For such a well selected group of patients a genetic diagnosis of only 5% stresses the fact that the cause for complex I deficiencies does not have to be in the structural subunits itself but can very well be sought and probably be found in for example the genes that encode protein transport, assembly factors, cofactors and others.

Screening of more patients for the *NDUFV2* and *NDUFV3* genes should be performed to answer the question whether these genes are also mutational targets in isolated complex I deficiency. Accurate determination of the deficiency with modified Western blotting techniques will improve the genetic screen yield.

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CHAPTER 12

General discussion

In the 1981 landmark paper, Sanger and co-workers presented the complete nucleotide sequence of human mtDNA (Anderson et al., 1981) consisting of 16,569 base pairs (bp) circular double-stranded DNA. Twenty years later the sequencing of the complete human genome of about 3 000 000 000 bp comes to a close (Dennis et al., 2001), illustrating the vast increase in the amount of data that now can be generated. In fact analysis of the entire mtDNA is now routinely being done as part of a diagnostic service in a few specialized laboratories and the amount of DNA sequence information rapidly increases. Initially normal mitochondrial DNA was operationally defined as absence of deletions and of mutations in a few protein-encoding genes and transfer RNA genes. However, this appeared to be an oversimplification as new mitochondrial DNA mutations were continuously found in numerous studies. This prompted us to devise new methodology to analyse the mtDNA in a more comprehensive manner. The first part of this general discussion describes the development of screening procedures – and part of the results – for rapid and cost effective determination of mutations in mitochondrial DNA.

The second part is focused on the characterization and screening of nuclear genes in mitochondrial disease with emphasis on complex I deficiency in mitochondrial encephalomyopathies. In the past, years of tedious, laborious work were required to assign a disease to a chromosomal region and isolate and characterize the underlying gene and gene defects from this region. Nowadays most of the genomic information can be found in the human genome databases and applied immediately. In this thesis the cDNA sequence, the chromosomal location and the intron-exon boundaries of the three flavoprotein genes of mitochondrial complex I were determined (de Coo et al., 1995; de Coo et al., 1997; de Coo et al., 1999a). Patients with isolated complex I deficiency and normal mitochondrial DNA were tested for mutations, and although no disease causing mutations were found in our group of patients, our initial hypothesis turned out to be correct and has been confirmed through follow-up investigations by people from the Nijmegen Center for Mitochondrial Disorders (the Netherlands) (Loeffen et al., 1998; van den Heuvel et al., 1998b; Schuelke et al., 1999b; Triepels et al., 1999 Loeffen et al., 2001) and others (Bénit et al., 2001; Petruzzella et al., 2001; Bénit et al., 2003b).

THE MITOCHONDRIAL GENOME

The sequence of the mitochondrial DNA is now known for over twenty years but many of its features are not yet fully understood, in spite of its

relatively small size. In 1988 the first studies by Holt et al. and Wallace et al. established a causal relationship between human disease and mutations in the mitochondrial genome (Holt et al., 1988; Wallace et al., 1988a). Since then the group of diseases that has been identified to be caused by or associated with mitochondrial genome mutations has grown massively. In the journal *Neuromuscular Disorders* (www.elsevier.com/locate/nmd) updates on "Mitochondrial encephalomyopathies: gene mutations" are reported regularly. In 2004, more than 100 pathogenic mtDNA mutations have been reported (Servidei, 2004). One may expect that more mutations and early- and late-onset diseases associated with mtDNA will be detected in the coming years. Different types of mutations have been described, varying from point mutations in tRNA genes (Wallace et al., 1988b; Goto et al., 1990), rRNA genes (Prezant et al., 1993) and in protein encoding genes (Wallace et al., 1988a; Manfredi et al., 1995) and rearrangements such as small deletions (Shoffner et al., 1995; Keightley et al., 1996; Comi et al., 1998; de Coo et al., 1999b), large deletions (Holt et al., 1988), multiple deletions (Zeviani et al., 1989; Suomalainen et al., 1995; Barrientos et al., 1996; Kaukonen et al., 1996; Kaukonen et al., 1999; Nishino et al., 1999), insertions (Santorelli et al., 1997), duplications (Poulton et al., 1989; Superti-Furga et al., 1993), inversions (Musumeci et al., 2000) and depletions (a diminished number of mtDNA-copies present compared to the amount of nuclear DNA) (Moraes et al., 1991).

The relation between the mtDNA genotype and the patient phenotype

Some "classical" clinical phenotype groups or syndromes can be assigned to certain types of mutations (see Table 1), but even different syndromic cases can be caused by the same mutation. For example the mutation that usually causes MERRF-syndrome can occasionally cause MELAS-syndrome. Also deletions in the mtDNA can have a variety of manifestations, which cannot be explained by the size or location of the deletion in the mtDNA. Often there is not a single phenotype but a spectrum of clinical features, each of which is found in other non-mitochondrial disorders as well. As a rule of thumb a mtDNA disorder may be considered, when a combination of at least 2 of these features co-exist in a patient or in a patient and his or her maternal relatives. Clinical investigations can be corroborated by familial, histological, enzymological, biochemical and/or molecular investigations (Munnich and Rustin, 2001; Bernier et al., 2002). The clinical heterogeneous expression of mutations in the mtDNA depends, amongst others, on the phenomenon of heteroplasmy and the mutation percentage can differ among tissues and in time (Poulton and

Marchington, 2002). For a given heteroplasmic mutation there is a tissue specific threshold, depending on the deleterious effect of that mutation and on the reliance of the cell on the mitochondrial energy supply, above which a biochemical or disease phenotype will develop. Moreover, the "background" nuclear genome also has a clear effect on the phenotypic manifestations of mtDNA mutations (Wallace, 1987; Chen et al., 1995).

The wide variety in disease manifestations and the many known but likely even more unknown associations with mtDNA mutations and possibly the entire spectrum of nuclear genes are a major challenge for diagnosis of and care for these patients. We propose a 2 step diagnostic procedure (see also Fig. 1) based on the results of our work (Chapters 1 to 6), on extensive experience with about hundred new patients with a mitochondrial disease per year, and on literature data (Chinnery and Turnbull, 1999; Jaksch et al., 2001; Bernier et al., 2002; Taylor et al., 2004). First, in case

Table 1. Some clinical phenotypes and their relation with the mtDNA mutation.

Clinical phenotype	mtDNA mutation		Hetero-plasmy	Homo-plasmy	Mode[1] of inheritance	References
AID	rRNA	A1555G		+	Maternal	Prezant et al., 1993
MELAS	tRNA	A3243G	+		Maternal	Goto et al., 1990
MIDD		A3243G	+		Maternal	van den Ouweland et al., 1995
MERRF		A8344G	+		Maternal	Shoffner et al., 1990
MiMyCa	tRNA	C3254G	+		Maternal	Kawarai et al., 1997
		A3260G	+		Maternal	Zeviani et al., 1991
		C3303T	+		Maternal	Silvestri et al., 1994
LHON	Protein genes	G3460A,	(+)	+	Maternal	Wallace et al., 1988a;
		G11778A,	(+)	+		Huoponen et al., 1991;
		T14484C	(+)	+		Johns et al., 1992
NARP/MILS		T8993C/G	+		Maternal	Shoffner et al., 1992; de Vries et al., 1993
		T9176C/G	+		Maternal	Campos et al., 1997
KSS	Large deletion		+		Sporadic	Holt et al., 1988
PEO	Large deletion		+		Sporadic	Holt et al., 1988
	Multiple large deletions		+		AD	Zeviani et al., 1989; Suomalainen et al., 1995; Kaukonen et al., 1996;
	Multiple large deletions		+		AR	Kaukonen et al., 1999 Bohlega et al., 1996
Pearson syndrome	Large deletion		+		Sporadic	Rotig et al., 1989
Fatal infantile myopathy	Depletion				Mendelian	Moraes et al., 1991
Fatal infantile hepathopathy	Depletion				Mendelian	Mazziotta et al., 1992

Notes: AD: Autosomal Dominant; AID: aminoglycoside-induced deafness; AR: Autosomal Recessive; KSS: Kearns-Sayre Syndrome; LHON: Leber's Hereditary Optic Neuropathy; MELAS: Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes; MERRF: Myoclonic Epilepsy with Ragged Red Fibres; MIDD: Maternal Inherited Diabetes and Deafness; MILS: Maternally-Inherited Leigh's Syndrome; MiMyCa: Mitochondrial Myopathy and Cardiomyopathy; NARP: Neuropathy, Ataxia, Retinitis Pigmentosa; PEO: Progressive External Ophthalmoplegia.

[1] Differences in penetrance may obscure the inheritance pattern.

of a classical syndrome the most frequent mutations and frequently involved genes in the mtDNA will be tested (Table 1) or the specific nuclear gene involved will be screened (see below). In case of a non-specific diagnosis but with clinical, biochemical and family evidence of being mitochondrial (Bernier et al., 2002) common mutations and the most frequently involved genes will be tested in the mtDNA. The number of tests will be extended depending on the symptoms involved (deafness, cardiomyopathy etc.). In general, the sensitivity of these tests is high, allowing testing of DNA from blood, hair root or urine sediment, saving the patient a biopsy of the affected tissue. In all cases however the affected tissue remains the most optimal material for identification of a mtDNA

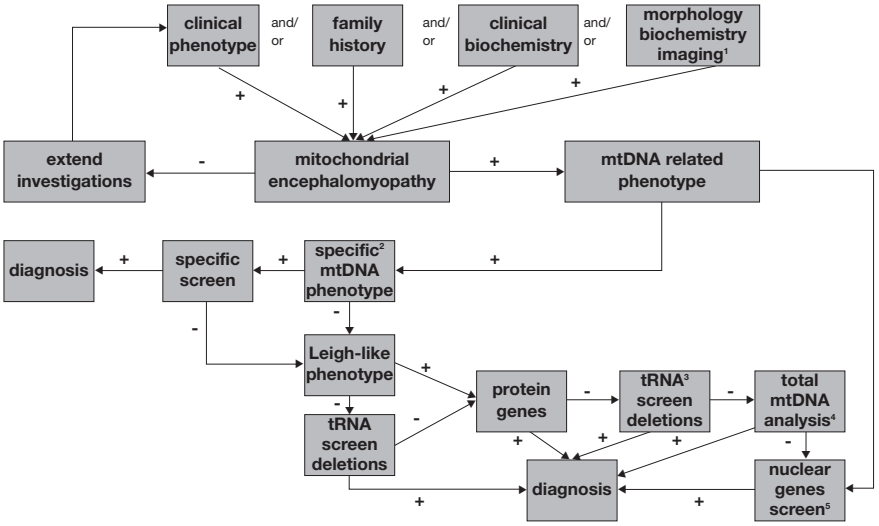

Fig. 1. Suspected mitochondrial encephalomyopathy; flowchart of the order of investigations after a negative (-) or a positive (+) result.
[1] Morphology biochemistry imaging or other studies that could contribute to the diagnosis e.g. NMR-spectroscopy, electrophysiological studies, genomics, metabolomics, proteomics.
[2] See Table 1; in less specific cases routinely the lab will test frequently encountered mutations and genes [e.g. $tRNA^{Leu(UUR)}$].
[3] If already done proceed to the next box.
[4] Not routinely performed.
[5] For specific phenotypes and known complex I gene mutations tests are done on a diagnostic basis (see Table 3-6). For the other group of patients investigations are done on a research basis.

mutation. If no common or symptom associated mutation is detected than (step 2) a complete analysis of the mtDNA will be performed on the affected (muscle) tissue by denaturing high performance liquid chromatography (D-HPLC) analysis or resequencing.

Diagnostic protocol for the investigation of the mtDNA

Step 1: Screening of (frequent) mtDNA mutations associated with specific syndromes or symptoms

In case of a classical syndrome or maternally inherited disorder (Table 1) the likelihood of finding a common genetic defect in the mtDNA is considerable. If the syndrome is usually caused by an mtDNA rearrangement, than the complete mtDNA will be screened entirely in a single assay. By applying "long PCR" kits, deletions are readily detectable at a very high sensitivity (Chapter 2) in blood DNA, thereby saving the patient a muscle biopsy. In case of an evident mtDNA deletion syndrome phenotype, and no deletion present in blood, a muscle biopsy has still to be performed to check the initial clinical observation. When a deletion is found by a non-quantitative PCR analysis it should be confirmed by conventional Southern blot analysis to determine level and significance. Alternatively the deletion can be confirmed in other tissues like hair roots (Bendall et al., 1997) or in material from a urine sample (McDonnell et al., 2004). When a single deletion is present, it is important to determine the presence of dimers or duplications as well. In general major single deletions have a 4% chance of being transmitted (Chinnery et al., 2004), only in case of duplications this risk is increased (Rotig et al., 1992). Contradictory results have been presented concerning the prognostic value of dimers and duplications (Jacobs et al., 2004). Another PCR problem is the disappearance of deletions by dilution of the solution in favour of the full-length, undeleted mtDNA molecule (Kajander et al., 1999). Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR may avoid these pitfalls (Agostino et al., 2003). Multiple deletions can be detected by the same PCR assay, but these are usually the result of defects in nuclear genes and the recurrence risks can be either 50% or 25% (Hirano et al., 2004; Kaukonen et al., 1999). In muscle tissue of older patients it can be difficult to discriminate these deletions from deletions in the mtDNA, occurring as part of the ageing process (Cortopassi and Wong, 1999; Wanrooij et al., 2004).

MtDNA depletion syndrome (MDS) (Table 1, 5) is caused by a quantitative abnormality in copy number of the mtDNA (Moraes et al., 1991). It occurs when the amount of mtDNA is less than 30% of controls, which can be determined by conventional Southern blot analysis or quantitative PCR.

MDS is transmitted as an autosomal recessive or dominant trait. The disorder is phenotypically heterogeneous (Nishino et al., 1999; Berger et al., 2003; Tesarova et al., 2004). Three genes, involved in the deoxyribonucleotide metabolism, have been reported in association with MDS. These genes are the *thymidine phosphorylase* gene in patients with a Mitochondrial Neuro-Gastro-Intestinal Encephalomyopathy (MNGIE) (Nishino et al., 1999), the *thymidine kinase 2* gene in early onset (encephalo-)myopathy (Mancuso et al., 2002) and the *deoxyguanidine kinase* gene in hepatoencephalopathy (Salviati et al., 2002). As secondary cause mtDNA depletion is seen in inclusion body myositis (Horvath et al., 1998) or as an iatrogenic cause in patients with acquired immunodeficiency syndrome treated with nucleoside analogs (Arnaudo et al., 1991). There will be more genes that cause MDS. Possible candidates will most likely be involved in mitochondrial dNTP metabolism and mtDNA replication and maintenance, and will overlap the group of genes associated with multiple deletions.

Step 2: Screening of frequent mutations or frequently affected genes in the mtDNA

A number of point mutations have been identified at relatively high frequency. Many pathogenic mutations are heteroplasmic. One of the examples that homoplasmy is associated with pathogeneity is the group of LHON mutations, which can be either homoplasmic or sometimes heteroplasmic. Three primary, usually homoplasmic mutations (Table 1) have been reported for LHON, and a number of secondary mutations with yet unknown significance (Servidei, 2004). The primary mutations are being found in about 94% of the patients with LHON in the Netherlands (Oostra et al., 1994a). As these mutations have a reduced penetrance, it is likely that additional factors are involved and that the mutations are obligate risk factors. More than 80 mutations have been found in the 22 tRNA genes, with 18 in the $tRNA^{Leu(UUR)}$ gene only. The most prevalent mutation in the $tRNA^{Leu(UUR)}$ gene is the A3243G MELAS mutation (Kogelnik et al., 1998; Servidei, 2004). In nearly all cases the MELAS mutation is screened on a routine basis because of its heterogeneous manifestation. In specific phenotypes screening occurs according to the clinical phenotype mentioned in Table 1. For less specific phenotypes, the MELAS, MERRF and the NARP/MILS mutations (protein genes), and the $tRNA^{Leu}$-, $tRNA^{Lys}$- and *ATPase6/8*-genes are screened because of the relatively high incidence of mutations (Table 1, Fig. 1). When these are not found, mutational hotspots, as reported for certain phenotypes, will be tested, like for example the $tRNA^{Leu}$ for MELAS and the *ND5* gene for Leigh-like or MELAS/Leigh

overlap phenotypes (Sudo et al., 2004) or for cardiomyopathy the $tRNA^{Leu(UUR)}$- and $tRNA^{Ile}$-genes (Merante et al., 1996). About half of the described mutations are detected in tRNA genes (Wallace et al., 1993) and occur as described earlier in a number of maternally transmitted disorders, like MELAS (Goto et al., 1990), MERRF (Shoffner et al., 1990) and myopathy-cardiomyopathy (Zeviani et al., 1991). Patients with single tRNA point mutations often show biochemical evidence of respiratory chain dysfunction with decreased activity of complex I and IV (Wallace, 1992). When patients presenting with respiratory chain defects are screened for mitochondrial tRNA mutations and for deletions in about 30% of the patients a pathogenic mutation was found by Jaksch et al. (Jaksch et al., 2001). The detection rate for adults (49%) was higher than in the paediatric age group (18%). We detected in about a 20% of the patients (n=170, children and adults) with a respiratory chain defect a pathogenic mutation by testing only for the deletions, the 3243 (MELAS) and 8344 (MERFF) mutations (unpublished data). The contribution of the protein and ribosomal genes in the group of patients with respiratory chain diseases has not been systematically investigated.

Step 3: Screening of the entire mtDNA

When no mutation is found in the first screening, one could either extend the number of known mutations tested with respect to specific clinical manifestations or one could perform a more general (wide-ranging) screening procedure. As many mutations appear to be private and their number increases rapidly, it becomes difficult to keep a symptom based protocol updated and the workload acceptable, as for every patient an increasing number of different tests has to be set up and carried out. In case the diagnosis of a mtDNA disorder is likely, or when excluding such a defect is important for counselling, one can screen the entire mtDNA. A variety of approaches have been used, like SSCP, sequence analysis, DGGE or D-HPLC analysis. SSCP analysis has a sensitivity of about 80-90%, but sequence analysis detects all mutations that have a heteroplasmy percentage of 30% or more. Both methods detect also (homoplasmic) variants. Methods, which only detect heteroplasmic mutations, like denaturing gradient gel electrophoresis (DGGE) or denaturing high performance liquid chromatography (D-HPLC), have the major advantage that homoplasmic polymorphisms are not being detected. In fact these methods use one of the characteristics of many pathogenic mtDNA mutations and that is their heteroplasmic state. The detection of heteroplasmic mutations is much more sensitive (>1-5% heteroplasmy) with these methods than with sequence analysis (>30% heteroplasmy), allowing also investigations in

case the target tissue is not available. However, homoplasmic pathogenic mutations may be missed. Specifically testing for these mutations can circumvent this or carefully checking the patterns generated for the different genes that contain the pathogenic homoplasmic variants (Fig. 2). A further refinement can be made by mixing the mtDNA from the mother or a control with the proband, but then also all polymorphisms and unclassified variants will be detected.

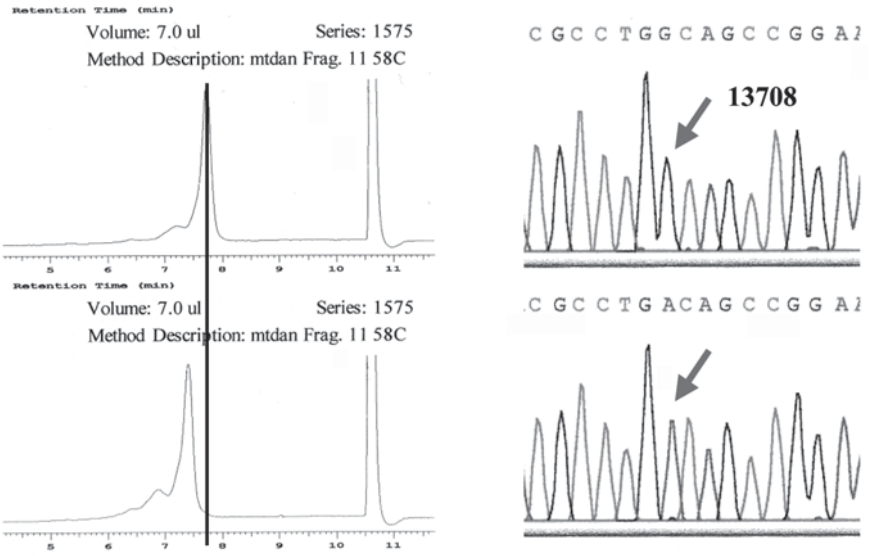

Fig. 2. Homoplasmic variant in mtDNA at position 13708 measured by HPLC. As can be seen from the left panel this homoplasmic variant causes a change in the retention time of the mtDNA fragment. The retention time is shorter in the lower part of the D-HPLC results. A sequence change from a G-to-A at position 13708 (right panel) causes a shortening of the retention time.

In the last couple of years we applied SSCP analysis of the tRNA genes, sequence analysis of the ND genes (de Vries et al., 1994), and D-HPLC analysis and recently the resequencing CHIP for the entire mtDNA (Booth et al., 2003). SSCP analysis was performed to screen 22 mitochondrial tRNA genes of 31 patients. In 19 of the patients at least one PCR fragment showed a mobility shift, which was/were subsequently sequenced. Because of the position of the primers, band shifts were not only found in the tRNA genes, but also in sequences flanking the tRNA genes (data not shown). A total of 11 different tRNA mutations was

found, 7 of which had been published before as non-pathogenic mutations (Byrne et al., 1991; Lauber et al., 1991; Brown et al., 1992; Obayashi et al., 1992; Houshmand et al., 1994) and 4 were not described yet. These latter mutations affected the tRNA genes for valine, alanine, histidine and threonine. A group of six patients carried more than one tRNA mutation and co-occurrence of the T10463C and G15928A was observed in the patients 11, 12, 13 with the MELAS phenotype and patient 6 with a complex I deficiency (Table 2).

Table 2. Mutations detected by SSCP in the mtDNA tRNA genes in the tested patient group.

tRNA	Mutation*	Hetero-plasmy	Evolutionary conservation**	Present in controls	Restriction enzyme	Patient Nr***	References
Val	G1642A	Yes	High	0/100	*Mbo*II	**14**	This study
Ala	A5592G	No	Moderate	0/100	*Bbv*I	**22**	This study
Lys	A8308G	No	Low	N.T.	*Esp*I	**23**	Obayashi et al., 1992
Gly	T10034C	No	High	4/100	*Spe*I	*15*	Marzuki et al., 1991
Arg	T10463C	No	High	4/50	*Xba*I[a]	**13**; **12**; **11**; *6*	Houshmand et al., 1994
His	A12171G	No	High	0/100	*Hin*fI	*6*	This study
Leu^{CUN}	A12308G	No	High	1/30	*Eco*RI[a]	**24**; **25**; *27*; *15*	Lauber et al., 1991; Houshmand et al., 1994
Thr	C15904T	No	Low	1/20	*Mse*I	*30*	Houshmand et al., 1994
Thr	T15916C	No	High	1/47	*Bsr*I	**9**	This study
Thr	A15924G	No	High	11/103	*Bsa*WI	*1*; **25**	Brown et al., 1992
Thr	G15928A	No	Low	5/40	*Mbo*II	**13**; **12**; **11**; *6*	Houshmand et al., 1994

N.T.= not tested

* Numbering of nucleotides are according to the Cambridge human mtDNA sequence (Anderson et al., 1981).

** Evol. cons.: Evolutionary conserved position. Conservation: low=human to bovine, moderate=human to mouse, high=human to octopus (Gadaleta et al., 1989).

*** Patient phenotype. *Italics* =complex I deficiency; *Italics and underscored* =multiple respiratory chain enzyme deficiencies; **Bold** = MELAS.

a Restriction enzyme site created by modified primers (Houshmand et al., 1994).

Direct sequence analysis was performed for the complex I and complex III genes. Eight patients with a complex I defect were tested for mutations in the ND genes, but no mutations were found (Roewer et al., 1996). Patients with an isolated complex III deficiency make up only 5 to 10% of the group of patients with an OXPHOS defect. A group of six patients was biochemically characterized and clinically compared, but there were no clinical findings specific for complex III deficiency (Mourmans et al., 1997). Five out of six of these patients were screened for mutations in the mtDNA cytochrome *b* gene. In three patients no mutation could be detected. In one patient a homoplasmic 15257G → A mutation was found. This mutation was already described and might have an attributive role in

Lebers Hereditary Optic Neuropathy (LHON) (Oostra et al., 1994b). In this case despite the complex III deficiency the base change is probably just a polymorphism. In a patient with Complex III deficiency a 4-bp out of frame deletion was detected. It was surprising to find such a drastic mutation in a very conserved gene (see also Chapter 4) (de Coo et al., 1999b).

D-HPLC analysis was performed for a group of 50 patients (see Chapter 6 for methods). Ten heteroplasmic mutations were identified, 8 of which were evidently pathogenic (3 in tRNA-genes and 5 in protein encoding genes). Also homoplasmic mutations were found, one of which was pathogenic. Not all homoplasmic variants were analysed by sequence analysis. In our experience, pre-screening by D-HPLC analysis followed by sequence analysis in case of a heteroplasmic variant or a homoplasmic variant in a critical fragment is a very sensitive way to identify pathogenic heteroplasmic mtDNA mutations in a rapid and automated way, which can easily be incorporated in a diagnostic service. The protocol is now further optimised, allowing a complete screening of the medina in a 6 hours D-HPLC run. The mitochondrial DNA resequencing CHIP (Affymetrix) is the next phase in screening the entire mtDNA (Fig. 3, Fig. 4). The reported detection level of heteroplasmy is 2-5% (Maitra et al., 2004). The drawback of the system is that only substitutions can be detected and not small deletions and insertions. For the mtDNA this is not a major issue, but for application on other nuclear genes it is. We are cur-

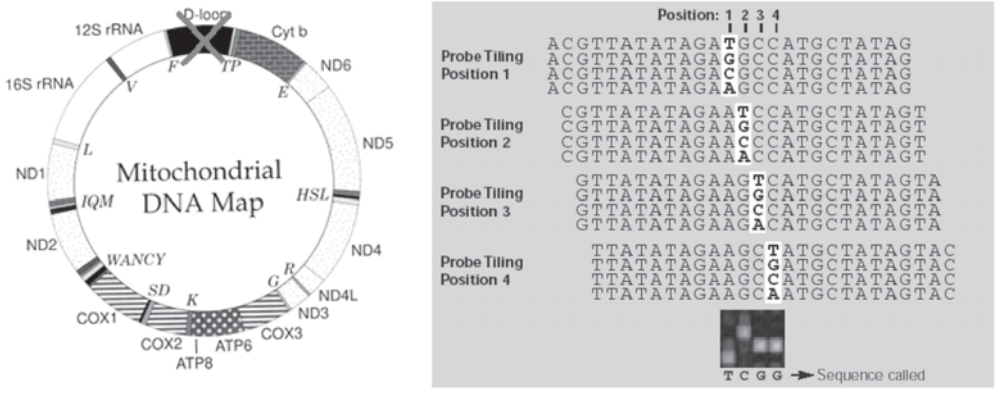

Fig. 3. Resequencing CHIP for the mtDNA. The left-hand panel shows the sequence contained on the CHIP and the right-hand panel the methodological approach to resequence by allele-specific oligonucleotidehybridization.

rently optimising software tools to circumvent this problem. We tested the mtDNA CHIP on 3 patients, which have been sequenced by conventional technology and results were identical. Next we tested 20 patients and identified with the suboptimal software (GDAS, Affymetrix) 345 variants, 171 of which were different. These were 90 SNPs, 5 pathogenic mutations and 71 unclassified variants, 5 of which were heteroplasmic, which have to be confirmed further. We also detected 2 single nucleotide insertions. A total of 5 mutations could not be confirmed by conventional sequence analysis, indicating that a mutation should always be confirmed by an independent technology. Our results indicate that complete analysis of the mtDNA should be a prerequisite, when a strong indication for an OXPHOS disorder exists. It can be expected that much of the future work will shift from identifying mutations to characterizing mutations to establish a possible pathogenic role. The pathogenicity of the new mutations was determined by studying the evolutionary conservation of the altered nucleotide or its position in the tRNA, the amount of heteroplasmy, the population frequency and, the segregation in the family (Lertrit et al., 1994).

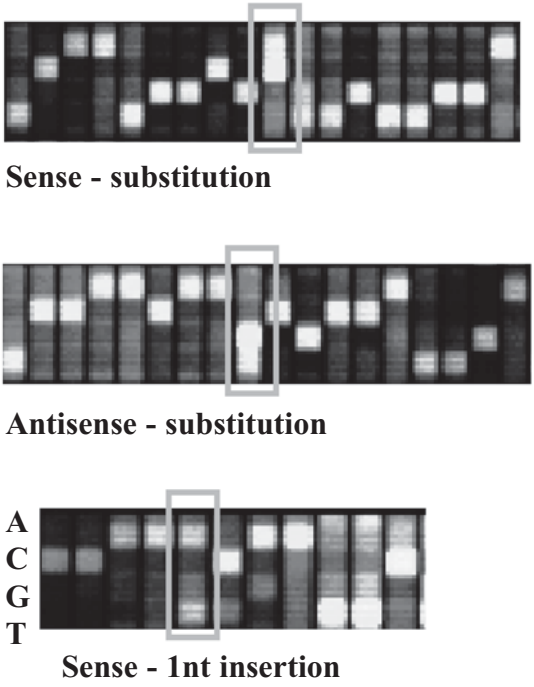

Fig. 4. Examples of the mtDNA resequencing CHIP. The upper and middle panel show a heterozygote for an A to C substitution, the lower panel a 1nt insertion.

MITOCHONDRIAL OXPHOS DISEASE CAUSED BY NUCLEAR GENE DEFECTS

Only a limited number of OXPHOS disorders have a direct clue for the nuclear gene involved. This can be based on a combination of clinical features and immunohistochemical and biochemical information from muscle biopsy material or on genetic information. For instance Leigh disease in combination with COX-negative fibres and a complex IV activity of less than 20% points to a defect in the *SURF1* gene. Mutations in this gene cause a loss of gene product and an assembly defect of complex IV. The assembly pattern can be shown on a Western blot. The level of the *SURF1* mRNA can be zero, reduced or normal (Pequignot et al., 2001). The vast majority of mitochondrial diseases show a heterogeneous clinical phenotype with aspecific signs of mitochondrial cell abnormalities in morphological studies and with moderate or combined deficiencies of OXPHOS complexes not pointing to a specific nuclear gene. Approaches to get more specific information that can contribute in finding the affected gene or the related gene product, involve antibody studies (e.g. this thesis), restrictive inclusion criteria (complex I deficiency) or biochemical tests [blue native gels (Ugalde et al., 2004)]. In case of isolated complex I deficiency one could test the structural subunits on a rational basis related to the specific function or previously identified mutations.

Interesting for gene-specific subclassification are the global approaches on gene expression and proteome, which have been used for classifying of at this point predominantly oncological disorders (Bocchetta and Carbone, 2004; Golub, 2004). A problem in case of mitochondrial disease may be the uniqueness of patients and gene defects precluding the generation of statistically significant groups of patients and data. In case of familial OXPHOS disease in large families or in many small clinically homogeneous families the genetic locus involved can be identified by linkage analysis or for example homozygosity mapping in AR-families with consanguinity. In case of small families complementation studies may be applied first to group patients with a similar genetic defect. The genetic locus can then be identified by linkage analysis, summing up the data of all those families or by complementation with fragmented chromosomal libraries (Tiranti et al., 1998). With the availability of the 50K SNP-CHIP (Affymetrix) it is possible to perform whole genome linkage analysis as part of a diagnostic service in suitable families.

Nuclear OXPHOS gene defects can be divided in the following groups (Zeviani, 2001):

1. Defects in genes encoding structural subunits of the OXPHOS complexes (Table 3);
2. Defects in genes encoding proteins involved in assembly of the OXPHOS complexes (Table 4);
3. Defects in genes decreasing the stability of mtDNA (Table 5);
4. Defects in genes encoding factors involved in metabolic pathways influencing the biogenesis of mitochondria, including OXPHOS (Table 6).

Groups 1, 2 and 3 are characterized by specific genetic defects influencing directly the function of the respiratory chain complexes, whereas group 4 includes proteins that are indirectly associated with the mitochondrial energy pathway.

1. Defects in genes encoding structural subunits of the OXPHOS complexes

In this group, defects of genes encoding subunits of complex I and II only have been reported so far.

Complex I

In case of an isolated complex I deficiency a mutation in the mtDNA as a cause for the disease has to be excluded first. Next, the search for gene defects in patients with isolated complex I deficiency was initially concentrated on the flavoprotein genes for functional reasons and based on data from immunodetection.

The 24 kDa subunit (NDUFV2)

Western blotting experiments by Morgan-Hughes and Schapira in 1988 (Morgan-Hughes et al., 1988; Schapira et al., 1988) showed that the 24 kDa band was lacking in two of their patients, suggesting a defect in the 24 kDa gene. At least one additional patient was detected with a diminished protein staining at the 24 kDa protein position with Schägger's blue native gel electrophoresis technique (Schägger, 1995). However, sequencing of the 24 kDa gene in these patients and RNA studies in one of them did not reveal any abnormalities (unpublished data; see also Chapter 11). The possible direct or indirect role of the 24 kDa subunit abnormalities in mitochondrial myopathies in two patients with a clinical phenotype of fatal infantile lactic acidosis (Procaccio et al., 1999). Immunoblotting for 6 nuclear DNA-encoded (20, 23, 24, 30, 49 and 51 kDa) and 1 mitochondrial DNA-encoded (ND1) complex I subunit, showed in one patient a reduced amount of the 24 kDa and the 51 kDa protein. In 2003 Bénit et al. (2003a) showed a 4bp deletion mutation in intron 2 to be associated with complex

I deficiency in a patient with early onset hypertrophic cardiomyopathy and encephalopathy. This mutation had been detected because of the combination of the clinical phenotype with lactic acidosis, the complex I deficiency and the decreased amounts of NDUFV2 protein on Western blot analysis that pointed to a defect in the *NDUFV2* gene.

10 kDa subunit (NDUFV3)

The 10 kDa subunit has been studied because of its possible role in electron transport as one of the three flavoproteins of complex I. There is not much known about the function of the protein. It is thought that it could play a supportive role for the 24 kDa subunit in the electron transfer (Skehel et al., 1991). For this process cysteine-residues are necessary. In contrast to the bovine and rat protein sequence, the human sequence of the 10kDa subunit contains a cysteine amino acid. One could hypothesize that in humans it could support another subunit in liganding an Fe-S cluster. But the human protein sequence does not show enough cysteines and is not positioned in a way that it can ligand an Fe-S cluster. It is very likely that the 2Fe-2S cluster is not located in the 10 kDa subunit but in the cysteine containing 24 kDa subunit. The 10 kDa gene was assigned by us to 21q22.3 and has been further refined to approximately 120 kb telomeric to *PDE9A* (OMIM 602973) (Berry et al., 2000; Michaud et al., 2000). No mutations in this gene have been identified by us or reported to date.

51 kDa subunit (NDUFVI)

The 51 kDa-subunit is the best characterized of the flavoprotein subunits. In Neurospora *crassa* the importance of the interaction of the 24 kDa-subunit with the 51 kDa subunit was shown for respiration (Almeida et al., 1999). The 51 kDa subunit is part of the electron flow in complex I according to the following route. Electrons are donated by NADH to the 51 kDa and the 24 kDa subunits [flavoprotein fraction (FP) of complex I] and subsequently transferred to the 75 kDa subunit [iron sulphur protein (IP) fraction of complex I] followed by transfer via the 23 and 20 kDa subunits [hydrophobic protein (HP) fraction of complex I] towards ubiquinone (Belogrudov and Hatefi, 1996). Characterization of the 51 kDa subunit (chapter 10) has been further refined by Schuelke (Schuelke et al., 1998). We did not detect any abnormalities in our group of 20 complex I deficient patients (see chapter 11), even though in one of these patients a decreased amount of the 24- and 51-kDa subunits was observed with normal amounts of the 20-, 23-, 30- and the 49-kDa subunits. Schuelke (Schuelke et al., 1999b) detected a homozygous mutation in one patient with a macrocephaly and a progressive leucodystrophy and a compound het-

erozygous mutation in two brothers with a progressive unspecified encephalomyopathy. Bénit et al. screened a group of 36 patients with isolated complex I deficiency for the six most conserved complex I genes *(NDUFV1*, *NDUFS1*, *NDUFS7*, *NDUFS8, NDUFA8* and *NDUFB6)* with denaturing high performance liquid chromatography (D-HPLC) and resequencing. They found three patients with a *NDUFS1* mutation and in three other unrelated patients six new mutations in *NDUFV1* (Bénit et al., 2001). The phenotypes were described as Leigh syndrome or ataxia and psychomotor retardation with hypotonia.

Several mutations in nuclear complex I genes in patients with a mitochondrial encephalomyopathy have been detected since the first mutation was found in 1998 (see introduction and discussion of Chapter 11 and Table 3). In only five patients out of 85 with an isolated complex I deficiency a mutation has been found in one of the complex I flavoprotein genes (Bénit et al., 2001; de Coo et al., 2004; Loeffen et al., 2000), indicating that either mutations are not tolerated in these important subunits or, more likely, that genetic heterogeneity is the rule and that no common complex I gene is involved. One should further keep in mind that yet only a small, highly selected minority of all patients with a complex I deficiency can be explained by a genetic defect. An additional confounding factor is that we do not know in how many cases the complex I deficiency is secondary to defects in other genes like assembly genes, or to conditions that give rise to decreased activity of OXPHOS complexes (Scholte, 1988). As shown, defects in structural subunits are not reliably predicted by a biochemical defect or absence of the specific subunit. This may be due to the way the different complexes become assembled and are dependent on each other. For example, our patient with a cytochrome *b* defect (complex III deficiency) showed biochemically not only a complex III, but also a complex I deficiency. This has been found in more patients with complex III defects. In contrast a defect of one of the complex I subunits only rarely appears to affect the assembly of complex III (Acin-Perez et al., 2004; Schägger, 2002; Schägger et al., 2004).

Complex II

The first nuclear OXPHOS mutation was found in a complex II gene by Bourgeron et al. in 1995. Other mutations in the complex II genes are listed in Table 3. Striking is the observation that defects in SDHB, SDHC and SDHD genes can lead to tumour formation.

Table 3. Mutations in genes coding for OXPHOS subunits.

Gene	Biochemical phenotype	Phenotype	References
NDUFS1	Complex I OXPHOS	Leigh syndrome	Bénit et al., 2001
NDUFS2	Complex I OXPHOS	Cardiomyopathy/ encephalopathy	Loeffen et al., 2001
NDUFS4	Complex I OXPHOS	Leigh syndrome	van den Heuvel et al., 1998a; Budde et al., 2000; Petruzzella et al., 2001
NDUFS6	Complex I OXPHOS	Neonatal lethal encephalopathy	Kirby et al., 2004
NDUFS7	Complex I OXPHOS	Leigh syndrome	Triepels et al., 1999
NDUFS8	Complex I OXPHOS	Leigh syndrome	Loeffen et al., 1998
NDUFV1	Complex I OXPHOS	Leucodystrophy and myoclonic epilepsy	Schuelke et al., 1999a; Bénit et al., 2001
NDUFV2	Complex I OXPHOS	Cardiomyopathy/ encephalopathy	Bénit et al., 2003a
SDHA (FP)	Complex II OXPHOS	Leigh syndrome	Bourgeron et al., 1995; Parfait et al., 2000
SDHA (FP)	Complex II OXPHOS	Late onset optic atrophy (AD)	Birch-Machin et al., 2000
SDHB	Complex II OXPHOS	Phaeochromocytoma and cervical paraganglioma	Astuti et al., 2001
PGL3 (SDHC) (cyt_{bL})	Complex II OXPHOS	Paraganglioma	Niemann and Muller, 2000
PGL1 (SDHD) (cyt_{bS})	Complex II OXPHOS	Paraganglioma	Baysal et al., 2000

FP=flavoprotein; cyt_{bL}= large cytochrome; cyt_{bS}= small cytochrome; PGL= hereditary paraganglioma.

2. Defects in genes encoding proteins involved in assembly of the OXPHOS complexes

Defects of genes encoding assembly factors mainly prohibit assembly of cytochrome *c* oxidase (COX, complex IV), which is a well-described process (Table 4). Only two mutations have been detected in genes involved in complex III and complex V assembly. Other assembly genes

Table 4. Mutations involved in the production and/or assembly of the OXPHOS complexes.

Gene	Biochemical defect	Clinical phenotype	References
BCS1L	Complex III assembly	Tubulopathy, hepatopathy, encephalopathy	de Lonlay et al., 2001
SURF-1	Complex IV assembly	Leigh syndrome	Tiranti et al., 1998; Zhu et al., 1998
SCO1	Complex IV assembly Copper transport	Neonatal hepatopathy and encephalopathy	Valnot et al., 2000a
SCO2	Complex IV assembly Copper transport	Neonatal cardioencephalomyopathy	Papadopoulou et al., 1999
COX 10	Complex IV assembly Haem modification	Tubulopathy and leucodystrophy	Valnot et al., 2000b
COX 15	Complex IV assembly	Cardiomyopathy	Antonicka et al., 2003
LRPPRC	Complex IV assembly?	Leigh syndrome	Mootha et al., 2003
ATP12	Complex V assembly	Lactic acidosis, methyl glutaconic aciduria and dysmorphic features	de Meirleir et al., 2004

are being searched for by using homology between yeast and human. Steinmetz (Steinmetz et al., 2002; Prokisch et al., 2004) created a database with deleted Saccharomyces *cereviciae* mutants selected for their impaired mitochondrial respiration. From the yeast genes human orthologues could be identified. Although the analysis of Saccharomyces *cereviciae* has been useful for complex III and IV, it is not possible to study complex I, as this yeast type lacks complex I. An alternative yeast model with complex I is Yarrowia *lipolytica* (Abdrakhmanova et al., 2004). Rhodobacter *capsulata,* E. *coli* and Neurospora *crassa* are also suitable models as complex I in these organisms have a similar structure to those in mammals (Schulte and Weiss, 1995; Dupuis et al., 1998; Rasmusson et al., 1998; Yagi et al., 1998; Almeida et al., 1999). Two possible assembly genes for complex I, the *CIA30* and *CIA84* (Kuffner et al., 1998; Schulte, 2001), have been derived in this way from Neurospora *crassa* studies. The human *CIA30* gene has been tested unsuccessfully in 13 complex I patients for mutations (Janssen et al., 2002).

3. Defects in genes altering the stability of mtDNA

A number of defects have been identified in genes involved in replication of the mtDNA, either as one of the components of the replication machinery or of the nucleoside metabolism. The gene defects may lead to fragmentation of the mtDNA (multiple deletions), a reduced amount of mtDNA (mtDNA depletion) or both (Table 5). Segregation can be autosomal dominant or recessive. The POLG gene is intriguing in this respect, as mutations have been associated with AD/AR-CPEO, SCA, ataxia, congen-

Table 5. Some examples of mutations of genes related to the mtDNA maintenance.

Defective protein (Gene)	Metabolic defect	Clinical phenotype	References
Thymidine phosphorylase (*TP*)	Nucleoside metabolism	Neurogastrointestinal encephalomyoneuropathy (AR)	Nishino et al., 1999
Twinkle (*C10ORF2*)	Helicase	Progressive external ophthalmoplegia (AD)	Spelbrink et al., 2001
Deoxyguanosine kinase (*DGUOK*)	Nucleoside metabolism	Hepatoencephalopathy	Mandel et al., 2001
Thymidine kinase 2 (*TK2*)	Nucleoside metabolism	Myopathy, mtDNA depletion syndrome	Saada et al., 2001
DNC (*SLC25A19*)	Deoxynucleotide carrier	Cong. Microcephaly (Amish type), α-ketoglutaric aciduria	Rosenberg et al., 2002
mtDNA polymerase γ (*POLG1*)	Replication	Male infertility, (AD- and AR-) PEO	Rovio et al., 2001; van Goethem et al., 2001
Elongation factor G1 (*EFG1*)	Translation	Hepatoencephalopathy	Coenen et al., 2004
Adenosine nucleotide translocator 1 (*ANT1*)	OXPHOS	ADPEO	Kaukonen et al., 2000

ital floppiness, hepatic failure and fertility problems (Mancuso et al., 2004; van Goethem et al., 2004; Taanman and Schapira, 2005). A mouse model carrying a proof-reading-deficient version of PolgA, the nucleus-encoded catalytic subunit of mtDNA polymerase, displays a phenotype of premature ageing and age-related diseases and an accumulation of mutations in the mtDNA (Trifunovic et al., 2004). A new class of gene defects is associated with the mitochondrial translation apparatus and mutations are found in neonates with progressive hepatoencephalopathy (Coenen et al., 2004).

4. Defects in genes encoding factors involved in metabolic pathways influencing the biogenesis of mitochondria, including OXPHOS

The list of genes with indirect effect on OXPHOS function is large and controversial. On this list are gene/protein defects such as paraplegin, frataxin, dynamin, *Tim9/10* and *ABC7* (Table 6). Their involvement is in a great variety of conditions, including normal aging, optic atrophy, (late-onset) neurodegenerative disorders and cancer.

Table 6. Some examples of mutations of mitochondrially expressed genes related to OXPHOS.

Gene	Biochemical defect	Clinical phenotype	References
SPG7	Zinc-metalloprotease (paraplegin)	Spastic paraplegia (AR)	Casari et al., 1998
FRDA	Iron metabolism (frataxin)	Ataxia (Friedreich's) (AR)	Rotig et al., 1997
Tim8 (DDP1)	Import	Deafness and dystonia (X-linked)	Koehler et al., 1999
ABC7	Iron transport	Ataxia and anemia (X-linked recessive)	Allikmets et al., 1999
OPA1	Dynamin related	Optic atrophy (AD)	Delettre et al., 2001
TAZ (tafazzin)	Cardiolipin metabolism	Dilated cardiomyopathy, neutropenia, and increased 3-methylglutaconic aciduria (X-linked)	Bione et al., 1996
MFN2 (mitofusin2)	Mitochondrial fusion	HMSN type 2a Axonal polyneuropathy	Zuchner et al., 2004

CONCLUDING REMARKS

For many patients it is hard to believe that in spite of all the progress that has been made in the group of mitochondrial encephalomyopathy (ME) disorders, a diagnosis with information about disease course and genetic consequences is too often not possible. Using our strategy (Fig. 1), ME-patients will know if they have a disease causing mtDNA mutation, or not.

If so, this means that these patients can get information about the disease course (however depending on the mutation this is not always straightforward) and therapeutic possibilities (sadly not many treatment strategies are available), and can receive genetic advice about risks for their relatives and transmission of the disorder. Defects in nuclear genes encoding the structural subunits are in most cases even more difficult to identify. From all the ME patients in between 23 to 33 percent have an isolated complex I deficiency (von Kleist-Retzow et al., 1998; Loeffen et al., 2000). Loeffen et al. detected mutations in 7 patients from a selected group of 20 complex I deficient patients (no exact data in the paper, referred to as an extensive screen) and Bénit et al. did so in 6 patients out of a group of 36 patients, by screening the 6 most conserved complex I genes (Bénit et al., 2001). We did not detect mutations in a group of 20 complex I patients by screening the *NDUFV1*, *NDUFV2* and the *NDUFV3* genes (see Chapter 11). These studies indicate that only in a small part of the well-selected patients we are able to detect a mutation. Therefore, we strive for better gene-predicting classification of the ME-disease group e.g. by Western blotting procedures in combination with monoclonal antibodies against the different subunits of complex I (proteomics) and gene expression profiling (genomics) and on the other hand we learn more about mitochondrial human genes (in particular the unknown) by investigations of the genes involved in complex I in other species [as has happened for example with assembly genes (Schulte, 2001)].

Initiatives are also taken to prevent the transmission and to improve the treatment of OXPHOS diseases. In 1999 an international workshop of the European Neuromuscular Society (ENMC) defined guidelines for counselling mitochondrial DNA associated diseases (Poulton and Turnbull, 2000). For large *de novo* deletions recurrence risks are low [1/25 (Chinnery et al., 2004)], but for point mutations this is not the case and recurrence risks are highly unpredictable due to the genetic bottleneck. Prenatal diagnosis is only reliable if the mtDNA mutations meet 3 criteria with respect to reliable genotype-phenotype relation, equal distribution of mutation load among all tissues and no change of mutation load in time. Frequent mutations like the A3243G mutation do not fulfil these requirements and only for the A8993G/C or A8344G mutation sufficient data are available to allow reliable analysis of fetal tissue. When the chances of affected children are high, PGD is a good alternative as the unaffected embryos can be selected, saving the couple multiple choices for termination of pregnancy (see Chapter 7). In the Netherlands this can only be carried out in the department of Clinical Genetics at the Academic Hospital Maastricht. New technologies, like oocyte selection and ooplasmic trans-

fer, arise and although in the Netherlands they are ethically accepted (Pennings and de Wert, 2003), it is not shown that these are completely safe, even if they have already been tested on patients (Barritt et al., 2001). Animal studies are needed to get more information about the potential and risk of these technologies. Therapy was not a topic in this thesis. However all chapters serve the final goal: How to improve the management of the patients with OXPHOS disease. This includes taking a medical history, making a diagnosis, performing investigations and providing prognosis, therapy, counselling and general care. Another initiative in 2003 by another international workshop supported by the ENMC tried to address the problems and to find possibilities for therapy in mitochondrial encephalomyopathies (Chinnery and Bindoff, 2003).

The impact of mitochondria and OXPHOS deficiencies to many aspects of human life and disease will continuously increase in the years to come. Especially as the borders between genetic and complex diseases will largely disappear, it can be expected that a fundamental process as energy metabolism, which is known to decline with age, will be a key factor. This can be illustrated by the role of OXHPOS defects in ageing, neurodegenerative diseases, cancer, cardiac disease, fertility and type 2 diabetes, but also as a side effect of antiviral therapies or antibiotics. The results obtained on the group of patients with a primary OXPHOS defect will set the stage for these common disorders as well. Mitochondria are intriguing organelles with great adaptive, symbiotic and disease causing capabilities. Much to diagnose, to learn, to study and to amaze has been left.

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Summary

This thesis is a contribution to the fast growing field devoted to the improvement of the diagnostics in patients with mitochondrial encephalomyopathies at the DNA level and is inspired amongst others by the hypothesis of intergenomic crosstalk between the nuclear genome (e.g. the 24 kDa subunit of complex I) and the mitochondrial genome. It presents the results of clinical, biochemical and molecular genetic studies that have been performed at the Department of Human Genetics, University Hospital Nijmegen, Nijmegen; at the Division of Genetics, University of Maastricht, Maastricht; at the Department of Neurology, Erasmus MC - University Medical Center Rotterdam, The Netherlands and at the Institute of Neurology, The National Hospital, Queen Square, London, UK *(Introduction, Chapter 1)*.

In this thesis, different strategies are described to discriminate the cause of a mitochondrial encephalomyopathy to be located either in the mitochondrial or in the nuclear genome *(Chapter 2-7)*.

First a PCR-based test is described to detect the whole spectrum of large deletions as can be found in Progressive External Ophthalmoplegia and in the Kearns-Sayre syndrome. The advantage of this PCR-based test compared to Southern blot analysis is the sensitivity of the method for detecting deletions. In many cases patient's leukocyte DNA is sufficient for making a diagnosis saving the patient a muscle biopsy procedure *(Chapter 2)*.

A single stranded conformation analysis (SSCP) screening method for mitochondrial tRNA mutations revealed in a patient with a mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS-phenotype), a mutation in the $tRNA^{Val}$ gene (G1642A) being the second report of this mutation. Our observation was done independently and our patient's phenotype is the same as in the former report confirming its likely pathogenicity. The phenotype of this patient differed from other MELAS patients because of the involvement of small cerebral arteries in the disease process. This involvement has not been reported before *(Chapter 3)*.

Next the analysis of five patients, with a biochemical phenotype of a complex III deficiency, for mutations in the only mitochondrially encoded subunit of complex III, the *cytochrome b* gene is described. In one patient a four base pair deletion-mutation at position 14787 and a homoplasmic

polymorphism are found. The mutation is heteroplasmic and present in 95% in muscle. In the clinically unaffected mother no mutation is detected. This frame shift mutation is predicted to cause a severe disruption of the synthesis of the cytochrome *b* protein. The phenotype of the patient, a Parkinsonism-MELAS overlap syndrome, has not been described before, neither in association with a complex III deficiency nor with a mutation in the *cytochrome b* gene. In two out of the four other patients the mutation analysis revealed two different homoplasmic polymorphisms *(Chapter 4)*.

In a patient with symptoms as seen in patients with a Leigh syndrome is a *de novo* arisen T8993C mitochondrial mutation reported. Hypotheses are formulated to explain this *de novo* event and the rise in mutant rate from 0% in the mother's muscle mtDNA to 79% in the patient. Nuclear encoded, modifier genes are very likely necessary to understand the high percentage of heteroplasmy for this mutation *(Chapter 5)*.

Hereafter is the use of denaturing high performance liquid chromatography (DHPLC) technology demonstrated as an answer to the increasing number of different tests to exclude all the possible mtDNA mutations. The DHPLC method is a fast, reliable and sensitive method to detect heteroduplexes that result from heteroplasmic strands. Therefore this method is particularly suited for mtDNA screening, because most mutations in the mtDNA are heteroplasmic. A mtDNA-DHPLC protocol was developed that enables a complete mtDNA mutation analysis within one day. Levels of heteroplasmy as low as 0.5% for the A8344G mutation can be detected. The first six mitochondrial encephalomyopathy patients screened with this method showed a mutation in three out of six patients tested. Exclusion of mtDNA involvement supports a subsequent investigation of nuclear genes and has important implications for counselling *(Chapter 6)*.

There are only a few therapeutic possibilities for patients with mitochondrial encephalomyopathies. In case of a mtDNA mutation as the causative factor for disease also the possibilities for prenatal diagnosis are limited because of the complicated way the mutation is transmitted. For the first time a prenatal diagnosis and transmission findings are reported in a family with Leigh syndrome associated with the T9176C (*ATPase6* gene) mutation *(Chapter 7)*.

In the second part of the thesis the characterization and mutation screening of the three flavoprotein fraction genes, the *NDUFV1, NDUFV2, NDUFV3* genes, of the complex I are described. Using chaotropic agents

complex I can be divided in three fractions. One of the fractions, the flavoprotein fraction, consists of three subunits of 51, 24 and 10 kDa and is functionally important for complex I and the oxidative phosphorylation (*Chapter 8-11)*.

First the cloning and mapping of the gene coding for the smallest of the three subunits the *NDUFV3* or 10 kDa-gene is described. The human cDNA sequence was elucidated by screening a human renal cDNA library with the known bovine 10-kDa cDNA. The 5' end of the cDNA was obtained with the rapid amplification of cDNA ends (RACE) procedure. Northern blot procedures showed the gene to be ubiquitously expressed. The gene contains three exons and spans about 20 kb. A Southern blot panel with human/hamster somatic cell hybrids showed that the gene is localized on chromosome 21. A 10 kDa gene containing cosmid was derived from a chromosome 21-specific cosmid library and used for a fluorescence *in situ* hybridization (Fish) procedure to refine the chromosome 21 location to 21q22.3 *(Chapter 8)*.

Next the cloning and characterization of the Fe-S cluster containing 24 kDa subunit gene is reported. The homologous bovine 24 kDa cDNA was used to screen a human cosmid library. The search was complicated by the presence of a pseudogene. The 24kDa cDNA cosmids were mapped by screening a Southern blot panel of human/hamster somatic cell hybrids to two different genes. One large fragment mapped to chromosome 19 and three smaller fragments to chromosome 18. Further refinement of the mapping was done with somatic cell hybrids containing either chromosome 18 or 19 fragments. With this procedure the locus could be assigned to 18p11.2-pter and to chromosome 19q13.3-qter. In a Fish procedure the loci were further refined to 18p11.2-11.31 and 19qter. The two genes were sequenced and revealed that the chromosome 19 locus represented a pseudogene and that the chromosome 18 locus represented the active 24 kDa gene. Northern blot analysis showed an ubiquitous gene expression *(Chapter 9)*.

Then the structure of the *NDUFV1* gene, encoding the 51 kDa flavoprotein subunit of complex I, is described. The structure of the gene was clarified by using the known bovine 51 kDa cDNA sequence. With primers derived from the cDNA, PCR fragments from genomic DNA were generated. The gene appeared to contain 10 exons coding for 464 amino acids and spanned about 5 kb of the human genome. Northern blot analysis showed ubiquitous gene expression with the highest expression in pan-

creas. For testis mRNA a unique mRNA length fragment was present *(Chapter 10)*.

Following the characterisation of the three flavoprotein subunit genes *NDUFV1*, *NDUFV2* and *NDUFV3* the mutation analysis is described. For this comprehensive mutation analysis twenty patients with a mitochondrial encephalomyopathy and an isolated complex I deficiency were selected. No mutations in this group of patients were detected. Three polymorphisms were found in the *NDUFV2* gene. This study supports the idea that the flavoprotein fraction of complex I is not a hotspot for mutations *(Chapter 11)*.

Finally the new findings presented in this thesis are put in perspective and directions for future research are discussed *(Chapter 12)*.

Samenvatting

Dit proefschrift is een bijdrage aan het snel groeiende kennisgebied gewijd aan de verbetering van de diagnostiek op DNA-niveau bij patiënten met mitochondriële encephalomyopathieën en is onder andere geïnspireerd door de hypothese van de communicatie over en weer tussen het kerngenoom (bijv. de 24 kDA subunit van complex I) en het mitochondriële genoom. Het presenteert de resultaten van klinische, biochemische en moleculair-genetische studies die uitgevoerd zijn op de Afdeling Humane Genetica, St.-Radboud Ziekenhuis Nijmegen; op de afdeling Genetica, Universiteit Maastricht; op de afdeling Neurologie, Erasmus MC - Universitair Medisch Centrum Rotterdam, en in het Institute of Neurology, The National Hospital, Queen Square, Londen, Engeland *(Introductie, hoofdstuk 1)*.

In dit proefschrift worden verschillende strategieën beschreven om de oorzaak van een mitochondriële encephalomyopathie of in het mitochondriële of in het kerngenoom te localiseren *(hoofdstukken 2-7)*.

Eerst wordt een PCR-test beschreven om het spectrum van grote deleties van het mtDNA, zoals gevonden kan worden bij patiënten met een progressieve externe ophthalmoplegie of het Kearns-Sayre syndroom, aan te tonen. Het voordeel van deze, op een PCR gebaseerde test in vergelijking met de 'Southern blotting'-techniek is de gevoeligheid van deze methode om deleties te detecteren. In veel gevallen is het DNA dat uit bloedleukocyten of uit haarwortels van de patiënt is verkregen, voldoende voor het stellen van de diagnose. Hierdoor kan de patiënt een spierbiopt worden bespaard *(hoofdstuk 2)*.

Met een ontwikkelde 'single stranded conformation polymorfism' (SSCP) analysemethode voor mitochondriële tRNA-mutaties is bij een patiënt met het klinische beeld van een mitochondriële encephalopathie met lactaatacidose en herseninfarctachtige episoden (MELAS-fenotype), een mutatie in het $tRNA^{Val}$ gen (G1642A) gevonden. Deze mutatie is eenmaal eerder gerapporteerd. Onze observatie is onafhankelijk van de eerdere rapportage gedaan. Het fenotype van onze patiënt is gelijk aan die van de eerdere, hetgeen de pathogeniciteit van deze mutatie waarschijnlijk maakt. Het fenotype van onze patiënt verschilt van andere MELAS-patiënten door de afsluiting van kleine cerebrale corticale arteriën. Betrokkenheid hiervan is niet eerder gerapporteerd *(hoofdstuk 3)*.

Vervolgens wordt de analyse op mutaties in het enige mitochondrieel gecodeerde complex III-gen, het *Cytochroom b*-gen, bij vijf geïdentificeerde patiënten met een biochemisch fenotype van een complex III-deficiëntie weergegeven. Bij een patiënt wordt een vier base paar deletiemutatie gevonden op positie 14787 en een homoplasmisch polymorfisme. De mutatie is heteroplasmisch en is aanwezig in 95% in spier. Bij de klinisch gezonde moeder is geen mutatie vastgesteld. Deze 'frameshift'-mutatie zal zeer waarschijnlijk zorgen voor een verstoring van de synthese van het cytochroom *b*-eiwit. Het fenotype van de patiënt is een overlappend beeld van Parkinsonisme en MELAS, wat niet eerder is beschreven noch geassocieerd met een complex III-deficiëntie, noch met een mutatie in het *Cytochroom b*-gen. Bij twee van de vier andere patiënten heeft de mutatieanalyse twee verschillende homoplasmische polymorfismen opgeleverd. Bij deze vier patiënten is geen mutatie gevonden. *(hoofdstuk 4)*.

Bij een patiënt met symptomen zoals gezien bij patiënten met een Leigh syndroom, wordt een *de novo* ontstane T8993C mitochondriële mutatie gerapporteerd. Hypothesen worden geformuleerd om deze *de novo*-gebeurtenis te verklaren en de stijging in mutatiepercentage van 0% bij het DNA in spier van de moeder naar 79% in spier van de patiënt. Kern gecodeerde, modificerende genen zijn waarschijnlijk nodig om het hoge percentage heteroplasmie van deze mutatie te begrijpen *(hoofdstuk 5)*.

Hierna wordt het gebruik van de 'denaturing high performance liquid chromatography' (DHPLC) technologie getoond als een antwoord op het toenemende aantal verschillende testen om alle mogelijke mtDNA-mutaties uit te sluiten. De DHPLC-methode is een snelle, betrouwbare en gevoelige methode om heteroduplexen te detecteren die resulteren van heteroplasmische DNA-strengen. Deze methode is bij uitstek geschikt voor mutatiedetectie van mtDNA, daar de meeste mutaties in dit DNA heteroplasmisch zijn. Een mtDNA-DHPLC protocol dat het mogelijk maakt om binnen een dag een complete mtDNA-mutatieanalyse te doen, is ontwikkeld. Een minimaal heteroplasmieniveau van 0,5% voor de A8344G-mutatie is gedetecteerd. Bij drie van de eerste zes mitochondriële encephalomyopathiepatiënten die gescreend werden met deze methode, is een mutatie aangetoond. Het uitsluiten van betrokkenheid van mtDNA-mutaties ondersteunt het doen van een vervolgonderzoek naar kerngenen en heeft belangrijke implicaties voor genetisch advies *(hoofdstuk 6)*.

Er zijn slechts enkele therapeutische mogelijkheden voor patiënten met mitochondriële encephalomyopathieën. In geval van een mtDNA-mutatie

als de oorzakelijke factor voor ziekte zijn de mogelijkheden voor prenatale diagnose beperkt vanwege de gecompliceerde manier waarop de mutatie wordt overgedragen. Voor de eerste keer wordt een prenatale diagnose en bevinding van een mutatieoverdracht gerapporteerd bij een familie met de T9176C (*ATPase6*-gen)-mutatie *(hoofdstuk 7)*.

Vervolgens wordt de karakterisering en mutatiescreening van de drie flavoproteïnen fractiegenen, de *NDUFV1, NDUFV2, NDUFV3* genen, van complex I gegeven. Met behulp van chaotrope agentia kan complex I in drie fracties worden verdeeld. Een van de fracties, de flavoproteïnenfractie, bestaat uit drie subunits van 51, 24 en 10 kDa en is functioneel belangrijk voor complex I *(hoofdstuk 8-11)*.

Eerst is de klonering en genomische localisering van de kleinste van de drie subunits, de *NDUFV3* of 10 kDa gen, beschreven. De menselijke cDNA-sequentie is opgehelderd met behulp van een menselijke nier cDNA-bibliotheek met runder 10kDa cDNA met bekende sequentie. Het 5' einde van het cDNA is verkregen met de snelle vermenigvuldigingsprocedure van cDNA-einden (RACE). Northern blot toonde aan dat het gen algemeen tot expressie komt. Het gen bevat drie exonen en bestrijkt ongeveer 20kb. Een Southern blot-procedure met een set humane/hamster somatische celhybriden toont dat het gen is gelocaliseerd op chromosoom 21. Een 10-kDa gen bevattende cosmide is verkregen van een chromosoom 21-specifieke cosmidenbibliotheek en gebruikt voor een fluorescentie *in situ* hybridisatie (Fish) procedure ter verfijning van de chromosoom 21-locatie naar een gebied beperkt tot 21q22.3 *(hoofdstuk 8)*.

Hierna wordt de klonering en karakterisering van het Fe-S cluster bevattende 24 kDa subunit gen beschreven. Het homologe runder 24 kDa cDNA is gebruikt om een humane cosmidenbibliotheek te screenen. De zoektocht is gecompliceerd geweest door de aanwezigheid van een pseudogen. De 24 kDa cDNA cosmiden zijn in kaart gebracht door het analyseren van de gebieden van hybridisatie met een Southern blot-panel met humane/hamster somatische celhybriden. Deze analyse verwijst naar twee genen. Een groot fragment is gelocaliseerd op chromosoom 19 en drie kleinere fragmenten op chromosoom 18. Verdere verfijning van de localisatie is verricht met chromosoomspecifieke somatische celhybriden voor chromosoom 18- en 19-fragmenten. Met deze procedure is het locus toegewezen aan 18p11.2-pter en aan chromosoom 19q13.3-qter. In een Fish-procedure zijn de loci verder verfijnd naar 18p11.2-11.31 en 19qter. De twee genen zijn gesequenced en onthullen dat de chromosoom 19 locus een

pseudogen representeert en de chromosoom 18 locus het actieve 24 kDa gen. De Northern blot toont een algemeen voorkomende genexpressie *(hoofdstuk 9)*.

Verder wordt de structuur van het *NDUFV1*-gen, coderend voor de 51 kDa flavoproteïne subunit van complex I, beschreven. De structuur van het gen is opgehelderd met behulp van de bekende runder 51kDa cDNA-sequentie. Met primers afgeleid van het cDNA, zijn PCR-fragmenten van genomisch DNA gegenereerd. Het gen blijkt 10 exonen te bevatten die coderen voor 464 aminozuren en strekt zich uit over 5kb. De Northern blot-analyse toont algemene expressie die het hoogste is in de pancreas. Voor testis mRNA is een uniek mRNA lengtefragment aanwezig *(hoofdstuk 10)*.

Vervolgens wordt de mutatieanalyse voor de drie flavoproteïnen subunits, namelijk de genen *NDUFV1*, *NDUFV2* en *NDUFV3*, beschreven. Voor deze mutatieanalyse zijn 20 patiënten met een mitochondriële encephalomyopathie en een geïsoleerde complex I-deficiëntie geselecteerd. Er zijn geen mutaties in deze groep patiënten gevonden. Drie polymorfismen zijn gevonden in het *NDUFV2*-gen. Deze studie ondersteunt het idee dat de flavoproteïnenfractie van complex I geen 'hotspot' is voor mutaties *(hoofdstuk 11)*.

Tot slot worden de nieuwe bevindingen, gepresenteerd in dit proefschrift, in perspectief gezet en aanwijzingen voor toekomstig onderzoek bediscussieerd *(hoofdstuk 12)*.

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Tijdens mijn middelbare school tijd werd mede door de docenten klassieke talen met hun verhalen over de filosofie van de Grieken mijn belangstelling gewekt voor de geneeskunde in het algemeen en de neurologie in het bijzonder. Tijdens de geneeskundestudie in Nijmegen bleef de neurologie mij boeien en wist onder meer een voortreffelijk docent als prof.dr. J.H. Lammers, anatoom, mij met zijn lichaampje van Zuckerkandl te intrigeren. Waarom daarop niet promoveren? Ik dank de docenten voor hun inspirerende lessen en colleges, ze stuurden mij verder in de richting van de neurologie.

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Mijn verdere opleiding tot kinderneuroloog onder leiding van de inspirator bij uitstek, prof.dr. Fons J.M. Gabreëls en zijn IKNC-collega's, prof.dr. Willie Renier, prof.dr Jan Rotteveel en dr. Reinier Mullaart, was het laatste zetje dat ik nodig had om in 1991 aan dr. Bernard van Oost te vragen of ik als clinicus mij in zijn laboratorium met de (neuro)genetica zou mogen bezighouden. Door zijn stimulerende en wetenschappelijke geest (hij had net in *Science* een artikel gelezen over het nut van een klinisch intermediair in het lab) werd ik aangenomen onder de voorwaarde dat ik, alvorens een promotietraject in zijn lab op het dynamische onderwerp van het bestuderen van "de rol van kerngenen bij patiënten met mitochondriële ziekten" in te gaan, een jaar naar Londen zou gaan om daar onder leiding van met name prof.dr. Anita Harding en dr. John A. Morgan-Hughes ervaring op te doen in laboratoriumtechnieken benodigd voor het beantwoorden van de gestelde vraag.

Na een 'tüchtige' stoomcursus DNA-technieken op het DNA-diagnos-

tieklab – waarvoor mijn grote dank aan alle medewerkers van het lab – en een supercursus in Utrecht bij prof.dr. B.A. van der Zeijst, bacterioloog, ging ik naar Engeland om als moleculair bioloog in spe aan de slag te gaan. Zeldzaam om na die korte cursussen jezelf zo goed vanuit Nederland in een vreemd lab in hartje Londen gesteund te voelen. Met uiteraard nog erg weinig moleculair-biologische bagage heb ik een jaar heel veel op dat gebied mogen leren. Naast de stimulans van de alom aanwezige, alleswetende prof. Harding en de topclinicus en neuromyoloog dr. Morgan-Hughes heb ik ook veel te danken aan de hoofden van het lab, dr. Mary Davis en dr. Mary Sweeney. Dank ook aan alle top lab- en pubmaten.

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Het onderzoek in de Nijmeegse jaren werd gesteund door subsidies van het Ter Meulen Fonds, het FBW Anthropogenetica en het Prinses Beatrix Fonds, waarvoor ik de wetenschappelijk adviesraden en de beheerders van deze fondsen zeer erkentelijk ben. Mede door het Prinses Beatrix Fonds was het mogelijk om een 'eigen' analist, Paul Buddiger, aan te stellen. Dit gaf de mogelijkheid om in het competitieve veld een vuist te maken. Vele humane complex I cDNA's konden hierdoor ontwikkeld worden en ook de mitochondriële DNA-diagnostiek met de databankontwikkeling en -ontsluiting voor verder onderzoek kregen een flinke stimulans. Paul, je was een voortreffelijke maat.

Vanaf september 1994 werkte ik parttime als kinderneuroloog in het AMC te Amsterdam, waar door het vroegtijdige overlijden van dr. Coby van den Bogert en het vertrek van dr. Piet Bolhuis het mitochondriële onderzoek niet verder tot bloei kwam. Ik ben dankbaar dat ik met beiden

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In 1997 verhuisde ik naar het Erasmus MC te Rotterdam om fulltime aan de slag te gaan op de afd. kinderneurologie, hoofd prof.dr. W.F.M. Arts. Toen werden goede contacten die al vanaf de vroegste Nijmeegse tijd dateerden, aangehaald. Met prof.dr. Herman F.M. Busch, prof.dr. (Jasper) H.R. Scholte en later met prof.dr. Pieter A. van Doorn waren al vele mitochondrieel getinte problemen uitgewisseld en soms tot op DNA-niveau toe 'opgelost'. Heel plezierig was het dan ook voor mij, dat het door Bernard van Oost en Fons Gabreëls geïnitieerde werk onder Jasper Scholte de kans kreeg om afgerond te worden. Met zijn humor, relativeringsvermogen en meesterschap in de klinische biochemie heb ik geboft. In de tussentijd werd de biochemie met dr. Wim Sluiter en dr. Kees Schoonderwoerd voor het mitochondriële research- en diagnostiekwerk uitgebreid. Dankbaar maak ik gebruik van hun aller expertise. Gelukkig stonden mijn collega's in de kinderneurologie, prof.dr. Willem Frans Arts, dr. Corine Catsman, Liesbeth Smit en neuropsychologe Femke Aarsen, mij toe om mijn eigen onderzoeksveld te veroveren, waarvoor mijn dank. Het inplannen en opvangen van de patiënten met mitochondriële aandoeningen wordt op de polikliniek kinderneurologie door Anke van der Linden, Els Noordzij, Margreet van Almenkerk en Betty van der Woude (coördinator polikliniek voor volwassenen) en in de kliniek, gecoördineerd door Lucienne Sas, met grote zorg gedaan. Dank ook voor de stimulansen vanuit de afdeling Klinische Genetica. Het opzetten en verbeteren van het neurogenetica-spreekuur, samen met dr. Grazia Mancini, is een constant genoegen en houdt mij scherp bij het mitochondriële handwerk. Ook de neurogenetica-assistenten Esther Brusse, Bregje van Wetten en Marie-Claire de Wit dank ik voor hun inzet.

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Curriculum vitae

Irenaeus Franciscus Maria de Coo werd op 28 juni 1954 geboren te Utrecht. In 1974 behaalde hij het eindexamen gymnasium aan de RK Scholengemeenschap Katwijk de Breul te Zeist, en volgde de cursus 'A' Level Science (physics, chemistry, botany, zoology) aan het Guildford County Technical College in Guildford, Engeland. De verdere opleiding werd onderbroken wegens het vervullen van de dienstplicht. In 1976 verliet hij de landmacht als luitenant telecommunicatie bij de Verbindingsdienst en ging geneeskunde studeren aan de Katholieke Universiteit te Nijmegen. Op 7 oktober 1983 behaalde hij het Artsexamen en begon met de opleiding tot neuroloog op de Afd. Neurologie (hoofd: Prof.dr. B.P.M. Schulte) in het Sint-Radboudziekenhuis te Nijmegen. Tijdens de studie in de geneeskunde en de neurologie vervulde hij stages in het vakgebied van de kinderneurologie op de Afdelingen Kinderneurologie (hoofd: Prof.dr. F.J.M. Gabreëls), Neuropediatrie (hoofd: Mw.dr. J.B. Krijgsman), en Kindergeneeskunde (hoofd: Prof.dr. G.B. Stoelinga). In 1987 werd hij neuroloog. Voor de verdere opleiding tot kinderneuroloog volgde hij in het Sophia Kinderziekenhuis te Rotterdam (hoofd: Prof.dr. H.K.A. Visser) een opleiding in de kindergeneeskunde. Hierna volgde een eenjarige opleiding op de Afd. Klinische Neurofysiologie van het Sint-Radboudziekenhuis te Nijmegen (hoofd: Prof.dr. S.L.H. Notermans) ter verkrijging van de aantekening in dit vak. Hij voltooide de opleiding tot kinderneuroloog aan het Interdisciplinair Kinderneurologisch Centrum (IKNC) van het Sint-Radboudziekenhuis te Nijmegen (hoofd: Prof.dr. F.J.M. Gabreëls), en bleef daar tot juni 1991 fulltime werken. Daarna werd de aanstelling parttime voortgezet naast een onderzoeksaanstelling bij de sectie DNA-diagnostiek (hoofd: Dr. B.A. van Oost) van het Anthropogenetisch Instituut Nijmegen (hoofd: Prof.dr. H.H. Ropers). In 1991 volgde hij de cursus Moleculairbiologische en recombinant-DNA technologie bij Prof.dr. B.A. van der Zeijst (Afdeling Bacteriologie, Faculteit der Diergeneeskunde, Rijksuniversiteit Utrecht). Vanaf augustus 1991 werkte hij een jaar als kinderneuroloog-onderzoeker in het moleculair-genetisch laboratorium van de University Department of Clinical Neurology, Institute of Neurology, Queen Square, in Londen, Verenigd Koninkrijk (Mw.Prof.dr. A.E. Harding, Prof.dr. J.A. Morgan-Hughes, Prof.dr. T.A. Schapira). Hier werd een eerste aanzet gegeven tot het bestuderen van de rol van kerngenen bij defecten in de oxidatieve fosforylering. Ook participeerde hij in de polikliniekken Neuromuscular Diseases en Neurogenetics van het National Hospital for Neurology en Great Ormond Street Hospital for Sick

Children. Het in Engeland gestarte project vervolgde hij vanaf september 1992 aan het Anthropogenetisch Instituut Nijmegen, waar hij vanaf september 1994 de moleculair-genetische defecten in complex I van de ademhalingsketen bestudeerde in parttime verband als neuroloog-onderzoeker op de Afd. DNA-diagnostiek (hoofd Dr. H.J.M. Smeets, na 1996: Dr. E.C. Mariman). Daarnaast was hij als staflid-kinderneuroloog parttime verbonden aan de Afd. Kinderneurologie (hoofd: Prof.dr. P.G. Barth) van de Afd. Kindergeneeskunde (hoofd: Prof.dr. H.S.A. Heymans) van het Academisch Medisch Centrum te Amsterdam. Vanaf maart 1997 is hij als staflid (kinder-)neuroloog verbonden aan de afdeling Neurologie van het Erasmus MC (hoofd: Prof.dr. F.G. van der Meché, vanaf 2003 Prof.dr. P.A. Sillevis Smitt). Hij participeert in meerdere (inter-)nationale projecten, werkgroepen en netwerken op zijn onderzoeksterrein, waaronder een KP5 Concerted Action 'Mitochondrial Biogenesis and Disease' (coördinator Prof.dr. H. Jacobs, Tampere, Finland), een KP6 STREP 'Mitochondrial diseases: From bedside to genome to bedside' (coördinator Dr. H.J.M. Smeets, Maastricht) en een nationaal samenwerkingsverband (RoMa) met de groep van Dr. H.J.M. Smeets (Capaciteitsgroep Genetica en Celbiologie) van de Universiteit Maastricht en met Prof.dr. H.R. Scholte en Dr. W. Sluiter (Afd. Biochemie) en Dr. G. Schoonderwoerd (Afd. Klinische Genetica) van het Erasmus MC, Rotterdam.

Hij is in 1979 gehuwd met Mechteld Wijgerinck en is vader van Jan Willem, Christianne, Hedwig, Winfried en Quinten.