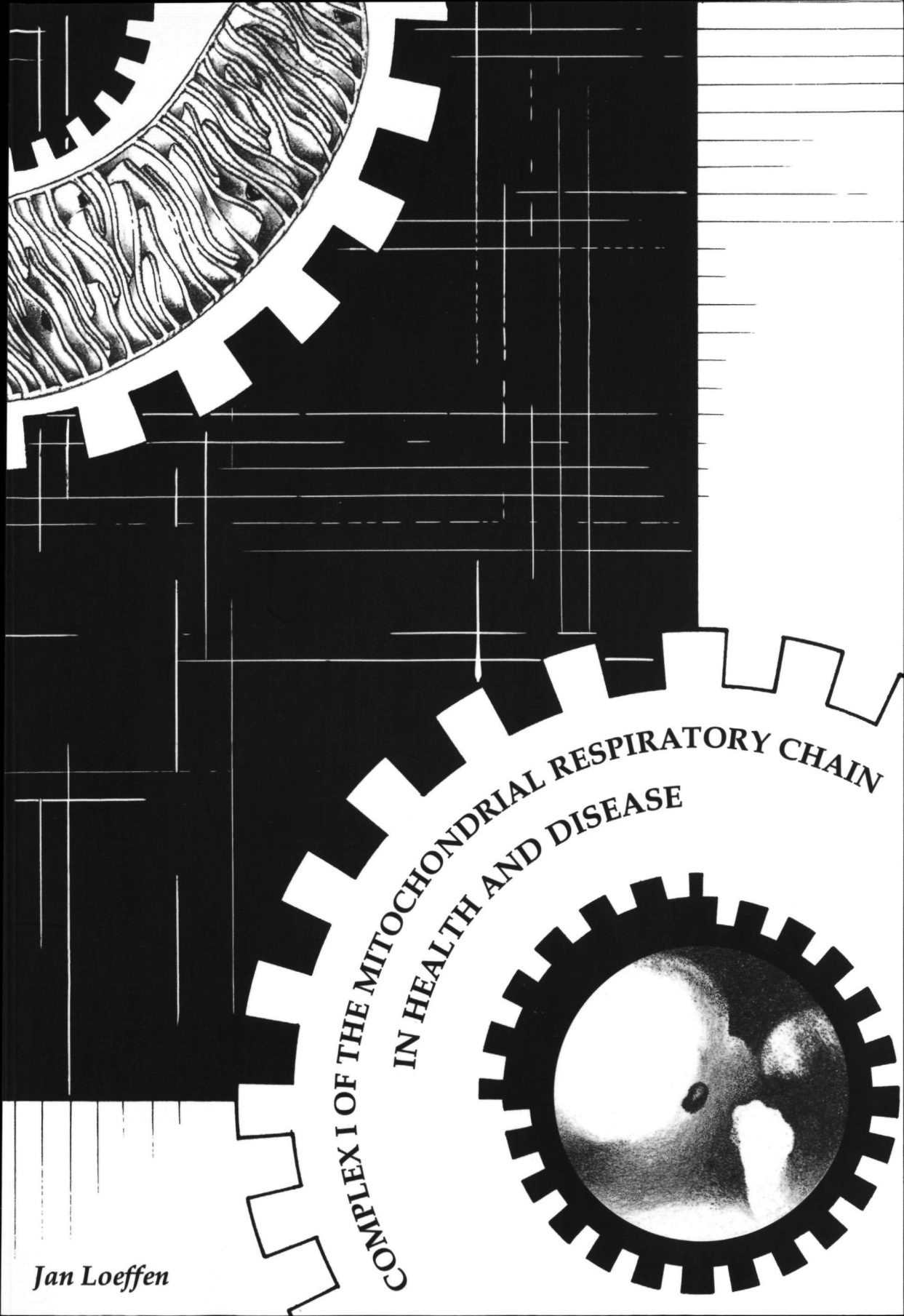

COMPLEX I OF THE MITOCHONDRIAL RESPIRATORY CHAIN IN HEALTH AND DISEASE

Jan Loeffen

COMPLEX I OF THE MITOCHONDRIAL RESPIRATORY CHAIN IN HEALTH AND DISEASE

MOLECULAR-GENETIC STUDIES

Een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

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Abbreviations

ACP	acylcarrier protein
acyl CoA	acyl-Coenzyme A
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BLAST	basic local alignment search tool
bp	base pair
c	cytochrome c
cAMP	cyclic AMP
cDNA	complementary DNA
CO_2	Carbon-dioxide
CoA	Coenzyme A
CoQ	Coenzyme Q (ubiquinone)
CrP	creatine phosphate
CSF	cerebrospinal fluid
CT	computed tomography
cyt	cytochrome
dCTP	deoxycytidine triphoshate
DIDMOAD	diabetes insipidus diabetes mellitus, optic atrophy and deafness
DNA	deoxyribonucleic acid
e	electron
EPR	electron paramagnetic resonance imaging
EST	expressed sequence tag
et al	et alii, and others
FAD	flavin adenine dinucleotide (oxidised form)
$FADH_2$	flavin adenine dinucleotide (reduced form)
Fe-S	Iron Sulphur
FILA	fatal infantile lactic acidosis
FMN	flavin mononucleotide (oxidised form)
FP	flavoprotein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
H^+	proton
HP	hydrophobic protein
IP	iron sulphur protein
IP_3	inositol triphosphate
kb	kilobase
kDa	kiloDalton
L/P	lactate pyruvate ratio
LHON	Leber s hereditary optic neuropathy
LLS	Leigh-like syndrome
LS	Leigh syndrome
MELAS	mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy with ragged red fibres

MILS	maternally inherited Leigh syndrome
min	minutes
MLD	macrocephaly with leukodystrophy
mM	millimolar
mmol/l	millimol per litre
MNGIE	myoneurogastrointestinal encephalomyopathy
M_r	calculated molecular weight
MRI	magnetic resonance imaging
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtETC	mitochondrial electron transport chain
mU	milliUnits
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NARP	neurogenic muscle weakness ataxia and retinitis pigmentosa
NCBI	national centre for biotechnology information
NCLA	neonatal cardiomyopathy with lactic acidosis
NDH I	NADH dehydrogenase I
NDH II	NADH dehydrogenase II
nDNA	nuclear DNA
ng	nanogram
Nuo	NADH ubiquinone oxidoreductase
OMIM	online Mendelian inheritance in man
ORF	open reading frame
OXPHOS	oxidative phosphorylation
P_i	phosphate with high energy transfer potential
PCR	polymerase chain reaction
PDHc	pyruvate dehydrogenase complex
PEO	progressive external ophthalmoplegia
PMR	psychomotor retardation
poly a	polyadenylation
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
RRFs	ragged red fibres
rRNA	ribosomal RNA
RT	reverse transcriptase
SSCP	single strand conformation polymorphism
STS	sequence tagged site
tRNA	transfer RNA
U	ubiquinone or units
UE	unspecified encephalomyopathy
µg	microgram
µL	microliter
µM	micromolar
µmol/l	micromol per litre
UTR	untranslated region

CHAPTER 1

GENERAL INTRODUCTION AND AIM OF THE STUDY

Partially based on:

Nuclear genes of human complex I of the mitochondrial electron transport chain: state of the art

Human Molecular Genetics **1998 7:1573-1579**

Energy production, storage and use are just as essential for cells as fuels are to the world economy. The provision of energy in mammalian cells is one of the most intriguing yet immense complex aspects of cellular function, with tentacles spreading to all corners of the cell. Mitochondria are the power plants of eukaryotic cells, which maintain an adequate cellular energy status. By means of a process called respiration, mitochondria convert nutrients into the energy-yielding compound adenosine triphosphate (ATP) to fuel the cell's activities.

ATP synthesis.

One of the main sources of ATP in the aerobic state of the cell is glucose. The complete oxidation of glucose to H_2O and CO_2 can be divided in three main parts:

I. Glycolysis,
II. Citric acid cycle,
III. Oxidative phosphorylation.

Glycolysis takes place in the cytoplasm of the cell. The overall reaction contains the conversion of the six-carbon compound glucose into two three-carbon molecules of pyruvate. A total of nine enzymatic steps are necessary to complete this conversion (Fig. 1.1). The net reaction of this transformation is:

$$\textbf{glucose} + 2P_i + 2ADP + 2NAD^+ \rightarrow \textbf{2pyruvate} + 2ATP + 2NADH + 2H^+ + 2H_2O$$

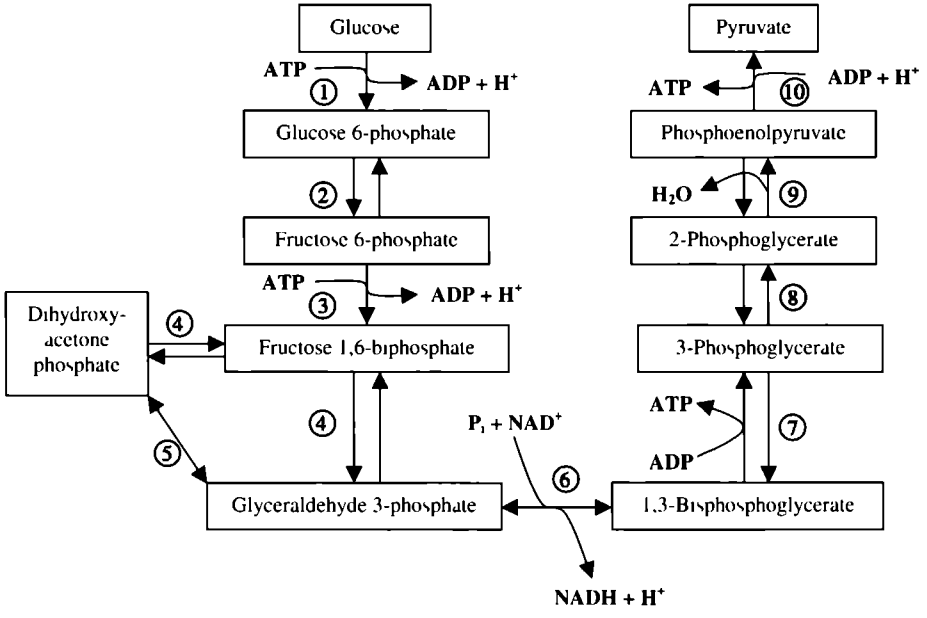

Figure 1.1 The glycolytic pathway 1=Hexokinase; 2=Phosphoglucose isomerase, 3=Phosphofructokinase-1, 4=Aldolase; 5=Triose phosphate isomerase, 6=Glyceraldehyde 3-phosphate dehydrogenase; 7=Phosphoglycerate kinase, 8=Phosphoglyceromutase, 9=Enolase, 10=Pyruvate kinase

Under anaerobic conditions, pyruvate is mainly converted into lactate by the enzyme lactate dehydrogenase, resulting in the overall reaction

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{Lactate} + \text{NAD}^+$$

The regeneration of NAD^+ maintains the operation of glycolysis under anaerobic conditions Lactic acid is mainly secreted into the bloodstream, after which it is processed by the liver Here the lactate is reoxidised into pyruvate, which is either further metabolised into CO_2 or converted into glucose The heart can maintain aerobic metabolism at times when skeletal muscle already converts pyruvate into lactate (e g during prolonged exercise) Therefore the heart can provide a serious contribution to the clearance of lactic acid from the blood

Under aerobic conditions, pyruvate is transported from the cytoplasm into the matrix of the mitochondrion Immediately on entering the matrix, pyruvate reacts with Coenzyme A to generate CO_2 and acetyl-CoA This highly exergonic conversion is catalysed by the Pyruvate Dehydrogenase complex (PDHc)

$$\text{pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

Acetyl-CoA enters the citric acid cycle (Krebs cycle) where it is further processed (Fig 1 2) Fatty acids are released into the blood, from which they are taken up and oxidised by most cells On the outer mitochondrial membrane, free fatty acids are coupled to Coenzyme A by

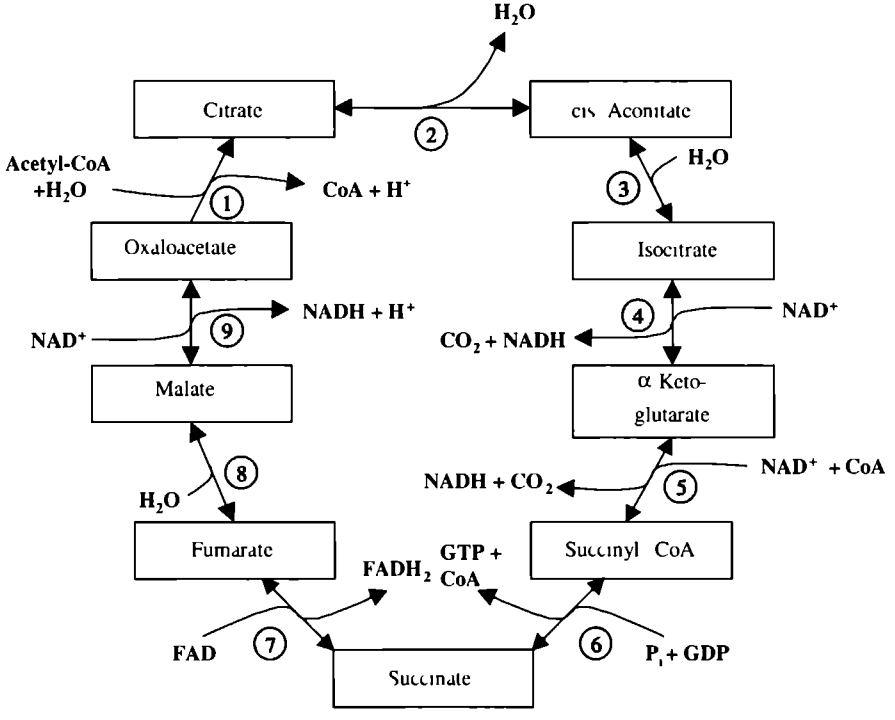

Figure 1 2 The citric acid cycle 1=Citrate synthase 2 + 3= Aconitase 4=Isocitrate dehydrogenase 5=α Ketoglutarate dehydrogenase complex 6=Succinyl CoA synthetase 7=Succinate dehydrogenase 8=Fumarase 9=Malate dehydrogenase

acyl-CoA synthetase to form an acyl-CoA. This is an endergonic reaction, which is linked to the hydrolysis of ATP. Long-chain acyl-CoA molecules cannot traverse the inner mitochondrial membrane and are carried across this membrane by carnitine. Carnitine palmitoyl transferase I transfers the acyl group from acyl-CoA to carnitine, after which the acyl-carnitine is transported across the inner mitochondrial membrane by carnitine acylcarnitine translocase. The acyl group is then reattached to CoA by carnitine palmitoyl transferase II, by which the acyl-CoA is regenerated. In a cyclic process, which is called ß-oxidation, the acyl-CoA is shortened by two carbon atoms (acetyl-CoA). Concomitantly, one molecule of NAD^+ and FAD are reduced to NADH and $FADH_2$. This cycle continues until all carbon atoms are converted into acetyl-CoA, which enters the citric acid cycle.

The citric acid cycle consists of nine reactions (Fig. 1.2) with the net result:

$$\textbf{acetyl-CoA} + 3NAD^+ + FAD + GDP + P_i + 2H_2O \rightarrow 2CO_2 + 3NADH + FADH_2 + GTP + 2H^+ + CoA$$

Several reactions of glycolysis, citric acid cycle and β-oxidation result in the reduction of NAD^+ and FAD to NADH and $FADH_2$, respectively. These reduced coenzymes are reoxidised by molecular oxygen in a multi-step process called oxidative phosphorylation (Fig. 1.3). The overall reaction of the oxidation of NADH is:

$$NADH + H^+ + \tfrac{1}{2}O_2 \rightarrow NAD^+ + H_2O$$

During the oxidation of NADH two electrons are released. In contrary to protons, electrons cannot exist in aqueous solutions. The electrons are therefore transported to oxygen via four multi-protein complexes together constituting the respiratory chain, which is embedded in the inner mitochondrial membrane. This transport is thermodynamically downhill and much of the released energy is conserved by the movement of protons from the mitochondrial matrix to the intermitochondrial membrane space.

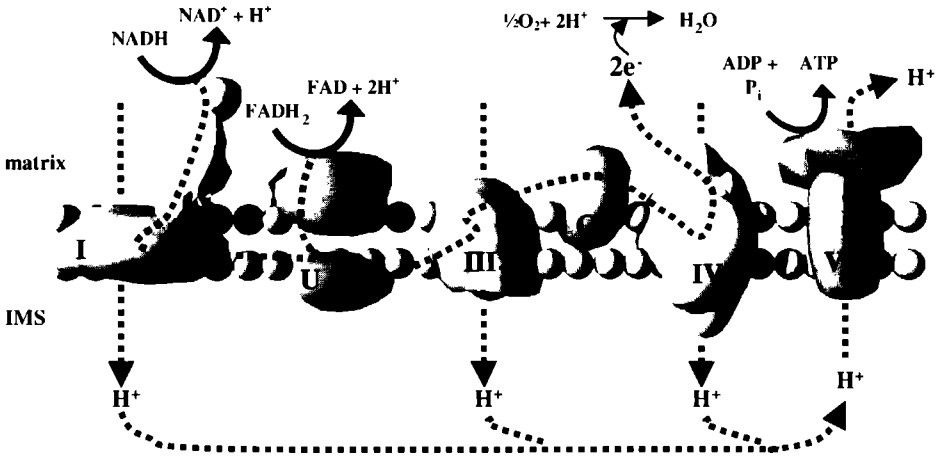

Figure 1.3 The oxidative phosphorylation system. I = complex I (NADH:ubiquinone oxidoreductase), II = complex II (succinate:ubiquinone oxidoreductase), U = ubiquinone, III = complex III (ubiquinone:cytochrome *c* oxidoreductase), C = cytochrome *c*, IV = complex IV (cytochrome *c* oxidase), V = complex V (ATP synthase), IMS = intermembrane space; e = electron.

Briefly, NADH donates two electrons to the first complex of the respiratory chain, which is called NADH ubiquinone oxidoreductase or complex I The electrons travel through the prosthetic groups of complex I towards ubiquinone (U) also called Coenzyme Q (CoQ) $FADH_2$ also transfers its electrons to ubiquinone but via succinate ubiquinone oxidoreductase or complex II Ubiquinone transports the electrons to ubiquinone cytochrome c oxidoreductase or complex III Cytochrome c accepts the electrons from complex III and transfers them to cytochrome c oxidase which catalyses the reduction of oxygen As mentioned previously, energy which is released by this electron transport is preserved by the translocation of protons across the inner mitochondrial membrane For each two electrons accepted from NADH, complex I transports four protons towards the intermembrane space, complex III two and complex IV four, which adds up to a total of ten protons Since complex II does not translocate protons, two electrons donated by $FADH_2$ result in the total translocation of six protons across the inner mitochondrial membrane The created proton gradient is used by ATP synthase or complex V to generate ATP The respiratory chain together with ATP synthase is called the oxidative phosphorylation (OXPHOS) system In the next section I will focus on the first and largest complex of the respiratory chain (also the main subject of this thesis) complex I

Complex I.

Evolution, composition and (spatial and functional) organisation of complex I

Although complex I studies have been performed in many species, most knowledge regarding the composition, spatial and functional organisation of the first mitochondrial electron transport chain (mtETC) enzyme complex has been obtained from *Escherichia coli*, *Neurospora crassa* and *Bos taurus* (Hatefi 1985, Weiss et al 1991, Walker 1992, Walker et al 1992, Weidner et al 1993, Guenebaut et al 1997, Grigorieff 1998) Many bacteria (like *E coli*) contain two NADH dehydrogenases, which are called NDH-I and NDH-II (Yagi 1993, Weidner et al 1993) NDH-I is like mitochondrial complex I, a multiple-protein enzyme complex and links electron transport to proton translocation It contains many redox centres, namely one flavine mononucleotide (FMN) and possibly up to nine Fe-S clusters (Friedrich 1998) NDH-I is built up of fourteen subunits, which have been named NADH ubiquinone oxidoreductase (Nuo) A to N (in some bacteria Nuo C and D are fused to NuoCD) and is generally considered to be the minimal form of a proton-pumping NADH ubiquinone oxidoreductase (Leif et al 1993, Table 1 1, page 16) NDH-II is a single polypeptide enzyme, which is in practice a non-proton pumping NADH ubiquinone oxidoreductase

Complex I of *E coli* can be fragmented into three parts, an NADH dehydrogenase fragment, a connecting fragment and a membrane fragment (Leif et al 1993) The NADH dehydrogenase part consists of NuoE, F and G containing the NADH binding site, the FMN and four electron paramagnetic detectable iron-sulphur clusters (Friedrich 1998), and is considered to be the electron input part of complex I The connecting fragment is made up of NuoB, CD and I and contains Iron-Sulphur cluster N2 (Leif et al 1995) Finally, the membrane fragment is built of NuoA, H and J-N Little is known about the functions of the membrane part of *E coli* complex I It probably participates in the binding of ubiquinone and the transport of protons

(Friedrich 1998)

Complex I of the fungus *N crassa* has a dual genetic origin The majority of its proteins are encoded by the nucleus, synthesised in the cytoplasm and imported into mitochondria, where they assemble together with several mitochondrial encoded proteins into the complete complex *N crassa* contains a complex I which probably consists of at least 35 different proteins of which 7 are encoded by mtDNA (Weiss et al 1991, Table 1 1) Currently, not all subunits are characterised at cDNA level All subunits which are present in NDH-I of bacteria are present in the eukaryotic complex I of *N crassa* As mentioned previously, *N crassa* complex I appears under the electron microscope as an L-shaped structure, with one arm embedded in the mitochondrial membrane, while the other arm protrudes into the matrix (Guenebaut et al 1997) So far, the FMN and only four Fe-S clusters have been identified Besides the subunits which are also present in the minimal form of complex I seen in bacteria, many additional proteins are present in complex I of *N crassa* In many cases they do not seem to have similarity to other proteins It has been speculated that many of these proteins isolate the complex in order to prevent electrons from escaping the enzyme (Friedrich and Weiss 1997)

Experiments performed with *N crassa* cells, in which radioactivity of pulse-labelled amino acids was followed, showed that the membrane arm and the arm protruding into the matrix are separately assembled (Tuschen et al 1990) These findings were confirmed in *N crassa* using disruption experiments of single complex I genes In addition, the membrane arm seems to be synthesised by fusion of two intermediates (Schulte et al 1994) The crystal structure of the *N crassa* complex has been determined and, by immunolabelling, the 49 kDa subunit has been pinpointed to the matrix-localised protruding arm of the complex (Guenebaut et al 1997) Immunolabelling of other subunits will give further insight into the interrelationships of the various subunits

The species evolutionary closest to man of which complex I has been studied extensively is *Bos taurus* Bovine complex I has ~42 subunits (Table 1 1) with a total molecular mass of ~900 kDa It consists of seven mitochondrial- and at least 35 nuclear-encoded subunits and has, like in *N crassa* an L-shaped configuration of which the peripheral arm protrudes in the mitochondrial matrix (Guenebaut et al 1997, Grigorieff 1998) The bovine complex differs from that of *N crassa* by having a thin stalk region linking the membrane-bound globular arm with the intrinsic membrane domain It is however clear that the form of complex I is highly conserved between prokaryotes like *E coli* and eukaryotes like *N crassa* and *Bos taurus* (Friedrich 1998, Grigorieff 1998) Most of the electron carriers, which exist of one flavine mononucleotide (FMN) and at least six Fe-S clusters (proven by electron paramagnetic resonance imaging (EPR)), are located in the peripheral arm With the use of chaotropic agents, the complex can be separated into three different fractions (Galante and Hatefi 1978 and 1979) The flavoprotein (FP) fraction and the iron-sulphur protein (IP) fraction are both water-soluble, whereas the hydrophobic protein (HP) fraction forms a water-insoluble aggregate The FP fraction (51, 24 and 10 kDa) and the IP fraction (75, 49, 30, 18, 15, 13 kDa, B13 and TYKY) form the principal catalytic sector and are located in the peripheral arm of the complex (Belogrudov and Hatefi 1994) Whether the TYKY subunit is present in the IP fraction or in the HP fraction is subject of debate Masui and co-workers located the TYKY

subunit in the IP fraction (Masui et al 1991), while others did not Since the prokaryotic TYKY equivalent (NuoI) is present in the connecting fragment (located between the dehydrogenase fragment and the membrane fragment), it could be possible that slight differences in the complex I purification and fractionation process result in the presence of TYKY in different fractions The HP fraction (containing the seven mitochondrial encoded subunits and ~24 nuclear encoded subunits) is probably involved in proton translocation (Ohnishi et al 1985, Belogrudov and Hatefi 1994) This fraction contains, besides hydrophobic subunits, also globular water-soluble ones and the presence of a particular subunit in this fraction should not be taken to imply that the protein is either hydrophobic or a component of the membrane part of the enzyme complex (Walker 1992).

Most aspects of complex I function regarding electron and proton transport are unknown, however some aspects have been elucidated The 51 kDa FP subunit carries the NADH-binding site (Chen and Guillory 1981, Deng et al 1990), contains an FMN and a tetranuclear iron-sulphur cluster (Pilkington et al 1991) The 24 kDa FP subunit contains a binuclear iron-sulphur cluster (Pilkington and Walker 1989) The 75 kDa IP subunit contains a tetranuclear and a binuclear iron-sulphur cluster (Runswick et al 1989) Sequencing of the bovine TYKY (23 kDa) IP subunit revealed that this subunit contains two cysteine motifs, which probably provide the ligands to accommodate two 4Fe-4S clusters (Dupuis et al 1991) Such a conserved cysteine motif has also been encountered in the PSST (20 kDa) HP subunit (Walker 1992) The FP and IP water-soluble fractions make contact through the 51 kDa FP and 75 kDa IP subunits The FP and IP subunit stoichiometry, which is a *sine qua non* for the study of the structure of an enzyme complex, and the substrate-induced conformational changes have been partly elucidated by Belogrudov and Hatefi in 1996 They showed that the proximity of the three FP subunits to one another, the proximity of the 51 kDa FP subunit to the 75 kDa IP subunit, and the proximity of all studied IP subunits to one another and to some of the HP subunits are altered when the catalytic sector of complex I is reduced by NADH or NADPH prior to the addition of a cross-linking reagent The authors speculated that these conformational changes may well be the device by which the energy derived from electron transfer through the catalytic components of complex I is transduced and conveyed to the subunits of the membrane sector Here, pK_a changes of appropriate residues induced by these conformational changes would result in proton uptake and release on opposite sides of the membrane

Table 1 1 lists the subunit composition of bacterial complex I (14 subunits), complex I of *N crassa* (~35 subunits, currently not all molecular-genetically characterised) and *B taurus* (~42 subunits) The prokaryotic subunits (NuoA-N) are present in all three species and seem to form the catalytic core of the complex *N crassa* has numerous additional proteins, several of which do not seem to be conserved in *B taurus*

In summary, the overall function of complex I of the mtETC is the dehydrogenation of NADH and the transportation of electrons to ubiquinone This electron transport device is linked by a yet unknown mechanism to proton transport across the inner mitochondrial membrane to the intermembrane space (~4 protons for each two electrons) This proton gradient is the force used for the production of ATP by F_1-F_0 ATP-synthase (complex V)

E. coli		*N. crassa*		*B. taurus*	
Name	***kDa***	***Name***	***kDa***	***Name***	***kDa***
NuoA	16.5	ND-3	6.0	ND-3	13.1
NuoB	25.1	19.3 kDa	25.0	PSST	23.8
NuoC[1]	68.7	31 kDa	32.3	30 kDa (IP)	30.3
NuoD[1]	68.7	49 kDa	54.0	49 kDa (IP)	49.2
NuoE	18.6	24 kDa	28.8	24 kDa (FP)	27.3
NuoF	49.3	51 kDa	54.3	51 kDa (FP)	50.7
NuoG	100.3	78 kDa	81.7	75 kDa (IP)	79.4
NuoH	36.2	ND-1	41.6	ND-1	35.7
NuoI	20.5	23 kDa	24.9	TYKY (IP)	23.9
NuoJ	19.9	ND-6		ND-6	19.2
NuoK	10.8	ND-L4	9.8	ND-4L	10.8
NuoL	66.4	ND-5	79.8	ND-5	68.3
NuoM	56.5	ND-4		ND-4	53.8
NuoN	45.7	ND-2	65.8	ND-2	39.3
-		-		10 kDa (FP)	11.9
-		21.3 kDa	24.5	AQDQ (IP)	19.8
-		-		15 kDa (IP)	12.7
-		-		13 kDa (IP)	13.4
-		29.9 kDa	30.9	B13 (IP)	13.3
-		-		42 kDa	39.3
-		40 kDa	43.0	39 kDa	42.8
-		-		B22	21.8
-		12.3 kDa		PDSW	21.0
-		-		KFYI	8.8
-		20.8 kDa	20.9	PGIV	20.1
-		-		ASHI	21.7
-		-		SGDH	21.6
-		-		B18	16.4
-		-		B17.2	17.1
-		-		B17	15.5
-		-		B15	15.2
-		14.8 kDa	14.8	B14	15.1
-		-		B14.5a	12.7
-		-		B14.5b	14.1
-		-		B12	11.1
-		9.3 kDa	9.3	B9	9.3
-		10.5 kDa	10.5	B8	11.1
-		9.6 kDa	14.5	SDAP	10.1
-		-		MLRQ	9.3
-		12kDa	12.3	AGGG	12.3
-		-		MWFE	8.1
-		-		MNLL	7.0
-		17.8 kDa	20.1	-	
-		21 kDa	21.0	-	
-		12 kDa	12.3	-	
-		21.3 kDa	21.3	-	
-		9.5 kDa	9.3	-	
-		21.3 kDa	21.4	-	
-		18.3 kDa	21.8	-	

Table 1.1 Complex I subunit composition of *E. coli*, *N. crassa* and *B. taurus*. All proteins were retrieved from the NCBI protein database, using Basic Local Alignment Search Tool (BLAST, Altschul et al 1990). [1] In some prokaryotes like *E. coli* NuoC and NuoD are fused to NuoCD. Subunits that are in the same row are homologous between the species.

Human complex I deficiency.

Among the group of mitochondriocytopathies (estimated incidence 1 10,000 live births, Bourgeron et al 1995), isolated complex I deficiency is frequently encountered (von Kleist-Retzow et al 1998) The primary genetic cause of the observed deficiencies may either be at the mtDNA or at the nDNA level Elucidation of the genetic defects of these disorders is important for genetic counselling and will extend our knowledge of the functional properties of the individual subunits A great variety in clinical presentation, age of onset, course of the disease and clinical-chemical abnormalities exists in complex I deficient patients It may therefore not come as a surprise that the diagnostic process is rather complicated and that the judgement of instituted treatments is almost impossible in individual patients

In general, most affected tissues are those with a high-energy demand, such as the brain, heart, kidney and skeletal muscle Ophthalmological signs like external ophthalmoplegia, ptosis, cataract and retinopathy are also frequently observed (Pitkanen et al 1996) The diagnosis 'enzymatic complex I deficiency' is often made in early childhood and, although exceptions exist, the course is often that of a multi-system disorder with a fatal outcome In most patients lactic acidemia (with increased lactate pyruvate ratios due to the altered redox state of the mitochondria) is present, although we are aware of several cases of complex I deficiency in which lactic acid, even after provocation (exercise, glucose tolerance test), is completely normal (this thesis) In order to correlate genotype to phenotype, a detailed clinical description of patients is of utmost importance In suspected patients, diagnosis can be made by enzymatic measurements, for which a skeletal muscle biopsy sample is the tissue of choice, cultured fibroblasts can also be used Noteworthy in this context is that not all deficiencies observed in skeletal muscle specimens are present in fibroblasts (Ruitenbeek et al 1996) This phenomenon, together with the diverse involvement of tissues and organs in affected patients, may be caused by differences in tissue expression of the individual subunits of the complex In nearly all patients, a considerable residual enzymatic activity is observed To the best of our knowledge, few patients have been described with an immeasurable low complex I activity

Under certain conditions prenatal diagnosis of respiratory chain disorders is possible at the enzymatic level and has been performed with success in specialised laboratories (Ruitenbeek et al 1992 and 1996) Because of the fact that the interpretation of the results of these measurements is difficult, it would be very helpful if prenatal diagnosis of complex I deficiency would be possible at DNA level One can easily obtain RNA and DNA from chorionic villi, on which mutational analysis can be performed by means of RT-PCR followed by direct sequencing, or in case of known mutations, by PCR combined with restriction fragment length polymorphisms (RFLP)

Genetics of OXPHOS disorders.

a. Mutations in mtDNA.

Since the first description of a patient with a disturbance in the mitochondrial energy-generating system by Ernster in 1959, numerous reviews about the mitochondrial electron transport chain (mtETC) and defects of its individual complexes have been published (Sengers et al 1984, DiMauro et al 1985, Trijbels et al 1988, Lombes et al 1989, Wallace 1992, Morgan-Hughes 1994, Johns 1995, Tein 1996, Schapira 1998a)
One of the most intriguing aspects of the OXPHOS system is its dual genomic control About 30 years ago, the first structural characterisation of human mitochondrial DNA (mtDNA) appeared in literature (Clayton and Vinograd 1967, Hudson and Vinograd 1967) The mtDNA, a 16,569 base-pair genome is present in several hundred copies per cell, encodes two rRNAs, 22 tRNAs and 13 mRNAs (Anderson et al 1981, Taanman 1999) The mRNAs encode subunits of three of four respiratory chain complexes (7 out of 42 complex I proteins, 1 out of 11 complex III proteins and 3 out of 13 complex IV proteins) as well as ATP synthase (2 out of 14 proteins) The remaining components of the OXPHOS system as well as all proteins involved in its assembly, maintenance and regulation are encoded on the nuclear DNA (nDNA)
Due to its easy accessibility and relatively small genome, mutational analysis studies of disorders of the OXPHOS system have the last ten years concentrated on the mtDNA Since 1988, numerous mtDNA mutations (rearrangements as well as point mutations) have been reported to occur in patients with mitochondriocytopathies, of which more than one hundred are considered pathogenic (Zeviani et al 1998a, Chinnery and Turnbull 1999) In general, mutations of mtDNA can be divided in major rearrangements and point mutations In the following sections a brief summary of the most important mtDNA mutations and their accompanying clinical phenotypes are described
Two aspects of the mitochondrial genome are especially important in the relation between mtDNA and OXPHOS deficiencies Firstly, mtDNA is transmitted only by the mother (Giles et al 1980) The mtDNA donated by sperm cells to the oocyte after fertilisation is actively degraded (Shitara et al 1998) This maternal inheritance pattern implicates that in the pedigree of patients with mtDNA mutations affected siblings can be expected only in the maternal lineage and only in the offspring of females Secondly, mutated and wild-type mtDNA can coexist within a single cell, especially in the case of point mutations This condition is termed heteroplasmy When there is heteroplasmy for a given mutation (rearrangements as well as point mutations), this suggests either that it arose relatively recent (no time to become homoplasmic) or the mutation cannot be tolerated in the homoplasmic state Different amounts of heteroplasmy can be present between adjacent cells It has been hypothesised that this feature of different amounts of mtDNA heteroplasmy between patients is responsible for the diversity in clinical phenotypes seen in carriers of the same mtDNA mutation Furthermore, it seems likely that a cell needs to cross a certain threshold for mutated mtDNA before it gets affected For many mtDNA mutations, a relation has been found between the amount of heteroplasmy and the phenotype (MELAS (Chinnery et al 1997), MERRF

(Hammans et al 1993), NARP (Uziel et al 1997) but other lack such a correlation (LHON (Ghosh et al 1996))

Large-scale rearrangements At the end of the eighties, large-scale rearrangements of mtDNA were for the first time linked to specific clinical phenotypes

Progressive external ophthalmoplegia (PEO, bilateral ptosis, ophthalmoplegia with or without general muscle weakness, OMIM 157640, 601226, 601227, 603280, 604005) has been linked to large deletions in mtDNA (Moraes et al 1989) It is transmitted in an autosomal-dominant manner with male to male transmission, indicating a nuclear gene predisposing to mtDNA deletions Linkage analysis mapped PEO to three different regions in different families while in a fourth family these three regions were excluded Therefore at least four different nuclear genes are involved in PEO (PEO type 1-4), which remain to be elucidated (Suomalainen et al 1995, Kaukonen et al 1996, Kaukonen et al 1999, Li et al 1999) PEO has also been described to inherit in an autosomal-recessive manner, associated with severe cardiomyopathy (Bohlega et al 1996) Kearns-Sayre syndrome (OMIM 530000), a disease characterised by ophthalmoplegia, pigmentary degeneration of the retina, cardiomyopathy, onset before age of 20 and a high protein content in CSF was also linked to a single large deletion of variable length in the mtDNA (Zeviani et al 1988, Lestienne and Ponsot 1988, Zeviani et al 1998b)

Patients with Pearson syndrome (OMIM 557000) suffer from refractory sideroblastic anemia with vacuolisation of bonemarrow precursors and exocrine pancreas dysfunction, which has been associated with deletions or duplications in the mtDNA (Rotig et al 1989, Superti-Furga et al 1993)

A disorder characterised by multiple deletions of mtDNA is myoneurogastrointestinal encephalopathy syndrome (MNGIE, OMIM 550900) (Hirano et al 1994) Recently, Nishino and colleagues revealed that the primary genetic defect of MNGIE is located in the gene coding for thymidine phosphorylase (Nishino et al 1999) Besides deletions, also partial duplications of the mtDNA are sometimes seen in mitochondrial myopathy (Poulton et al 1989) Finally, a clinical phenotype with diabetes insipidus, diabetes mellitus, optic atrophy and deafness (DIDMOAD, Wolfram syndrome, OMIM 598500) may be linked to large-scale rearrangements (Rotig et al 1993, Barrientos et al 1996) Collier and colleagues however, found linkage in families with DIDMOAD patients to chromosome 4p16 1 (Collier et al 1996) and in 1998 Inoue located the gene responsible for a large percentage of DIDMOAD patients, WFS1 (Inoue et al 1998), which has been confirmed by other groups (Strom et al 1998, Hardy et al 1999)

Usually the inheritance pattern observed in patients with major mtDNA rearrangements is sporadic, probably because they occur in the germ-line In rare cases however, a heteroplasmic duplication of mtDNA and a large deletion is maternally transmitted in diabetes and deafness (Ballinger et al 1992, Dunbar et al 1993)

Leber's hereditary optic neuropathy (LHON, OMIM 535000) Many mtDNA point mutations have been reported in literature Several of these mutations are linked to specific clinical phenotypes At the end of the eighties, Wallace and colleagues found the G11778A (ND-4) mutation in patients with Leber's hereditary optic neuropathy (LHON) (Wallace et al 1988a) LHON patients present in mid-life with acute or subacute central vision loss leading to central scotoma Other important mutations linked to LHON are the G3460A (ND-1) (Huoponen et al

1991, Howell et al 1991), G14459A (ND-6) (Jun et al 1994), T14484C (ND-6) (Mackey and Howell 1992) and G15257A (CYT B, Johns and Neufeld 1991) LHON mutations are maternally transmitted and are traditionally considered being familial, yet several patients have been reported as individual cases Biochemical analysis of the respiratory chain enzymes showed that complex I activity can be deficient in patients with LHON Since there seems to be a male preponderance present in patients with LHON, influence of an X-linked nuclear gene has been suggested (Vilkki et al 1991)

LHON and dystonia The combination of LHON and dystonia is considered to be a separate clinical phenotype Two mutations have been found in these patients, namely the G14459A (ND-6) (Jun et al 1994) and the A11696G (ND-4) (De Vries et al 1996)

Myoclonic epilepsy with ragged red fibres (MERRF, OMIM 545000) MERRF syndrome is a maternally transmitted clinical phenotype linked by Shoffner in 1990 to the mtDNA mutation A8344G ($tRNA^{lys}$) (Shoffner et al 1990) This mutation accounts for 80-90% of the MERRF patients (Shoffner and Wallace 1992) Biochemical studies of the respiratory chain in these patients often reveal combined deficiencies, with most prominently affected complex I and complex IV (Wallace et al 1988b) The common MERRF mutation has also been found in patients with Leigh syndrome, isolated myoclonus, familial lipomatosis and isolated myopathy (Silvestri et al 1993)

Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS, OMIM 540000) Another important clinical syndrome that has been connected to a mitochondrial encoded tRNA gene ($tRNA^{leu}$) is MELAS Goto and colleagues found in several MELAS patients the A3243G mutation, which turned out to be the most common MELAS mutation (Goto et al 1990) The clinical phenotype of patients with MELAS is extremely diverse (de Vries et al 1994) Diversity is also encountered in the phenotypes associated with the A3243G mutation, which can be MELAS, PEO, isolated myopathy, cardiomyopathy and diabetes mellitus with deafness (Zeviani et al 1998a) As counts for the mutation found in the $tRNA^{lys}$ gene seen in MERRF, mutations in the $tRNA^{leu}$ gene often result in multiple enzyme deficiencies of the respiratory chain, with a preference for complex I and IV deficiency

NARP (OMIM 551500) / MILS (OMIM 516060) Holt et al found a T to G transversion at nucleotide pair 8993 of the mtDNA (ATPase 6 subunit) in a family with adult-onset maternally inherited neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) (Holt et al 1990) The clinical phenotype varied according to the amount of heteroplasmy found in each individual patient This T8993G mutation has also been found in patients with Leigh syndrome (Tatuch et al 1992, Shoffner et al 1992) This form of Leigh syndrome caused by a mutation in the ATPase 6 gene, is also indicated in literature by the acronym MILS (maternally inherited Leigh syndrome) NARP and MILS can coexist within the same family In 1994 hypertrophic cardiomyopathy was added to the list of clinical phenotypes associated with the T8993G mutation (Pastores et al 1994) De Vries and colleagues found in 1993 a T to C transition at position 8993 in patients with juvenile Leigh syndrome (De Vries et al 1993) It is believed that the T8993C mutation results in a milder dysfunction of complex V compared to the T8993G mutation The different phenotypes associated with the same mutation could be caused by a different amount of heteroplasmy of the mutations in tissues It has been

suggested that a higher percentage of mutated mtDNA (T8993G) results in Leigh syndrome, while lower amounts might result in NARP (Tatuch et al 1992)

Parkinson disease (PD). Mitochondrial involvement in PD was a fact when a complex I deficiency was found in tissue of the substantia nigra of PD patients (Schapira et al 1989) The substantia nigra is the origin of the main clinical features of PD due to degeneration of dopaminergic neurons Studies of OXPHOS function in PD patients in other parts of the brain and other tissues revealed conflicting results (Schapira 1998b) There is however a uniform opinion that there is a complex I deficiency in platelets of PD patients. The active metabolite 1-methyl-4-phenylpyridinium (MPP) of the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a selective complex I inhibitor which induces parkinsonism in humans This again stresses the possible involvement of complex I in the pathogenesis of PD Several studies have been performed to locate possible mutations in mitochondrially encoded complex I subunits, however with no consistent results Fusion studies between ρ_0 cells and platelets showed complex I deficiency in cybrids with PD patient platelets compared to normal complex I activity when control platelets were used This proves that the complex I deficiency in PD patients is of mitochondrial nature (Gu et al 1998) The exact genetic cause remains to be elucidated

b. Mutations in nDNA.

Mutations in nuclear encoded subunits of the OXPHOS system were at the start of our study in 1997 limited to one Bourgeron and colleagues found an R554W substitution in the Fp subunit of succinate ubiquinone oxidoreductase (complex II) This mutation was found in two siblings born from consanguineous parents, suffering from Leigh syndrome (Bourgeron et al 1995)

In case of complex I, no mutations were described in nuclear encoded complex I genes at the start of our study At that time, only a few human nuclear encoded complex I subunits were characterised at mRNA and amino acid level, so naturally, mutational analysis studies were scarce

Aim of the study.

Many patients and tissue specimens of patients (mainly skeletal muscle and skin fibroblasts tissue) are referred to our centre under suspicion of malfunctioning of the OXPHOS system These patients mainly concern children who present their initial symptoms in the first years of life Isolated complex I deficiency is a large contributor to this group, however the exact percentage at the start of our studies was unknown Patients diagnosed with complex I deficiency are always checked for common mtDNA mutations These mutations are seldom encountered, especially in young children The impression exists that isolated complex I deficient patients (without common mtDNA mutations) express an autosomal recessive inheritance pattern instead of maternal Furthermore, there seems to be a high frequency of consanguinity in the families presented to our centre All these factors led to the hypothesis that nuclear-genetic causes may be present in our complex I deficient patients, e g situated in the nuclear encoded subunits of complex I The principle of our complex I enzyme assay is measurement of the amount of NADH which is oxidised to NAD^+ by complex I in the presence of ubiquinone This measurement is performed in the absence and presence of rotenone, which is a specific complex I inhibitor Therefore it mainly measures whether the flux of electrons from NADH to ubiquinone is working properly Studies performed with *N crassa* complex I showed that isolates of the peripheral arm of complex I are capable of transporting electrons from NADH to ubiquinone This fragment (of which the IP fraction is the main contributor) is therefore one of the main candidates to host defective subunits in our complex I deficient patients

Currently, therapy for the often fatal developing complex I deficiency is not available It is therefore of utmost importance to elucidate the molecular-genetic cause of isolated complex I deficiency to offer the possibility of genetic counselling and prenatal diagnostics Furthermore, when the genetic cause(s) of complex I deficiency is (are) elucidated, this could help us forward in the process of understanding complex I deficiency, which eventually should lead to a rational and effective therapy

At the start of this study the aims were as follows

1 Selection and clinical characterisation of an isolated complex I deficient patient group
2 Exclusion of common mtDNA point mutations and major rearrangements within this group
3 Elucidation of the cDNA and amino acid sequence of all human nuclear encoded complex I subunits
4 Investigation of the presence of mutations in the nuclear genes coding for Iron sulphur protein (IP) fraction subunits of complex I in our patients

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ISOLATED COMPLEX I DEFICIENCY IN CHILDREN: CLINICAL, BIOCHEMICAL AND GENETIC ASPECTS

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Abstract.

We retrospectively examined clinical and biochemical characteristics of 27 patients with isolated enzymatic complex I deficiency (established in cultured skin fibroblasts) in whom common pathogenic mtDNA point mutations and major rearrangements were absent Clinical phenotypes present in this group are Leigh syndrome (n=7), Leigh-like syndrome (n=6), fatal infantile lactic acidosis (n=3), neonatal cardiomyopathy with lactic acidosis (n=3), macrocephaly with progressive leukodystrophy (n=2) and a residual group of unspecified encephalomyopathy (n=6) subdivided into progressive (n=4) and stable (n=2) variants Isolated complex I deficiency is one of the most frequently observed disturbances of the OXPHOS system In general, respiratory chain enzyme assays performed in cultured fibroblasts and skeletal muscle tissue reveal similar results, but for complete diagnostics we recommend enzyme measurements to be performed in at least two different tissues to minimise the possibility of overlooking the enzymatic diagnosis Lactate levels in blood and CSF and cerebral CT/MRI studies are highly informative, although normal findings do not exclude complex I deficiency With the discovery of mutations in nuclear encoded complex I subunits, adequate pre- and postnatal counselling becomes available Finally, considering information currently available, isolated complex I deficiency in children seems in the majority to be caused by mutations in nuclear DNA

Introduction.

Since the first report of a patient with a decreased activity of NADH:ubiquinone oxidoreductase (complex I, OMIM 252010) of the mitochondrial respiratory chain by Morgan-Hughes and co-workers in 1979, over 100 cases followed, which placed isolated complex I deficiency on the map as an important inborn error of energy metabolism. Recent studies revealed that within the group of respiratory chain disorders, isolated complex I deficiency is a major contributor (33%; von Kleist-Retzow et al 1998).
Complex I is a large multi-protein assembly, which is partly located in the mitochondrial inner membrane and partly protruding into the matrix (Fig. 2.1). Its main function is transport of electrons from NADH to ubiquinone with simultaneous shunting of protons across the inner mitochondrial membrane to the intermembrane space. The subunit composition of complex I has been elucidated in several eukaryotes, such as *Neurospora crassa* (Weiss et al 1991) and *Bos taurus* (Walker 1992, Skehel et al 1998), which appears to consist of ~35 and ~42 proteins, respectively. Of the 42 subunits present in human complex I, seven are encoded by mitochondrial DNA (mtDNA) (Chomyn et al 1986). Complex I can be fragmented by chaotropic agents in three different parts (Galante and Hatefi 1979). These parts represent the Flavoprotein fraction (FP; composed of three subunits, which contain the NADH-, Flavin mononucleotide- and several Fe-S cluster binding sites), the Iron-Sulphur protein fraction (IP; composed of approximately seven subunits, which contain several Fe-S cluster binding sites) and the residual membrane part called the Hydrophobic protein fraction (HP; contains among

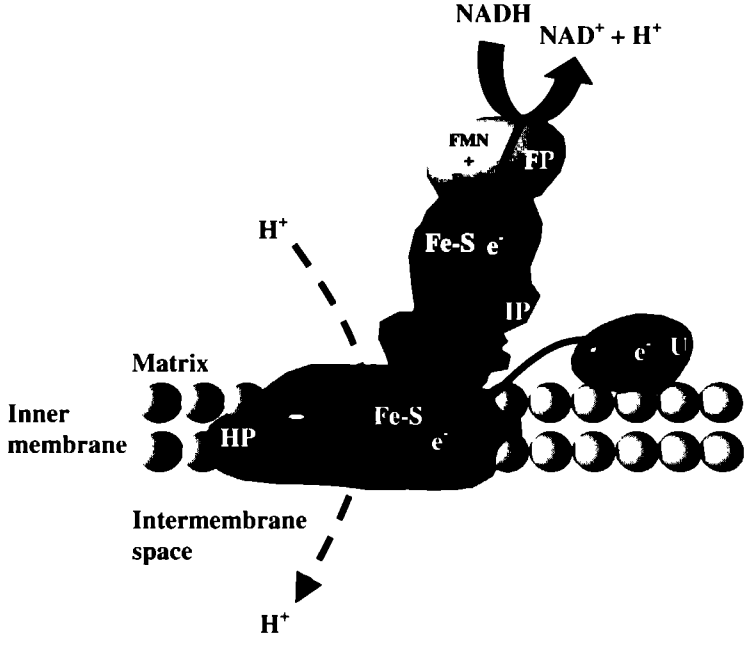

Figure 2.1 Human complex I Schematic overview of human complex I Electrons are transferred from NADH to ubiquinone through three distinct parts of complex I, namely the Flavoprotein (FP) fraction, Iron-Sulphur protein (IP) fraction and the Hydrophobic protein (HP) fraction. Simultaneously with this electron transport, protons are transported towards the intermembrane space. U = ubiquinone; FMN = flavin mononucleotide; Fe-S = Iron-Sulphur cluster, e = electron

others all mitochondrial encoded subunits) In the past few years, 34 of at least 35 human nuclear encoded complex I subunits have been characterised (Loeffen et al 1998a, Smeitink and van den Heuvel 1999)

Disorders of the oxidative phosphorylation (OXPHOS) system express a great variety in clinical symptoms (De Vivo 1993, Morris et al 1995, Jackson et al 1995, Munnich et al 1996) First presentation can occur in infancy with severe encephalopathy leading to early death, as well as in adulthood with mild exercise intolerance Similarly, the heredity patterns are also diverse varying from evident maternal, autosomal-dominant, autosomal-recessive to sporadic and X-linked In the early 1990s, Robinson and colleagues published about 40-isolated complex I deficient patients (Robinson 1993, Pitkanen et al 1996a) These patients expressed mainly four clinical phenotypes *severe neonatal lactic acidosis* (OMIM 245400 Hoppel et al 1987), *Leigh syndrome* (OMIM 256000, Leigh 1951), *cardiomyopathy with cataract and lactic acidosis* (OMIM 212350, Pitkanen et al 1996b) and *hepatomegaly with renal tubulopathy* (Robinson 1993)

Some of these well-described clinical entities are clearly associated with mutations in the mtDNA Important characteristics of mtDNA mutations are the maternal inheritance pattern (Giles et al 1980), age of disease presentation (often late childhood and adulthood) and variability in clinical course and intrafamilial presentation Previously identified mtDNA defects causing isolated complex I deficiency are listed in Table 2 1 In general, these abnormalities can be divided in two important groups, namely major rearrangements and point mutations The mtDNA point mutations linked to isolated complex I deficiency seem to concentrate in the $tRNA^{Leu}$ gene (OMIM 590050), $tRNA^{Lys}$ gene (OMIM 590060) and the ND genes (OMIM 516000-516006) No mutations in nuclear genes have been previously reported to cause isolated complex I deficiency

Our centre examines many tissue specimens of children suspected of OXPHOS disorders Respiratory chain enzyme activities are preferably measured in fresh skeletal muscle tissue and additionally in cultured skin fibroblasts Evaluation of enzymatic OXPHOS system measurements in skeletal muscle over a five year period revealed in 23% of the patients an isolated complex I deficiency, which makes it the most frequently found deficiency of the OXPHOS system diagnosed in our centre (Table 2 2) Family data concerning these patients usually points to autosomal-recessive traits In our experience, screening for common mtDNA mutations in these patients is unsatisfactory (in less than 5% of patients suspected of OXPHOS disorders, common mtDNA mutations are encountered) Therefore, we started a research project concerning mutational analyses studies in nuclear encoded complex I subunits as a first step in resolving the suspected nuclear cause of isolated complex I deficiency This approach has been successful since we encountered up to now mutations in five different pedigrees (OMIM 602141 Loeffen et al 1998b, OMIM 602694 van den Heuvel et al 1998, OMIM 601825 Triepels et al 1999a, OMIM 161015 Schuelke et al 1999)

In the present communication we review clinical, biochemical and genetic characteristics of our isolated complex I deficient patient group as established in cultured skin fibroblasts (n = 27), who all presented in infancy and young childhood In addition, we summarise data concerning mutations in nuclear encoded subunits as encountered in this group up to now and discuss the possible role of mtDNA and nuclear DNA (nDNA) mutations in isolated complex I deficiency

mtDNA abnormality	*Clinical phenotype*	*Reference*
Deletion	• diabetes, Fanconi syndrome • cerebellar ataxia, hypogonadotropic hypogonadism, choroidal dystrophy	Luder and Barash 1994 Barrientos et al 1997
A3243G $tRNA^{leu}$	• MELAS	Goto et al 1992
T3250C $tRNA^{leu}$	• isolated myopathy	Ogle et al 1997
A3251G $tRNA^{leu}$	• myopathy with lactic acidosis	Houshmand et al 1996
T3271C $tRNA^{leu}$	• MELAS	Kirby et al 1999
C3303T $tRNA^{leu}$	• isolated myopathy	Kirby et al 1999
T3394C ND1	• long QT syndrome	Matsuoka et al 1999
G3460A ND1	• LHON	Howell et al 1991, Majander et al 1991
A8344G $tRNA^{lys}$	• MERFF	Shoffner et al 1990
G8363A $tRNA^{lys}$	• MERRF	Arenas et al 1999
G11778A ND4	• LHON	Larsson et al 1991
G14459A ND6	• Leigh syndrome	Kirby et al 1999
T14484C ND6	• LHON	Oostra et al 1995

Table 2.1 mtDNA deletions and point mutations related to isolated complex I deficiency.

Isolated deficiencies	*%*	*Combined deficiencies*	*%*
Complex I	22.7%	**Complex I+IV**	7.9%
PDHc	19.9%	**Complex I+III**	3.0%
Complex III	10.3%	**Complex I+III+IV**	3.9%
Complex IV	10.0%	**Complex I+PDHc**	3.6%
Suc. cyt. c oxidoreductase	3.3%	**Complex I+IV+PDHc**	3.3%
Complex II	1.3%	**Complex IV+PDHc**	4.8%
		Other combinations	6.0%
Total	67.5%	**Total**	32.5%

Table 2.2 Occurrence percentages of isolated and combined OXPHOS disorders (and combinations with Pyruvate Dehydrogenase complex (PDHc) deficiency) diagnosed in skeletal muscle tissue, assembled at our centre over a five year period (n=331 diagnosed patients). Suc. cyt. c = succinate:cytochrome *c*.

Clinical phenotypes.

We studied clinical, biochemical and genetic aspects of 27 patients (21 males, 6 females), all diagnosed with isolated complex I deficiency in fibroblasts Several distinct phenotypes could be distinguished Clinical, biochemical and genetic details of these patients are listed in Table 2 3 The group Fatal Infantile Lactic Acidosis (FILA) is classified separately since they suffered from isolated complex I deficiency in fibroblasts, but combined complex I and IV deficiency in muscle tissue

Isolated complex I deficiency in cultured fibroblasts.

Leigh syndrome (LS)[01 07] and Leigh like syndrome (LLS[08 13])
Clinical criteria for the diagnosis of Leigh syndrome (Rahman et al 1996) are summarised in Table 2 4 We did not use the criteria concerning increased lactate levels in blood and/or CSF, because several of our patients, some with neuropathologically proven Leigh syndrome, do not have increased lactate levels in blood and/or CSF The main symptoms observed in our patients are listed in Table 2 3 Leigh syndrome was confirmed by the histopathological findings post mortem in seven families, but no neuropathological studies were available in six patients who are classified as suffering from Leigh-like syndrome All but one patient died within the first 5 years of life, six of them died within the first year Two patients showed normal lactate levels in blood and CSF (van den Heuvel et al 1998, Triepels et al 1999a), and in one of them (05), lactate levels remained within normal limits even after glucose loading

Macrocephaly with progressive leukodystrophy (MLD)
Macrocephaly in association with progressive leukodystrophy was the major clinical feature in two of our patients (Table 2 3) Clinical details of these patients have been reported previously (Dionisi-Vici et al 1997, Schuelke et al 1999)

Neonatal cardiomyopathy with lactic acidosis (NCLA)
Cardiomyopathy, congestive as well as hypertrophic, is a not uncommon sign in respiratory chain disorders (Antozzi and Zeviani 1997) Three patients presented at the first day of life with symptoms of hypertrophic cardiomyopathy and lactic acidosis One child died after 4 days, one at the age of 1 5 months, whereas the third child is currently alive at the age of 6 months The metabolic acidosis together with the neonatal cardiomyopathy are the only abnormalities observed in this latter patient In all three patients there was no sign of cataract (the combination of cardiomyopathy, lactic acidosis and cataract has been described as a separate clinical entity in complex I deficiency (Pitkanen et al 1996b))

Unspecified encephalomyopathy (UE)
Six of our patients showed variable neuromuscular symptoms that could not be assigned to a specific phenotype Virtually all of these patients were psychomotor retarded and had decreased muscle tone Four of these patients deteriorated and died within 3 years after presentation Two patients with a milder disease course expressed relatively unusual phenotypes Patient 23 presented at 7 4 years with extrapyramidal signs, muscle weakness and

Patient	Sex	Age of onset (mths)	Age of death (mths)	PMR	Seizures	Hypotonia	Pyramidal signs	Ataxia	Nystagmus	Macrocephaly	Microcephaly	Cardiomyopathy	Resp. disturbance	+ Family history	Consanguinity	Abnormal CT/MRI	RRFs	Blood lactate [3]	L/P ratio [4]	CSF lactate [5]	Fibroblasts % [6]	Fresh muscle % [7]	Frozen muscle % [8a+b]	Affected gene	Mutation
Isolated complex I deficiency in cultured fibroblasts																									
Leigh and Leigh-like syndrome (LS and LLS)																									
01	m	4	7	×	×		×						×			×		3.7	21	3.8	67		n[a]		
02	m	2	6	×	×	×	×		×				×	×		×		7.3	32	3.6	64				
03	f	0.5	3	×		×			×			×	×	×		×		6.1			25		13[a]		
04	m	1	2	×	×	×	×					×	×			×		3.4	20	5.6	63		23[a]	NDUFS8	P79L, R102H
05	m	11	60	×		×	×						×	×		×		n	n	n	62	23		NDUFS7	V122M
06	f	0	1		×	×							×			×		5.4			33	26			
07	m	26	28	×		×			×				×			×		4.2		3.2	59				
08	m	8	16	×	×	×							×			×		n		n	68			NDUFS4	5 bp dupl.
09	m	6	24	×		×	×		×			×	×	×	×	×		9.9		7.8	39		39[b]		
10	f	0.3	4			×					×			×	×	×		5.5	34	7.6	54				
11	f	0	23	×	×	×	×					×[9]	×		×	×	×	6.2	50	5.8	35		24[a]		
12	m	9	-	×	×	×	×		×							×		6.7	41	5.0	38	44			
13	m	9	48	×	×	×							×			×		3.6	52	3.0	33		38[a]		
Neonatal Cardiomyopathy with Lactic Acidosis (NCLA)																									
14	m	0	1.5			×						×	×		×		×	4.9	38	3.8	61				
15	m	0	-									×						4.3	51	5.0	55	14			
16	m	0	4d									×						24			36	24			

Table 2.3 continued.

Patient	Sex	Age of onset (mths)	Age of death (mths)	PMR	Seizures	Hypotonia	Pyramidal signs	Ataxia	Nystagmus	Macrocephaly	Microcephaly	Cardiomyopathy	Resp. disturbance	+ Family history	Consanguinity	Abnormal CT/MRI	RRFs	Blood lactate [3]	L/P ratio [4]	CSF lactate [5]	Fibroblasts % [6]	Fresh muscle % [7]	Frozen muscle % [8b+c]	Affected gene	Mutation
Clinical characteristics																		**Biochemistry**					**Enzyme activity**	**Genetics**	
Macrocephaly with progressive Leukodystrophy (MLD)																									
17	m	2	6	×	×	×	×			×		×				×		11		17	24		19[b]		
18	f	6	-	×	×	×				×						×		n		4.0	36			NDUFV1	A341V
Unspecified encephalomyopathy (UE)																									
a. Progressive																									
19[1]	m	5	14	×	×	×						×				×		2.3		3.1	73	35		NDUFV1	R59X, T423M
20[1]	m	7	17	×	×	×						×						3.4		3.0	64	45		NDUFV1	R59X, T423M
21	m	10	36	×		×				×			×	×	×			5.0		3.3	45	68			
22	f	0	14	×	×	×							×		×			3.3	14		42		33[b]		
b. Stable																									
23	m	89	-	×														n	39	3.4	35		20[b]		
24	m	9	-	×		×	×	×										3.4	44	2.4	70	41			
Isolated complex I deficiency in cultured fibroblasts and combined complex I and IV deficiency in muscle tissue																									
Fatal Infantile Lactic Acidosis (FILA)																									
25[2]	m	0	0.5			×							×	×				24	167		41				
26[2]	m	0	0.5				×						×	×				11	126		55		9[c]		
27	m	0	2	×	×								×					13	102	4.7	45	51			
		4.6	**12.7**																		**49**	**32**[10]			

Table 2.3 Clinical, biochemical and genetic characteristics as encountered in our patient group. PMR = psychomotor retardation; Resp. = respiratory; RRFs = ragged red fibres; mths = months. [1+2] patients are brothers; [3] control range 0.6-2.1 mmol/l; [4] control range 11.5-16.5; [5] control range 1.4-1.9 mmol/l; [6] control range 0.11-0.28 mU/mU cytochrome *c* oxidase (n=14); [7] control range 0.070-0.25 mU/mU citrate synthase (n=20); [8a] control range 0.084-0.273 mU/mU citrate synthase (n=13) (homogenate); [8b] control range 0.101-0.389 mU/mU cytochrome *c* oxidase (600×*g*); [8c] control range 8.9-27 mU/mg protein (600×*g*); [9] congestive; [10] average of residual complex I activity in fresh and frozen muscle tissue. All complex I activities are expressed as percentage of the lowest reference value. Bottom row lists mean values.

Criteria of Leigh syndrome.	
Clinical (Leigh and Leigh-like syndrome):	***Neuropathological (Leigh syndrome):***
1. Progressive neurological disease with motor and intellectual developmental delay	1. Bilateral, symmetrical spongiform lesions
2. Signs and symptoms of brainstem and/or basal ganglia disease	2. Vacuolation of the neuropil
3. Characteristic features of Leigh disease on neuroimaging	3. Relative sparing of neurons
	4. Demyelination
	5. Gliosis
	6. Capillary proliferation

Table 2.4 Clinical and neuropathological criteria of Leigh syndrome as published by Rahman et al 1996. Patients whom we classified under Leigh syndrome met the clinical as well as the neuropathological criteria, while in patients with Leigh-like disease no neuropathological studies were performed.

optic nerve atrophy. The previous history was unremarkable except for mild psychomotor retardation. CSF lactate was elevated, but brain MRI and muscle histochemical studies revealed no abnormalities. He has remained relatively stable and is currently 10 years old. The other patient presented at the age of 9 months with severe ataxia and mild psychomotor retardation. Brain MRI showed severe cerebellar destruction while other brain parts were completely intact. The boy has subsequently remained stable and is currently 6 years old.

Isolated complex I deficiency in cultured fibroblasts, combined I and IV deficiency in muscle tissue.

Fatal infantile lactic acidosis (FILA).

One of the most serious phenotypes associated with OXPHOS disorders in general and complex I deficiency in particular is FILA. This phenotype is characterised by extremely high lactate levels in blood and CSF and high L/P ratios, indicating a severely disturbed NADH/NAD^+ ratio and demanding rigorous intervention of respiratory complications and severe metabolic acidosis. Three of our patients (all males, two are siblings) presented shortly after birth with tachypnoea and extreme metabolic acidosis caused by persistently high levels of lactate (Table 2.3). The two brothers rapidly deteriorated, eventually resulting in severe cardiorespiratory insufficiency and died within two weeks after birth. The third male infant (described previously by Bentlage et al in 1996) presented at birth with similar characteristics. All three infants expressed isolated complex I deficiency in fibroblasts (complex IV activity was repeatedly completely normal in different fibroblast cultures); however in skeletal muscle tissue severe complex I and IV deficiencies were found.

Enzyme studies.

Activities of respiratory chain enzyme complexes, pyruvate dehydrogenase complex (PDHc) and citrate synthase in muscle cells (quadriceps muscle biopsy) and skin fibroblasts (culture) were measured using the following enzyme assays (slightly modified): NADH:ubiquinone oxidoreductase and succinate:cytochrome *c* oxidoreductase as described by Fischer et al (1985 and 1986); cytochrome *c* oxidase by Cooperstein and Lazarow (1951); citrate synthase by Srere (1969); and PDHc according to van Laack et al (1988). In muscle tissue, enzyme

assays were performed in crude homogenates or 600*g* supernatants of crude homogenates, while in fibroblasts mitochondrial enriched fractions were used The enzyme assays performed in cultured skin fibroblasts were slightly modified according to procedures as described by Bentlage and co-workers in 1996 We considered a patient isolated complex I deficient when the activity was evidently decreased below the reference interval (0 11-0 28 mU/mU cytochrome *c* oxidase) in two different fibroblast preparations In contrast to the complex I activity in muscle tissue which is usually referenced to citrate synthase activity, cytochrome *c* oxidase has been used as marker enzyme in fibroblasts This is because the activity of citrate synthase can be artificially decreased during the mitochondria enrichment process of fibroblasts due to leakage out of the mitochondrial matrix The other fibroblast respiratory chain enzyme activities had to be within normal limits as determined in a representative control population Complex I activity in fibroblasts was reduced in all patients (inclusion criterion), with a mean residual activity of 49% of the lowest control value (range 24% to 73%, Table 2 3) The complex I activity in skeletal muscle has been measured in 20 patients In half of these patients, enzyme assays were performed in fresh muscle tissue, while in the other half muscle tissue has been deep frozen in liquid nitrogen after biopsy Measurements in fresh tissue preserve the possibility to measure oxidation rates and ATP and CrP production rates All but one patient showed reduced complex I activity (mean 32% of the lowest control value, range 9% to 68%, Table 2 3) When residual complex I activities of muscle tissue are compared to those of fibroblasts, 8 patients showed a more severely decreased activity in skeletal muscle (>15% additional decrease compared to fibroblasts), 10 patients expressed comparable values (between 0% to 15% variation), while in only 2 patients complex I activity was actually higher in skeletal muscle (>15%)

Molecular-genetic studies.

All patients included in our study were screened for major mtDNA rearrangements by a previously developed long template PCR technique (Li et al 1995) This revealed a fragment of approximately 16 5 kb in all patients Furthermore, the following common pathogenic mtDNA mutations were excluded in fibroblasts in our patient group by restriction enzyme analysis A3243G ($tRNA^{Leu}$), T3271C ($tRNA^{Leu}$), G3460A (ND 1), T4160A (ND-1), A4317G ($tRNA^{Gln}$), A8344G ($tRNA^{Lys}$), T8356C ($tRNA^{Lys}$), T8993G/C (ATPase 6), G11778A (ND-4), G14459A (ND-6), T14484C (ND-6)

Up till now, 10 nuclear encoded complex I subunits have been screened in twenty of our patients by a combination of RT-PCR and direct DNA sequencing Candidate genes for mutational analysis studies were chosen on the basis of strong conservation during evolution and/or speculated functional aspects The *NDUFV1* (*51 kDa*) gene revealed an A341V mutation in a patient with macrocephaly and progressive leukodystrophy (18) and compound heterozygous mutations resulting in a R59X and T423M in two brothers (19 and 20) suffering from a progressive unspecified encephalomyopathy (Schuelke et al 1999) The *NDUFS4* (*18 kDa*) gene contained a 5 bp duplication in a patient with Leigh-like syndrome (08, van den Heuvel et al 1998) A mutation was also found in the *NDUFS7* gene (V122M) in two brothers with Leigh syndrome (05, Triepels et al 1999a) and in the *NDUFS8* gene (compound heterozygous mutations resulting in P79L and R102H in a patient with Leigh syndrome (04,

Loeffen et al 1998b) Direct sequencing of the *NDUFS5* (*15 kDa*) (Loeffen et al 1999), *NDUFA1* (*MWFE*) (Loeffen et al 1998c), *NDUFB3* (*B12*), *NDUFB6* (*B17*) (Smeitink et al 1998a), *NDUFAB1* (*SDAP*) (Triepels et al 1999b) and the *NDUFA8* (*PGIV*) (Triepels et al 1998) cDNAs revealed no mutations

Discussion.

During the last decade substantial progression has been made in the elucidation of complex I composition and its physiological aspects of many prokaryotes and eukaryotes, including man (for a review see Smeitink et al 1998b) The cDNA structure has been elucidated for almost all known human complex I subunits, while the gene structure has been solved of about seven genes Many important genetic aspects of the complex I subunits remain to be elucidated, like transcription and translation regulation, possible tissue specificity, but also interaction processes between the nuclear and mitochondrial genomes Also largely unknown is the targeting of complex I proteins towards and into mitochondria, the assembly of complex I and the implantation into the inner mitochondrial membrane

Information concerning pathophysiological aspects of complex I deficiency remains also scarce Studies discussing clinical aspects of complex I deficient patients published by the group of Robinson and very recently by the group of Thorburn and Dahl (Robinson 1993, Pitkanen et al 1996a, Kirby et al 1999) describe important clinical aspects, however differences with our patient population remain

In our group of 27 patients a strong male preponderance is present (male female = 3 5 1) Male predominance in complex I deficiency has been reported previously (Zhuchenko et al 1996, von Kleist-Retzow et al 1998, Kirby et al 1999) Only one complex I subunit is known to be encoded on the X-chromosome (NDUFA1), in which no mutations have been reported The mean age at presentation in the entire group was 4 6 months All but five patients have died within the first five years of life (mean age of survival after first presentation among the deceased patients was 8 6 months) 67% of our patients died in the first two years of life Four of the survivors express a relatively stable course though severely disabled From this data it can be concluded that life expectancy of patients with an isolated complex I deficiency is very poor

We distinguished six different phenotypes within our patient group Leigh syndrome, Leigh-like syndrome, macrocephaly with progressive leukodystrophy, neonatal cardiomyopathy with lactic acidosis, unspecified encephalomyopathy (progressive or stable) and fatal infantile lactic acidosis (FILA) Compared to the phenotypes described by Robinson and Thorburn similarities, but also differences can be observed Both studies describe in keeping with our study Leigh syndrome as an important, if not the most important phenotype in isolated complex I deficiency 26% of our patient group was neuropathologically proven to suffer from this clinical entity When Leigh-like patients are added to this group the percentage increases to 48% Leigh syndrome is more and more recognised as an important phenotype in disorders of energy metabolism in general and complex I deficiency in particular (Morris et al 1996, Rahman et al 1996) It is possible that most if not all patients present in our Leigh-like group are actually patients suffering from Leigh syndrome Neuropathological studies, which provide the ultimate evidence, were not performed in these patients

Cardiomyopathy is an important and often observed finding in disorders of energy metabolism In this study, eight patients suffered from cardiomyopathy (30%), which was in 7 patients hypertrophic and in 1 patient congestive (Table 2 3) The only two patients in our group with signs of RRFs suffered from cardiomyopathy RRFs are often associated with mutations in structural genes of the mtDNA We excluded the most common mtDNA mutations, however the possibility of unrecognised mutations remains The facts that family history in both patients (especially the maternal lineage) was negative for neuromuscular disorders and parents were consanguineous points in the direction of autosomal-recessive inheritance The phenotype of the two patients with RRFs did not differ significantly from the other patients in their group Robinson and colleagues have published several patients with cardiomyopathy, cataract and complex I deficiency All eight patients with cardiomyopathy in our group showed no signs of lens opacities Several patients have been described by our centre with a characteristic phenotype of cardiomyopathy and cataract but without complex I deficiency (Sengers et al 1985, Smeitink et al 1989) Patients classified in our residual group presented mainly with symptoms of encephalomyopathy Additional diagnostic studies could not provide further clues for a more defined phenotype and autopsy has not been performed Two patients in this group are alive at this moment and express a relatively stable course It is possible that mtDNA mutations, which tend to cause a more prolonged stable course are present in these latter two patients, yet family history did not provide evidence for maternal inheritance and common mtDNA mutations were not encountered

An important diagnostic determinant in disorders of energy metabolism is the lactate concentration in blood and CSF Twenty-three of our patients (85%) clearly expressed elevated blood lactate levels, while in four patients blood lactate remained within normal limits CSF lactate was measured in twenty-one patients, and turned out elevated in all but two patients (90%) The two patients with normal lactate levels in CSF also expressed normal lactate levels in blood Importantly, in both of these patients mutations have been found in nuclear encoded subunits of complex I (*NDUFS7* and *NDUFS4*, respectively) Two of the patients with normal lactate levels in blood actually did have elevated lactate levels in CSF Strikingly, both patients expressed a relatively stable course It is possible that lactate levels change during the disease process They can be normal in relatively "good" disease periods, while they increase during disease worsening Many of the patients admitted to our centre have been referred by physicians from general hospitals Increased lactate levels in blood or CSF remain an important determinant whether or not to refer to a metabolic disease centre It is therefore quite possible that the percentage of elevated lactate levels in complex I deficiency is an overestimation of the actual situation Respiratory chain disorders with normal lactate levels could remain undetected, although generally lactic acid elevation is the rule rather than the exception The results of our study indicate that lactate measurements have to be performed in blood as well as CSF However, normal values in blood and CSF certainly do not exclude complex I deficiency

Neuroimaging studies performed in patients suspected of or suffering from disorders of energy metabolism are generally considered to be very useful Many abnormalities have been reported varying from general brain destruction with wide cavitations, spotlike hypodense (CT) or hyperdense (T2 weighted MRI) lesions, cortical atrophy and generalised demyelination In our patient group, CT and/or MRI scans have been made in eighteen

patients These resulted in typical lesions e g in basal ganglia in all patients with Leigh and Leigh-like syndrome Cortical atrophy was observed in five patients (distributed in different clinical subgroups) One patient with clear ataxic symptoms only expressed destructive lesions in the cerebellum Brain scans were normal in only one patient Since abnormalities seen with neuroimaging are often present in patients with disorders of energy metabolism, it is not generally used for specific diagnostic purposes The importance of (successive) neuroimaging is to provide information about the extend of disease and disease progression as well as therapeutic effects

Differences in residual respiratory chain enzyme activities between tissues have been previously reported (Bentlage et al 1996) This is especially the case in the three FILA patients with isolated complex I deficiency in fibroblasts (complex IV activity repeatedly normal), whereas complex I and IV were decreased in muscle tissue Fatal infantile lactic acidosis (FILA) is characterised by extremely high lactate levels in blood and CSF and high L/P ratios, indicating a severely disturbed NADH/NAD^+ ratio and demanding rigorous intervention of respiratory complications and severe metabolic acidosis There are several explanations possible for these intriguing tissue specific phenomena Tissues can express isoforms of proteins involved in complex I function as, for example, has been described for complex IV This may concern proteins, which are actually within the complex, but also assembly or chaperone proteins In addition, differences in tissue stoichiometry of individual subunits may result in different residual complex I activities Since virtually all pathogenic mtDNA mutations are heteroplasmic it is also possible that tissues express different amounts of mutated mtDNA resulting in different residual activities (especially tRNA mutations are prone to result in combined deficiencies) Furthermore, it is possible that complex IV is secondarily affected by complex I (e g by oxygen radical production due to malfunctioning of complex I), which could be more pronounced in muscle tissue due to its higher energy demand Several of these intriguing phenomena are subjects of future studies In several patients presented to our centre, respiratory chain enzyme deficiencies were found in muscle tissue, while enzyme assays performed in cultured fibroblasts turned out completely normal It is therefore important to perform diagnostic studies of the OXPHOS system in at least two different tissues

From the end of the eighties up to now, mutational analysis studies performed in patients with OXPHOS disorders mainly concerned mtDNA Shoffner (1996) reported common pathogenic mtDNA point mutations and rearrangements in patients with OXPHOS disorders to occur in 30% of investigated adults and only in 4% of children Many reports of pathogenic mtDNA mutations describe patients who clinically presented in late childhood and adulthood, nevertheless onset in infancy has also been reported A literature study concerning mtDNA deletions and point mutations which have been related to isolated complex I deficiency revealed 14 mutations (two deletions and twelve point mutations) However, several patients have been described with mtDNA mutations in which respiratory chain enzyme assays were not performed Most mutations in mitochondrial encoded tRNAs and rRNAs result in multiple respiratory chain complex deficiencies, but the possibility of single affected complexes in individual patients always remains Mutations in the mitochondrial encoded $tRNA^{Leu}$ gene can result in isolated complex I deficiency In some of our patients the complete mitochondrial encoded $tRNA^{Leu}$ gene has been sequenced which revealed no

mutations (unpublished results) In the study performed by Kirby and colleagues (Kirby et al 1999) sequencing of the $tRNA^{Leu}$ of 51 complex I deficient patients revealed just one novel mutation and five known mutations

Many pedigrees in our patient population indicate autosomal-recessive inheritance and family history did not reveal patients with similar phenotypes in the maternal lineage Seven non-related patients were born from consanguineous parents (26%) As a start to elucidate the hypothesised nuclear cause of isolated complex I deficiency we sequenced the highly conserved structural complex I genes of twenty isolated complex I deficient patients, which revealed mutations in seven patients (five pedigrees) Strikingly, five of these patients (three pedigrees) expressed a Leigh(-like) phenotype, which was also the case in the published patient with a complex II mutation, as well as in the patients with mutations in the Surfeit-1 gene (Bourgeron et al 1995, van den Heuvel et al 1998, Loeffen et al 1998b, Tiranti et al 1998, Zhu et al 1998, Triepels et al 1999a) In both families with more than one affected child in which nuclear mutations have been found (V122M in NDUFS7 subunit and R59X and T423M in NDUFV1 subunit) the brothers expressed a strongly resembling clinical phenotype This is in contrast with mutations found in mtDNA, where affected relatives often express variable clinical phenotypes

In summary, we presented, biochemical, genetic and clinical data concerning 27 patients with isolated complex I deficiency expressed in cultured fibroblasts OXPHOS system enzyme assays in cultured fibroblasts and skeletal muscle tissue in general reveal similar results, but for complete diagnostics, we recommend enzyme measurements to be performed in at least two different tissues to minimise the possibility of overlooking the enzymatic diagnosis Lactate levels in blood and CSF and cerebral CT/MRI studies are highly informative, though normal findings do not exclude complex I deficiency With the discovery of mutations in nuclear encoded complex I subunits, adequate pre- and postnatal counselling becomes available Finally, considering information currently available, isolated complex I deficiency in children seems in the majority to be caused by mutations in the nuclear DNA

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THE X-CHROMOSOMAL *NDUFA1* GENE OF COMPLEX I IN MITOCHONDRIAL ENCEPHALOMYOPATHIES: TISSUE EXPRESSION AND MUTATION DETECTION

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Introduction.

The electron transport chain, the final step in the generation of ATP, consists of four protein enzyme complexes, of which NADH:ubiquinone oxidoreductase (complex I) is the largest. Complex I contains at least 42 subunits, seven of which are encoded by the mitochondrial DNA (ND1-6 and ND4L), nuclear genes encode the remainder (Hatefi 1985, Walker et al 1992, Robinson 1993). Complex I catalyses the transfer of electrons from NADH to ubiquinone, which is coupled to the translocation of hydrogen atoms across the inner mitochondrial membrane. Patients described with a (partially) complex I deficiency can generally be categorised in two major clinical phenotypes: an isolated myopathy, and a multisystem disorder, with predominantly encephalopathy.
Respiratory chain defects may be inherited as autosomal or X-linked Mendelian traits (Orstavik et al 1993, Zhuchenko et al 1996), or in case of certain mutations in mitochondrial DNA, as maternal traits. To date, no mutations in a nuclear encoded subunit of complex I have been described. In our biochemically proven complex I deficient patients as well as among the affected sibs, (latter actually not all biochemically evaluated), we observed a strong male preponderance, suggestive for X-linked inheritance.
Recently, the *NDUFA1* gene, one of the nuclear encoded complex I genes, was isolated, and mapped to chromosome Xq24 (Zhuchenko et al 1996). The *NDUFA1* gene is composed of three exons, and spans about 5.0 kb of genomic DNA. It shows 80% homology to the bovine MWFE subunit of complex I. The knowledge of function of the human NDUFA1, and the bovine MWFE subunit is very limited. The bovine MWFE subunit is thought to be situated in the extrinsic membrane domain of complex I (Walker 1992).
We now report the results of a mutation analysis study of the *NDUFA1* gene in a patient group with an isolated decreased activity of complex I in cultured skin fibroblasts. We describe the offspring distribution of patient families, and we give an outline of the pattern of tissue distribution of the mRNA transcript from the *NDUFA1* gene.

Patients, material and methods.

Seventeen patients (three females, and fourteen males, sex ratio 0.18), with an isolated decreased activity of NADH:ubiquinone oxidoreductase measured in cultured skin fibroblasts, were selected for a mutation analysis study of the cDNA sequence of the *NDUFA1* gene. Frequently encountered clinical features in our patient group were hypotonia (generalised, and/or axial), failure to thrive, seizures, psychomotor retardation, and respiratory distress. Lactic acidaemia, increased blood lactate/pyruvate ratio's, and increased cerebrospinal fluid lactate, were present in most patients. The patients come from fifteen families (two families have two sons represented in the patient group). In eleven families the pedigree could be retrieved. Three families (two male, and one female patient) were known with consanguinity.
The enzymatic activity of complex I was measured in cultured skin fibroblasts according to established techniques (Bentlage et al 1996). Reference values ranged from 0.11-0.28 mU/mU cytochrome *c* oxidase. Patient values ranged from 0.026 to 0.080 mU/mU cytochrome *c* oxidase.

To examine whether mutations in the *NDUFA1* gene are a common cause of isolated complex I deficiency, skin fibroblasts from all patients were cultured, and harvested after reaching confluency Total RNA was extracted, and 5 µg of RNA was reverse transcribed to cDNA by Superscript II RNase H reverse transcriptase (Life Technologies, The Netherlands), using oligo(dT), and random hexanucleotides (Ploos van Amstel et al 1996). A sample of the cDNA was amplified by Polymerase Chain Reaction (PCR), using primers designed according to the published sequence of the *NDUFA1* gene The generated PCR fragment spanned the entire coding region of the *NDUFA1* cDNA Both strands were screened for the presence of mutations in the cDNA of the NDUFA1 subunit by direct sequencing, performed by automated sequencing (ABI Prism, model 377 version 2 1 2), using the ABI Prism Taq DyeDeoxy terminator cycle sequencing ready reaction kit (Perkin Elmer, The Netherlands)
A master blot, containing poly(A)$^+$ RNA isolated from various human tissues was purchased from Clontech, The Netherlands The quantity of mRNA spotted for each tissue onto the master blot was normalised using eight different housekeeping gene transcripts as probes (ribosomal protein S9, ubiquitin, 23 kDa highly basic protein, hypoxanthine guanine phosphoribosyl transferase, tubulin, β-actin, phospholipase, and glyceraldehyde triphosphate dehydrogenase) A cDNA probe homologous to human *NDUFA1* was prepared by random primed labelling (Sambrook et al 1989), and hybridised to the master blot The filter was washed under stringent conditions, and analysed by autoradiography

Results.

The offspring distribution could be determined in eleven families, and consisted of seventeen subjects Seven were female, all healthy, ten were male, two healthy, and eight with proven decreased activity of complex I, or expressing a clinical phenotype strongly resembling the symptoms of the brother/sister in the patient group.
We analysed the cDNA of the *NDUFA1* gene of all patients with complex I deficiency for abnormalities by direct sequencing, which revealed no mutations, nor polymorphisms We compared the transcription levels of *NDUFA1* mRNA in foetal, and adult human tissues to quantify the relative abundance of *NDUFA1* mRNA We used a dot blot containing carefully normalised quantities of poly (A$^+$) RNA from a wide range of human tissues In foetal stages, *NDUFA1* mRNA was detected in all tissues tested without marked differences, but a clearly high amount was observed in foetal heart In adult stages dots with higher intensity were seen in (in descending order) human heart, skeletal muscle, liver, kidney, and gland tissues (see Fig 3 1) A diversity of brain structures expressed a moderate activity of the NDUFA1 subunit compared to human heart and skeletal muscle

	1	2	3	4	5	6	7	8
a	whole brain	amygdala	caudate nucleus	cerebel-lum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
b	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	subthal. nucleus	spinal cord	
c	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
d	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
e	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
f	appendix	lung	trachea	placenta				
g	foetal brain	foetal heart	foetal kidney	foetal liver	foetal spleen	foetal thymus	foetal lung	
h	yeast RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	poly r(A) 100 ng	human C_0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

Fig. 3.1a Multiple tissue Master blot. The type and position of poly(A^+) RNAs and controls dotted on the positively charged nylon membrane. subthal. = subthalamic.

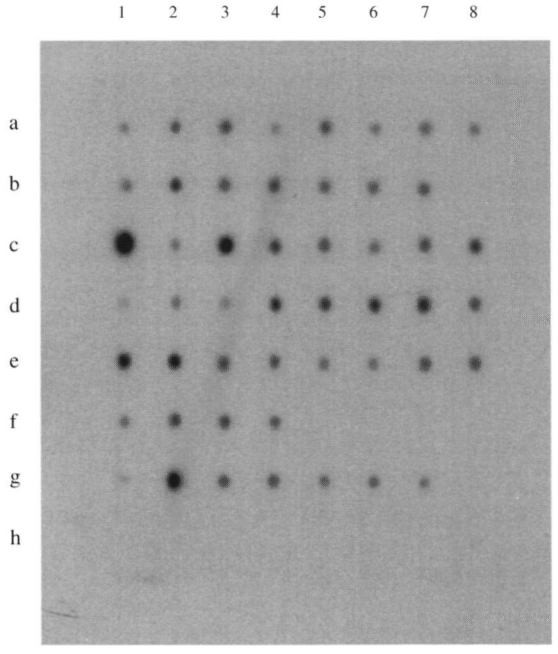

Figure 3.1b Human RNA multiple tissue blot data. Hybridisation was performed with a ^{32}P-labelled cDNA probe of the NDUFA1 subunit.

Discussion.

The purpose of this mutation analysis study was to investigate the hypothesis of X-linked inheritance patterns in complex I disease as suggested by the strong male preponderance in our patientgroup and among the affected children in the sibs group.
In literature many mutations in mitochondrial encoded genes of subunits of NADH:ubiquinone oxidoreductase have been described and linked to specific clinical presentations (for a review see Zeviani and Taroni 1994). The fact that in many patients with an isolated decreased activity of complex I, a molecular defect in mtDNA has not been found, combined with the fact that at least 35 subunits are encoded on the nuclear genome, make defects in nuclear encoded subunits of the NADH:ubiquinone oxidoreductase certainly possible.
Several human nuclear genes encoding for subunits of complex I have been cloned, and mapped to a specific chromosome (Procaccio et al 1997, Pata et al 1997, Gu et al 1996, Hyslop et al 1996, Hattori et al 1995, de Coo et al 1995, Ali et al 1993), but until now only one gene has been located on the X chromosome (Zhuchenko et al 1996). Up to now, no mutation analysis studies in nuclear encoded NADH:ubiquinone oxidoreductase subunits have been described in literature.
We selected patients with an isolated decreased activity of complex I measured in cultured skin fibroblasts. The mutation analysis study was performed on cDNA obtained from RNA isolated out of cultured skin fibroblasts. This study revealed no mutations in the *NDUFA1* cDNA, so we conclude that mutations in this gene are not a frequent cause of isolated complex I deficiency. For many genes encoding subunits of NADH:ubiquinone oxidoreductase the sequence, and chromosomal localisation remain to be elucidated, so other genes responsible for complex I disease might be located on the X chromosome.
We studied the tissue, and developmental expression of the *NDUFA1* mRNA because measurements of complex I activity in different tissues are not always conclusive. Some patients express reduced complex I activity in fibroblasts, others only in muscle or liver. In addition, isolated complex I disease gives a variety of clinical phenotypes, possibly because of tissue specific, or developmental expression of complex I subunits. The human RNA master blot expressed the *NDUFA1* mRNA in all human tissues present on the blot, and as expected a high expression was found in human heart, foetal heart, skeletal muscle, liver, and kidney (tissues with high metabolic activity). The central nervous system, known for it's high energy demand, is very often affected in complex I disease. Especially the basal nuclei are frequently involved in the disease process, which could be reflected in an increased dot intensity seen in the putamen compared to whole brain. An alternative explanation for differences of cDNA expression of the *NDUFA1* gene seen on the master blot, is the fact that it reflects the mRNA expression of the *NDUFA1* gene, so there may be a discrepancy between the mRNA expression, and the actual enzyme activity.
We remain looking for other nuclear encoded genes in general, and X-linked genes in particular, as candidate for complex I deficiency in our patients.

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MOLECULAR CHARACTERISATION AND MUTATIONAL ANALYSIS OF THE HUMAN NDUFB6 (B17) SUBUNIT OF THE MITOCHONDRIAL RESPIRATORY CHAIN COMPLEX I

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Abstract.
Bovine NADH ubiquinone oxidoreductase (complex I) of the mitochondrial respiratory chain consists of about 35 nuclear-encoded subunits We review the current knowledge of the 15 human complex I subunits cloned so far, and report the 567-bp cDNA sequence, the chromosomal localisation and the tissue expression of an additional subunit, the NDUFB6 subunit The cDNA open reading frame of *NDUFB6* comprises 387 bp and encodes a protein of 128 amino acids (calculated M_r 15 5 kDa) There is 82 7% and 78 1% homology, respectively, at the cDNA and amino acid level with the bovine counterpart The gene of the NDUFB6 subunit has been mapped to chromosome 2 A multiple-tissue dot-blot showed ubiquitous expression of the mRNA with relatively higher expression in tissues known for their high-energy demand Of these, kidney showed the highest expression Mutational analysis of the subunit revealed no mutations or polymorphisms in 20 patients with isolated enzymatic complex I deficiency in cultured skin fibroblasts

Introduction.

The molecular biology of complex I (NADH ubiquinone oxidoreductase), the first multi-subunit enzyme complex of the mitochondrial respiratory chain, has been thoroughly investigated in *Bos taurus* The complex has about 42 subunits with a combined molecular mass of 970 kDa (Fearnley et al 1989, Walker 1992, Fearnley et al 1994, Walker 1995, Walker et al 1995, Skehel et al 1998) Of these, seven subunits are encoded by the mitochondrial genome (ND1 6, ND4L) (Gibb and Ragan 1990) The remaining subunits are encoded by the nuclear genome The results of studies with the fungus *Neurospora crassa* and bovine studies have revealed important information concerning the spatial and functional organisation of complex I (Hatefi 1985, Walker 1992, Belogrudov and Hatefi 1994, Guenebaut et al 1997) It is likely that, as in *N crassa* bovine complex I has an L-shaped configuration with an extrinsic globular domain attached to an intrinsic domain (Walker 1995, Guenebaut et al 1997) In the late 1960s, it was found that most of the electron carriers of complex I, which are predominantly located in the extramembranous part of complex I, can be isolated as two water-soluble complexes a flavoprotein (FP) and an iron-sulphur protein (IP) fraction (Hatefi and Stempel 1967) Both subcomplexes make contact through the 51- and 75-kDa subunits (Belogrudov and Hatefi 1994) The FP fraction (51, 24 and 10 kDa) has, in contrast to the IP and the water-insoluble aggregate HP (hydrophobic protein fraction), catalytic properties (Ohnishi et al 1985) The IP fraction contains at least the 75-, 49-, 30-, 18-, 15-, 13 kDa and B13 subunits The HP fraction contains the seven mitochondrial (mt) DNA-encoded subunits and about 25 nuclear encoded subunits In man the cDNA or gene structure of complex I has currently been determined for 15 nuclear encoded subunits (Pilkington and Walker 1989, Chow et al 1991, Spencer et al 1992, Hyslop et al 1996, Zhuchenko et al 1996, Kim et al 1997, Pata et al 1997, Procaccio et al 1997, Ton et al 1997, van den Heuvel et al 1998) These human complex I subunits, and, if known, their chromosomal localisation are summarised in Table 4 1 Information about the function of the individual human subunits is limited in man, and only a few mutational analyses have been performed (van den Heuvel et al 1998, Loeffen et al 1998) Our most important reasons for investigating the nuclear encoded complex I subunits in man are (1) to unravel the origin of the phenotypic complexity encountered in patients with inherited complex I deficiency and (2) to develop reliable tools for prenatal diagnosis Furthermore, assessment of nuclear mutations in complex I-deficient patients will extend our knowledge of the functional properties of the individual subunits

The incidence of mitochondrial disorders, to which complex I deficiencies of the respiratory chain belong is approximately 1 10,000 living births (Bourgeron et al 1995) The clinical presentation and course of complex I-deficient patients vary greatly from neonatal death due to a multisystem disorder to mild exercise intolerance (Pitkanen et al 1996a) The observed differences in complex I-deficient phenotypes may, at least partially, be explained by genetic differences in the various complex I subunits So far, only a few mtDNA mutations have been associated with complex I deficiency (Pitkanen et al 1996b) The exact composition of complex I, the number of individual subunits and their functional properties may differ in various developmental stages as well as in various tissues Clay and Ragan (1988) for example, showed that rat liver, kidney and lung were lacking the 17- and 18 kDa proteins

found in brain, heart and skeletal muscle.

In an attempt to elucidate the molecular defects in complex I-deficient patients we recently, started a research program in order to (1) determine the human cDNA and gene structure of all nuclear-encoded subunits of the respiratory chain complex I, (2) determine the chromosomal localisation of the various genes of these subunits, (3) obtain information regarding the tissues in which this complex is expressed, and, finally, (4) perform a mutational analysis of our complex I-deficient patients. By this approach we very recently found the first patient with a pathological mutation in one of the nuclear-encoded subunits of this intriguing enzyme complex (van den Heuvel et al 1998). Here, we review the present knowledge of human nuclear-encoded complex I subunits, and report the characterisation and the results of a mutational study of the human NDUFB6 subunit of this complex.

Human name	*Bovine name*	*Chromosome*	*Reference*
NDUFV1	51 kDa	11q13	Spencer et al 1992
NDUFV2*	24 kDa	18p11.31-11.2	Pilkington & Walker 1989
NDUFV3	10 kDa	21q22.3	de Coo et al 1997
NDUFS1	75 kDa	2q33-34	Chow et al 1991
NDUFS4	18 kDa (AQDQ)	5	van den Heuvel et al 1998
NDUFS7	PSST	19p13	Hyslop et al 1996
NDUFS8	TYKY	11	Procaccio et al 1997
NDUFA1	MWFE	Xq24-25	Zhuchenko et al 1996
NDUFA2	B8		Ton et al 1997
NDUFA4	MLRQ		Kim et al 1997
NDUFA5	B13	7q32	Pata et al 1997
NDUFA6	B14		Ton et al 1997
NDUFB3	B12		Ton et al 1997
NDUFB5	SGDH		Ton et al 1997
NDUFC1	KFYI		Ton et al 1997

Table 4.1 Details of the presently known nuclear-encoded subunits of human complex I (human gene nomenclature according to (White et al 1997). * pseudogene known (*NDUFV2P1*: chromosome 19q13.3-ter).

Material and methods.

Cloning of the human NDUFB6 cDNA.

Human *NDUFB6* cDNA was obtained by reverse transcription using oligo(dT) and random hexamer primers on poly(A)$^+$ RNA isolated from human heart tissue (Clontech, USA). Amplification by the polymerase chain reaction (PCR) was performed with cDNA as template

according to the protocol for Taq polymerase (Life Technologies, The Netherlands) using oligonucleotides 5 AACTAGTCCGTAGTTCGAGGG 3 and 5 GGAACAAACTTAGGCTCATAAG 3 Oligonucleotide sequences were based on several human expressed sequences tags (ESTs) resembling the bovine 5 and 3' open reading frame (ORF) of the NDUFB6 subunit The PCR product was purified for direct DNA sequencing DNA sequence analysis of both DNA strands was performed using Dyedeoxy terminators on an automated ABI 377 sequencer according to a protocol provided by the manufacturer (Perkin Elmer, The Netherlands)

Chromosomal localisation of the human NDUFB6 gene

We searched the Genbank sequence-tagged site (STS) database using the cDNA sequence of the human NDUFB6 subunit This revealed an STS (Genbank accession number G30177) of 75 bp (100% match with the corresponding part of the human *NDUFB6* transcript)

Tissue distribution

To determine the tissue- and developmental stage specific expression of the *NDUFB6* mRNA, we used a Human RNA Master Blot (Clontech, USA) The blot was hybridised with an [α^{32}P]dCTP-labelled *NDUFB6* cDNA probe using Standard Hybridisation Solution (Sambrook et al 1989), washed twice with 2× SSC, 1% SDS at 65°C, and subjected to autoradiography The quantity of mRNA spotted for each tissue on the Master Blot was normalised using eight different housekeeping gene transcripts as probes (ribosomal protein S9, ubiquitin, highly basic 23-kDa protein, hypoxanthine guanine phosphoribosyl transferase, tubulin, β-actin, phospholipase and glyceraldehyde triphosphate dehydrogenase)

Protein analysis

The average hydropathy of the human NDUFB6 subunit was calculated according to the procedure described by Kyte and Doolitte (1982), using computer software provided by CAOS/CAMM, University of Nijmegen, The Netherlands

Mutation detection

Total RNA was extracted from cultured skin fibroblasts of 20 patients (16 males, 4 females) with isolated complex I deficiency (Loeffen et al 1998) A 5-µg sample of RNA was reverse transcribed to cDNA in 1 h at 42°C with 200 U of Superscript II reverse transcriptase (Life Technologies, The Netherlands), using oligo(dT) and random hexamer primers (Ploos van Amstel et al 1996) One microliter of cDNA was subjected to PCR amplification A fragment encompassing the entire coding region was generated using the synthetic oligonucleotide primers described previously The PCR program consisted of 35 cycles of 60 s 94°C, 60 s 55°C and 60 s 72°C The cycles were preceded by an initial denaturation step of 2 min at 94°C and were followed by a final extension of 10 min at 72°C The products of the PCR were analysed on a 2% TBE agarose gel with 0 5 µg/ml ethidium bromide The nucleotide sequences of the PCR products were analysed by direct sequencing using the Taq Dye Deoxy Terminator Cycle Sequencing Kit according to the manufacturer s recommendations (Perkin Elmer, The Netherlands)

Results.

By reverse transcription-PCR using human heart RNA and oligonucleotide primers based upon the *NDUFB6* cDNA sequence from cow and human ESTs, we obtained a 489-bp cDNA fragment of the human nuclear-encoded *NDUFB6* gene. This comprises the ORF of 387 bp, which encodes a protein of 128 amino acids (Genbank accession number AF035840). The human *NDUFB6* ORF showed 82.7% homology with the bovine equivalent (Fig. 4.1). The amino acid sequence showed 78.1% homology with the bovine NDUFB6 amino acid sequence. The calculated molecular weight of the protein encoded by the human *NDUFB6* ORF is 15.5 kDa.

In contrast to, for example, the 18-kDa subunit (van den Heuvel et al 1998), the human NDUFB6 subunit contains no phosphorylation consensus site. Furthermore, no cysteine-rich motifs were present in the amino acid sequence. The prediction of the hydropathy of the protein, essentially calculated as described by Kyte and Doolittle (1982), revealed that approximately 20% of the protein has hydrophobic properties (data not shown). A search of the Genbank STS database using the determined human *NDUFB6* cDNA sequence revealed a specific STS (Genbank accession number G30177) of 75 bp (100% match with the corresponding part of the human NDUFB6 subunit transcript), which has been mapped to chromosome 2.

Determination of the tissue distribution showed the highest mRNA level in kidney (Fig. 4.2). Expression was also high in putamen, heart, skeletal muscle, liver and lung. In our group of 20 isolated complex I-deficient patients, none of the following mtDNA mutations were present: A3243G (MELAS), T3271C (MELAS), A4317G (MELAS), A8344G (MERFF), T8993G/C (NARP/Leigh syndrome). Deletions of mtDNA were excluded by means of the long template PCR technique (Li et al 1995), which revealed a single band of 16.5-kb representing the full-length linearised mtDNA. Furthermore, no mutations or polymorphisms were detected in any patient by complete sequencing of the human *NDUFB6* ORF. Mutations in the nuclear-encoded NDUFA1 and 18-kDa (AQDQ) subunits have been previously excluded in this cohort of patients.

```
          1           11          21          31          41
Human     MTGYTPDEKL  RLQQLRELRR  RWLKDQELSP  REPVLPPQKM  GPMEKFWNKF
          :.:::::.::  ::::::::::  ::::::::::  ::::::::..  .:.:.:::::
Bovine    MSGYTPEEKL  RLQQLRELRR  RWLKDQELSP  REPVLPPQRV  SPVERFWNKF

          51          61          71          81          91
Human     LENKSPWRKM  VHGVYKKSIF  VFTHVLVPVW  IIHYYMKYHV  SEKPYGIVEK
          :.. . :...  .. .:.:::::  .:::::.:::  :::::.::::  ..:::.::::
Bovine    LQDGALWKNV  IYKTYRHSIF  AFTHVLIPVW  IIHYYIKYHV  TTKPYTIVEK

          101         111         121
Human     KSRIFPGDTI  LETGEVIPPM  KEFPDQHH
          :.:::::::::  ::::::::::  ::::::::
Bovine    KPRIFPGDTI  LETGEVIPPM  KEFPDQHH
```

Figure 4.1 (continued)

```
                                                    -70         -60
Human                                                 cgcgctta    agtaactagt
          -50         -40         -30         -20         -10
Human     ccgtagttcg  agggtgcgcc  gtgtcctttt  gcgttggtac  cagcggcgac
           1          11          21          31          41
Human     ATGACGGGGT  ACACTCCGGA  TGAGAAACTG  CGGCTGCAGC  AGCTGCGAGA
          ::: ::::::  : :: :: ::   :::::::::  ::::::::::  :::: :::::
Bovine    ATGTCGGGGT  ATACGCCCGA  GGAGAAACTG  CGGCTGCAGC  AGCTTCGAGA
          51          61          71          81          91
Human     GCTGAGAAGG  CGATGGCTGA  AGGACCAGGA  GCTGAGCCCT  CGGGAGCCGG
          ::: ::::::  ::::::::::  : :: :::::  :::::::::  ::::: :: :
Bovine    GCTAAGAAGG  CGATGGCTGA  AAGATCAGGA  GCTGAGCCCC  CGGGAACCCG
          101         111         121         131         141
Human     TGCTGCCCCC  ACAGAAGATG  GGGCCTATGG  AGAAATTCTG  GAATAAATTT
          ::::::: ::   :::: : ::   :::: :::  ::  ::::::  ::::::::::
Bovine    TGCTGCCTCC  GCAGAGGGTG  TCGCCTGTGG  AGCGATTCTG  GAATAAATTT
          151         161         171         181         191
Human     TTGGAGAATA  AATCCCCTTG  GAGGAAAATG  GTCCATGGGG  TATACAAAAA
          ::: :  :    : : :  ::  :: :::  ::  :: ::  :    :::   : :
Bovine    TTGCAAGACG  GAGCTCTCTG  GAAGAACGTG  ATCTATAAGA  CATATCGACA
          201         211         221         231         241
Human     GAGTATCTTT  GTTTTCACTC  ATGTACTTGT  ACCTGTCTGG  ATTATTCATT
           :::::::::  : ::: ::::  :::::::: :  :::::::::  ::::::::::
Bovine    CAGTATCTTT  GCTTTTACTC  ATGTACTTAT  TCCTGTCTGG  ATTATTCATT
          251         261         271         281         291
Human     ATTACATGAA  GTATCATGTT  TCTGAAAAAC  CATATGGCAT  AGTTGAAAAG
          ::::  : ::  ::::::  ::   ::  :::::  :::::  :::   :::::::::
Bovine    ATTATCTCAA  GTATCACGTG  ACTACAAAAC  CATATACCAT  CGTTGAAAAG
          301         311         321         331         341
Human     AAGTCCAGAA  TATTCCCTGG  TGATACAATT  CTGGAGACTG  GAGAAGTAAT
          ::: ::::::  :::: :: ::  ::::::::::  ::::::::::  ::::::::::
Bovine    AAGCCCAGAA  TATTTCCAGG  TGATACAATT  CTGGAGACTG  GAGAAGTAAT
          351         361         371         381         391
Human     TCCACCAATG  AAAGAATTTC  CTGATCAACA  TCATTAAaga  ttatgtaaaa
           :::::  :::  ::::::::::  : ::::::::  :::::  :
Bovine    CCCACCCATG  AAAGAATTTC  CCGATCAACA  TCATTGA
          401         411         421         431         441
Human     agttaaaagg  cttatgagcc  taagtttgtt  cctatattac  catatttact
          451         461         471         481         491
Human     gaattttctg  gaaaagtaac  tttaataaag  tttaatctca  gaaattgtca
          501                      poly a signal
Human     aaaaaaaaaa
          poly a site
```

Figure 4.1 Human and bovine amino acid and cDNA sequences of the NDUFB6 subunit of NADH·ubiquinone oxidoreductase. The human and bovine cDNA sequences show 82.7% homology. The bovine sequence was obtained from Genbank, USA (accession number X63212). Primers used to generate fragments encoding the complete sequence of the human *NDUFB6* cDNA with the polymerase chain reaction are underlined. The open reading frame obtained after sequencing is supplemented with bases prior to the ATG and after the TGA with the use of expressed sequence tags obtained via Genbank. In the part of the figure, which shows amino acid similarity between human and bovine, one dot indicates that the amino acids are not identical but have similar chemical characteristics.

	1	2	3	4	5	6	7	8
a	whole brain	amygdala	caudate nucleus	cerebel-lum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
b	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	subthal. nucleus	spinal cord	
c	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
d	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
e	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
f	appendix	lung	trachea	placenta				
g	foetal brain	foetal heart	foetal kidney	foetal liver	foetal spleen	foetal thymus	foetal lung	
h	yeast RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	poly r(A) 100 ng	human C_0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

Figure 4.1a Multiple tissue Master blot. The type and position of poly(A^+) RNAs and controls dotted on the positively charged nylon membrane. subthal. = subthalamic.

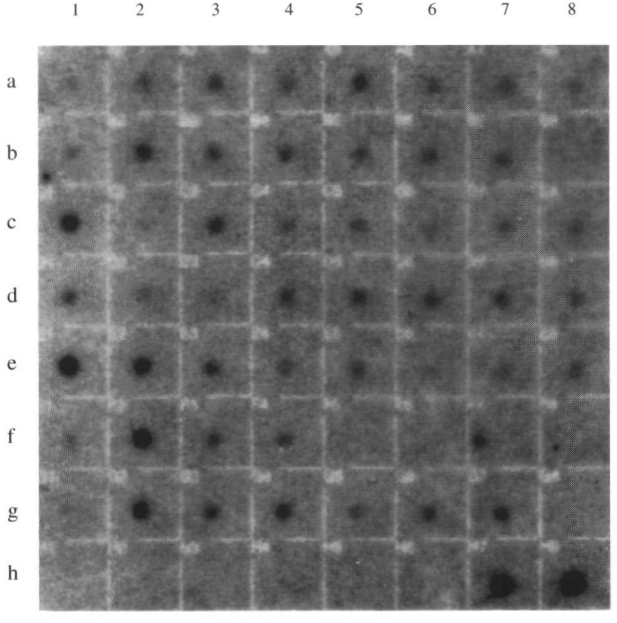

Figure 4.1b Human RNA multiple tissue Master blot data. Hybridisation was performed with a ^{32}P-labelled cDNA probe for the *NDUFB6* gene.

Discussion.

Complex I of the respiratory chain is embedded in the lipid bilayer of the inner mitochondrial membrane In *N crassa* the multiple subunits of this complex form an L-shaped configuration of which the extrinsic peripheral arm protrudes into the mitochondrial matrix (Guenebaut et al 1997) The three-dimensional structure of the human counterpart is not known The bovine NDUFB6 subunit is located in the intrinsic membrane domain of the complex (Walker 1992) Based upon the predicted protein analysis, revealing an important hydrophobic segment sufficiently long to form a transmembrane α-helix, we assume that, as in bovine, the human NDUFB6 subunit is part of the intrinsic membrane domain of complex I Presumably, the human NDUFB6 subunit is, as assumed for bovine (Walker 1992), anchored into the inner mitochondrial membrane The more hydrophilic parts of the sequence probably interact with globular proteins in the large extrinsic membrane domain of the complex, which protrudes into the mitochondrial matrix (Walker 1992)

Presently, information concerning the function of the human NDUFB6 subunit is lacking Based on the obtained results we assume that this subunit has no phosphorylation site Owing to the lack of cysteine-rich motifs it is highly unlikely that the human NDUFB6 subunit contains a binding site for an Iron-Sulphur cluster The human *NDUFB6* ORF is 387 bp long and its nucleotide sequence has 82 7% homology with that of the cow The molecular weight calculated from the cDNA deducted amino acid sequence is 15 5 kDa The use of STSs has enabled the localisation of the *NDUFB6* gene to chromosome 2 Multiple tissue dot-blot analysis showed the highest mRNA expression of the NDUFB6 subunit in organs known for their high-energy demand, with kidney showing the highest expression The latter result differs from the mRNA expression we observed in the NDUFA1 NADH ubiquinone oxidoreductase subunit (Loeffen et al 1998)

Deficient activity of complex I of the respiratory chain may result directly from mutations in the mtDNA- or nuclear DNA-encoded subunits Apart from the recently found mutation in the nuclear-encoded complex I NDUFS4 subunit, various mtDNA mutations have been associated with complex I deficiency (Wallace 1992, Robinson 1993) Deletions and common mtDNA mutations were not present in our patients However, defects in other classes of genes, such as those involved in the regulation and assembly of the complex may also be involved We performed a mutational detection study at the RNA level in 20 complex I-deficient patients Neither mutations nor polymorphisms were detected in the ORF of the *NDUFB6* cDNA of these patients However, the promoter region of the *NDUFB6* gene was not included in this study At the moment we are in the process of mutational analysis of the presently known nuclear-encoded subunits and characterising presently unknown human nuclear-encoded complex I cDNA sequences in order to extend our mutation detection studies By this approach we are trying to extend indirectly our knowledge of the function of the individual subunits of this vital complex Elucidation of the molecular defects in patients may be of great importance for genetic counselling and prenatal diagnosis Currently this is only possible by enzymatic measurements in chorionic villi and amniocytes Interpretation of the results of mutational analysis studies will be more reliable

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THE HUMAN NADH:UBIQUINONE OXIDOREDUCTASE NDUFS5 (15 kDa) SUBUNIT: cDNA CLONING, CHROMOSOMAL LOCALISATION, TISSUE DISTRIBUTION AND THE ABSENCE OF MUTATIONS IN ISOLATED COMPLEX I DEFICIENT PATIENTS

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Abstract.

We have cloned the cDNA of the NDUFS5 subunit (15 kDa) of the human mitochondrial respiratory chain complex NADH ubiquinone oxidoreductase (complex I) The open reading frame consists of 321 base-pairs, coding for 106 amino acids, with a calculated molecular weight (M_r) of 12 5 kDa There is an 81 0% identity with the bovine equivalent on cDNA level and 75 5% identity on amino acid basis PCR analysis of rodent-human somatic cell hybrids revealed that the human *NDUFS5* gene maps to chromosome 1 The *NDUFS5* mRNA is expressed ubiquitously in human tissues, with a relative higher expression in human heart, skeletal muscle, liver, kidney and foetal heart A mutation detection study of twenty isolated enzymatic complex I deficient patients revealed no mutations, or polymorphisms

Introduction.

NADH ubiquinone oxidoreductase (complex I), the first multisubunit inner membrane protein complex of the mitochondrial electron transport chain, transfers two electrons with a high energy transfer potential from NADH to ubiquinone Simultaneously to this electron transfer, protons are translocated from the mitochondrial matrix, across the inner mitochondrial membrane, to the intermembrane space Subsequently, protons are returned to the mitochondrial matrix by complex V, resulting in the generation of ATP, the main carrier of free energy in pro- and eukaryotic cells

Complex I consists of about 42 polypeptides, seven of which are encoded by the mitochondrial genome, nuclear genes encode the remainder (Walker 1992, Walker et al 1992, Robinson 1993, Walker 1995) Defects in mitochondrial encoded subunits of complex I have been described (Zeviani and Taroni 1994) Up till now, only one mutation in a nuclear encoded subunit of complex I has been reported in literature (this thesis, van den Heuvel et al 1998)

Bovine complex I contains a number of prosthetic groups which are involved in the transfer of electrons, namely flavin mononucleotide (FMN), and at least six and possibly eight iron-sulphur clusters (binuclear and tetranuclear) (Runswick et al 1991, Yamaguchi and Hatefi 1993, Albracht et al 1996) The bovine 15 kDa subunit is thought to be part of the complex I iron-sulphur protein fraction (IP) which is built up of ~8 subunits (75, 49, 30, 18, 15, and 13 kDa subunit, TYKY and the B13 subunit) (Hatefi 1985, Ragan and Hatefi 1986, Masui et al 1991) It is hypothesised that subunits of the IP fraction play a part in the redox reactions catalysed by the iron-sulphur clusters

In the past decade numerous patients with a deficient enzymatic activity of complex I have been reported Clinical phenotypes associated with complex I deficiency are fatal neonatal lactic acidosis, myopathy with exercise intolerance and lactic acidosis, MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes), MERRF (myoclonus epilepsy, and ragged red fibres), encephalomyopathy of childhood and adult life, Leigh syndrome, and Alpers disease (Morris et al 1995) In patients clinically suspected to suffer from a deficiency of complex I (or one of the other complexes of the respiratory chain), definitive confirmation is usually obtained by biochemical analysis of a skeletal muscle biopsy.

Molecular characterisation of nuclear encoded complex I subunits is of utmost importance, since in many isolated complex I deficient patients, no mutations in mitochondrial encoded genes related to complex I have been found Mutational analysis studies could reveal nuclear defects, which hopefully will increase the understanding of the function of complex I and its individual subunits Genetic diagnostics in chorion villi as well as in patient skin fibroblasts (preferred over biochemical diagnostics) then become available

The cDNA of the Flavoprotein group (FP) subunits (NDUFV1, NDUFV2 and NUDFV3) was sequenced by Buddiger et al in 1997, which revealed no pathogenic mutations in a group of 20 complex I deficient patients We focus in our patient group on the second important subgroup, namely the Iron-Sulphur protein fraction (IP) The human cDNA sequence is known for the NDUFS4 (18 kDa) subunit of complex I (van den Heuvel et al 1998), and a mutation

detection study revealed the first mutation in a nuclear encoded subunit in our group of isolated complex I deficient patients This finding suggests other nuclear encoded complex I subunits should be taken into consideration as well

In order to extend our current knowledge regarding the molecular defects of complex I deficiency, cloning of the human nuclear encoded complex I subunits is warranted In the present communication we report the cDNA sequence, and chromosomal localisation of the human NDUFS5 subunit, we describe the tissue distribution of the messenger RNA transcript, and data of a mutation detection study performed in twenty patients with an isolated deficiency of NADH ubiquinone oxidoreductase determined in cultured skin fibroblasts

Patients, material and methods.

Patient group Twenty patients (four females, sixteen males, sex ratio 1 4) with an isolated decreased activity of complex I (measured in all cases in cultured skin fibroblasts), were selected for mutation detection on RNA level in the NDUFS5 subunit Age of clinical onset varied from a few days after birth, to the age of ten months Thirteen patients deceased before the age of two years Clinical phenotypes varied from severe neonatal metabolic acidosis, followed by cardiorespiratory distress proceeding to death, to hypotonia and psychomotor retardation at the age of eight months Four families were known with consanguinity Strikingly, four patients (20%) had a cardiomyopathy (Loeffen et al 1998) Neither mitochondrial DNA deletions, nor common mtDNA pathogenic mutations (A3243G (MELAS), T3271C (MELAS), A4317G (MELAS), A8344G (MERRF), T8993G/C (NARP/Leigh syndrome)) were present in this patient group In previous studies we searched for mutations in the cDNA of the nuclear encoded NDUFA1 (MWFE) and NDUFS4 (18 kDa) subunits in our patient group (Loeffen et al 1998, van den Heuvel et al 1998)

Tissue culture and enzyme measurements Deep-frozen skin fibroblasts of patients were cultured in M-199 medium, after addition of foetal calf serum, glutamine and antibiotics (penicillin and streptomycin) according to established procedures Cells were harvested after reaching confluency The enzymatic activity of complex I was measured according to procedures described by Bentlage et al in 1996 Reference values used ranged from 0 11-0 28 mU/mU cytochrome *c* oxidase Patient values ranged from 0 026 to 0 080 mU/mU cytochrome *c* oxidase

Cloning of the human NDUFS5 subunit Wild type mRNA was obtained from a human heart poly A^+ RNA pool (Clontech, USA) The pool consisted of seven normal Caucasian males and females, age range 20-78 First-strand cDNA was obtained by reverse transcription of mRNA by Superscript II Rnase H reverse transcriptase (Life Technologies, The Netherlands), during 1 hour at 42°C in a total volume of 40 µl according to established procedures (Ploos van Amstel et al 1996)

Human ESTs (retrieved from Genbank, USA, accession no W72456, W76515), resembling the bovine 15 kDa cDNA were used for PCR primer design Amplification was carried out in a total volume of 25 µl, containing 1 µl of cDNA, 2 5 µl 10× PCR buffer, 50 ng forward

(5'-GCCAGAGAAGAGTCAAGGGC-3'), and reverse primer (5'-CGTAAACAACCTTTCCAGTGG-3'), 0.25 mM dNTPs, 1.5 mM $MgCl_2$, and 0.5 unit of Taq Polymerase (Life Technologies, The Netherlands). Amplification parameters were as follows: 35 cycles, 1 min. denaturation at 94°C, 1 min. annealing at 55°C, and 1 min. primer extension at 72°C. The cycles were preceded by an initial denaturation step of 1 min. at 94°C, and were followed by a final extension of 10 min. at 72°C.

Mutation detection studies in complex I deficient patients. Patient total RNA was isolated from cultured skin fibroblasts using RNAzol[tm] B (Biosolve, The Netherlands) (Chomczynski and Sacchi 1987). RNA was reverse transcribed to first strand cDNA as described before. PCR conditions were similar as those described for cloning of the human NDUFS5 subunit. Patient PCR product was purified from a 1% agarose gel, using the QIAquick GEL purification kit according to procedures as described by the manufacturer (Qiagen, The Netherlands). Both strands were screened for the presence of mutations by direct sequencing (ABI Prism, model 377 version 2.1.2), using the ABI Prism[tm] Taq DyeDeoxy terminator cycle sequencing ready reaction kit (Perkin Elmer, The Netherlands).

Chromosomal localisation of the human NDUFS5 gene

To determine the chromosomal localisation of the *NDUFS5* gene, we performed a PCR on genomic DNA extracted from human-rodent somatic hybrid cells, containing defined human chromosomes (NIGMS mapping panel 2, Coriell Cell Repository, USA). The forward and reverse oligonucleotide used were respectively 5'-CACATTGGCAAGGGGGAG-3' and 5'-ATTTTTACACACTTTGACAAGGAGG 3'. PCR product was visualised using a 4% agarose gel.

Tissue distribution of the NDUFS5 mRNA. For specific tissue and developmental stage expression of the *NDUFS5* mRNA, a Human RNA Master Blot[tm] (Clontech, USA) was used. The quantity of RNA spotted for each tissue onto the master blot was normalised using eight different housekeeping gene transcripts as probes (ribosomal protein S9, ubiquitin, 23 kDa highly basic protein, hypoxanthine guanine phosphoribosyl transferase, tubulin, β-actin, phospholipase, and glyceraldehyde triphosphate dehydrogenase). Blots were hybridised with a α-^{32}P-dCTP labelled cDNA probe, using Standard Hybridisation Solution (Sambrook et al. 1989), washed twice with 2× SSC, 1% SDS at 65°C, and were subjected to autoradiography.

Calculated hydropathy of the human NDUFS5 subunit. We calculated the average hydropathy of the NDUFS5 subunit, using a method based on the procedure described by Kyte and Doolittle (1982). Computer software was used from the National Centre for Computer Aided Chemistry and Bioinformatics (CAOS/CAMM, Nijmegen, The Netherlands).

Results.

cDNA cloning of the human NDUFS5 subunit

We performed a blast search (Genbank, USA) with the bovine cDNA sequence of the 15 kDa subunit of complex I, which revealed several human ESTs, which we used for designing a forward and reverse oligonucleotide to clone the human homologue cDNA obtained after reverse transcription of mRNA from a Human Heart Poly A^+ RNA pool was used as template for PCR We produced a 428 base-pair product (Genbank accession no AF020352), which constituted the full-length open reading frame of the human *NDUFS5* transcript Fifteen expressed sequence tags (ESTs) retrieved from Genbank, which were not available at time of sequencing, were used to complete the 5'UTR and 3'UTR (Fig 5 1a) The human NDUFS5 subunit was named according to guidelines for Human Gene Nomenclature (White et al 1997)

cDNA sequence and protein characteristics of the human NDUFS5 subunit

The human as well as the bovine (15 kDa) *NDUFS5* open reading frame consist of 321 nucleotides coding for 106 amino acids Comparison of the open reading frame of the bovine 15 kDa cDNA with the human equivalent revealed an 81 0% homology on nucleotide sequence level (Fig 5 1a) Concerning the amino acid sequence, there was a 75 5% homology (Fig 5 1b) When amino acids with similar characteristics are considered equal, there is a 95 3% homology The molecular weight calculated from the amino acid sequence (M_r) is 12 5 kDa, the hypothetical isoelectric point (pI) is 9 8 The prediction of the hydropathy showed an overall hydrophilic protein The 3'UTR contains an AATAAA polyadenylation signal at nucleotide position 419 to 424 A poly A site is positioned 16 base-pairs downstream of the poly adenylation signal

A blast search with the human *NDUFS5* cDNA-, and amino acid sequence revealed the bovine sequence mentioned above and several human ESTs A blast search in mouse and residual EST databases (Genbank), revealed several significant matches for mouse (82% identity in 152 base pair overlap, Genbank accession no AA529094) and rat (83% identity in 119 base pair overlap, Genbank accession no H35001)

Mutation detection in isolated complex I deficient patients

Mutation detection on cDNA level was performed by PCR using primers specific for the human NDUFS5 subunit, followed by automatic DNA sequence analyses of the entire open reading frame of both DNA strands No mutations or polymorphisms were observed in the *NDUFS5* transcript

Chromosomal localisation of the human NDUFS5 gene

To determine the chromosomal localisation of the *NDUFS5* gene, we amplified an intronless 130 base pair part of the 3' region by PCR with genomic DNA extracted from human-rodent somatic hybrid cells as template The obtained PCR product was specific for man, and equal in size compared to the cDNA fragment A signal was observed in the lane containing chromosome 1 (results not shown)

```
          -50         -40         -30         -20         -10
Human     agtgggagcg  gcggccagag  aagagtcaag  ggcacgagca  tcgggtagcc
          1           11          21          31          41
Human     ATGCCTTTCT  TGGACATCCA  GAAAAGGTTC  GGCCTTAACA  TAGATCGATG
          ::::::::::  : ::  : ::  :::::::: :  ::  :: ::  ::::::: ::
Bovine    ATGCCTTTCT  TTGATGTGCA  GAAAAGGCTG  GGTGTTGACC  TAGATCGCTG
          51          61          71          81          91
Human     GTTGACAATC  CAGAGTGGTG  AACAGCCCTA  CAAGATGGCT  GGTCGATGCC
          : :::::::::  ::::::: ::  : :::::  :  ::::::  :     :::::::
Bovine    GATGACAATC  CAGAGTGCTG  AGCAGCCTCA  CAAGATCCCA  TCCCGATGCC
          101         111         121         131         141
Human     ATGCTTTTGA  AAAAGAATGG  ATAGAATGTG  CACATGGAAT  CGGTTATACT
          ::::::::::  :: ::::::  ::::: ::::  ::::::::::  :::  ::
Bovine    ATGCTTTTGA  GAAGGAATGG  ATAGAGTGTG  CACATGGAAT  CGGCAGTATC
          151         161         171         181         191
Human     CGGGCAGAGA  AAGAGTGCAA  GATAGAATAT  GATGATTTCG  TAGAGTGTTT
          :: :::::::  : :::::::::  :::::::: :  :: ::::::  :::::::: :
Bovine    CGAGCAGAGA  AGGAGTGCAA  AATAGAATTT  GAGGATTTCA  GAGAGTGTCT
          201         211         221         231         241
Human     GCTTCGGCAG  AAAACGATGA  GACGTGCAGG  TACCATCAGG  AAGCAGCGGG
          ::::::: :::  ::::::::::  ::::      : ::::::::  :  :::::::
Bovine    GCTTCGACAG  AAAACGATGA  AACGTCTGCA  TGCCATCAGG  AGACAGCGGG
          251         261         271         281         291
Human     ATAAGCTGAT  AAAGGAAGGA  AAGTACACCC  CTCCACCTCA  CCACATTGGC
          : ::::: ::   :::::::::  :::::::: :  : ::::::::  ::::   :::
Bovine    AAAAGCTAAT  CAAGGAAGGG  AAGTACACAC  CCCCACCTCA  CCACTCAGGC
          301         311         321         331         341
Human     AAGGGGGAGC  CTCGGCCCTG  Aacagagcag  ctgctgatgt  ctggaggctg
           ::: ::: :  ::::: ::::  :
Bovine    CAGGAGGAAC  CTCGGTCCTG  A
          351         361         371         381         391
Human     attttcctgt  tctctgttct  ccactggaaa  ggttgtttac  gacaaacctc
          401         411         421         431         441
Human     cttgtcaaag  tgtgtaaaaa  taaaggattg  ctccatccta  aaaaaaaaaa
```

Figure 5.1a Human and bovine cDNA sequence of the NDUFS5 subunit of complex I. Comparison of human and bovine cDNA sequence of the NDUFS5 subunit of complex I (Genbank accession numbers AF020352 and X63220, respectively) revealed 81.0% homology. Primers used to generate fragments encoding the complete open reading frame of the human *NDUFS5* cDNA by PCR, are underlined. Primers used for chromosomal localisation of the *NDUFS5* gene are double underlined. The 5'UTR, and 3'UTR of the *NDUFS5* cDNA were completed with sequences of human ESTs resembling the human *NDUFS5* open reading frame.

```
          1           11          21          31          41
Human     MPFLDIQKRF  GLNIDRWLTI  QSGEQPYKMA  GRCHAFEKEW  IECAHGIGYT
          :::.:.::::  :...::::.::  ::.::::.:..  .:::::::::  :::::::: .
Bovine    MPFFDVQKRL  GVDLDRWMTI  QSAEQPHKIP  SRCHAFEKEW  IECAHGIGSI
          51          61          71          81          91
Human     RAEKECKIEY  DDFVECLLRQ  KTMRRAGTIR  KQRDKLIKEG  KYTPPPHHIG
          ::::::::::.  .:: ::::::  :::::  .::  .::.::::::  :::::::: :
Bovine    RAEKECKIEF  EDFRECLLRQ  KTMKRLHAIR  RQREKLIKEG  KYTPPPHHSG
          101
Human     KGEPRP
          ..:::.
Bovine    QEEPRS
```

Figure 5.1b Human and bovine amino acid sequence of the NDUFS5 subunit of complex I. Comparison of human and bovine amino acid sequence of the NDUFS5 subunit of complex I revealed a 75.5% homology (: = identical amino acid, . = amino acid with similar characteristics).

Tissue distribution of the NDUFS5 subunit mRNA

The multiple tissue dot blot was hybridised using a ^{32}P-labeled *NDUFS5* cDNA probe, and was subjected to autoradiography (Fig 5.2). All human tissues expressed the *NDUFS5* mRNA. A signal was also seen at the 100 and 500 ng human genomic DNA dots. High intensity dots were seen in human tissues as (in descending order) heart, kidney, lung, liver, ovary, and skeletal muscle. The foetal tissues loaded on the blot all expressed the *NDUFS5* mRNA, dots with high intensity are foetal heart, and foetal lung.

Discussion.

Mitochondrial respiratory chain deficiency, with isolated complex I deficiency as a major contributor, occurs in approximately 1/10,000 live births (Bourgeron et al 1995). Complex I consists of seven mitochondrial encoded subunits (Chomyn et al 1985 and 1986), the remainder are nuclear encoded of which sixteen have been cloned, and reported in literature (Chow et al 1991, Spencer et al 1992, Hattori et al 1995, de Coo et al 1995, Zhuchenko et al 1996, Gu et al 1996, Hyslop et al 1996, Pata et al 1997, de Coo et al 1997, Procaccio et al 1997, Kim et al 1997, Ton et al 1997, van den Heuvel et al 1998). Recently, the first mutation has been described in a nuclear encoded complex I subunit, which consisted of a five base pair duplication in the 18 kDa (AQDQ) subunit (van den Heuvel et al 1998). Hence, it seems worthwhile to analyse the nuclear encoded complex I genes for mutations in complex I deficient patients.

The human NDUFS5 subunit contains four cysteine residues, but not in a motif that provides ligands for binuclear and tetranuclear iron-sulphur centres. There is therefore no indication that the NDUFS5 subunit is an iron-sulphur protein. The same conclusion was drawn for the bovine 15 kDa subunit (Walker et al 1992). Alternatively, a ubiquinone binding protein can be isolated from bovine complex I with a molecular weight of 15 kDa. It is suggested, based on molecular weight resemblance, that this ubiquinone binding protein is the NDUFS5 subunit (Suzuki and Ozawa 1986). In the IP fraction however there are two proteins with a molecular weight of 15 kDa, namely the NDUFS5, and the NDUFA5 (B13) subunit (Walker 1992). So whether the NDUFS5 subunit plays a direct role in the transfer of electrons or in the binding of ubiquinone remains to be elucidated.

The bovine 15 kDa subunit consists of 106 amino acids, which are not post translationally modified (Walker 1992). The knowledge of the three dimensional structure and function of complex I in general, and from the NDUFS5 subunit in particular remains very limited. The calculated hydropathy curve of the human NDUFS5 subunit expresses an overall hydrophilic protein, strongly resembling the curve published by Walker et al (1992) for the bovine equivalent. This suggests that the NDUFS5 subunit is part of the extrinsic membrane domain of complex I. This is in agreement with the NDUFS5 subunit being part of the 1α fraction, which is thought to represent the external membrane part of complex I (Finel et al 1992).

Because antibodies for Western Blotting were not available for the NDUFS5 subunit, we studied the tissue and developmental expression of the mRNA using a multiple tissue RNA master blot. In our experience (Sperl et al 1990, Bentlage et al 1996), measurement of

complex I activity for diagnostics in only one tissue (e g heart, liver, muscle, fibroblasts) is not always conclusive Some patients express enzyme defects only in muscle, others in fibroblasts In 1996 Pitkanen et al published patients with a complex I deficiency in fibroblasts and heart tissue, but not in skeletal muscle (Pitkanen et al 1996) With ^{32}P-labeled *NDUFS5* cDNA as a probe, we studied the expression of the mRNA in a diversity of human tissues, different human developmental stages, and some lower organisms (yeast and *E coli*) The probe was expressed in all human tissues present on the blot Dots with relative high expression were human heart, kidney, skeletal muscle, liver, lung, ovary, and foetal heart, all tissues with high metabolic activities, which are often clinically affected in complex I deficient patients The frequent involvement of basal ganglia seen in complex I deficient patients, is in agreement with the observed relative high expression of the *NDUFS5* mRNA transcript in the putamen (as compared to whole brain) Also, the human genomic DNA dots display a hybridisation signal This may be caused by the presence of pseudogenes, or homology of the *NDUFS5* cDNA sequence with repetitive nucleotide sequences in the human genome RNA samples are probably devoid of large quantities of genomic DNA contamination

Our centre has wide experience with measurements of respiratory chain enzyme activities in different tissues, but especially difficulties with interpreting the measured values in chorion villi and amniocytes remain, making prenatal diagnosis rather complicated It would facilitate diagnostic procedures, and genetic counselling when molecular defects on genomic DNA level in complex I deficient patients could be established We are especially interested in the chromosomal localisation of the complex I subunits, considering the strong male preponderance present in our patient group In a previous study we sequenced therefore, the only known X-linked complex I subunit, namely the NDUFA1 This revealed neither mutations nor polymorphisms (Loeffen et al 1998) The NDUFS5 subunit has been mapped on chromosome 1

We have chosen for RT-PCR followed by direct sequencing as mutation detection method, because the possibility of missing mutations using this method is significant lower as with Single Strand Conformation Polymorphism (SSCP) We found no mutations in the *NDUFS5* cDNA We therefore hypothesise that mutations in the open reading frame of the NDUFS5 subunit are not a frequent cause of complex I deficiency We remain searching for mutations in other nuclear encoded subunits of NADH ubiquinone oxidoreductase in our patient group

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	1	2	3	4	5	6	7	8
a	whole brain	amygdala	caudate nucleus	cerebel-lum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
b	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	subthal. nucleus	spinal cord	
c	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
d	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
e	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
f	appendix	lung	trachea	placenta				
g	foetal brain	foetal heart	foetal kidney	foetal liver	foetal spleen	foetal thymus	foetal lung	
h	yeast RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	poly r(A) 100 ng	human C_0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

Fig. 5.2a Multiple tissue Master blot. The type and position of poly(A^+) RNAs and controls dotted on the positively charged nylon membrane.

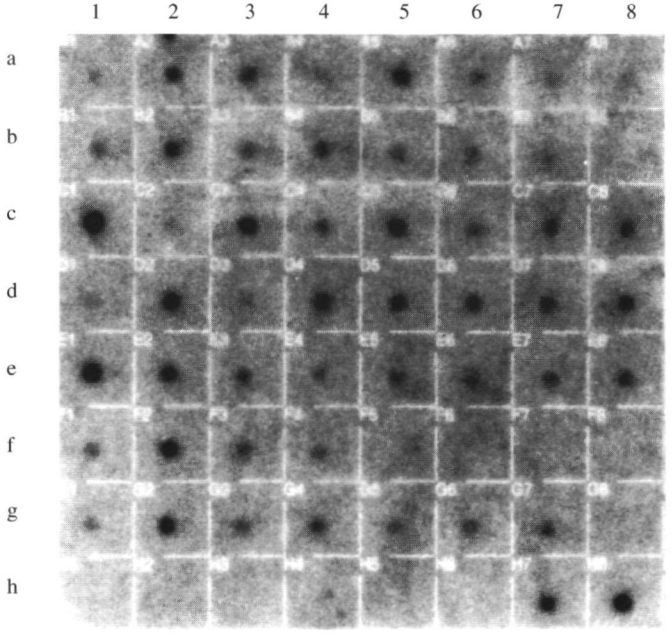

Fig. 5.2b mRNA tissue distribution of the NDUFS5 subunit. The multiple tissue dot blot (Clontech, USA) was hybridised using a ^{32}P-labeled *NDUFS5* cDNA probe, and was subjected to autoradiography. The probe was ubiquitously expressed, especially in tissues with high-energy demand.

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cDNA SEQUENCE AND CHROMOSOMAL LOCALISATION OF THE REMAINING THREE HUMAN NUCLEAR ENCODED IRON SULPHUR PROTEIN (IP) SUBUNITS OF COMPLEX I: THE HUMAN IP FRACTION IS COMPLETED

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Abstract.

NADH ubiquinone oxidoreductase (complex I) of the mitochondrial respiratory chain can be fragmented in a Flavoprotein (FP), Iron-Sulphur protein (IP) and Hydrophobic protein (HP) subfraction The IP subfraction is hypothesised to be significant, since it contains important prosthetic groups highly conserved among species We cloned the cDNA of three remaining human NADH ubiquinone oxidoreductase subunits of this IP fraction the NDUFS2 (49 kDa), NDUFS3 (30 kDa), and NDUFS6 (13 kDa) subunit All presented cDNAs include the complete open reading frame (ORF), which consists of 1392, 795, and 375 base pairs, coding for 463, 264, and 124 amino acids, respectively The latter show 96%, 90%, and 83% homology with the corresponding bovine translation products The 3' untranslated regions (UTR) are complete in all three cDNAs

Polymerase chain reaction performed with DNA isolated from somatic human-rodent cell hybrids containing defined human chromosomes as template, gave a human specific signal which mapped the *NDUFS2* and *NDUFS3* gene to chromosome 1 and 11, respectively In case of the NDUFS6 subunit a pseudogene may be present since signals were seen in the lanes containing chromosome 5 and 6

The NDUFS2 contains a highly conserved protein kinase C phosphorylation site and the NDUFS3 subunit contains a highly conserved casein kinase II phosphorylation site which make them strong candidates for future mutation detection studies in enzymatic complex I deficient patients

Introduction.

The energy generation of human cells is highly dependent on an adequate functioning of the respiratory chain, which is situated in the mitochondrial inner membrane Four multiple-subunit enzyme complexes, ubiquinone, and cytochrome *c* together constitute the respiratory chain, of which NADH·ubiquinone oxidoreductase (complex I) is the largest complex Each complex, with the exception of succinate dehydrogenase (complex II), has one or more subunits encoded by the mitochondrial DNA (mtDNA) Bovine complex I consists of about 42 subunits (Walker et al 1995, Walker 1995), seven of which are encoded by mtDNA (Chomyn et al 1985 and 1986) The remainder are nuclear encoded, and must be transported from the cytosol to the inner mitochondrial membrane At present, almost all cDNA sequences coding for bovine NADH ubiquinone oxidoreductase subunits have been cloned (Walker 1992, Walker et al 1992, Arizmendi et al 1992) Complex I of the fungus *Neurospora crassa* is also almost completely elucidated, and consists of approximately 35 subunits (Weiss et al 1991), purple bacteria as *Escherichia coli* contain a less complex variant with only 14 subunits (Weidner et al 1993)

Complex I accepts electrons from the electron carrier NADH and transfers them to ubiquinone This electron transport is accompanied by the translocation of protons across the inner mitochondrial membrane to the intermembrane space Complex V (F_1F_0-ATP synthase) uses this proton gradient to generate ATP Several prosthetic groups facilitate this transport of electrons At present, these are hypothesised to be one, or two Flavine Mononucleotides (FMN) molecules, and six to eight binuclear and tetranuclear Iron-sulphur clusters (Runswick et al 1991, Yamaguchi and Hatefi 1993, Albracht et al 1996) For the majority of the complex I subunits, position within the complex as well as the function are unknown

Bovine complex I can be fragmented with chaotropic agents, like perchlorate This reveals a soluble component, consisting of the Flavoprotein fraction (FP) and the Iron-Sulphur protein fraction (IP), and a precipitate known as the hydrophobic protein fraction (HP) (Galante and Hatefi 1978 and 1979) The FP fraction contains three subunits, namely the NDUFV1 (51 kDa), NDUFV2 (24 kDa) and NDUFV3 (10 kDa) subunit, of which the cDNA and nDNA have been cloned in man (Spencer et al 1992, Ali et al 1993, Hattori et al 1995, de Coo et al 1995, de Coo et al 1997, Schuelke et al 1998) The IP fraction consists of the NDUFS1 (75 kDa), NDUFS2 (49 kDa), NDUFS3 (30 kDa), NDUFS4 (18 kDa), NDUFS5 (15 kDa), NDUFS6 (13 kDa), NDUFS8 (TYKY), and the NDUFA5 (B13) subunit, of which NDUFS1, NDUFS4, NDUFS5, and NDUFA5 have been cloned (Chow et al 1991, Pata et al 1997, Procaccio et al 1997, van den Heuvel et al 1998, Loeffen et al 1999) The HP fraction, which contains globular water-soluble as well as hydrophobic subunits, consists of about 24 subunits The (partial) cDNA structure of ten of the 24 subunits (NDUFA1, NDUFA2, NDUFA4, NDUFA6, NDUFB3, NDUFB5, NDUFB6, NDUFB9, NDUFS7, NDUFC1) has been reported in literature (Zhuchenko et al 1996, Gu et al 1996, Hyslop et al 1996, Ton et al 1997, Kim et al 1997, Smeitink et al 1998)

The clinical phenotype expressed in complex I deficient patients is very heterogeneous, ranging from neonatal respiratory distress with extreme lactic acidosis at birth, to hypotonia with psychomotor retardation in the first years of life Since several studies mention a possible

male preponderance in respiratory chain deficiencies (Orstavik et al 1993, Zhuchenko et al 1996, Loeffen et al 1998) suggesting an X-linked inheritance pattern, chromosomal mapping of complex I subunits is warranted.

Most proteins incorporated in complex I are encoded by the nuclear DNA. In the past, much research has been performed on mtDNA in relation to complex I disease (Shoffner et al 1990, Goto et al 1990, Holt et al 1990). Recently, Buddiger published 20 complex I deficient patients who had no mutations in mitochondrial encoded subunits of complex I as well as in the three subunits situated in the FP subfraction (Buddiger et al 1997). We focus on the second important subgroup of complex I, the IP subfraction, which contains subunits with important prosthetic groups highly conserved among species. Therefore, elucidation of the cDNA structures and determination of the chromosomal localisation of all IP subunits is a rational prerequisite for future mutation detection studies in enzymatic complex I deficient patients.

In the present communication we report the cloning and chromosomal mapping of the remaining three so far not characterised nuclear encoded IP subunits (NDUFS2, NDUFS3, and NDUFS6) of human complex I.

Material and methods.

Material. The following materials were obtained from commercial sources. Molecular size markers, Taq DNA polymerase, superscript II RNAse H^- reverse transcriptase, and custom made oligonucleotide primers were purchased from Life Technologies Inc., the dRhodamine Dye Terminator Sequencing kit from Perkin Elmer, gel purification systems from Qiagen, poly A^+ RNA pool from Clontech, NIGMS mapping panel 2 from Coriell Cell Repositary, and RNAzol from Biosolve BV. All other chemicals used were of analytical grade or as previously reported (van den Heuvel et al 1998).

Software. Sequence alignments were performed with Sequence Navigator of Perkin Elmer. Homology searches were performed with the Basic Local Alignment Search Tool (BLAST) on the Internet at the server of the National Centre for Biotechnology Information (NCBI) (Altschul et al 1990). Molecular characteristics of cDNAs, and amino acid sequences were calculated using the OMIGA molecular biological software package (Hitachi). The average hydropathy was calculated using a window of 17 amino acids, according to the method described by Kyte and Doolittle (1982). We searched for amino acid motifs with use of PROSITE (http://www.embl-ebi.ac.uk/searches/prosite_input.html). Mitochondrial import sequences were determined with the program MitoProtII (Claros and Vincens 1996).

cDNA cloning of the human NDUFS2, NDUFS3 and NDUFS6 subunit. Bovine cDNA sequences of the NDUFS2, NDUFS3 and NDUFS6 subunits (Genbank accession no. X14338,

M58469, X63221) were used for a BLAST search in the Genbank (USA) human Expressed Sequence Tag (EST) database This revealed for each subunit several human ESTs, which were used in designing forward and reverse subunit specific oligonucleotides (Table 6 1) These oligonucleotides were applied in polymerase chain reaction
mRNA obtained from a human heart poly A^+ RNA pool (twelve normal Caucasian males and females, age range 20-78), was reverse transcribed to cDNA This process was carried out in 1 hour at 42°C, and catalysed by superscript II RNAse H reverse transcriptase. The reaction mix (total volume 40 µl) contained RNA, oligo(dT) and random hexanucleotide primers (Ploos van Amstel et al 1996)
PCR amplification was carried out in a reaction volume of 25 µl, containing 1 µl template, 2 5 µl 10× PCR buffer, 50 ng forward and reverse primer, 0 25 mM $MgCl_2$, 0 25 mM dNTPs, and 0 5 unit of Taq DNA Polymerase Polymerase chain reaction parameters are listed in Table 6 1 PCR product was purified using the QIAquick PCR purification kit Wild type reaction products were cycle sequenced (ABI Prism, model 377 version 2 1 2) according to procedures as described by the manufacturer

Chromosomal localisation of the NDUFS2, NDUFS3 and NDUFS6 gene To determine the chromosomal localisation of the *NDUFS2*, *NDUFS3*, and *NDUFS6* gene, we designed a forward oligonucleotide primer in the 3' terminal part of the open reading frame, and a reverse oligonucleotide primer in the 3' untranslated region (UTR) of each subunit With these oligonucleotides (reaction conditions are listed in Table 6 1), PCR was performed with as template genomic DNA extracted from human-rodent somatic cell hybrids, containing defined human chromosomes (NIGMS mapping panel 2) PCR product was analysed using a 4% agarose gel The obtained cDNA fragments were sequenced using the ABI Prism, model 377 version 2 1 2 in combination with the ABI[tm] Taq Dyedeoxy terminator cycle ready reaction kit

Results and discussion.

Isolated complex I disease is a major contributor to respiratory chain deficiencies, which are estimated to occur in 1 10,000 live births (Bourgeron et al 1995) Molecular biological studies performed on mitochondrial encoded complex I subunits revealed in some patients a mutation in one of the mitochondrial tRNAs (Tiranti et al 1995, Houshmand et al 1996) or in one of the mitochondrial genes coding for complex I subunits (Oostra et al 1995, de Vries et al 1996) Yet many patients remain genotypic undiagnosed Recently we described a patient with a 5 bp duplication present in the *NDUFS4* gene (18 kDa, van den Heuvel et al 1998) Both findings stress the need to characterise all nuclear encoded complex I subunits, especially those with a putative important function like the various IP subunits

Subunits	Forward oligonucleotides		Reverse oligonucleotides	
NDUFS2 ***(49 kDa)***	NDUFS2F1	TAAGATGGCGGCGCTGAGG	NDUFS2R1	AGCTTCTCAGTGCCTCGGTG
	NDUFS2F2	GATTCAGCCCAGCAGAGGTG	NDUFS2R2	GACACTCGCTCGTAGAACTC
	NDUFS2F3	ATGGTGCGGAAGTGTGATCCTCA	NDUFS2R3	ATCATAGGGCTGGGTCTTCC
	NDUFS2F4	CCCTTTCTTCTGGCTGTTTG	NDUFS2R4	GGTAGCCCTCAGTATACAAC
	NDUFS2F5	GACAATTGACATTGGGGTTG	NDUFS2R5	CACAGAAGAAGCTGATAGGC
	NDUFS2F6	AAGCGAGCAGAGATGAAGAC		
	NDUFS2F7	CAGATGTCGTTGCCATCATAGG		
NDUFS3 ***(30 kDa)***	NDUFS3F1	CATCTGAGTAACATGGCGG	NDUFS3R1	CTGTGCATTGGTGTGATCCC
	NDUFS3F2	GTTAGAGGTCTGTATCCATCC	NDUFS3R2	GGATCCTTCTTAGATCAGGG
	NDUFS3F3	AGCCAACTGGTATGAAAGGG	NDUFS3R3	CGCTGTCTAGGATCCACATGC
	NDUFS3F4	GAGAGTCTCAAGCTTGAAGCC		
NDUFS6 ***(13 kDa)***	NDUFS6F1	AAATGGCGGCGGCGATGACC	NDUFS6R1	CATAAACCTGGCCAGTGTGCG
	NDUFS6F2	AAGACTACAGGAGAATTCGG	NDUFS6R2	GCACAGAACCAGCGAGCTTCAC
	NDUFS6F3	CCAGTTCAGACAGCACCACCAC		

PCR conditions	
NDUFS2F1 + NDUFS2R5	94°C/1 min.; 94°C/1 min., 58 °C/1 min., 72 °C/1 min. (34 cycles); 72 °C/10 min.
NDUFS3F1 + NDUFS3R3	94°C/1 min.; 94°C/1 min., 57 °C/1 min., 72 °C/1 min. (34 cycles); 72 °C/10 min.
NDUFS6F1 + NDUFS6R2	94°C/1 min.; 94°C/1 min., 57 °C/1 min., 72 °C/1 min. (34 cycles); 72 °C/10 min.
NDUFS2F7 + NDUFS2R5	94°C/1 min.; 94°C/1 min., 60 °C/1 min., 72 °C/5 min. (34 cycles); 72 °C/10 min.
NDUFS3F4 + NDUFS3R3	94°C/1 min.; 94°C/1 min., 60 °C/1 min., 72 °C/5 min. (34 cycles); 72 °C/10 min.
NDUFS6F3 + NDUFS6R2	94°C/1 min.; 94°C/1 min., 60 °C/1 min., 72 °C/5 min. (34 cycles); 72 °C/10 min.

Table 6.1 Oligonucleotides listed here were used for cloning of the *NDUFS2*, *NDUFS3* and *NDUFS6* cDNAs (NDUFS2F1 + NDUFS2R5, NDUFS3F1 + NDUFS3R3, NDUFS6F1 + NDUFS6R2), for direct DNA sequencing reactions (NDUFS2F1, NDUFS2F2, NDUFS2F3, NDUFS2F4, NDUFS2F5, NDUFS2F6, NDUFS2R1, NDUFS2R2, NDUFS2R3, NDUFS2R4, NDUFS2R5, NDUFS3F1, NDUFS3F2, NDUFS3F3, NDUFS3R1, NDUFS3R2, NDUFS3R3, NDUFS6F1, NDUFS6F2, NDUFS6R1, NDUFS6R2), and in PCR with DNA isolated from human-rodent somatic cell hybrids as template (NDUFS2F7 + NDUFS2R5, NDUFS3F4 + NDUFS3R3, NDUFS6F3 + NDUFS6R2) F = Forward primer; R = Reverse primer. The lower part of Table 6.1 contains the PCR conditions for each oligonucleotide pair used. Listed are respectively: preceding denaturation step; denaturation, annealing, extension; final extension step.

The human *NDUFS2*, *NDUFS3*, and *NDUFS6* cDNAs were amplified with PCR using the following oligonucleotide combinations: NDUFS2F1 + NDUFS2R5, NDUFS3F1 + NDUFS3R3, and NDUFS6F1 + NDUFS6R2 (Table 6.1 and Fig. 6.1). The oligonucleotides listed in Table 6.1 with exception of NDUFS2F7 and NDUFS3F4 were used in direct DNA sequencing reactions using the Dyedeoxy terminators. All three cDNAs contain the complete ORF (1392, 795, and 375 base pairs), coding for 463, 264, and 124 amino acids, respectively

(Table 6.2). Molecular weights calculated from amino acid sequences are respectively 52.5, 30.2, and 13.7 kDa. The 3' untranslated regions (UTR) were completed using several human ESTs retrieved from Genbank (poly A signals (AATAAA) and poly A tails are present). Comparison of amino acid sequences of bovine and human subunit equivalents revealed a similarity of 96% (NDUFS2), 90% (NDUFS3), and 83% (NDUFS6). High similarity between human and bovine housekeeping proteins is not uncommon (similarity of published nuclear encoded complex I proteins ranges from 79% to 95%). It can be hypothesised that high similarity percentages reflect strong conservation among species and therefore possible functional importance. cDNA similarity is highest in the region coding for the mature protein. We calculated the theoretical existence of a mitochondrial targeting sequence (MTS) with MitoProtII software, and compared the results with the experimental bovine data published by Walker et al 1992 (the bovine N-terminal part of the mature protein was sequenced by Edman degradation prior to the complete ORF). In man, possible cleavage sites are present between amino acid positions 20 and 21 in the NDUFS2, 36 and 37 in the NDUFS3 and 28 and 29 in the NDUFS6 subunit. In case of the bovine NDUFS2 subunit, the N-terminus of the mitochondrial targeting sequence is incomplete. For the NDUFS3 and NDUFS6 the cleavage site between MTS and mature protein was similar as predicted for man. The untranslated regions, as well as MTS coding regions clear reflect lower similarity percentages between man and *Bos taurus*. The NDUFS3 and NDUFS6 MTS are 55.6% and 60.7% similar, while the mature proteins are 94.7% and 89.6% similar.

PCR performed with DNA extracted from somatic human-rodent cell hybrids containing defined human chromosomes as template gave signals specific for man in all three subunits. In case of the NDUFS2 subunit this fragment contained two (partial) exons and an intron, for the *NDUFS3* and *NDUFS6* the fragment was equal to the fragment when cDNA was used as template. The *NDUFS2* fragment was present in the lane containing chromosome 1, the *NDUFS3* in the lane containing chromosome 11, and unfortunately the *NDUFS6* fragment was present in both the lanes containing chromosome 5, as chromosome 6. One of the bands is likely caused by a pseudogene. We could not differentiate which of the two was the pseudogene, since both bands contained the same nucleotide sequence in this short fragment.

Subunit	Genbank accession no.	cDNA size	ORF size	No. a.a.	M_r kDa	Signal peptide	H B	H N	H E	No. cysteine			
										H	B	N	E
NDUFS2	AF050640	1565	1392	463	52.5	1-20	96%	62%	38%	7	6	6	4
NDUFS3	AF067139	887	795	264	30.2	1-36	90%	46%	30%	2	3	3	0
NDUFS6	AF044959	515	375	124	13.7	1-28	83%	-	-	6	4	-	-

Table 6.2 Characteristics of the NDUFS2, NDUFS3, and NDUFS6 subunit.
ORF = open reading frame, a.a. = amino acids; M_r = calculated molecular weight; H = *H. sapiens*; B = *B. taurus*; N = *N. crassa*; E = *E. coli*.

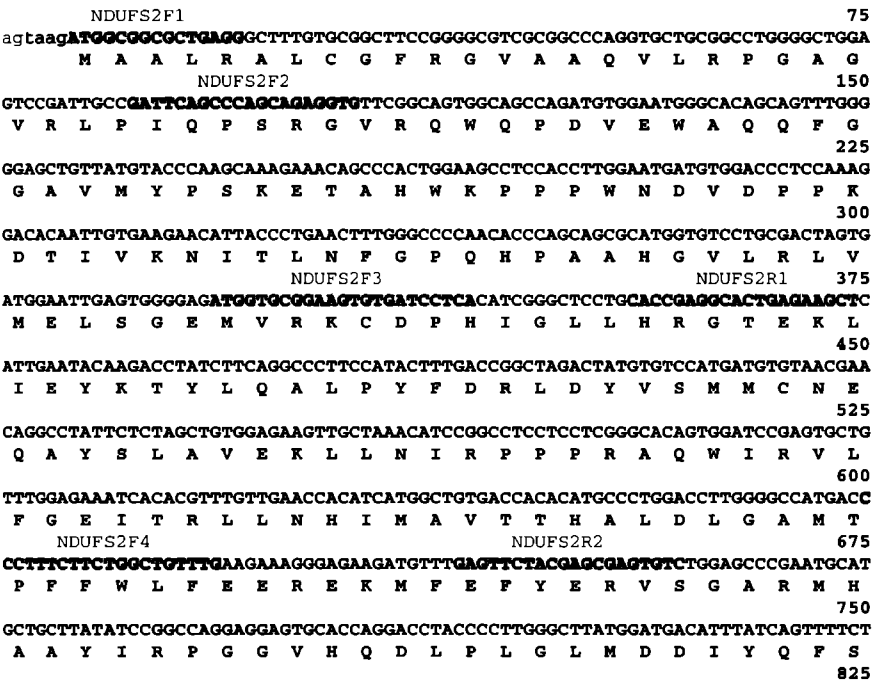

Fig 6.1 (continued) cDNA and amino acid sequence of the NDUFS2 subunit

```
      NDUFS3F1                                                                  75
catctgagtaacATGGCGGCGGCGGCGGTAGCCAGGCTGTGGTGGCGCGGGATCTTGGGGGCCTCGGCGCTGACC
              M  A  A  A  A  V  A  R  L  W  W  R  G  I  L  G  A  S  A  L  T
                                                                           150
AGGGGGACTGGGCGACCCTCCGTTCTGTTGCTGCCGGTGAGGCGGGAGAGCGCCGGGGCCGACACGCGCCCCACT
 R  G  T  G  R  P  S  V  L  L  L  P  V  R  R  E  S  A  G  A  D  T  R  P  T
                                                                           225
GTCAGACCACGGAATGATGTGGCCCACAAGCAGCTCTCAGCTTTTGGAGAGTATGTGGCTGAAATCTTGCCCAAG
 V  R  P  R  N  D  V  A  H  K  Q  L  S  A  F  G  E  Y  V  A  E  I  L  P  K
                                                 NDUFS3F2                  300
TATGTCCAACAAGTTCAGGTGTCCTGCTTCAATGAGTTAGAGGTCTGTATCCATCCTGATGGCGTCATCCCAGTG
 Y  V  Q  Q  V  Q  V  S  C  F  N  E  L  E  V  C  I  H  P  D  G  V  I  P  V
                     NDUFS3R1                                              375
CTGACTTTCCTCAGGGATCACACCAATGCACAGTTCAAATCTCTGGTTGACTTGACAGCAGTGGACGTCCCAACT
 L  T  F  L  R  D  H  T  N  A  Q  F  K  S  L  V  D  L  T  A  V  D  V  P  T
                                                                           450
CGGCAAAACCGTTTTGAGATTGTCTACAACCTGTTGTCTCTGCGCTTCAACTCACGGATCCGTGTGAAGACCTAC
 R  Q  N  R  F  E  I  V  Y  N  L  L  S  L  R  F  N  S  R  I  R  V  K  T  Y
                                                    NDUFS3F3               525
ACAGATGAGCTGACGCCCATTGAGTCTGCTGTCTCTGTGTTCAAGGCAGCCAACTGGTATGAAAGGGAGATCTGG
 T  D  E  L  T  P  I  E  S  A  V  S  V  F  K  A  A  N  W  Y  E  R  E  I  W
                                          NDUFS3R2                         600
GACATGTTTGGAGTCTTCTTTGCTAACCACCCTGATCTAAGAAGGATCCTGACAGATTATGGCTTCGAGGGACAT
 D  M  F  G  V  F  F  A  N  H  P  D  L  R  R  I  L  T  D  Y  G  F  E  G  H
                                                                           675
CCTTTCCGGAAAGACTTTCCTCTATCTGGCTATGTTGAGTTACGTTATGATGATGAAGTGAAGCGGGTGGTGGCA
 P  F  R  K  D  F  P  L  S  G  Y  V  E  L  R  Y  D  D  E  V  K  R  V  V  A
                                                                           750
GAGCCGGTGGAGTTGGCCCAAGAGTTCCGCAAATTTGACCTGAACAGCCCCTGGGAGGCTTTCCCAGTCTATCGC
 E  P  V  E  L  A  Q  E  F  R  K  F  D  L  N  S  P  W  E  A  F  P  V  Y  R
                    NDUFS3F4                                               825
CAACCCCCGGAGAGTCTCAAGCTTGAAGCCGGAGACAAGAAGCCTGATGCCAAGTAGctccagggaacgcatgtg
 Q  P  P  E  S  L  K  L  E  A  G  D  K  K  P  D  A  K  *
NDUFS3R3                                 poly a signal          poly a site 900
gatcctagacagcgccttatctatgattgagtgtccgtgtaaataaattcctacttagacttaaaaaaaaaaaaa
```

Fig 6.1 (continued) cDNA and amino acid sequence of the NDUFS3 subunit

```
                 NDUFS6F1                                                  75
cggcgcaaaATGGCGGCGGCGATGACCTTCTGCCGGCTGCTGAACCGGTGTGGCGAGGCGGCGCGGAGCCTGCCC
          M  A  A  A  M  T  F  C  R  L  L  N  R  C  G  E  A  A  R  S  L  P
                                                                NDUFS6R1   150
CTGGGCGCCAGGTGTTTCGGGGTGCGGGTCTCGCCGACCGGGGAGAAGGTCACGCACACTGGCCAGGTTTATGAT
 L  G  A  R  C  F  G  V  R  V  S  P  T  G  E  K  V  T  H  T  G  Q  V  Y  D
          NDUFS6F2                                                         225
GATAAAGACTACAGGAGAATTCGGTTTGTAGGTCGTCAGAAAGAGGTGAATGAAAACTTTGCCATTGATTTGATA
 D  K  D  Y  R  R  I  R  F  V  G  R  Q  K  E  V  N  E  N  F  A  I  D  L  I
                                                                           300
GCAGAGCAGCCCGTGAGCGAGGTGGAGACTCGGGTGATAGCGTGCGATGGCGGCGGGGGAGCTCTTGGCCACCCA
 A  E  Q  P  V  S  E  V  E  T  R  V  I  A  C  D  G  G  G  G  A  L  G  H  P
                                                              NDUFS6F3 375
AAAGTGTATATAAACTTGGACAAAGAAACAAAAACCGGCACATGCGGTTACTGTGGGCTCCAGTTCAGACAGCAC
 K  V  Y  I  N  L  D  K  E  T  K  T  G  T  C  G  Y  C  G  L  Q  F  R  Q  H
                                                                           450
CACCACTAGagcgtgtggcacgccgggggtcccgcagcatcctgtgagcatttccgcggggaagctgagcacgtg
 H  H  *
    NDUFS6R2                              poly a signal              poly a site 525
aagctcgctggttctgtgcgaagggtattcctggtgctgaataaagggtgttgctgtcatggctgaaaaaaaaaa
```

Fig 6.1 (continued) cDNA and amino acid sequence of the NDUFS6 subunit Primers used for PCR and sequence reactions are shaded Primers are also listed in Table 6 1 Positions of poly a signal and site have been indicated for each subunit 5'UTR and 3'UTR were completed (when possible) with use of ESTs retrieved from Genbank, USA

	Consensus amino acid patterns			
	Name	**No.**	**A.A. position**	**Also present in:**
NDUFS2 (*49 kDa*)	**N-glycosylation site** (NITL; NFSL; NRTI)	3	79	*B. taurus*
			250	*B. taurus*
			270	*B. taurus*
	Casein kinase II phosphorylation site (TAEE; SRGD; TSME; TAIE)	4	279	*B. taurus, N. crassa*
			322	*B. taurus*
			373	*B. taurus*
			398	*B. taurus, N. crassa*
	N-myristoylation site (GVVTAE; GATYTA)	2	276	*B. taurus, N. crassa*
			394	*B. taurus*
	Protein kinase C phosphorylation site (TEK; SLR; SLR; RGD)	4	120	*B. taurus, N. crassa*
			252	*B. taurus*
			340	*B. taurus, N. crassa, E. coli*
			416	*B .taurus, N. crassa*
	Tyrosine phosphorylation site (KLIEYKTY)	1	122	*B. taurus, N. crassa*
NDUFS3 (*30 kDa*)	**cAMP phosphorylation site** (RRES)	1	37	*B. taurus*
	Casein kinase II phosphorylation site (SLVD; TAVD; TYTD; TPIE; SPWE)	5	110	*B. taurus*
			115	*B. taurus, N. crassa, E. coli*
			145	*B. taurus, N. crassa*
			151	*B. taurus*
			237	*B. taurus, N. crassa*
	N-myristoylation site (GILGAS, GVFFAN)	2	13	-
			175	*B. taurus, N. crassa*
	Protein kinase C phosphorylation site (TGR; TVR; SLR; SLK)	4	24	*N. crassa*
			46	*B. taurus*
			134	*B. taurus, N. crassa*
			251	*B. taurus, N. crassa*
NDUFS6 (*13 kDa*)	**Casein kinase II phosphorylation site** (SEVE)	1	78	-
	N-myristoylation site (GGGGAL; GGALGH)	2	89	*B. taurus*
			91	*B. taurus*

Table 6.3 Amino acid consensus patterns were searched with PROSITE. A consensus amino acid sequence pattern was titled conserved when in multiple alignment a similar consensus pattern was present. All data listed in this Table concern the human subunits.

In order to speculate about functional properties of the human NDUFS2, NDUFS3 and NDUFS6 subunit we searched for conserved consensus amino acid patterns among eu- and prokaryotes. For this search we included besides man the subunit equivalents of *B. taurus*, *N. crassa* and *E. coli*, since complex I has been almost completely characterised in these organisms. The Swissprot accession numbers for *B. taurus*, *N. crassa* and *E. coli* are: NDUFS2: P17694, P22142, P33600; NDUFS3: G163416, P23710, P33599; NDUFS6: P23934. NDUFS2 and NDUFS3 are represented in man, *B. taurus*, *N. crassa*, and *E. coli*, while the NDUFS6 is not present in *N. crassa*, and *E. coli*. Since *E. coli* contains a complex I consisting of only 14 subunits, a large amount of the nuclear encoded subunits of human complex I have no representative in bacteria. The *N. crassa* complex I contains about 35

subunits, and yet there is no representative present of the NDUFS6 Subunits with no obvious equivalent present in lower life forms could have emerged late in evolution when these species had already diverged or they diverged fast so that detection of homology is impossible In multiple alignment we considered besides identical patterns, a pattern with different amino acids coding for the same consensus motif conserved The pattern search performed with PROSITE revealed for each subunit several consensus amino acid motif patterns (Table 6 3), but only two were conserved among all four species The NDUFS2 subunit contains a conserved SLR protein kinase C phosphorylation site at the C terminus of the protein The *NDUFS3* transcript contains a conserved casein kinase II phosphorylation site (TAVD) at amino acid position 115 This high conservation among eukaryotes and prokaryotes is suggestive for functional significance The NDUFS6 subunit has a conserved N-myristoylation site between amino acid 89 and 96 In the past suggestions were made that the NDUFS2, and NDUFS3 subunit could contain Fe-S clusters (Preis et al 1990, Fearnley and Walker 1992), which are commonly reflected by consensus cysteine patterns The human NDUFS2 contains seven cysteine residues, six of which are conserved in *B taurus*, and three in *N crassa* The NDUFS3 has two cysteine residues, both conserved in *B taurus*, but none in *N crassa* The human *NDUFS6* transcript contains six cysteine residues, four are conserved in *B taurus* Considering the consensus patterns for Fe-S clusters, none of the three subunits seems to meet the criteria, but further experimental studies are warranted

The average hydropathy was calculated using the OMIGA molecular biological software package (Hitachi) All subunits express an overall hydrophilic pattern The NDUFS2 subunit contains a hydrophobic segment of about 25 amino acids, which is long enough to form a transmembrane α helix Bovine NDUFS2, NDUFS3 and NDUFS6 are located in the 1α fraction of complex I (which is likely to be the part protruding into the mitochondrial matrix) (Finel et al 1992), which is in accordance to the overall hydrophilic pattern of the human equivalents In addition, the NDUFS2 has been localised by immuno-labelling on the peripheral arm (protruding into the mitochondrial matrix) of complex I in *N crassa* (Guenebaut et al 1997)

Mammalian NADH ubiquinone oxidoreductase has proven to be a very large and complicated protein complex The NDUFS2 and NDUFS3 subunits are highly conserved in prokaryotes and eukaryotes, since homologues are found in many species and even in chloroplast (Fearnley et al 1989, Dupuis et al 1991) Furthermore they contain highly conserved phosphorylation sites which might be involved in regulatory function We therefore consider the *NDUFS2* and *NDUFS3* strong candidates for mutation detection studies in isolated complex I deficient patients

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cDNA OF EIGHT NUCLEAR ENCODED SUBUNITS OF NADH:UBIQUINONE OXIDOREDUCTASE: HUMAN COMPLEX I cDNA CHARACTERISATION COMPLETED?

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Abstract.
NADH:ubiquinone oxidoreductase (complex I) is an extremely complicated multi-protein complex located in the inner mitochondrial membrane. Its main function is the transport of electrons from NADH to ubiquinone, which is accompanied by translocation of protons from the mitochondrial matrix to the intermembrane space. Human complex I appears to consist of forty-one subunits of which thirty-four are encoded by nDNA. Here we report the cDNA sequences of the hitherto uncharacterised eight nuclear encoded subunits, all located within the Hydrophobic Protein (HP) fraction of complex I. Now all currently known forty-one proteins of human NADH:ubiquinone oxidoreductase have been characterised and reported in literature, which enables more complete mutational analysis studies of isolated complex I deficient patients.

Introduction.

NADH ubiquinone oxidoreductase of the mitochondrial respiratory chain is one of the most complicated multi-protein enzyme complexes present in the human cell (Smeitink et al 1998a) Its main function is transport of electrons by oxidation of NADH followed by reduction of ubiquinone This process is accompanied by the translocation of protons from the mitochondrial matrix to the intermembrane space, a process of which much has to be learned Several prosthetic groups catalyse these electron transport reactions, which are hypothesised to be at least one Flavin Mononucleotide (FMN) and six to eight Iron-Sulphur clusters (Albracht et al 1996, Ohnishi 1998) One of the most extensively studied mammals with regard to complex I is *Bos taurus* Bovine complex I consists of about forty-one subunits, which have mainly been characterised by the research group of Walker (Walker 1992 and references therein) Seven are encoded by mitochondrial DNA (ND1-ND6, ND4L, (Chomyn et al 1985 and 1986), the remainder are nuclear encoded and imported into mitochondria
By using chaotropic agents, complex I can be fragmented into three different fractions (Galante and Hatefi 1978 and 1979) The Flavoprotein (FP) fraction contains three subunits all of which have been characterised in man on complementary DNA (cDNA) as well as nuclear DNA (nDNA) level, namely NDUFV1 (*51 kDa*), NDUFV2 (*24 kDa*) and NDUFV3 (*10 kDa*) (Spencer et al 1992, Ali et al 1993, de Coo et al 1995, Hattori et al 1995, de Coo et al 1997, Schuelke et al 1998) The NADH binding site of complex I has been assigned to the NDUFV1 subunit (Chen and Guillory 1981, Deng et al 1990) In addition, the NDUFV1 contains the FMN and a tetranuclear Iron-Sulphur cluster (Pilkington et al 1991) The NDUFV2 subunit contains a binuclear Iron-Sulphur cluster (Pilkington and Walker 1989), while the NDUFV3 does not contain any known redox centres The second fraction is the so-called Iron-Sulphur Protein (IP) fraction The IP fraction contains at least seven subunits all of which are fully characterised on cDNA level, namely the NDUFS1 (*75 kDa*), NDUFS2 (*49 kDa*), NDUFS3 (*30 kDa*), NDUFS4 (*18 kDa*), NDUFS5 (*15 kDa*), NDUFS6 (*13 kDa*) and NDUFA5 (*B13*) (Chow et al 1991, Pata et al 1997, Loeffen et al 1998, van den Heuvel et al 1998, Loeffen et al 1999) Several functional properties have been postulated concerning the IP subunits The NDUFS1 contains a tetra-nuclear and probably also a bi-nuclear Iron-Sulphur cluster (Runswick et al 1989) Furthermore, an active cAMP consensus phosphorylation site has been encountered in the NDUFS4 subunit (Papa et al 1996)
The final subgroup of complex I is the Hydrophobic Protein (HP) fraction This is a large group containing 24 nuclear encoded subunits, of which in man the cDNA has been characterised for 16 subunits NDUFA1 (*MWFE*), NDUFA2 (*B8*), NDUFA4 (*MLRQ*), NDUFA6 (*B14*), NDUFA8 (*PGIV*), NDUFA9 (*39 kDa*), NDUFAB1 (*SDAP*), NDUFB1 (*MNLL*), NDUFB3 (*B12*), NDUFB5 (*SGDH*), NDUFB6 (*B17*), NDUFB7 (*B18*), NDUFB9 (*B22*), NDUFS7 (*PSST*), NDUFS8 (*TYKY*), and NDUFC1 (*KFYI*) (Wong et al 1990, Baens et al 1994, Zhuchenko et al 1996, Gu et al 1996, Hyslop et al 1996, Ton et al 1997, Kim et al 1997, Procaccio et al 1997, Triepels et al 1998a, Triepels et al 1998b, Smeitink et al 1998) A complete list of Genbank accession numbers for all human complex I subunits is presented in Table 7 1 The complete nDNA structure of subunits present in the HP fraction has only been elucidated for the *NDUFS8* and *NDUFA1* gene (Zhuchenko et al 1996, de Sury et al 1998)

Electron Paramagnetic Resonance (EPR) spectroscopy studies performed with bovine complex I revealed that all known Iron-Sulphur clusters are located in the extra membranous part (Finel et al 1992), which accommodates the FP and IP subfractions Little is understood about functional properties of the individual subunits of the HP fraction, which also accounts for functional properties of this fraction as a unit Worth mentioning are the two consensus tetranuclear Iron-Sulphur cluster patterns present in NDUFS8 (Dupuis et al 1991a), a consensus binuclear Iron-Sulfur cluster pattern in NDUFS7 (Arizmendi et al 1992) and NDUFA8 (Dupuis et al 1991b) and the NDUFAB1 subunit being an acyl-carrying protein (Runswick et al 1991)

Up to now, isolated complex I deficiency has been mostly connected to mutations in mtDNA, of which several have been described (Zeviani et al 1998) Yet, in our isolated complex I deficient patient group (measured in cultured skin fibroblasts) consisting of mainly young children, common mtDNA mutations and major mtDNA rearrangements cannot be found It is therefore likely, that many defects of respiratory chain disorders will be at nuclear level As a prerequisite to mutational analysis studies of nuclear encoded subunits of complex I in our patient group, we cloned all remaining uncharacterised complex I subunits

Here we report the cDNA sequences of these remaining eight uncharacterised subunits, which are all part of the HP fraction Now all known mammalian complex I cDNAs have been cloned in man Furthermore we examined to what extend these subunits are conserved in evolution and speculate about functional properties

Material and methods.

Commercial purchased materials

Oligonucleotide primers, Taq DNA polymerase, Superscript II RNAse H reverse transcriptase were purchased from Life Technologies Inc (Breda, The Netherlands), the dRhodamine Dye Terminator Sequencing kit was purchased from Perkin Elmer (Warrington, England), PCR purification systems were purchased from Qiagen (Leusden, The Netherlands), RNAzol was purchased from Biosolve BV (Watford, England), and a human heart poly A^+ RNA pool was purchased from Clontech (Palo Alto, USA)

Software

Homology searches in protein and nucleotide databases were performed with the Basic Local Alignment Search Tool (BLAST) on the internet at the server of the National Centre for Biotechnology Information (NCBI) (Altschul et al 1990) Hydropathy was plotted using methods based on the procedures described by Kyte and Doolittle (1982) Computer software was used from the National Centre for Computer Aided Chemistry and Bioinformatics (CAOS/CAMM, Nijmegen, The Netherlands)

H. sapiens / Bos taurus	Genbank accession no.	Reference
NDUFV1 / 51 kDa	AF053070	Schuelke et al 1998
NDUFV2 / 24 kDa	M25484	De Coo et al 1995, Hattori et al 1995
NDUFV3 / 10 kDa	X99726-8	De Coo et al 1997
NDUFA1 / MWFE	X81900	Zhuchenko et al 1996
NDUFA2 / B8	AF047185	Ton et al 1997
NDUFA3 / B9	AF044955	CP
NDUFA4 / MLRQ	U94586	Kim et al 1997
NDUFA5 / B13	U53468	Pata et al 1997
NDUFA6 / B14	AF047182	Ton et al 1997
NDUFA7 / B14.5A	AF050637	CP
NDUFA8 / PGIV	AF044953	Triepels et al 1998b
NDUFA9/ 39 kDa	G189048	Baens et al 1994
NDUFA10 / 42 kDa	AF087661	CP
NDUFAB1 / SDAP	AF087660	Triepels et al 1998a
NDUFB1 / MNLL	AF054181	-
NDUFB2 / AGGG	AF050639	CP
NDUFB3 / B12	AF047183	Ton et al 1997
NDUFB4 / B15	AF044957	CP
NDUFB5 / SGDH	AF047181	Ton et al 1997
NDUFB6 / B17	AF035840	Smeitink et al 1998
NDUFB7 / B18	M33374	Wong et al 1990
NDUFB8 / ASHI	AF044958	CP
NDUFB9 / B22	S82655	Gu et al 1996
NDUFB10 / PDSW	AF044954	CP
NDUFS1 / 75 kDa	X61100	Pilkington et al 1991
NDUFS2 / 49 kDa	AF050640	Loeffen et al 1998, Procaccio et al 1998
NDUFS3 / 30 kDa	AF067139	Loeffen et al 1998
NDUFS4 / 18 kDa	AF020351	Van den Heuvel et al 1998
NDUFS5 / 15 kDa	AF020352	Loeffen et al 1999
NDUFS6 / 13 kDa	AF044959	Loeffen et al 1998
NDUFS7 / PSST	-	Hyslop et al 1996
NDUFS8 / TYKY	U65579	Procaccio et al 1997
NDUFC1 / KFYI	AF047184	Ton et al 1997
NDUFC2 / B14.5B	AF087659	CP

Table 7.1 Complete list of all published human nuclear encoded complex I subunit cDNAs. CP = current paper. In case of the NDUFV3 and NDUFS7, an accession no. of the cDNA sequence could not be retrieved. We listed the exon accession no. of the NDUFV3 subunit instead. The NDUFB1 cDNA sequence is not published, but has been submitted to Genbank.

cDNA cloning.

200 ng heart poly A^+ RNA obtained from a pool of twelve healthy male and female Caucasians, age range 20-78 years, was reverse transcribed to cDNA by superscript II RNAse H^- reverse transcriptase in one hour at 42°C (Ploos van Amstel et al 1996). Bovine cDNA sequences of the remaining eight nuclear encoded subunits of complex I were BLASTed in

the NCBI human Expressed Sequence Tags (EST) database, which revealed for each subunit several hits. Oligonucleotide primers were chosen (when possible) in the 5' and 3' untranslated region (UTR) in order to clone the complete open reading frame (ORF). Primers used for cloning of the cDNAs as well as for direct sequencing reactions are listed in Table 7.2. PCR reactions were performed in a reaction volume of 50 µl, after addition of 1µl cDNA template, 5 µl 10× PCR buffer, 100 ng Forward and Reverse primer, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, and 1 U Taq DNA polymerase. PCR products were purified using the QIAquick PCR purification kit. Both strands were analysed by direct sequencing (ABI Prism, model 377 version 2.1.2), using the ABI Prism^{Tm} dRhodamine terminator cycle sequencing ready reaction kit.

Subunits	Forward oligonucleotides		Reverse oligonucleotides	
NDUFA3 B9	**NDUFA3F**	CCCGCCGCGGAGACAAAG	**NDUFA3R**	GGCCGCCTCTGTCAGTGGAG
NDUFA7 B14.5A	**NDUFA7F**	CGCGGACGGAAGATGGC	**NDUFA7R**	GAGGAAATCCAAGGAGGCAAAGTAG
NDUFA10 42 kDa	**NDUFA10F1**	TCCTGAGCTGACCGGGTAG	**NDUFA10R1**	TGTCTGGATAATGAATCCCC
	NDUFA10F2	GTGGCATTTCCTACTTGGGG	**NDUFA10R2**	GGTTGTACATCGCCTCCAGG
	NDUFA10F3	CTGGTTGTACAGCAGTCGCC	**NDUFA10R3**	TTTTCACTCATCTCAGGGAG
	NDUFA10F4	AGGCGGATTCAGAAGAAAGG	**NDUFA10R4**	CTGAACTGATGTAAGACACGG
	NDUFA10F5	CAGGATAAGTTTGAGGTGCTG	**NDUFA10R5**	TGGCCTTTCACAGCAGACC
NDUFB2 AGGG	**NDUFB2F**	GAAGCGAAGTAGGCAGGGGC	**NDUFB2R**	CCTTGAAATTCTCACCTGGCTCTTG
NDUFB4 B15	**NDUFB4F1**	GCTCCCAAGATGTCGTTCCC	**NDUFB4R1**	GGCAGGATTTTCGATGAGC
	NDUFB4F2	CATGGGAGCTCTGTGTGGATTTG	**NDUFB4R2**	GGCAGGAATACATATAGTCATCATTG
NDUFB8 ASHI	**NDUFB8F1**	GGCATGTGGAAGAAGGTG	**NDUFB8R1**	CAGGGAGCTTCGGGTAGTC
	NDUFB8F2	TAGGACCCCAGAAGAACGG	**NDUFB8R2**	AAGCCAGGAAACCGAAGAGC
			NDUFB8R3	TTCTAGGAATGAGGGAGTCC
NDUFB10 PDSW	**NDUFB10F1**	AGGCCCGGGACCCGGACGG	**NDUFB10R1**	GTTGATAATTTCTTGGTCGACTTTG
	NDUFB10F2	CAGTACCGCCGCGTGCCAG	**NDUFB10R2**	CAGTCATACAGAGCCACACAGCAG
NDUFC2 14.5B	**NDUFC2F1**	TCGTCGTGGTTTTCCTTGTAG	**NDUFC2R1**	GGCCGTAATATATAGAAGCTGG
	NDUFC2F2	CTACCTGTATGCTGTGAGGG	**NDUFC2R2**	CAAATTCAGAAACAGCAGGTATCAG

Table 7.2 List of oligonucleotide primers used in PCR amplification and direct DNA sequencing reactions of all eight nuclear encoded complex I cDNAs presented in this study.

Results and discussion.

NADH:ubiquinone oxidoreductase has been fully characterised on cDNA level in several species like *B. taurus* (Walker 1992), *Neurospora crassa* (Weiss et al 1991) and *Escherichia coli*, (Weidner et al 1993). The complexity of this multi-protein complex varies from a minimal structural unit of fourteen subunits present in many bacteria like *E. coli* to the currently known maximum of at least 41 subunits present in cow. With the additional eight cDNAs presented in this study, all known 41 subunits of human NADH:ubiquinone oxidoreductase have been characterised, which clears the way for more complete mutational analysis studies of nuclear encoded subunits in isolated complex I deficient patients.

The HP fraction to which all eight presented subunits belong is a precipitate, which remains after fragmentation with chaotropic agents. At least 31 subunits belong to this fraction

including the seven, which are mitochondrially encoded. Little is known about the retained functional integrity of this precipitate. There is speculation that the HP fraction participates in the transfer of protons from the mitochondrial matrix to the intermembrane space (Belogrudov and Hatefi 1994).

Human subunit	Size ORF	No. a.a.	M_r (kDa)	Leader peptide in B	H / B identity (a.a.)	Homology
NDUFA3	255	84	9.3	-	83.3%	*Bos taurus*
NDUFA7	342	113	12.6	-	88.5%	*Bos taurus* *Caenorhabditis elegans*
NDUFA10	1068	355	40.8	+	80.2%	*Bos taurus* *Caenorhabditis elegans*
NDUFB2	318	105	12.1	+	83.8%	*Bos taurus* *Caenorhabditis elegans*
NDUFB4	390	129	15.2	-	73.6%	*Bos taurus* *Gallus gallus*
NDUFB8	561	186	21.8	+	84.9%	*Bos taurus*
NDUFB10	519	172	20.8	-	82.4%	*Bos taurus* *Caenorhabditis elegans*
NDUFC2	360	119	14.2	-	73.9%	*Bos taurus*

Table 7.3 Molecular characteristics of the presented eight human complex I subunits. ORF = open reading frame, M_r = calculated molecular weight, a.a. = amino acids, H = *Homo sapiens*, B = *Bos taurus*. Homology between man and other species was investigated with use of BLAST search at the server of NCBI.

PCR amplification with subunit specific oligonucleotide primers revealed the complete Open Reading Frame (ORF) of all eight remaining subunits: *NDUFA3* (*B9*) 255 bp, *NDUFA7* (*B14.5A*) 342 bp, *NDUFA10* (*42 kDa*) 1068 bp, *NDUFB2* (*AGGG*) 318 bp, *NDUFB4* (*B15*) 390 bp, *NDUFB8* (*ASHI*) 561 bp, *NDUFB10* (*PDSW*) 519 bp and *NDUFC2* (*B14.5B*) 360 bp (Fig. 7.1). Molecular characteristics of these subunits are listed in Table 7.3. Several of the eight subunit amino acid chains are different in length in man compared to *B. taurus*. The first three amino acids of the bovine NDUFB2 (*AGGG*) subunit are not present in man, in case of the NDUFB10 (*PDSW*) subunit, man misses six amino acids on the N-terminal side whereas there are an extra two at the C-terminal side, the NDUFA10 (*42 kDa*) of man is twelve amino acids longer (situated at the N-terminal side) and finally, bovine NDUFC2 (*B14.5B*) starts with two methionine residues, whereas man contains only one. The identity on amino acid level between man and *B. taurus* ranges between 73% and 89%. These identity percentages are slightly less than seen in nuclear encoded subunits located in the minimal structural unit as e.g. present in *E. coli*, where identity percentages between man and *Bos taurus* vary from 90% to 97%. Yet, for many subunits of NADH:ubiquinone oxidoreductase the interspecies conservation is extremely high, which underlines the functional importance of this complex. Average hydropathy calculation using a range of 17 amino acids revealed an overall hydrophilic pattern for five subunits (*NDUFA7, NDUFA10, NDUFB2, NDUFB4* and *NDUFB10*). The other three subunits contain one or more continuous hydrophobic parts of at least twenty amino acids. The presence of hydrophilic proteins in the insoluble hydrophobic fraction of complex I has been reported previously for *Bos taurus* (Walker 1992). One can speculate that the hydrophilic proteins present in the HP fraction contribute to the outer

sections of the membranal part of complex I which communicate with mitochondrial matrix or intermembrane space, while the purely hydrophobic proteins present in this fraction like the mtDNA encoded ND genes are actually in the lipid bilayer In cow, three of eight subunits presented in this paper appear to have a leader peptide It is reasonable to assume that these Mitochondrial Targeting Sequences (MTS) will also be present in man Interestingly, in cow all FP fraction subunits and five of seven IP fraction subunits (71%) appear to have an N-terminal targeting sequence whereas this applies for only nine of twenty-four (38%) of the HP fraction subunits

EPR spectroscopy studies of bovine, *N crassa* and *E coli* complex I revealed several Iron-Sulfur redox centres (Ohnishi 1998) Six nuclear encoded subunits contain conserved cysteine patterns corresponding with consensus Iron-Sulfur clusters as listed in the introduction Three of those cysteine-rich subunits are present in the HP fraction, namely NDUFS7, NDUFS8 and NDUFA8 Of all eight subunits presented in this paper, the NDUFA10 and NDUFB10 are the only two with more than two cysteine residues among its amino acids The NDUFA10 subunit contains six cysteine residues and the NDUFB10 five, all of which are conserved in *B taurus*, with the exception of the first NDUFA10 cysteine residue Yet, the cysteine residues are not grouped resembling a consensus Iron-Sulfur cluster pattern

When three-dimensional structures of *N crassa* (at least 35 subunits) and *E coli* (14 subunits) are compared, the main difference appears to be the size (Guenebaut et al 1998) The core of fourteen proteins seems to be equal, higher species appear to have more subunits moulded around this core The eight characterised cDNAs presented in this study do not appear to be highly conserved among species A BLAST search with the amino acid sequences of these subunits revealed (apart from the obvious matches with bovine subunits) only matches (although weak in some occasions) in *Caenorhabditis elegans* (NDUFA7, NDUFA10, NDUFB2 and NDUFB10) and *Gallus gallus* (NDUFB4)

Isolated complex I deficiency is a major cause of abnormalities in the energy generating system present in mitochondria Great efforts have been made in the past to link a genetic cause to this enzyme defect Several mtDNA mutations have been described, but these certainly cannot explain all isolated complex I deficiencies, especially not the most frequently observed phenotype which presents in infancy, expressing a highly progressive lethal course Therapeutic possibilities regarding complex I deficiency are out of reach for the time being Therefore, mutational analysis offers the possibility for early detection and if desired for prenatal diagnosis We presented the remaining eight nuclear encoded complex I cDNAs, which completes human complex I on cDNA level

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```
      NDUFA3F                                                                  75
cccgccgcggagacaaagATGGCTGCGAGAGTCGGCGCCTTCCTCAAGAATGCCTGGGACAAGGAGCCAGTGCTG
                    M  A  A  R  V  G  A  F  L  K  N  A  W  D  K  E  P  V  L
                                                                            150
GTCGTGTCCTTCGTCGTCGGGGGCCTCGCTGTAATTCTGCCCCCATTGAGCCCCTACTTCAAGTACTCCGTCATG
 V  V  S  F  V  V  G  G  L  A  V  I  L  P  P  L  S  P  Y  F  K  Y  S  V  M
                                                                            225
ATCAACAAGGCCACGCCCTACAACTACCCAGTGCCCGTCCGTGATGATGGGAACATGCCCGACGTGCCCAGCCAC
 I  N  K  A  T  P  Y  N  Y  P  V  P  V  R  D  D  G  N  M  P  D  V  P  S  H
                                                        NDUFA3R             300
CCCCAGGACCCTCAGGGCCCCAGCCTGGAGTGGCTGAAGAAACTGTGAgcacctccactgacagaggcggccccct
 P  Q  D  P  Q  G  P  S  L  E  W  L  K  K  L  *
                                   340
cccacggctcccaataaaaatgtgaaaaccaaaaaaaaaa
        poly A signal        poly A site
```

Fig 7.1 (continued) cDNA and amino acid sequence of the NDUFA3 subunit

```
     NDUFA7F                                                                 75
cgcggacggaagATGGCGTCCGCCACCCGTCTCATCCAGCGGCTGCGGAACTGGGCGTCCGGGCATGACCTGCAG
              M  A  S  A  T  R  L  I  Q  R  L  R  N  W  A  S  G  H  D  L  Q
                                                                           150
GGGAAGCTGCAGCTACGCTACCAGGAGATCTCCAAGCGAACTCAGCCTCCTCCCAAGCTCCCTGTGGGTCCTAGC
 G  K  L  Q  L  R  Y  Q  E  I  S  K  R  T  Q  P  P  P  K  L  P  V  G  P  S
                                                                           225
CACAAGCTCTCCAACAATTACTATTGCACTCGCGATGGCCGCCGGGAATCTGTGCCCCCTTCCATCATCATGTCG
 H  K  L  S  N  N  Y  Y  C  T  R  D  G  R  R  E  S  V  P  P  S  I  I  M  S
                                                                           300
TCGCAGAAGGCGCTGGTGTCAGGCAAGCCAGCAGAGAGCTCTGCTGTAGCTGCCACTGAGAAGAAGGCGGTGACT
 S  Q  K  A  L  V  S  G  K  P  A  E  S  S  A  V  A  A  T  E  K  K  A  V  T
                                                                           375
CCAGCTCCTCCCATAAAGAGGTGGGAGCTGTCCTCGGACCAGCCTTACCTGTGAcactgcaccctcacggccacc
 P  A  P  P  I  K  R  W  E  L  S  S  D  Q  P  Y  L  *
          NDUFA7R                                                          450
cgactactttgcctccttggatttcctccagggagaatgtgacctaatttatgacaaatacgtagagctcaggta
                                                  500
tcacttctagttttactttaaaaaataaaaaaatagagacagaaaaaaaa
                  poly A signal       poly A site
```

Figure 7.1 (continued) cDNA and amino acid sequence of the NDUFA7 subunit

```
      NDUFB2F                                                                 75
gaagcgaagtaggcaggggcgaggcggctggggaccgcggggcggacgggagcgagtATGTCCGCTCTGACTCGG
                                                        M  S  A  L  T  R
                                                                           150
CTGGCGTCTTTCGCTCGCGTTGGAGGCCGCCTTTTCAGAAGCGGCTGCGCACGGACTGCTGGAGATGGTGGAGTC
 L  A  S  F  A  R  V  G  G  R  L  F  R  S  G  C  A  R  T  A  G  D  G  G  V
                                                                           225
CGTCATGCCGGTGGTGGTGTGCACATTGAGCCCCGGTATAGACAGTTCCCCCAGCTGACCAGATCCCAGGTGTTC
 R  H  A  G  G  G  V  H  I  E  P  R  Y  R  Q  F  P  Q  L  T  R  S  Q  V  F
                                                                           300
CAGAGCGAGTTCTTCAGCGGACTCATGTGGTTCTGGATTCTCTGGCGCTTTTGGCATGACTCAGAAGAGGTGCTG
 Q  S  E  F  F  S  G  L  M  W  F  W  I  L  W  R  F  W  H  D  S  E  E  V  L
                                                                           375
GGTCACTTTCCGTATCCTGATCCTTCCCAGTGGACAGATGAAGAATTAGGTATCCCTCCTGATGATGAAGACTGA
 G  H  F  P  Y  P  D  P  S  Q  W  T  D  E  E  L  G  I  P  P  D  D  E  D  *
                                   NDUFB2R                                 450
aggtgtagactcagcctcactctgtacaagagccaggtgagaatttcaaggattatcgacttcatattgcacatt
                                   490
aaagttacaaattaaagtggcttggtcaagaatgaaaaaa
      poly A signal          poly A site
```

Figure 7.1 (continued) cDNA and amino acid sequence of the NDUFB2 subunit

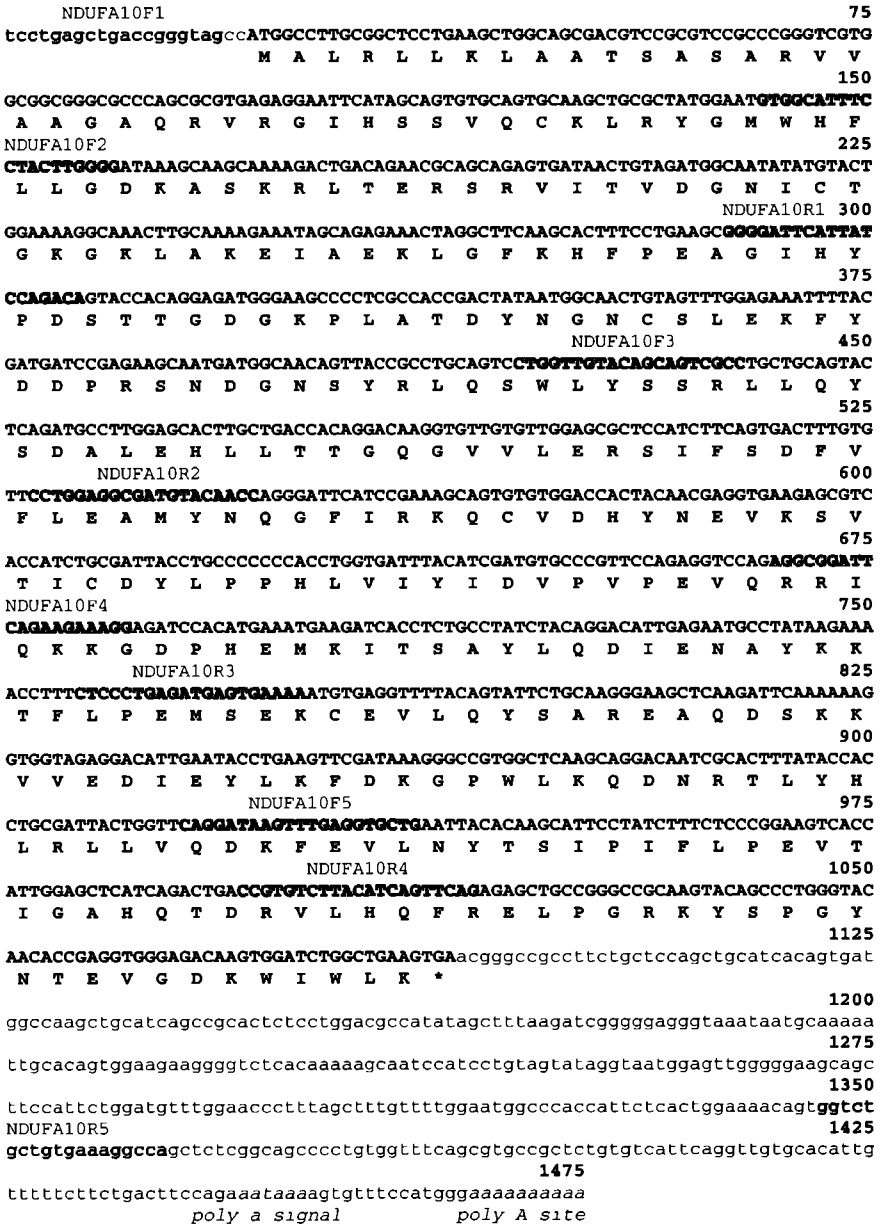

Figure 7.1 (continued) cDNA and amino acid sequence of the NDUFA10 subunit.

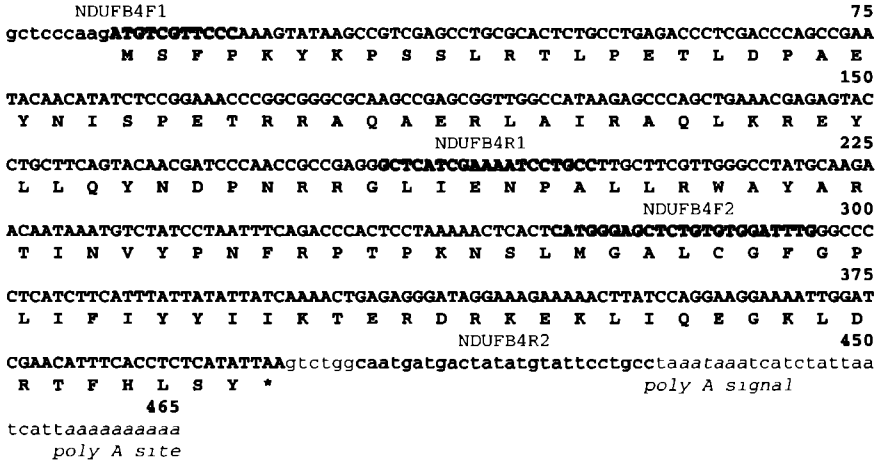

Figure 7.1 (continued) cDNA and amino acid sequence of the NDUFB4 subunit

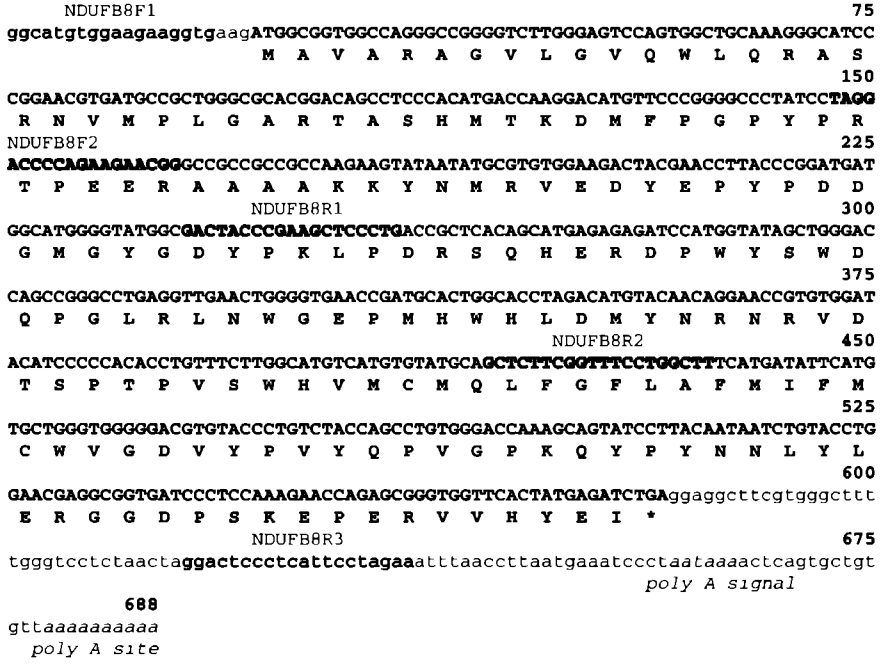

Figure 7.1 (continued) cDNA and amino acid sequence of the NDUFB8 subunit

```
     NDUFB10F1                                                                  75
aggcccgggacccggacggaggtagaggccagggcagcgcgtccgggagcggagtccgcgcccgccgccgccATG
                                                                             M
                                                                           150
CCGGACAGCTGGGACAAGGATGTGTACCCTGAGCCCCCGCGCCGCACGCCGGTGCAGCCCAATCCCATCGTCTAC
 P  D  S  W  D  K  D  V  Y  P  E  P  P  R  R  T  P  V  Q  P  N  P  I  V  Y
                                                                           225
ATGATGAAAGCGTTCGACCTCATCGTGGACCGACCCGTGACCCTCGTGAGAGAATTTATAGAGCGGCAGCACGCA
 M  M  K  A  F  D  L  I  V  D  R  P  V  T  L  V  R  E  F  I  E  R  Q  H  A
                                NDUFB10F2                                   300
AAGAACAGGTATTACTACTACCACCGGCAGTACCGCCGCGTGCCAGACATCACTGAGTGCAAGGAGGAGGACATC
 K  N  R  Y  Y  Y  Y  H  R  Q  Y  R  R  V  P  D  I  T  E  C  K  E  E  D  I
                                                NDUFB10R1                   375
ATGTGCATGTATGAAGCCGAAATGCAGTGGAAGAGGGACTACAAAGTCGACCAAGAAATTATCAACATTATGCAG
 M  C  M  Y  E  A  E  M  Q  W  K  R  D  Y  K  V  D  Q  E  I  I  N  I  M  Q
                                                                           450
GATCGGCTCAAAGCCTGTCAGCAGAGGGAAGGACAGAACTACCAGCAGAACTGTATCAAGGAAGTGGAGCAGTTC
 D  R  L  K  A  C  Q  Q  R  E  G  Q  N  Y  Q  Q  N  C  I  K  E  V  E  Q  F
                                                                           525
ACCCAGGTGGCCAAGGCCTACCAGGACCGCTATCAGGACCTGGGGGCCTACAGTTCTGCCAGGAAGTGCCTGGCC
 T  Q  V  A  K  A  Y  Q  D  R  Y  Q  D  L  G  A  Y  S  S  A  R  K  C  L  A
                                                                           600
AAACAGAGGCAGAGGATGCTGCAAGAGAGAAAAGCTGCAAAAGAGGCCGCCGCTGCCACCTCCTGAggcagctgt
 K  Q  R  Q  R  M  L  Q  E  R  K  A  A  K  E  A  A  A  A  T  S  *
                NDUFB10R2                                                  670
gggtgccccctgctgtgtggctctgtatgactgttgctgaaatataaagccctgcaacctgaaaaaaaaaa
                                                                poly A site
```

Figure 7.1 (continued) cDNA and amino acid sequence of the NDUFB10 subunit.

```
       NDUFC2F1                                                                 75
tcgtcgtggttttccttgtagttcgtggtctgagaccaggcctcaagtggaaacggcgtcaccATGATCGCACGG
                                                                       M  I  A  R
                                                                           150
CGGAACCCAGAACCCTTACGGTTTCTGCCGGATGAGGCCCGGAGCCTGCCCCCGCCCAAGCTGACCGACCCGCGG
 R  N  P  E  P  L  R  F  L  P  D  E  A  R  S  L  P  P  P  K  L  T  D  P  R
                                                                           225
CTCCTCTACATCGGCTTCTTGGGCTACTGCTCCGGCCTGATTGATAACCTGATCCGGCGGAGGCCGATCGCGACG
 L  L  Y  I  G  F  L  G  Y  C  S  G  L  I  D  N  L  I  R  R  R  P  I  A  T
                          NDUFC2R1                                         300
GCTGGTTTGCATCGCCAGCTTCTATATATTACGGCCTTTTTTTTTGCTGGATATTATCTTGTAAAACGTGAAGAC
 A  G  L  H  R  Q  L  L  Y  I  T  A  F  F  F  A  G  Y  Y  L  V  K  R  E  D
     NDUFC2F2                                                              375
TACCTGTATGCTGTGAGGGACCGTGAAATGTTTGGATATATGAAATTACATCCAGAGGATTTTCCTGAAGAAGAT
 Y  L  Y  A  V  R  D  R  E  M  F  G  Y  M  K  L  H  P  E  D  F  P  E  E  D
                                                                           450
AAGAAAACATATGGTGAAATTTTTGAAAAATTCCATCCAATACGTTGAagtcttcaaaatgcttgctccagtttc
 K  K  T  Y  G  E  I  F  E  K  F  H  P  I  R  *
        NDUFC2R2                                500
actgatacctgctgtttctgaatttgatggaacatgtttcttatgacagt
```

Figure 7.1 (continued) cDNA and amino acid sequence of the NDUFC2 subunit
Primers used for PCR and sequence reactions are shaded. Primers are also listed in Table 7.1

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DEMONSTRATION OF A NEW PATHOGENIC MUTATION IN HUMAN COMPLEX I DEFICIENCY: A 5-bp DUPLICATION IN THE NUCLEAR GENE ENCODING THE NDUFS4 (18 kDa) SUBUNIT

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Abstract.

We report the cDNA cloning, chromosomal localisation, and a mutation in the human nuclear gene encoding the NDUFS4 (18 kDa) subunit of the mitochondrial respiratory chain complex I The cDNA has an open reading frame of 175 amino acids and codes for a protein with a molecular mass of 20 1 kDa Its gene was mapped to chromosome 5 A homozygous 5-bp duplication, destroying a consensus phosphorylation site, in the *NDUFS4* cDNA was found in a complex I-deficient patient The patient showed normal muscle morphology and a remarkably non-specific fatal progressive phenotype without increased lactate concentrations in body fluids The child's parents were heterozygous for the mutation In 19 other complex I-deficient patients, no mutations were found in the *NDUFS4* gene

Introduction.

Among the inborn errors of metabolism, disturbances in the mitochondrial energy metabolism form a quantitatively major group, occurring in 1 10,000 live births (Bourgeron et al 1995) In case of a defective pyruvate oxidation, skeletal muscle and brain tissue are often involved In these mitochondrial encephalomyopathies (OMIM 251900), the most frequently observed enzyme deficiencies are located in the pyruvate dehydrogenase complex and complexes I (OMIM 252010) and/or IV of the respiratory chain (Wallace 1992, Ruitenbeek et al 1996) Numerous patients with isolated complex I deficiency have been reported (Korenke et al 1990, DiMauro and Moraes 1993, Shoffner and Wallace 1994) Their clinical presentation in general is that of a very severe, often fatal, multisystemic disorder frequently dominated by brain and skeletal muscle abnormalities Less severe phenotypes have also been described (Wallace 1992, Trijbels et al 1996) Until now, only one mutation in a nuclear gene has been described in a patient with a defective respiratory chain enzyme complex a point mutation in the flavoprotein (FP) subunit of complex II (Bourgeron et al 1995). No mutations in the nuclear genome of one of the other respiratory chain complexes have been reported The relatively high frequency of complex I-deficient patients prompted us to investigate the molecular cause of this enzyme deficiency Complex I or NADH.ubiquinone oxidoreductase, the first multisubunit enzyme complex of the mitochondrial respiratory chain, plays a vital role in cellular ATP production, the primary source of energy for many crucial processes in living cells (Walker 1992, Pilkington et al 1993, Trijbels et al 1996) It removes electrons from NADH and passes them via a series of different protein-coupled redox centres (FMN and FeS clusters) to the electron acceptor ubiquinone (Yamaguchi and Hatefi 1993) In well-coupled mitochondria, the electron flux leads to ATP generation via the building of a proton gradient over the inner membrane

Complex I is composed of ≥41 subunits, of which 7 are encoded by the mitochondrial genome (NDI-6, ND4L) and the remaining by nuclear genes (Fearnley and Walker 1992) Limited information is available concerning the precise location and function of the individual subunits Very recently, a low-resolution three-dimensional structure of complex I from *Neurospora crassa* has been elucidated (Guenebaut et al 1997) A number of different subunits contribute to binding sites for FMN, NADH, and 4Fe-4S and 2Fe-2S centres (Walker 1992, Pilkington et al 1993) It is hypothesised that subunits of the FP fraction play a role in FMN and NADH binding Proteins in the IP (iron-sulphur protein) fraction likely function in reduction-oxidation reactions via different iron-sulphur clusters Very recently, it has been shown that the nuclear-encoded 18-kDa subunit of bovine heart complex I can be phosphorylated by a mitochondrial cyclic adenosine 5'-monophosphate (cAMP)-dependent protein kinase (Papa et al 1996) It is well established that the 18-kDa (AQDQ) subunit is in the extrinsic domain of complex I (Walker 1992)

In some patients (i e in Leber hereditary optic neuropathy) a mutation in the ND4 subunit of the mitochondrial genome has been established (Brown et al 1997) So far, the human cDNA sequences of seven nuclear-encoded subunits (e g NDUFV1, NDUFV2, NDUFS1, NDUFS7, NDUFA1, NDUFA5, NDUFB9) have been published (Chow et al 1991, Spencer et al 1992, de Coo et al 1995, Hattori et al 1995, Gu et al 1996, Hyslop et al 1996, Zhuchenko et al 1996,

Pata et al 1997) The availability of the bovine cDNA sequence for the 18-kDa (AQDQ) protein (Walker et al 1992) enabled us to apply a PCR-based strategy for generating the cDNA for the human NDUFS4 (18-kDa) protein from human heart

In this article, we present the human cDNA sequence of the nuclear-encoded NDUFS4 subunit, the chromosomal localisation of the gene, and the results of mutation detection in 20 complex I-deficient patients all exhibiting a multisystem disorder Detailed information is presented about the clinical symptoms of one patient in whom we found a 5-bp duplication in the *NDUFS4* gene This is the first report of a pathological mutation in a nuclear complex I gene of the mitochondrial respiratory chain

Patient, material and methods.

Case history

A male patient, born after an uncomplicated pregnancy as the second child of healthy nonconsanguineous Caucasian parents, was first admitted at the age of 8 mo with severe vomiting, failure to thrive, and hypotonia Despite supportive care, his clinical condition deteriorated in the following months At the age of 13 mo, he showed severe psychomotor retardation, convulsions, bradypnoea, cyanosis, hypotonia, and depressed tendon reflexes Results of routine clinical chemical blood and urine investigations, as well as amino acid and organic-acid analyses in blood and urine, were normal Lactic acid concentrations in blood and cerebrospinal fluid were within the range of controls Electromyographic parameters were normal Cerebral magnetic resonance imaging revealed generalised brain atrophy and symmetrical basal ganglia abnormalities When the patient was 14 mo of age, a skeletal muscle biopsy (musculus quadriceps femori) was performed to investigate the mitochondrial energy-generating system (see Biochemical methods) Light and electron microscopy and histochemistry did not reveal abnormalities No ragged-red fibres were present The child's condition deteriorated severely, and he died of cardiorespiratory failure at the age of 16 mo The family history is normal for neuromuscular disorders A younger brother and older sister of the proband are healthy

Cloning of the human NDUFS4 cDNA

Human *NDUFS4* cDNA was obtained by reverse transcription using oligo(dT) and random hexamer primers on poly A^+ RNA isolated from human heart (Clonetech) PCR amplification was performed with cDNA as template according to the protocol for *Taq* polymerase (Life Technologies) by use of oligonucleotides 5-ATGGCGGCGGTCTCAATGTC 3 and 5 CTATTTTGTGGATACTCTTGTTC 3 Oligonucleotide sequences were based on homology with bovine 18-kDa cDNA sequence (Walker et al 1992) and known human expressed sequence tags (ESTs) The PCR product was purified for direct DNA sequencing DNA sequence analysis of both DNA strands was performed using the dyedeoxy-terminators on an automated ABI 377 sequencer according to a protocol provided by the manufacturer (Applied Biosystems)

Chromosomal localisation of the NDUFS4 gene

Genomic DNA was isolated from human-rodent somatic cell hybrids containing all 24 human chromosomes (Coriell Cell Depositories) digested with *Eco*RI, fractionated, and blotted to Hybond nylon membrane The blot was hybridised with α^{32}P-dCTP-labeled human *NDUFS4* cDNA probe (of 528 bp) Binding of the probe was visualised by autoradiography

Mutation detection

Total RNA was extracted (Chomczynski and Sacchi 1987) from cultured skin fibroblasts (Bentlage et al 1996) and stored as ethanol precipitate at -80°C A 5-µg sample of RNA was reverse transcribed to cDNA in 1 h at 42°C with 200 U of Superscript II reverse transcriptase (RT) (Life Technologies), using oligo(dT) and random hexamer primers One microliter of cDNA was subjected to PCR amplification By PCR we generated one fragment covering the entire coding region, using the synthetic oligonucleotide primers described before The PCR program consisted of 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C The cycles were preceded by an initial denaturation step of 3 min at 95°C and were followed by a final extension of 10 min at 72°C The PCR reaction was analysed on a 1 0% agarose gel with 0 5 µg/ml ethidium bromide in 1× Trisborate EDTA The nucleotide sequences of the PCR products were analysed by direct sequencing using the *Taq* Dye Deoxy terminator cycle sequencing kit according to the manufacturer s recommendations (Applied Biosystems)

To confirm the mutation at the genomic DNA level, a fragment consisting of 96 bp was amplified in the same PCR buffer as described above, using forward primer 5 TATGACATTGAAGAGAGGAAGG 3 and reverse primer as described above PCR fragments were resolved in a 20% polyacrylamide gel Ethidium bromide was used to visualise the PCR fragments, and the presence of the duplication could be checked by size

Results.

Biochemical results

From the decreased activity of NADH ferricyanide oxidoreductase as well as NADH cytochrome c reductase, a deficiency in complex I in skeletal muscle mitochondria can be concluded (see Table 8 1) The NADH Q_1 oxidoreductase assay revealed a complex I deficiency in cultured skin fibroblasts also The activities of the other respiratory chain enzyme complexes and Mg^{2+}-ATPase were all normal

Cloning of the human NDUFS4 cDNA

We obtained a 528-bp cDNA fragment of the human nuclear-encoded NDUFS4 subunit by RT-PCR using human heart RNA and oligonucleotide primers based on the *NDUFS4* cDNA sequence from cow (Walker et al 1992) and known human ESTs (Adams et al 1995) Sequence analysis of the human *NDUFS4* cDNA clone revealed an open reading frame which encodes 175 amino acids (Fig 8 1) The amino acid sequence showed 91% homology with the bovine 18-kDa amino acid sequence (data not shown) The calculated molecular weight of the protein encoded by the human *NDUFS4* cDNA is 20 1 kDa As in the cow, the protein contains a leader sequence (amino acids 1-42) for import into the mitochondria In the cow,

this signal peptide is removed after import. There is a phosphorylation consensus site (RVS) at amino acid positions 171-173 (Pearson and Kemp 1991, Kemp et al 1994). The human protein has no cysteine residues.

	Enzyme activity	
Skeletal muscle	**Patient**	**Control range**
• cytochrome *c* oxidase	7.2[a]	2.8-11.8[a]
• NADH:ferricyanide oxidoreductase	1.16[b]	1.7-4.1[b]
• NADH:cytochrome *c* oxidoreductase	27[c]	63-529[c]
• succinate:cyt *c* oxidoreductase	100[c]	39-249[c]
• citrate synthase	1.28[b]	1.0-2.1[b]
• Mg^{2+}-ATPase	634[d]	213-553[d]
• pyruvate dehydrogenase complex	18.3[c]	27-61[c]
Fibroblasts		
• NADH:Q_1 oxidoreductase	0.075[e]	0.11-0.28[e]
• Succinate:cyt *c* oxidoreductase	0.23[e]	0.21-0.44[e]
• decylubiquinol:cyt *c* oxidoreductase	1.22[e]	1.25-2.62[e]
• cytochrome *c* oxidase	239.0[c]	147-252[c]
• citrate synthase	311.0[c]	144-257[c]

Table 8.1 Respiratory chain enzyme activities in index patient's tissue specimens. Patient's individual respiratory-chain enzyme and PDHc activities measured in skeletal muscle tissue and cultured skin fibroblasts. Complex I activity is reduced in both tissue specimens.
[a] = in k/mg protein; [b] = in U/mg protein; [c] = in mU/mg protein; [d] = in mU/g protein; [e] = in mU/mU cytochrome *c* oxidase.

Chromosomal localisation of the human NDUFS4 gene.
A Southern blot with genomic DNA from human-rodent somatic cell hybrids containing all human chromosomes has been used for hybridisation with human *NDUFS4* cDNA as probe. A specific hybridisation signal was detected only for chromosome 5, indicating that the nuclear-encoded *NDUFS4* (18 kDa) gene maps to this chromosome (Fig. 8.2).

Mutation detection in the human NDUFS4 subunit.
A template of human cDNA was produced by first-strand synthesis of human fibroblast RNA originating from 20 patients with a complex I deficiency enzymatically established in fibroblasts and, when available, also in skeletal muscle and other tissues. One set of oligonucleotide primers enabled us to amplify the entire coding region of the *NDUFS4* cDNA. Direct sequencing of this region revealed a novel homozygous 5-bp duplication of the cDNA sequence at positions 466-470 (AAGTC) in one patient (Fig. 8.3a). No other sequence aberrations could be detected in the *NDUFS4* cDNA of all other patients. The 5-bp duplication results in a frameshift at codon K158 and destroys a phosphorylation site present at the carboxy-terminus of the NDUFS4 protein. Furthermore, the duplication changes the amino acid sequence of the NDUFS4 protein from amino acid 158 to the carboxy terminus and expands the length of the protein by 14 amino acids (Fig. 8.3b). The mutation was

confirmed on the genomic DNA level by PCR combined with PAGE. Both parents appeared to be heterozygous for the mutation (Fig. 8.3c). The segregation of this mutation in the family is consistent with an autosomal recessive mode of inheritance. The duplication was not present in 100 control alleles and the 19 other complex I-deficient patients.

```
                                                                      75
gcagcaagATGGCGGCGGTCTCAATGTCAGTGGTACTGAGGCAGACGTTGTGGCGGAGAAGGGCAGTGGCTGTA
          M  A  A  V  S  M  S  V  V  L  R  Q  T  L  W  R  R  R  A  V  A  V
                                                                     150
GCTGCCCTTTCCGTTTCCAGGGTTCCGACCAGGTCGTTGAGGACTTCCACATGGAGATTGGCACAGGACCAGACT
 A  A  L  S  V  S  R  V  P  T  R  S  L  R  T  S  T  W  R  L  A  Q  D  Q  T
                                                                     225
CAAGACACACAACTCATAACAGTTGATGAAAAATTGGATATCACTACTTTAACTGGCGTTCCAGAAGAGCATATA
 Q  D  T  Q  L  I  T  V  D  E  K  L  D  I  T  T  L  T  G  V  P  E  E  H  I
                                                                     300
AAAACTAGAAAAGTCAGGATCTTTGTTCCTGCTCGCAATAACATGCAGTCTGGAGTAAACAACACAAAGAAATGG
 K  T  R  K  V  R  I  F  V  P  A  R  N  N  M  Q  S  G  V  N  N  T  K  K  W
                                                                     375
AAGATGGAGTTTGATACCAGGGAGCGATGGGAAAATCCTTTGATGGGTTGGGCATCAACGGCTGATCCCTTATCC
 K  M  E  F  D  T  R  E  R  W  E  N  P  L  M  G  W  A  S  T  A  D  P  L  S
                                                                     450
AACATGGTTCTAACCTTCAGTACTAAAGAAGATGCAGTTTCCTTTGCAGAAAAAAATGGATGGAGCTATGACATT
 N  M  V  L  T  F  S  T  K  E  D  A  V  S  F  A  E  K  N  G  W  S  Y  D  I
                                                                     525
GAAGAGAGGAAGGTTCCAAAACCCAAGTCCAAGTCTTATGGTGCAAACTTTTCTTGGAACAAAAGAACAAGAGTA
 E  E  R  K  V  P  K  P  K  S  K  S  Y  G  A  N  F  S  W  N  K  R  T  R  V
                                                                     600
TCCACAAAATAGgttggcactgactatatctctgcttgactgtgaataaagtcagctatgcagtatttatagtcc
 S  T  K  *                                                         663
atgtataataaatacatctcttaatctcctaataaattggacctttaaactacaaaaaaaaaa
   poly a signal                                          poly a site
```

Figure 8.1 cDNA and amino acid sequence of the human NDUFS4 subunit. The cDNA and amino acid sequence are submitted to Genbank (accession no. NM002495).

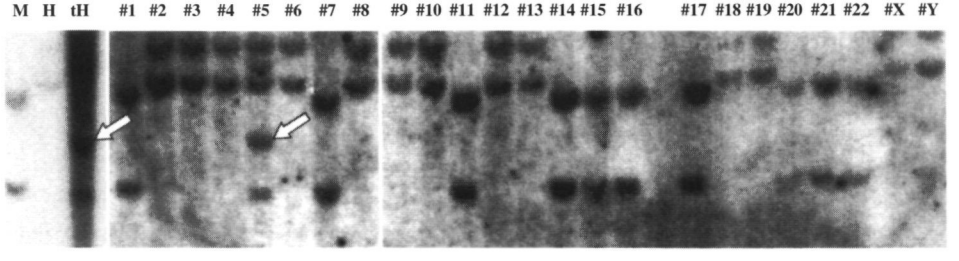

Figure 8.2 Chromosomal localisation of the human *NDUFS4* (18 kDa) gene. The *NDUFS4* gene was localised to chromosome 5 by chromosomal somatic cell hybrid analysis. Genomic DNA was isolated from a panel of mouse or hamster/human cells, each containing a single human chromosome. The DNA was digested with *Eco*RI, fractionated, transferred to Hybond nylon, and probed with a 528-bp *NDUFS4* cDNA. A positive signal was observed in the sample of total DNA of human origin (left arrow). Mouse (M) and hamster (H) genomic DNA showed no NDUFS4 signal. A strong signal for the NDUFS4 was seen only on hybrids containing chromosome 5 (right arrow).

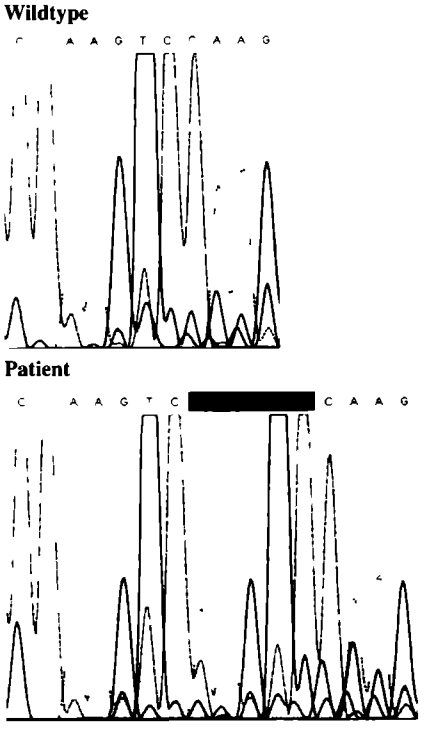

Figure 8.3a DNA sequence of the *NDUFS4* cDNA segments for wild type (top) and patient with the mutation (bottom) In case of the patient, a 5 bp duplication (AAGTC) was observed, resulting in a frameshift and extension of the C terminus of the NDUFS4 protein

```
Wildtype NGWSYDIEER KVPKPKSKSY GANFSWNKRT RVSTK*
Patient  NGWSYDIEER KVPKPKSSPS LMVQTFLGTK EQEYPQNRLA LTISLLDCE*
               150        160        170        180        190
```

Figure 8.3b Primary C-terminal amino acid sequence of wild-type and patient's NDUFS4 protein

Figure 8.3c Molecular analysis of the *NDUFS4* (18 kDa) gene Segregation of the 5-bp duplication in the *NDUFS4* gene in the family, detected by PCR on genomic DNA and by PAGE Mutant and wild-type alleles are 101 bp and 96 bp, respectively Lane 1 contains the molecular size marker, lane 2 brother, lane 3 father, lane 4 mother and lane 5 index patient.

Discussion.

Complex I deficiencies, whether or not as part of multiple enzyme deficiencies, are a major cause of mitochondrial encephalomyopathies. So far, no mutation in a nuclear gene of one of the numerous complex I subunits has been described. The multi-systemic progressive clinical presentation of our patient was highly suggestive for a mitochondriocytopathy (Munnich et al 1992, DiMauro and Moraes 1993, Shoffner and Wallace 1994, Ruitenbeek et al 1996). Despite normal clinical chemical features, biochemical analyses indeed revealed a reduced complex I activity both in skeletal muscle and cultured skin fibroblasts. In addition, a 5-bp duplication in the cDNA and genomic DNA encoding the nuclear-encoded NDUFS4 (18 kDa) subunit of complex I of the respiratory chain could be demonstrated. Both parents were heterozygous for this mutation.

We started our mutation analysis in complex I-deficient patients with examination of the *NDUFS4* gene, because Papa et al (1996) found that the bovine 18-kDa subunit can be phosphorylated. A mitochondrial inner membrane-bound kinase acts via a cAMP-dependent mechanism. The presence of a phosphorylation/dephosphorylation site is frequently associated with regulation of biological processes. In the case of phosphorylation of the *NDUFS4* subunit, one can hypothesise an influence on complex I activity or a role in posttranslational processes The cDNA, encoding the 18-kDa subunit of bovine complex I, had already been cloned. We cloned the human *NDUFS4* subunit by RT- PCR, using human heart mRNA as template. The protein encoded by the human *NDUFS4* gene is 175 amino acids long, and its calculated molecular mass is 20.1 kDa. The amino acid sequence of the human NDUFS4 protein is highly conserved. There is ~91% identity between the human and bovine counterparts. The bovine consensus phosphorylation site (RVS) is also present in the human subunit. As in the cow, the human NDUFS4 subunit has a leader sequence for import into the mitochondria. Although the human, like the bovine, NDUFS4 protein is supposed to be part of the IP fraction (Walker et al 1992), it has no cysteine residues that might provide ligands

for iron-sulphur centres A Blast search for homologous sequences revealed two different EST clones (AA258924 and N29566) originating from different human tissues

We have included in this study 20 patients with a complex I deficiency in fibroblasts and, in most patients, in at least one other tissue as well Direct sequencing of the entire *NDUFS4* cDNA of these patients revealed in one patient a homozygous 5-bp duplication A mistake made by the RT or *Taq* DNA polymerase in the RT-PCR was excluded by repeating the RT-PCR and DNA sequencing of this patient, all of which revealed the homozygous duplication in the patient's cDNA The presence of the duplication in the patient was confirmed on the genomic DNA level The 5-bp duplication causes a shift in the translational reading frame The shift results in (1) an elongation of the mature protein by 14 amino acids and (2) destroys the phosphorylation consensus site Although the gene product belongs to the IP fraction and therefore is probably involved in crucial redox reactions, its relation to the adjacent subunits and its exact function are still obscure Our results, a mutation with an autosomal recessive mode of inheritance, associated with a fatal multisystemic complex I deficiency biochemically established in two different tissue types, stress the physiological importance of this subunit in humans Because the phosphorylation site in the NDUFS4 subunit is disrupted in the patient, we speculate that the mutation in this vital site for regulation of protein function in eukaryotic cells is responsible for the observed complex I deficiency Alternatively, the obviously changed C-terminus of the NDUFS4 subunit of the patient has caused the complex I deficiency The protein has been elongated by 14 amino acids, and amino acids 158-175 have been modified A proper transport and/or folding and orientation of the NDUFS4 protein in the enzyme complex or in the mitochondrial inner membrane may be hindered Perhaps the alterations in the patient NDUFS4 subunit also lead to instability and premature breakdown of the protein

In conclusion, we present the first mutation in a nuclear gene of one of the subunits of complex I of the human respiratory chain Although the frequency of the described mutation must be further established, the finding improves the potentials for genetic counselling and prenatal diagnosis These preventive tools are of utmost importance, because therapeutic intervention is unsuccessful in the majority of complex I deficient patients Mutation analysis of other nuclear genes in patients with a complex I deficiency is in progress

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THE FIRST NUCLEAR-ENCODED COMPLEX I MUTATION IN A PATIENT WITH LEIGH SYNDROME

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Abstract.
Nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase (complex I) is the largest multi-protein enzyme complex of the respiratory chain. The nuclear-encoded NDUFS8 (TYKY) subunit of complex I is highly conserved among eukaryotes and prokaryotes and contains two 4Fe4S ferredoxin consensus patterns, which have long been thought to provide the binding site for the iron-sulphur cluster N-2. The *NDUFS8* cDNA contains an open reading frame of 633 bp, coding for 210 amino acids. Cycle sequencing of amplified *NDUFS8* cDNA of 20 patients with isolated enzymatic complex I deficiency revealed two compound heterozygous transitions in a patient with neuropathologically proven Leigh syndrome. The first mutation was a C236T (P79L), and the second mutation was a G305A (R102H). Both mutations were absent in 70 control alleles and co-segregated within the family. A progressive clinical phenotype proceeding to death in the first months of life was expressed in the patient. In the 19 other patients with enzymatic complex I deficiency, no mutations were found in the *NDUFS8* cDNA. This article describes the first molecular genetic link between a nuclear-encoded subunit of complex I and Leigh syndrome.

Introduction.

The oxidative phosphorylation (OXPHOS) system of mammals consists of five complicated multi-protein complexes (complexes I-V) Complexes I and II accept electrons from the reduced coenzymes nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), respectively These electrons are transported along the electron transport chain, and coupled to this transport a proton gradient is created by complexes I, III, and IV across the inner mitochondrial membrane ATP synthase (complex V) couples proton movement down its electrochemical gradient with the synthesis of ATP from adenosine diphosphate and P_i

Complex I has been widely studied in prokaryotes as well as eukaryotes Genetic characteristics of NADH ubiquinone oxidoreductase are best documented in *Bos taurus* (Walker 1992), *Neurospora crassa* (Weiss et al 1991), and *Escherichia coli* (Weidner et al 1993, Friedrich 1998) *E coli* complex I consists of the 14 subunits that together are considered to be the minimal structural unit for enzymatic activity In humans, the exact number of complex I subunits is unknown (for a review see Smeitink et al 1998a) *B taurus* is the mammal nearest to humans whose complex I has been structurally elucidated (Walker 1992, Grigorieff 1998) It contains ~42 subunits (Walker 1995, Walker et al 1995), 35 of which are encoded by the nucleus and the remaining 7 by mtDNA (Chomyn et al 1985 and 1986) Complex I contains several redox centres to facilitate electron transport These are hypothesised to be one non-covalently bound flavin mononucleotide and 6-8 iron-sulphur clusters (Yamaguchi and Hatefi 1993, Albracht et al 1996, Ohnishi 1998)

One of the most frequently encountered syndromes associated with defects of the OXPHOS system was named after the man who first described a young patient suffering from a progressive neurodegenerative disorder with characteristic neuropathology, namely, Leigh syndrome (MIM 256000, Leigh 1951) The estimated incidence of Leigh syndrome is ~1 40,000 live births (Rahman et al 1996) Several inheritance patterns can be differentiated in Leigh syndrome (DiMauro and De Vivo 1996) autosomal recessive, with defects described in biotinidase (Baumgartner et al 1989), pyruvate dehydrogenase complex (PDHc), OXPHOS complex II (Bourgeron et al 1995) and complex IV (Willems et al 1977), X-linked recessive, with defects in the $E_{1\alpha}$ subunit of PDHc (Matthews et al 1993), and maternal, with defects in subunit 6 of ATP synthase (Shoffner et al 1992, Tatuch et al 1992), tRNA(Lys) (Hammans et al 1993), tRNA(Trp) (Santorelli et al 1997), tRNA(Val) (Chalmers et al 1997), and tRNA(Leu) (Graeber and Muller 1998), MERFF mutation A8344G (Berkovic et al 1991), and A3243G (Rahman et al 1996) Recently, respiratory-chain enzyme assays performed in patients with Leigh syndrome revealed that complex I deficiency (MIM 252010) is a major contributor to this disease entity (Morris et al 1996, Rahman et al 1996), but until now molecular genetic defects have not been described

The NDUFS8 (TYKY) is a highly conserved nuclear-encoded complex I subunit with equivalents present in many species, best documented in humans (Procaccio et al 1997), *B taurus* (Walker 1992), *N crassa* (Duarte et al 1996), *E coli* (Weidner et al 1993), and *Rhodobacter capsulatus* (Chevallet et al 1997) The position of the NDUFS8 subunit in different fractions of complex I (flavoprotein [FP] fraction, iron-sulphur protein [IP] fraction,

and hydrophobic protein [HP] fraction) has not been firmly established Masui et al (1991) located the bovine TYKY subunit in the IP fraction, but it is also considered to belong to the HP fraction (Walker 1992) The amino acid sequence contains two 4Fe4S ferredoxin consensus patterns (PROSITE pattern P500198 CIACKLCEAICP and CIYCGFCQEACP) Whether these consensus patterns provide the binding site for iron-sulphur cluster N-2 is still under debate (Dupuis et al 1991, Albracht et al 1997, Duarte et al 1997) Albracht and coworkers proposed in 1997 that the NDUFS8 subunit has functional significance in the reduction of ubiquinone coupled to the pumping of protons Recently, homologous recombination studies performed with the NuoI subunit of *R capsulatus* (equivalent to human NDUFS8) showed that NuoI plays an important role in the assembly of complex I, which emphasises the importance of this subunit (Chevallet et al 1997)

Herein we report the results of a mutational analysis study of the NDUFS8 subunit performed in 20 patients with isolated complex I deficiency Our study revealed two new compound heterozygous transition mutations in one patient with Leigh syndrome Detailed information concerning the clinical characteristics of this patient is presented This is the first molecular genetic link between Leigh syndrome and a nuclear-encoded subunit of NADH ubiquinone oxidoreductase In addition, we studied the mRNA tissue distribution of the NDUFS8 subunit and discuss possible connections between tissue expression and the clinical phenotype of the patient

Patients, material, and methods.

Case report

In May 1990, a 5-wk-old male infant presented with poor feeding and episodes of apnoea and cyanosis The symptoms, which had existed since the first day of life, escalated in the course of an acute gastro-enteritis He was the second child of healthy, non-consanguineous parents of Dutch origin Pregnancy, birth, and first hours of life were uneventful The family history was negative for major neurological disorders At admission, the main symptoms were mild cyanosis, severe hypercarbia, a cardiac murmur (grade 2/4), drowsiness with absent optical and acoustical blink, eye flutter, intense hypotonia, brisk tendon reflexes with ankle clonus, and erratic seizures A weak cry, rudimentary sucking and primitive reflexes were still present The occipitofrontal circumference was normal Dysmorphic features or hepatomegaly were absent Cardiac investigation revealed a moderate hypertrophic obstructive cardiomyopathy Computed tomography of the brain showed extensive white-matter hypodensity, mild ventricular enlargement, and, at a later occasion, also hypodense symmetric lesions in putamen and mesencephalon Evoked potentials were delayed for all modalities tested

Blood tests showed increased lactate (3 4 mmol/l, normal range 0 6-2 1 mmol/l) and pyruvate (167 µmol/l, normal range 30-80 µmol/l) levels Cerebrospinal fluid (CSF) lactate and pyruvate levels were elevated to 5 6 mmol/l (normal range 1 4-1 9 mmol/l) and 193 µmol/l (normal range 96-145 µmol/l), respectively The CSF lactate/pyruvate ratio was also increased (29 0, normal range 11 7-16 5), and the CSF protein content was slightly increased (504 mg/l, normal range 245-460 mg/l)

On the basis of these findings, a clinical diagnosis of subacute necrotising

encephalomyelopathy, or Leigh syndrome, was made Light microscopy studies of a quadriceps femoris muscle biopsy sample obtained when the infant was 10 wk of age showed a reduced number of small type 1 fibres (16%, normal 50%), ragged red fibres were not seen Electron microscopy (EM) studies were not performed At the age of 11 wk, the infant died of cardiorespiratory failure Biochemical examinations performed on skin fibroblasts, skeletal muscle, heart muscle, and liver and brain tissue, obtained at autopsy performed <1 h after death, revealed severely reduced activity of NADH ubiquinone oxidoreductase (39% residual activity of the lowest reference value in muscle tissue, see Table 9 1), whereas all other complexes of the respiratory chain expressed normal activity PDHc activity was slightly reduced in muscle In cultured skin fibroblasts, an isolated NADH ubiquinone oxidoreductase deficiency was observed (69% residual activity of the lowest reference value, see Table 9 1) Biochemical studies of heart, liver, and brain tissue (also obtained at autopsy) revealed an overall reduced activity of NADH ubiquinone oxidoreductase, with respectively, virtually 0%, 53%, and 3% residual activity of the lowest reference value (Table 9 1) The completely deficient complex I activity in heart tissue was repeatedly confirmed Macroscopically, the brain had a normal appearance Microscopic investigation of the brain showed extensive bilateral symmetrical degeneration, predominantly in the rostral and caudal brain stem, diencephalon, and central nuclei, but also elsewhere, including the spinal cord and semioval centre The lesions consisted of spongiform degeneration, capillary proliferation with endothelial swelling, demyelination, and gliosis Neuronal damage was variable in different brain slides, partly because there was a relative sparing of neurons in comparison to the surrounding tissue EM studies of heart and liver tissue were not performed On the basis of data obtained at autopsy, the patient was neuropathologically diagnosed with Leigh syndrome

Patient group

Twenty patients with isolated complex I deficiency (4 females and 16 males) were included in this study Concise clinical characteristics have been published elsewhere (Loeffen et al 1998), and a detailed report of our complex I deficient patient group will be published elsewhere (Loeffen et al 2000) In this patient group the *NDUFA1*, *NDUFS4*, *NDUFS5*, and *NDUFB6* cDNA were previously examined by direct DNA sequencing (Loeffen et al 1998, Smeitink et al 1998b, van den Heuvel et al 1998, Loeffen et al 1999) In one patient a mutation was found in the *NDUFS4* gene Neither major mitochondrial DNA rearrangements nor common pathogenic mtDNA mutations (A3243G [MELAS], T3271C [MELAS], A4317G [MELAS], A8344G [MERRF], T8993G/C [NARP/Leigh syndrome]) were present in this patient group

Commercially purchased materials

Molecular size markers, *Taq* DNA polymerase, Superscript II RNAse H reverse transcriptase, *Bal*I restriction endonuclease, and custom-made oligonucleotide primers were obtained from Life Technologies, *Pin*AI restriction endonuclease from Promega, a d-Rhodamine DyeDeoxy Terminator Sequencing Kit from Perkin Elmer, gel purification systems from Qiagen a poly A^+ RNA pool and a Poly A^+ RNA master blot from Clontech, and RNAzol from Biosolve BV

Software

Consensus-pattern searches in the NDUFS8 amino acid sequence were performed with PROSITE Sequence alignments were performed with Sequence Navigator (Perkin Elmer) Hydropathy and secondary structure were plotted by means of methods based on the procedures described by Chou and Fasman 1978, Kyte and Doolittle 1982, and Nishikawa 1983 Computer software was obtained from the National Centre for Computer-Aided Chemistry and Bioinformatics (CAOS/CAMM)

Tissue culture and enzyme measurements

In our patient group, biochemical enzyme measurements of respiratory-chain complexes were performed on skin fibroblasts cultured according to standard procedures Enzyme assay procedures were slightly modified from those used for muscle tissue (Bentlage et al 1996)
The autopsy tissue materials of the patient described in this article were obtained <1 h after death and immediately deep frozen in liquid nitrogen and kept at -80°C until enzyme assays were performed The enzyme assays described in this paper were based on the following methods (slightly modified) NADH ubiquinone oxidoreductase according to Fischer et al (1986), succinate cyt *c* oxidoreductase according to Fischer et al (1985), cytochrome *c* oxidase according to Cooperstein and Lazarow (1951), citrate synthase according to Srere (1969), and PDHc according to van Laack et al (1988) Control tissue specimens were handled and stored under conditions similar to those of the patient In our experience, loss of respiratory-chain enzyme activities in deep-frozen tissue samples obtained <2 h after death is negligible Control values and number of controls are listed in Table 9 1 Values in fibroblasts are expressed on cytochrome *c* oxidase base (cytochrome *c* oxidase and citrate synthase are expressed on a protein base) We chose cytochrome *c* oxidase as a marker instead of citrate synthase, because in the presented patient enzyme activities were measured in fibroblasts mitochondria enriched fractions In our experience, citrate synthase can be artificially decreased in mitochondria enriched fractions, since it is a mitochondrial matrix enzyme

Mutational analysis of the NDUFS8 cDNA

Patient RNA was extracted from cultured skin fibroblasts according to methods described by Chomczynski and Sacchi 1987 Wild-type RNA was commercially obtained and consisted of pooled poly A^+ RNA from 12 healthy Caucasian males and females, age range 12 78 years The RNA was reverse transcribed to cDNA by superscript II RNAse H reverse transcriptase with oligo(dT) and random hexamer primers (Ploos van Amstel et al 1996)
Oligonucleotide primers were designed according to the *NDUFS8* cDNA sequence published by Procaccio et al (1997, GenBank accession number U65579) The primer oligonucleotides F(orward)1 (5 TGGCCGAATGGCAGCGTC 3) and R(everse)3 (5 TTTTATTGGGCAGCAGGGGC 3) were applied in PCR to amplify the complete open reading frame (ORF) PCR was performed in a reaction volume of 25 µl containing 1 µl of cDNA template, 0 5 unit of *Taq* DNA polymerase, 2 5 µl of 10× PCR buffer, 1 5 mM of $MgCl_2$, 50 ng of F1 and R3, and 2 5 mM of dNTPs PCR parameters were 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C, and 1 min extension at 72°C The cycles were preceded by an initial denaturation step of 1 min at 94°C and were followed by a final extension step of 10 min The amplification

product was purified from a 2% agarose gel. Sequence reactions using the DyeDeoxy Terminator Sequencing Kit were performed with oligonucleotide primers F1, F2 (5'-CCTACAAGTATGTGAACATGC-3'), F3 (5'-CATTGCCTGCAAGCTCTGCG-3'), R1 (5'-AGTTGATGGTGGCCGGTTCC-3'), R2 (5'-GATGTCATAGCGGGTGGTCC-3'), and R3 (Fig. 9.1). The cDNA sequence analysis was performed on the automated ABI 377 sequencer according to the protocol provided by the manufacturer.

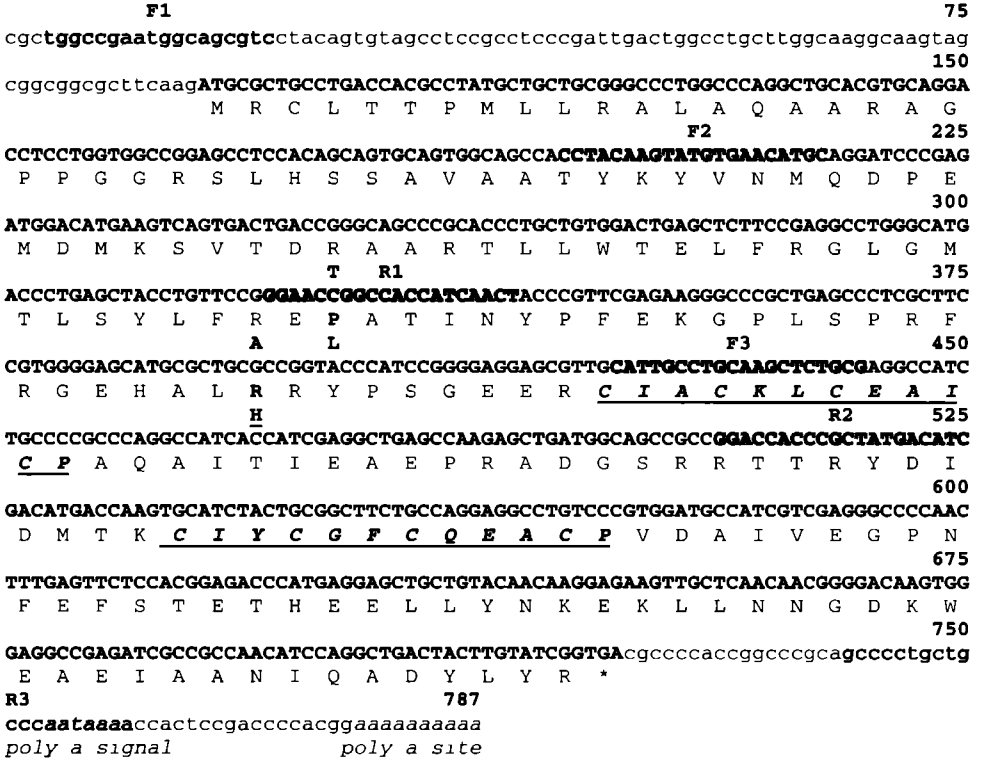

Fig. 9.1 Human *NDUFS8* cDNA and amino acid sequence (Procaccio et al 1997). Primers used for amplification of the ORF were F1 and R3; all primers were used in DNA sequence reactions. The ORF is printed bold and is capitalised, and both consensus 4Fe4S ferredoxin clusters are in italic and underlined. Mutations are shown above the cDNA sequence, substitutions below the amino acid sequence.

Restriction enzyme endonuclease analysis.

Both mutations were confirmed by restriction endonuclease analysis of the *NDUFS8* cDNA fragment obtained with oligonucleotide primers F1 and R3. C236T was cleaved with restriction enzyme *Bal*I (recognition site 5'-TGG/CCA-3') and G305A with *Pin*AI (recognition site 5'-A/CCGGT-3') (The forward slash [/] indicates the break point as created by the restriction enzymes). The transition mutations present in our patient (position underlined in the digestion recognition site) introduce the restriction sites. For both digestions, conditions were used as recommended by the manufacturer.

Tissue distribution of the NDUFS8 mRNA.

A human RNA master blot was used to quantify the expression of *NDUFS8* mRNA in a range of human adult and foetal tissues The quantity of RNA spotted for each tissue onto the master blot was normalised using eight different housekeeping gene transcripts as probes (ribosomal protein S9, ubiquitin, 23 kDa highly basic protein, hypoxanthine guanine phosphoribosyl transferase, tubulin, β-actin, phospholipase, and glyceraldehyde triphosphate dehydrogenase) Using a standard hybridisation solution (Sambrook et al 1989), we hybridised the master blot with an α[^{32}P]-dCTP-labelled cDNA probe The blot was washed twice with 2× SSC, 1% SDS, at 65°C and was subjected to autoradiography

Results.

Biochemical measurements

In our patient group (n=20), residual complex I activity measured in cultured skin fibroblasts was 24%-73% of the lowest reference value (expressed on a cytochrome *c* oxidase base) The other respiratory-chain complexes showed normal activities in all patients In most patients, respiratory-chain complex activities were also measured in other tissues apart from fibroblasts In the case of the patient with two compound heterozygous transition mutations in the *NDUFS8* gene, enzyme activities were measured in a quadriceps muscle biopsy specimen, in cultured skin fibroblasts, and in liver, brain, and heart tissue In all of these tissues complex I activity was markedly reduced, whereas other complexes of the electron transport chain were essentially within the normal range (Table 9 1)

Mutational analysis of the NDUFS8 cDNA

PCR with oligonucleotide primers F1 and R3 produced a cDNA fragment of 757 bp, including the complete ORF of the NDUFS8 subunit Direct DNA sequence analysis of amplified pooled control *NDUFS8* cDNA confirmed the published wild-type cDNA sequence Amplified *NDUFS8* patient cDNA was then cycle sequenced and analysed In one patient this revealed two heterozygous transitions The first mutation, a C→T at bp position 236, resulted in an amino acid change of proline into leucine (amino acid position 79) In the second mutation, a G→A at bp position 305, arginine had been replaced by histidine (amino acid position 102). The presence of the first and second mutation in the common population was checked by restriction endonuclease analysis In 35 controls (70 alleles), C236T and/or G305A were not present The other 19 patients with enzymatic complex I deficiency all displayed the wildtype *NDUFS8* cDNA sequence

	Muscle tissue		Skin fibroblasts		Heart tissue		Liver tissue		Brain tissue	
	PAT	CON	PAT	CON	PAT	CON	PAT	CON	PAT	CON
Complex I	1.7[a]	4.4-19 (31)	0.069[b]	0.11-0.28 (14)	± 0[a]	4.5-41 (6)	2.5[a]	4.7-9.2 (7)	0.16[a]	5.1-19 (5)
Complex IV	170[a]	73-284 (21)	181[a]	147-252 (14)	174[a]	73-412 (7)	53[a]	13.9-108 (11)	58[a]	26-181 (14)
Suc. cyt c oxidored.	24[a]	22-78 (39)	0.33[b]	0.21-0.44 (14)	26.3[a]	11.2-62 (7)	14[a]	6.0-51 (9)	14[a]	11-40 (6)
Citrate synthase	87[a]	48-146 (43)	154[a]	144-257 (14)	316[a]	208-490 (7)	37[a]	13.7-96 (10)	216[a]	53-207 (10)
PDHc	2.5[a]	2.8-8.0 (15)	-	-	9.1[a]	2.3-21 (6)	1.1[a]	0.38-7.4 (24)	-	-

Table 9.1 Respiratory chain enzyme activities in index patient's tissue specimens. Patient's individual respiratory-chain enzyme and PDHc activities measured in skeletal muscle tissue, cultured skin fibroblasts, heart tissue, liver tissue, and brain tissue. Complex I activity is severely reduced in all tissue specimens. Numbers in parentheses denote how many controls were used for each enzyme assay. Suc. cyt *c* oxidored. = succinate:cytochrome *c* oxidoreductase.[a] = mU/mg protein; [b] = mU/mU cytochrome *c* oxidase.

Confirmation of mutations by restriction endonuclease analyses.

RNA isolated from whole blood of both parents and three healthy siblings (two boys, one girl) was reverse transcribed to cDNA. Oligonucleotide primers used for cloning of the complete ORF of the NDUFS8 subunit were applied in PCR. The PCR fragment was digested with restriction enzymes *Bal*I (C236T) and *Pin*AI (G305A). The results were visualised on a 4% agarose gel. Since both restriction endonucleases cut in the region containing the mutation, the wild-type *NDUFS8* cDNA fragment remained intact. In the patient both mutations were heterozygous, and after cleavage with *Bal*I they expressed three bands (a wild-type cDNA fragment, one fragment of 325 bp, and one fragment of 432 bp). Incubation with *Pin*AI again revealed three fragments (a wild-type cDNA fragment, one band of 365 bp, and one band of 392 bp). When *Bal*I *and Pin*AI were added simultaneously, the corresponding four digestion fragments were seen, which implies that the two mutations are on different alleles (data not shown). Three bands corresponding to the patient's were expressed in the father's cDNA after incubation with *Bal*I, and a single wild-type band was expressed when *Pin*AI was applied. The mother's cDNA showed an undigested wild-type band after incubation with *Bal*I and three bands (similar to the patient's) when *Pin*AI was used. An older brother had the same digestion pattern as the father, a younger sister the same fragment pattern as the mother, and the youngest son showed only wild-type bands after digestion with *Bal*I and *Pin*AI (Fig. 9.2).

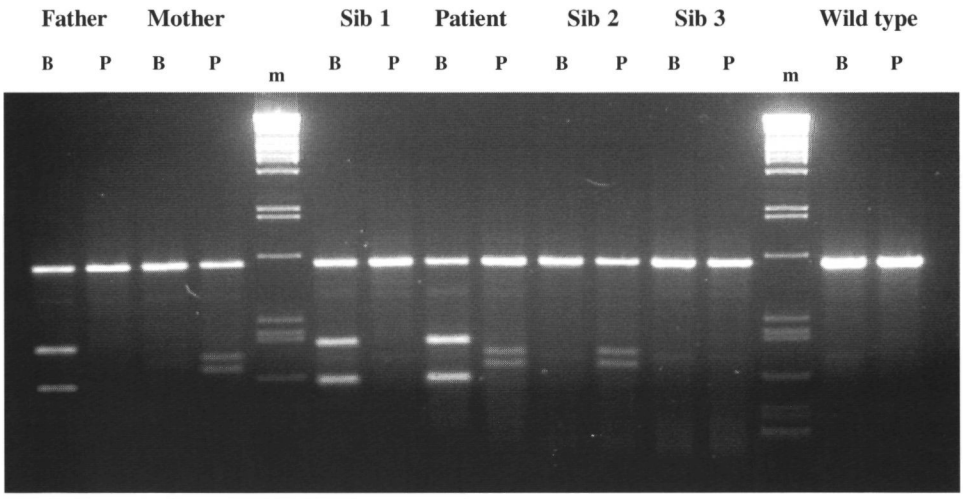

Fig. 9.2 Restriction endonuclease analysis pattern. Wild-type and Sib 3 *NDUFS8* cDNA is undigested; patient is a carrier of C236T and G305A, father and sib 1 are carriers of C236T, and mother and sib 2 are carriers of G305A. The marker (m) is Lambda *Pst*I. For each person, the first lane contains the fragments after digestion with *Bal*I (B), and the second lane the fragments after *Pin*AI (P) was applied.

Tissue distribution of the NDUFS8 mRNA.

Hybridisation of the [^{32}P]-dCTP-labelled *NDUFS8* cDNA probe to the RNA master blot revealed a ubiquitous expression pattern in human adult and foetal tissues. Adult tissue dots with relatively higher intensities are, in decreasing order, heart, skeletal muscle, pituitary gland, adrenal gland, kidney, caudate nucleus, putamen, substantia nigra, and lung. Among foetal tissues, heart expressed the highest dot intensity (Fig. 9.3).

Discussion.

The clinically, biochemically, and genetically heterogeneous group of OXPHOS disorders (estimated incidence 1:10,000 live births (Bourgeron et al 1995) has a main contributor in Leigh syndrome combined with isolated complex I deficiency. In the late 1980s and early 1990s, several mtDNA mutations associated with complex I deficiency were identified (Wallace et al 1988, Holt et al 1989, Poulton and Gardiner 1989, Zeviani et al 1989, Goto et al 1990, Holt et al 1990, Tanaka et al 1990, Zeviani et al 1991, Shoffner et al 1992). Yet, in many patients with isolated complex I deficiency, mtDNA mutations cannot be found (Buddiger et al 1997, Loeffen et al 1999). Recently, we found the first mutation (a 5-bp duplication in the *NDUFS4* (18 kDa) gene to be identified in a nuclear-encoded subunit of complex I (van den Heuvel et al 1998). These findings make further studies of mutations in nuclear-encoded subunits of complex I necessary.

	1	2	3	4	5	6	7	8
a	whole brain	amygdala	caudate nucleus	cerebel-lum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
b	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	subthal. nucleus	spinal cord	
c	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
d	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
e	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
f	appendix	lung	trachea	placenta				
g	foetal brain	foetal heart	foetal kidney	foetal liver	foetal spleen	foetal thymus	foetal lung	
h	yeast RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	poly r(A) 100 ng	human C_0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

Fig. 9.3a Multiple tissue master blot. The type and position of poly(A^+) RNAs and controls dotted on the positively charged nylon membrane.

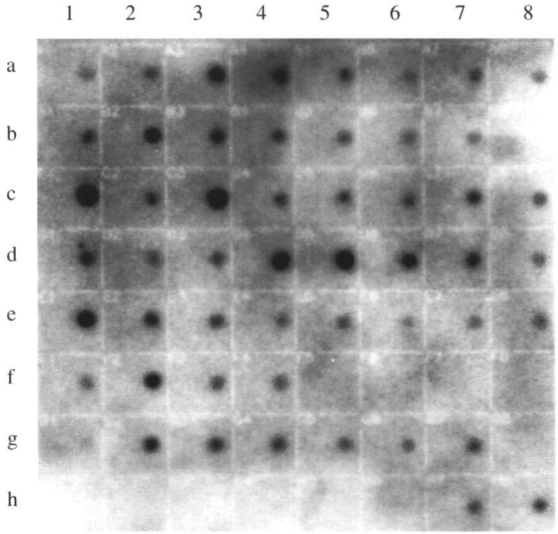

Figure 9.3b Tissue distribution of *NDUFS8* mRNA with a poly A^+ RNA master blot. NDUFS8 is ubiquitously expressed in human tissues, with a relatively higher expression in the human heart, skeletal muscle, gland tissue, kidney, caudate nucleus and putamen.

We chose the NDUFS8 subunit for mutational analysis studies for several reasons In the first place, the NDUFS8 subunit is highly conserved among species, with, for example, 93% amino acid identity between humans and *B taurus* It is also one of the 14 subunits that together constitute the minimal structural unit for enzymatic activity, as found in *E coli*, which stresses its possible functional significance Finally, it contains two consensus 4Fe4S ferredoxin patterns, which have long been considered to be binding sites for iron-sulphur cluster N-2 These findings make this protein a strong candidate gene for isolated complex I deficiency

Cycle sequencing of the *NDUFS8* cDNA in our complex I-deficient patient group revealed two compound heterozygous mutations in a patient neuropathologically diagnosed with Leigh syndrome The clinical phenotype of this patient and the results of additional laboratory and imaging studies are highly suggestive of an OXPHOS disorder, and diagnosis (isolated complex I deficiency) was biochemically confirmed in muscle tissue, cultured skin fibroblasts, and heart, liver, and brain tissue The first transition (C236T) resulted in an amino acid substitution of leucine for proline It is well known that proline markedly influences protein architecture A Chou-Fasman plot comparison of NDUFS8 wild types and NDUFS8 containing this specific mutation clearly revealed the disturbance of an α helix by a β-sheet Although it remains purely speculative which conformation the NDUFS8 protein will have when incorporated in the entire complex, proline is very important for secondary and tertiary structure, and thus the disappearance of this amino acid will likely disturb the protein structure The second mutation (G305A) replaced arginine with histidine Histidine contains an imidazole side chain that can readily switch between uncharged and positively charged to catalyse the making and breaking of bonds A Basic Local Alignment Search Tool (BLAST) search at the server of the National Centre for Biotechnology Information with the human NDUFS8 amino acid sequence revealed an extremely strong conservation of the region containing the arginine, which is mutated in the patient This region of 24 amino acids is ± 100% conserved in, among others, *B taurus, N crassa Paracoccus denitrificans Rhodobacter capsulatus* and *Nicotiana tabacum* This stresses the possible significance of this region, which is located just prior to the first cysteine-rich 4Fe-4S ferredoxin consensus pattern (eight amino acid residues between the mutated arginine and the consensus cluster) An arginine-to-histidine mutation is the main genetic cause in another disease associated with complex I deficiency, namely, Leber hereditary optic neuropathy (Wallace et al 1988, Singh et al 1989) Although both amino acids are basic, they are clearly not, in all cases, exchangeable Since neither mutation is present in 70 control alleles and in the remaining 19 complex I-deficient patients, it is unlikely that these mutations represent population polymorphisms These two mutations with an autosomal recessive inheritance pattern, combined with the lethal clinical course within the early months of life, provide further evidence for the functional importance of this iron-sulphur cluster binding pattern containing nuclear-encoded complex I subunit

Restriction endonuclease analysis performed on *NDUFS8* cDNA of all members of the patient's family showed that mutation C236T was inherited from the father and mutation G305A from the mother In the oldest son (carrier of C236T), the activity of respiratory chain enzyme complexes was measured in a skeletal muscle biopsy specimen, and that of each

complex was within the normal range While the mother was pregnant with the younger sister (carrier of G3O5A), chorionic villi were obtained for biochemical measurements of the respiratory-chain complex activities, which were again within normal limits (no muscle biopsy was performed in the younger sister) It seems likely, therefore, that both mutations are necessary to reduce the enzymatic activity of NADH ubiquinone oxidoreductase

Leigh syndrome has been linked, by biochemical enzyme assays to several single enzyme and enzyme complex deficiencies, including deficiencies in biotinidase, PDHc, NADH ubiquinone oxidoreductase, succinate dehydrogenase, cytochrome c oxidase, and ATP synthase However, molecular genetic defects have been described in only a few subunits of PDHc, the FP subunit of succinate dehydrogenase, subunit 6 of ATP synthase, and some mitochondrially encoded tRNAs Recent mutational analysis of all 10 nuclear encoded subunits of cytochrome *c* oxidase (complex IV of the OXPHOS system) in a Leigh syndrome patient with cytochrome *c* oxidase deficiency revealed no mutations (Adams et al 1997) The patient described in the present article provides the first genetic link between a nuclear-encoded subunit of complex I and Leigh syndrome Since complex I deficiency is a large contributor to Leigh syndrome, other patients with isolated complex I deficiency in whom a similar clinical phenotype is expressed should be screened for *NDUFS8* mutations as well

The tissue expression of the *NDUFS8* cDNA showed dots with relatively high intensity in heart, skeletal muscle, kidney, pituitary gland, adrenal gland, caudate nucleus, putamen, and lung All of these tissues have a relatively high energy demand and are therefore likely to be affected by reduced energy availability The patient with Leigh syndrome reported here had symptoms that strongly correlate with the high-intensity dots present on the NDUFS8 master blot, such as hypertrophic cardiomyopathy hypotonia, convulsions and episodes of respiratory distress The specific neuropathology seen in Leigh syndrome can be hypothesised to be the result of oxidative stress caused by a diminished activity of complex I It would be reasonable to assume that oxygen radicals are generated in the vicinity of an iron sulphur cluster A defective NDUFS8 subunit, as seen in our patient, is a likely source of malfunctioning electron transport and therefore a possible source of free radicals The review by Robinson (1998) concerning oxygen free radicals and human complex I deficiency states that NDUFS8 would be a prime candidate for an electron donor for superoxide formation Future research is necessary to investigate the possible relationship between the specific neuropathology present in Leigh syndrome and malfunction of the NDUFS8 subunit

In conclusion, we have described herein the first mutations identified in the nuclear-encoded NDUFS8 subunit of complex I (possible binding site for iron-sulphur cluster N-2) in a patient with isolated complex I deficiency whose condition was neuropathologically diagnosed as Leigh syndrome Our analysis of these compound heterozygous transitions is the second report of genetic defects in nuclear-encoded subunits of complex I The frequency of these mutations in complex I deficient patients in general and, more specifically, in patients diagnosed with Leigh syndrome must be further established These findings contribute to possibilities for genetic counselling and prenatal diagnosis, which are of utmost importance since therapeutic intervention in complex I disease is very unsatisfactory We will continue to search for other candidate genes for complex I deficiency

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MUTATIONS IN THE COMPLEX I *NDUFS2* GENE ARE ASSOCIATED WITH HYPERTROPHIC CARDIOMYOPATHY AND ENCEPHALOMYOPATHY

Submitted

Abstract.
Human complex I is built up and regulated by genes encoded by the mitochondrial DNA (mtDNA) as well as the nuclear DNA (nDNA) In recent years, attention mainly focussed on the relation between complex I deficiency and mtDNA mutations However, several aspects of our isolated complex I deficient patient group make a nuclear-genetic cause likely (high percentage of consanguinity, autosomal-recessive mode of inheritance, absence of common mtDNA mutations) The nuclear encoded NDUFS2 (49 kDa, NuoD) protein is part of complex I of many pro- and eukaryotes Hypothesised functions of the NDUFS2 are participation in proton translocation and ubiquinone binding The gene coding for the NDUFS2 protein is therefore an important candidate for mutational detection studies in enzymatic complex I deficient patients Screening of patient *NDUFS2* cDNA by RT-PCR in combination with direct DNA sequencing revealed three missense mutations resulting in the substitution of conserved amino acids in three families

Introduction.

The genetic causes of oxidative phosphorylation (OXPHOS) disorders in general and more specific of isolated complex I deficiency (OMIM 252010) remain largely to be elucidated The mitochondrial DNA encodes for several structural components of the OXPHOS system (13 polypeptides which are incorporated in complex I, III, IV and V) as well as 22 tRNAs and 2 rRNAs (Anderson et al 1981) The nucleus however, encodes for the majority of the structural complex components, but also for all non-structural OXPHOS system proteins, like e g transcription and translation factors, chaperone proteins and assembly proteins This dual genetic control makes adequate genetic counselling in OXPHOS disorders difficult Several mtDNA mutations have clearly been linked to important OXPHOS system disorders, but in the majority of adult patients (60%) and children (>90%) common mtDNA mutations are not encountered (Shoffner 1996)

Isolated complex I deficiency is a serious inborn error of metabolism, often presenting in infancy or early childhood A diversity of clinical phenotypes has been described to occur in complex I deficient patients like Leigh(-like) syndrome, fatal infantile lactic acidosis, cardiomyopathy and lactic acidosis with or without cataract, macrocephaly with leukodystrophy and hepatopathy with tubulopathy (Rahman et al 1996, Pitkanen et al 1996, Kirby et al 1999, Loeffen et al 2000) The common divisor in these patients is the prominent place of symptoms of central nervous system and muscular disease The mean age of presentation encountered in our patient group is ~5 months (Loeffen et al 2000) The inheritance pattern in the majority of families (no affected children in the maternal lineage, high percentage of consanguinity) makes autosomal-recessive inheritance and therefore a nuclear-genetic cause likely

Human complex I consists of at least 42 different proteins (~35 nuclearly encoded, seven mitochondrially encoded (Chomyn et al 1986), which makes it one of the most complex multi-protein constructions in the human cell (Smeitink and van den Heuvel 1999) The main function of complex I is oxidation of NADH Electrons, which are freed by this oxidation, are transported to free-moving ubiquinone This electron transport is coupled to a yet unknown mechanism, by which protons are translocated from the mitochondrial matrix into the intermembrane space Several important aspects of these functions have (partly) been elucidated NADH binds to the NDUFV1 subunit of complex I (Chen and Guillory 1981, Deng et al 1990), electrons are then transported through the flavin mononucleotide (FMN, Pilkington et al 1991) and at least six Iron-Sulphur (Fe-S) clusters which have been detected by electron paramagnetic resonance studies (Ohnishi 1998) The proton translocation process as well as the ubiquinone binding and reduction process remain poorly understood

The NDUFS2 (49 kDa, OMIM 602985) subunit of complex I is a highly conserved protein, with homologues present in many pro- and eukaryotes In *Neurospora crassa*, the 49 kDa subunit has been located in the peripheral part of complex I near the membrane domain (Guenebaut et al 1997) This is further illustrated by characterisation studies performed with *Escherichia coli* complex I which showed that NuoD (the equivalent of NDUFS2 in prokaryotes) is situated in the connecting part, which possibly functions as a bridge between the NADH dehydrogenase fragment and the membrane fragment (Braun et al 1998) The

following two findings concerning the NDUFS2 subunit are indicative for possible functions of this protein At first, there may be an evolutionary relationship between the NDUFS2 and hydrogenases thought to function as proton translocators, which is suggestive for the NDUFS2 subunit to play a role in the proton translocation process (Friedrich 1998) Secondly, Darrouzet and colleagues presented a piericidin-resistant mutant of the bacterium *Rhodobacter capsulatus*, which contained a V407M substitution in the highly conserved C-terminal part of NuoD (Darrouzet et al 1998) The complex I inhibiting properties of piericidin probably result from antagonism of the ubiquinone binding site (Degli Esposti 1998) It is therefore well possible that NuoD participates in the binding of ubiquinone Hence, the NDUFS2 subunit is a major candidate gene for mutational analysis studies in isolated complex I deficient patients

In this study we present the results of mutational detection studies performed with cDNA of isolated complex I deficient patients in whom major mtDNA rearrangements and eleven common mtDNA mutations have been excluded This revealed missense mutations in four patients (three families) These findings increase our possibilities for adequate genetic counselling and prenatal diagnostics and in the future may also result in more information concerning the proton translocation and ubiquinone binding process

Patients, Material and Methods.

Family A Family A consists of three children whose parents are consanguineous (Fig 10 1). Two of these children suffered from an isolated complex I deficiency The oldest child in this family is healthy and currently alive (A1, female) The early neonatal period of the second child (A2, male) was normal, but after six months neurological regression was noted He stopped smiling and holding objects and was unable to track moving items Investigation of the eyes revealed a fine horizontal nystagmus and bilateral optic atrophy Muscle tone was axially decreased while this was increased in all extremities with brisk tendon reflexes An EEG was normal, while a brain CT scan revealed bilateral hypodensities in basal ganglia An echocardiogram showed left ventricular hypertrophy Lactate levels were severely increased in blood (9 9 mM, normal 0 6-2 1 mM) and CSF (7 8 mM, normal 1 4-1 9 mM) The patient's neurologic condition deteriorated steadily and he became gradually lethargic with episodes of apnoea eventually leading to death at the age of 24 months The third child (A3, female) expressed a phenotype resembling her brother's, though the age of presentation was earlier and the course of deterioration faster The blood lactic acid level was found elevated at the second day of life (12 mM) She suffered from severe neurological regression, axial hypotonia with pyramidal signs and failure to thrive. Prolonged latencies seen in Visual Evoked Potentials (VEP) and an abnormal electroretinogram (ERG) pointed towards optic atrophy and problems with the photoreceptors, respectively Brainstem Auditory Evoked Potentials (BAEP) showed prolonged conduction in the brain stem region The cerebral CT scan disclosed marked generalised atrophy with ventriculomegaly An echocardiogram made at birth was normal, but at the age of three months a hypertrophic cardiomyopathy was demonstrated. She died at the age of 18 months due to cardiorespiratory failure Both patients expressed an isolated complex I deficiency in cultured skin fibroblasts (Table 10 1) Enzyme

assays were also performed in skeletal muscle tissue of patient A2 which again revealed isolated complex I deficiency (muscle tissue of patient A3 was not available).

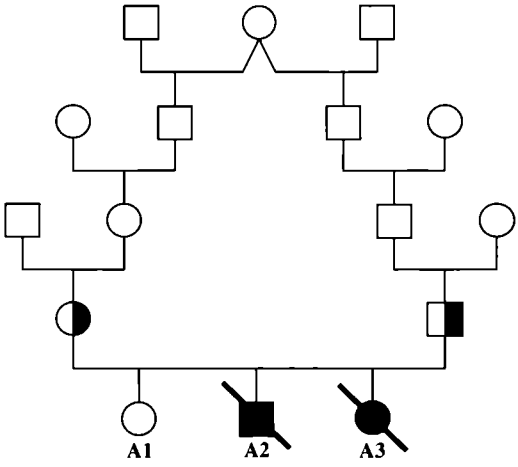

Figure 10.1 Family tree of family A. The oldest child is not affected (A1). Patient A2 and A3 suffered from a complex I deficiency and were homozygous for the G683A mutation while both parents are heterozygous.

Family B. There were no signs of genetic diseases within this family. Patient B1 (male; family tree Fig. 10.2) exhibited respiratory insufficiency during the first day of life. Lactic acid was severely increased in blood (24 mM) leading to acidosis (pH 6.9). Echocardiography performed in the early neonatal period showed a hypertrophic cardiomyopathy, which was confirmed at autopsy. The child died after four days due to cardiorespiratory failure. Biochemical investigation of the OXPHOS system revealed an isolated complex I deficiency in cultured skin fibroblasts and skeletal muscle tissue (Table 10.1).

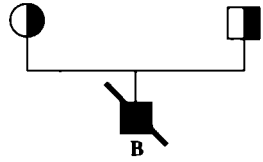

Figure 10.2 Family tree of family B. The complex I deficient patient is homozygous for the C686A mutation while both parents are heterozygous.

Family C. Family C (consanguineous parents (Fig. 10.3), negative family history for neuromuscular disorders) consists of four children, three of whom died of a clinical phenotype resembling an OXPHOS disorder. Measurement of enzyme activities of the respiratory chain, PDHc and citrate synthase revealed an isolated complex I deficiency in the second child (C2) in cultured fibroblasts as well as muscle tissue (Table 10.1). Unfortunately, tissue specimens of patient C1 and C3 were not available. The first patient (C1; male) presented at 7 months

with repeated vomiting and failure to thrive Physical examination showed horizontal nystagmus, mild ataxia, muscle hypotonia and pallor of the optic discs Cognitive development was age appropriate, yet motor delay was prominent Lactate level was increased in blood (4 2 mM) A brain CT scan disclosed areas of white matter hypodensities The patient died at the age of 18 months The second index patient (C2, male) presented at the age of 10 months with a phenotype strongly resembling the phenotype of his brother Recurrent vomiting, failure to thrive, nystagmus and generalised hypotonia with mild cognitive impairment but marked motor delay were present Lactic acid was elevated in blood (5 0 mM), as well as in CSF (3 3 mM) From the age of 2 5 years he developed recurrent episodes of sleep apnoea Brain CT scan and MRI revealed progressive hypodensity of basal ganglia and midbrain The course deteriorated steadily necessitating mechanical ventilation The child died at the age of 3 years The third patient (C3, male) presented at birth with severe metabolic acidosis (lactic acid 12 5 mM) The clinical characteristics and course were similar to the phenotype of his brothers and he died at the age of two years The fourth child of this family was born after egg cell donation and is alive and healthy

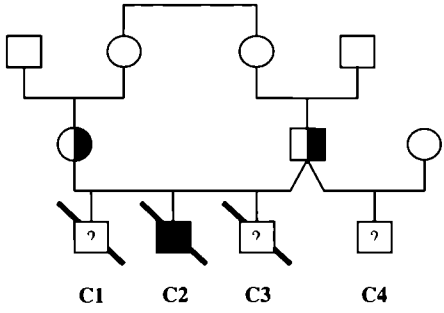

Figure 10 3 Family tree of family C The first three sons died of complex I deficiency The fourth son was born after egg cell donation and is alive and healthy Patient C2 is homozygous for the T1237C mutation while both parents are heterozygous Unfortunately DNA and RNA from C1 C3 and C4 were not available

Biochemical enzyme assays

The respiratory chain enzyme complex activities, PDHc and citrate synthase in skeletal muscle tissue and skin fibroblasts (culture) were assayed using the following methods (slightly modified) NADH ubiquinone oxidoreductase and succinate cytochrome *c* oxidoreductase as described by Fischer et al 1985 and 1986, cytochrome *c* oxidase by Cooperstein and Lazarow 1951, citrate synthase by Srere 1969 and PDHc according to van Laack et al 1988 In case of cultured fibroblasts, enzyme measurements were performed in mitochondria enriched fractions (Bentlage et al 1996), while in case of muscle tissue 600×*g* supernatant of crude homogenates was used

Mutational analysis

Patient total RNA isolated from cultured fibroblasts was reverse transcribed to cDNA by superscript II RNAse H reverse transcriptase after addition of oligo(dT) and random hexamer

primers (Ploos van Amstel et al 1996) Wildtype and patient *NDUFS2* cDNA was amplified and sequenced as published previously (Loeffen et al 1998a) All patients have been screened for major mtDNA rearrangements by a previously developed long template PCR technique (Li et al 1995) Furthermore, the following common pathogenic mtDNA mutations have been excluded in fibroblasts in our patient group by a combination of PCR and RFLP 14484T→C (ND-6), 14459G→A (ND-6), 11778G→A (ND-4), 8993T→G/C (ATPase 6), 8356T→C ($tRNA^{Lys}$), 8344A→G ($tRNA^{Lys}$), 4317A→G ($tRNA^{Gln}$); 4160T→A (ND-1); 3460G→A (ND-1), 3271T→C ($tRNA^{Leu}$), 3243A→G ($tRNA^{Leu}$) Procedures were followed as recommended by the restriction enzyme manufacturer

Restriction Fragment Length Polymorphism (RFLP).
An *NDUFS2* nDNA fragment of 294 base pairs (Genbank accession no AF183172) of all members of family A and B was amplified using oligonucleotides 5'-AAGATGTTTGAGTTCTACGAGCG-3' (forward) en 5'-ACTGATAAATGTCATCCATAAGCC-3' (reverse) The 683G→A mutation destroys an *Msp*I restriction site (recognition site C\CGG), while the 686C→A mutation destroys an *Hae*III restriction site (recognition site GG\CC) In case of the parents of family C as well as patient C2 a nDNA fragment of 342 base pairs was amplified (Genbank accession no AF183173) using oligonucleotides 5'-AGACCATATGTAGAGTAGGTAGC-3' (forward) and 5'-TTAGTTAAGACTTGCAAGGTGGG-3' (reverse) The 1237T→C mutation introduces a *Ban*I restriction site (recognition site G\GYRCC) In all RFLP assays, conditions were used as recommended by the manufacturer

Northern blot and multiple tissue dot blot
In order to check the specificity of an $\alpha[^{32}P]$-dCTP-labelled *NDUFS2* cDNA probe, we hybridised the probe to a multiple tissue Northern blot commercially purchased from Clontech, USA (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas), using a standard hybridisation solution based on procedures described in Sambrook et al 1989 The blot was washed twice with 2× SSC, 0 1% SDS, at 65°C and was subjected to autoradiography. To visualise the distribution of *NDUFS2* mRNA in a large range of human adult and foetal tissues, the same probe was hybridised to a multiple tissue RNA Master blot The quantity of RNA spotted for each tissue on the Master blot was normalised using eight different housekeeping gene transcripts as probes (ribosomal protein S9, ubiquitin, 23 kDa highly basic protein, hypoxanthine guanine phosphoribosyl transferase, tubulin, β-actin, phospholipase, and glyceraldehyde triphosphate dehydrogenase)

Results.

Biochemical enzyme assays
The enzyme assays of citrate synthase and respiratory chain enzymes were performed in cultured skin fibroblasts of all patients and additionally in fresh muscle tissue in case of patient B and C2 and in muscle tissue which has been stored in liquid nitrogen of patient A2 (in muscle tissue of patient A2 and B PDHc activity was also measured and found normal)

This revealed in all cases an isolated complex I deficiency (complex I enzyme activities of all patients are listed in Table 10.1).

	NADH:ubiquinone oxidoreductase (complex I)	
	Fibroblasts 0.11-0.28[a] (14)	**Muscle 70-250[b] (20) 101-389[c] (13)**
Patient A2	0.043	40[c]
Patient A3	0.060	-
Patient B	0.040	17[b]
Patient C2	0.045	48[b]

Table 10.1 Residual complex I activity of the patients in cultured fibroblasts and muscle tissue. All patients expressed an isolated complex I deficiency. Complex I activity is referred to cytochrome *c* oxidase base in case of fibroblasts[a] (mU/mU cytochrome *c* oxidase), to citrate synthase (mU/U citrate synthase) when measured in fresh muscle tissue[b] and to cytochrome *c* oxidase (U/mU cytochrome *c* oxidase) when measured in 600×*g* supernatant of muscle tissue which has been deep frozen in liquid nitrogen[c]. Numbers of controls used for each assay are listed in parentheses.

Mutational analysis studies.

RT-PCR followed by direct DNA sequencing of the *NDUFS2* cDNA revealed missense mutations in four patients (three families). In family A we found a homozygous G→A transition at position 683 in both complex I deficient patients (A2 and A3). As a consequence of this mutation a conserved arginine is substituted into a glutamine (Table 10.2). The *NDUFS2* cDNA of the index patient in family B contained a homozygous 686C→A transversion, which leads on protein level to the substitution of a conserved proline into a glutamine (Table 10.2). Finally, in patient 2 of family C, we encountered a homozygous T→C transition at position 1237. This mutation results in the replacement of a conserved serine residue by a proline (Table 10.2).

Restriction Length Polymorphism (RFLP) analysis.

We confirmed the presence of the mutations found in the index patients on nDNA by means of RFLP analysis (Fig. 10.4). To prove the presence of the mutation found in patient A2 and A3, we amplified and sequenced an *NDUFS2* nDNA fragment of the region containing the mutation (294 base pairs; Genbank accession no. AF183172), which revealed two recognition sites for *Msp*I in control cDNA, one of which is destroyed by the 683G→A mutation. Complete digestion with *Msp*I results in three bands in the control and the healthy sib A1 (203, 57 and 34 base pairs), confirming the presence of two wildtype alleles. Two bands were found in both patients (203 and 91 base pairs), who contain two mutated alleles and finally four bands were seen in case of the heterozygous parents (203, 91, 57 and 34 base pairs).

The same wildtype *NDUFS2* nDNA fragment as generated for all members of family A (Genbank accession no. AF183172) contains three *Hae*III recognition sites. Since one *Hae*III site is destroyed by the 686C→A mutation, only two cleavage sites remain in patient B1.

Complete digestion of control *NDUFS2* nDNA therefore results in four bands (161, 60, 57 and 16 base pairs, Fig 10 4), in case of our patient three bands are seen (161, 117 and 16 base pairs, two mutant alleles), while the heterozygously mutated parents express five bands (161, 117, 60, 57 and 16 base pairs) The 60 and 57 bands are not separated from each other and the 16 base pair band is not visible.

The 1237T→C mutation found in patient C2 introduces a *Ban*I restriction site A control *NDUFS2* nDNA fragment (342 base pairs, Genbank accession no AF183173) remains therefore undigested, while the nDNA fragment of the patient is cleaved in two fragments of 175 and 167 base pairs The nDNA fragment of both parents is partly digested resulting in three bands of 342, 175 and 167 base pairs The 175 and 167 bands are not separated Unfortunately, nDNA as well as cDNA of patient C1 and C3 are not available.

All three mutations have been excluded in nDNA fragments of 85 controls (170 alleles)

	226	227	228	229	230
683G→A	Y	I	**R**	P	G
Wildtype	tat	atc	**cgg**	cca	gga
Patients A	tat	atc	**cag**	cca	gga
	Y	I	**Q**	P	G
	227	228	229	230	231
686C→A	I	R	**P**	G	G
Wildtype	atc	cgg	**cca**	gga	gga
Patient B	atc	cgg	**caa**	gga	gga
	I	R	**Q**	G	G
	411	412	413	414	415
1237T→C	L	V	**S**	D	G
Wildtype	ctg	gtg	**tct**	gat	ggc
Patient C	ctg	gtg	**cct**	gat	ggc
	L	V	**P**	D	G

Table 10.2 *NDUFS2* wildtype and patients cDNA and amino acid sequence of the regions containing the mutations

RNA tissue distribution

Hybridisation of the radioactively labelled *NDUFS2* cDNA probe to the multiple tissue Northern blot revealed a single band for each tissue present on the blot, which confirms the specificity of the probe (data not shown) In order to evaluate the *NDUFS2* RNA distribution in a variety of tissues, we hybridised the same probe to a multiple tissue Master blot (Fig 10 5) This revealed a similar pattern (slight differences) as seen for other nuclear encoded complex I subunits The *NDUFS2* cDNA probe is ubiquitously expressed, with a relative higher expression in the following tissues adult and foetal heart, kidney, skeletal muscle, adrenal gland and liver Also the human and *E coli* DNA dots (H4, H7 and H8) were positive

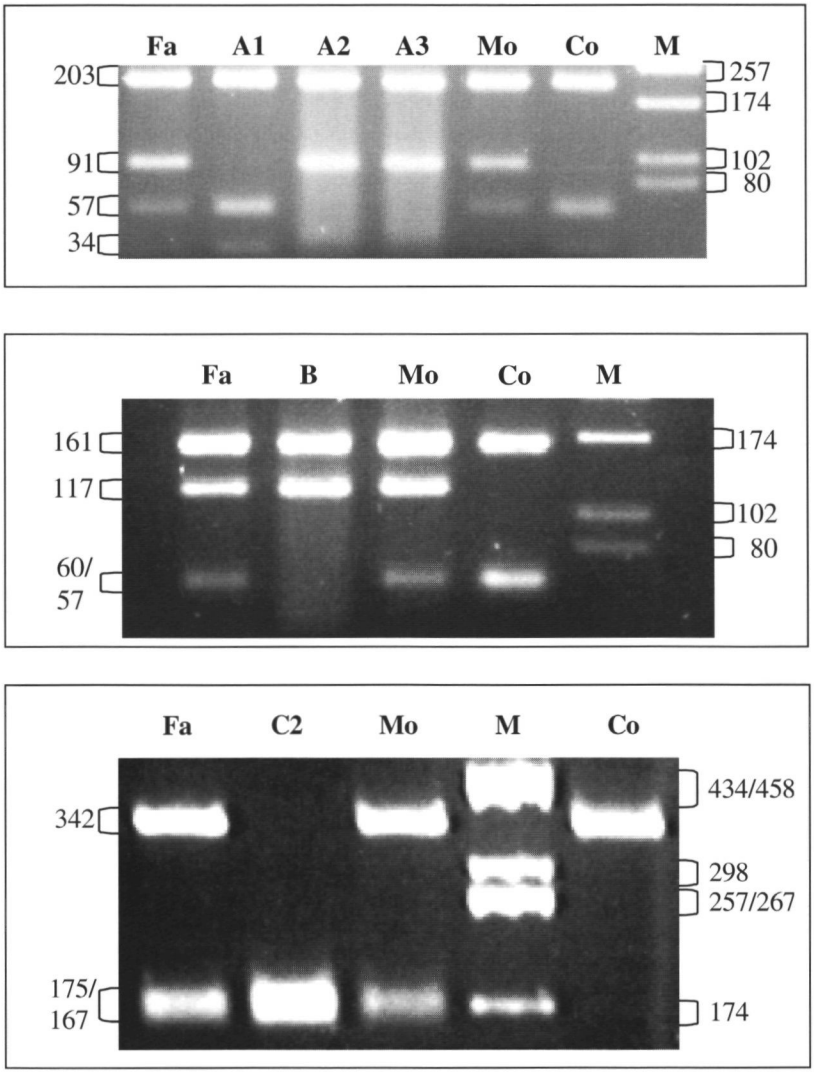

Figure 10.4 *Top:* RFLP pattern of family A after digestion with *Msp*I. Complete digestion results in three bands in the control and A1 (203, 57 and 34 bp (latter band faint), two bands in the index patients (203 and 91 bp) and four in case of their parents (203, 91, 57 and 34 bp). *Middle:* The family B nDNA RFLP pattern after digestion with *Hae*III. Complete digestion results in four bands when control DNA is used as template (161, 60, 57 and 16 bp), three bands in case of our index patient (161, 117 and 16 bp) and five when nDNA of the parents is used as template (161, 117, 60, 57 and 16 bp). The 60 and 57 bands are not separated from each other, the 16 bp band is not visible). *Bottom:* The 1237T→C mutation introduces a *Ban*I restriction site. The control *NDUFS2* nDNA fragment (342 bp, Genbank accession no. AF183173) remains therefore undigested, while the nDNA fragment of the index patient is cleaved in two fragments of 175 and 167 bp. Nuclear DNA fragments of both parents are partly digested resulting in three bands of 342, 175 and 167 bp. The 175 and 167 bands are not separated. The DNA size marker (M) is Puc18/*Hae*III (band sizes are listed right from the image), Mo = mother, Fa = father, Co = control, bp = base pairs.

	1	2	3	4	5	6	7	8
a	whole brain	amygdala	caudate nucleus	cerebel-lum	cerebral cortex	frontal lobe	hippo-campus	medulla oblongata
b	occipital lobe	putamen	substantia nigra	temporal lobe	thalamus	subthal. nucleus	spinal cord	
c	heart	aorta	skeletal muscle	colon	bladder	uterus	prostate	stomach
d	testis	ovary	pancreas	pituitary gland	adrenal gland	thyroid gland	salivary gland	mammary gland
e	kidney	liver	small intestine	spleen	thymus	peripheral leukocyte	lymph node	bone marrow
f	appendix	lung	trachea	placenta				
g	foetal brain	foetal heart	foetal kidney	foetal liver	foetal spleen	foetal thymus	foetal lung	
h	yeast RNA 100 ng	yeast tRNA 100 ng	*E. coli* rRNA 100 ng	*E. coli* DNA 100 ng	poly r(A) 100 ng	human C_0t1 DNA 100 ng	human DNA 100 ng	human DNA 500 ng

Figure 10.5a Multiple tissue Master blot. The type and position of poly(A^+) RNAs and controls dotted on the positively charged nylon membrane. subthal. = subthalamic.

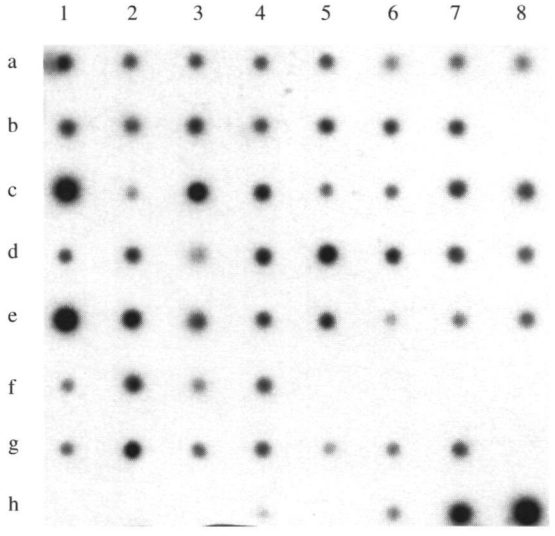

Figure 10.5b Tissue distribution of *NDUFS2* mRNA visualised on a poly A^+ RNA master blot. The *NDUFS2* cDNA probe is ubiquitously expressed in human tissues, with a relatively higher expression in the human and foetal heart, kidney, skeletal muscle, adrenal gland tissue, whole brain and liver tissue.

Discussion.

Mitochondriocytopathies have an incidence of about 1 in 10,000 live births (Bourgeron et al 1995) The exact prevalence of one of the mitochondrial disorders, complex I deficiency, is at the moment unknown However, we know that complex I deficiency is one of the most frequently occurring OXPHOS enzyme disorders in children (Loeffen et al 2000)

The clinical phenotype observed in patients with complex I deficiency, often develops devastating, with early onset, rapid progression and death within a few years after birth Successful therapeutic possibilities are currently not available It is therefore important to locate the genetic causes of this disease to offer at least the possibility of adequate genetic counselling and prenatal diagnostics In previous years, several nuclear mutations resulting in deficient activity of one of the respiratory chain complexes, have been found These genes concerned structural genes in case of complex I deficiency (van den Heuvel et al 1998, Loeffen et al 1998b, Schuelke et al 1999, Triepels et al 1999), and complex II deficiency (Bourgeron et al 1995), but also mutations in assembly genes have been described (in relation to complex IV deficiency, Zhu et al 1998, Tiranti et al 1998, Coenen et al 2000, Smeitink et al 2000) Yet, for a large part of our complex I deficient patients, gene defects remain to be identified

The NDUFS2 subunit is the third largest complex I subunit Its mRNA contains an open reading frame of 1392 base pairs, coding for 463 amino acids. Electron microscopy studies performed in *N crassa* using Fab fragments of antibodies directed against the 49 kDa subunit located this protein in the extramembraneous part of complex I, near the membrane domain (Guenebaut et al 1997) Disruption of this subunit results in the complete absence of the peripheral arm of complex I (Guenebaut et al 1997) Recent studies revealed interesting aspects of the NDUFS2 subunit The identification of a *R capsulatus* mutant containing a V407→M substitution in NuoD (prokaryotic equivalent of NDUFS2), which is partially resistant to piericidin, is an indication for a possible role of NuoD in ubiquinone binding (Darrouzet et al 1998) This amino acid substitution is located in the conserved C-terminal part of the protein. The structure of piericidin is similar to the structure of ubiquinone, both containing a polar and an aliphatic part It is therefore possible, that the inhibiting function of piericidin results from antagonism of the ubiquinone-binding site Darrouzet and colleagues hypothesise that NuoD interacts with the polar part of piericidin while the aliphatic component interacts with NuoH, which is located in the membranous part of complex I The piericidin resistant mutants are also partially resistant to rotenone. It seems likely that piericidin and rotenone share at least partially a common binding site This was also seen in competition experiments performed by Okun and co-workers, which showed that the hydrophobic part of piericidin and rotenone share a common binding domain with partially overlapping sites (Okun et al 1999)

The NDUFS2 protein is highly conserved in many species (95% in *Bos taurus*, 62% in *Neurospora crassa* and 38% in *Escherichia coli*) which indicates its overall functional importance (Loeffen et al 1998a) The NDUFS2 subunit has also weak homology with several proteins involved in the transport of protons, like HycE (subunit of formate hydrogenlyase)

and HyfG (subunit of Ni-Fe hydrogenase), so there might be an evolutionary relationship between these proteins. This implicates that there might be a role for the NDUFS2 subunit in proton transport (Friedrich and Weiss 1997).

Hybridisation of the *NDUFS2* cDNA probe to the RNA Master blot showed spots at all adult and foetal tissues present on the blot. Dots with a relative higher intensity are tissues with a high metabolic activity like heart, kidney and skeletal muscle tissue. Hybridisation of the probe to the multiple tissue Northern blot confirmed the probe's specificity. The human DNA dots (H7 and H8) were also positive on the RNA Master blot. This can be caused by the presence of relatively large exons, which hybridise to a substantial part of the probe or the presence of pseudogenes. Also the *E. coli* dot (H4) showed a faint signal. This can be caused by the high percentage of homology between the *NDUFS2* subunit and NuoD. The hybridisation of the probe to the DNA dots is however not a problem for the interpretation of the RNA dots, since the RNA dots on the blot contain virtually no DNA (guaranteed by the manufacturer).

The amino acid substitution present in family A (R228Q) is located just prior to the amino acid, which has been changed in family B (P229Q). These substituted amino acids are conserved in many pro- and eukaryotes (Table 10.3). The high conservation of the bases flanking these substituted amino acids (RPGGV) is reflected by their conservation (partially) in some proteins, which are involved in proton pumping like e.g. HycE of *Mycobacterium tuberculosis*. However, functional aspects of such an amino acid pattern have not been described. Chou-Fasman plots (Chou and Fasman 1978) calculated with the NDUFS2 wildtype and mutated proteins (R228Q as well as P229Q), show replacement of α-helix by β-sheet in the region prior to the proline at position 229. When this region is functionally important, it is thinkable that conformational peptide changes alter the overall function of the NDUFS2 protein. The amino acid which is substituted in family C (S413P) is also conserved in many species (Table 10.3). The biochemical characteristics of the substituted amino acids clearly differ from those of their substitutes. These different characteristics can have serious consequences for the functionality of the NDUFS2 protein and therefore on the overall complex I activity. It is unlikely that the missense mutations encountered in our index patients are population polymorphisms since they were not encountered in 170 control alleles. In addition, a BLAST search in the human EST database of the National Centre for Biotechnology Information (NCBI) with a cDNA fragment containing the mutations, only revealed wildtype EST fragments (5 ESTs were present containing the region which is mutated in family A and B, and 30 in the region which is mutated in family C). Definite proof of the pathogenicity of the mutations found in our index patients has to come from rescue of the complex I activity by introducing a vector containing the wildtype *NDUFS2* cDNA into our patient fibroblasts. These experiments are however rather difficult and time consuming since we need up to 10 million cells to perform our enzyme assays accurately.

Several clinical aspects of the phenotypes found in the patients described in this article are worth discussing. The patients of family A (A2 and A3) and the patient in family B suffered from a hypertrophic cardiomyopathy. In patient B the cardiomyopathy was present at birth, while in patient A3 it developed in the first months of life (in case of patient A2 the presence

or absence of the cardiomyopathy at birth was not investigated). The multiple tissue dot blot autoradiograph showed the highest expression of *NDUFS2* mRNA in cardiac tissue. Cardiomyopathy is not an uncommon clinical finding in patients with mitochondrial encephalomyopathies. In our group of isolated complex I deficient patients, 30% suffered from cardiomyopathy (mainly hypertrophic). Besides the cardiomyopathy, ocular symptoms were frequent among our index patients. Both patients in family A (A2 and A3) as well as patient C1 showed signs of optic atrophy. Horizontal nystagmus was seen in patient A2, A3, C1, C2 and C3. Since patient B1 died four days after birth, time was too short to detect possible visual defects.

Species	*Mutation A (R228Q)* ↓	*Mutation B (P229Q)* ↓	*Mutation C (S413P)* ↓
PT	222 **MHAAYI**Q**PGGVHQ** 234	223 **HAAYIR**Q**GGVHQD** 235	407 **FGVYLV**P**DGSSRP** 419
WT	222 **MHAAYIRPGGVHQ** 234	223 **HAAYIRPGGVHQD** 235	407 **FGVYLVSDGSSRP** 419
BT	187 **MHAAY**V**RPGGVHQ** 199	188 **HAAY**V**RPGGVHQD** 200	372 **FGVYLVSDGSSRP** 384
NC	237 L**HAAY**V**RPGGVHQ** 249	238 **HAAY**V**RPGGVHQD** 250	422 MGVYVVSDGSERP 434
WT	153 **MHA**SF**IRPGGVAQ** 165	154 **HA**SF**IRPGGVAQD** 166	338 **FGV**FL**VSNGSNRP** 350
MT	183 **M**NS**AYIRPGGVAQ** 195	184 NS**AYIRPGGVAQD** 196	384 LGVHM**VSDG**GT**RP** 396
SM	151 **MHAAY**F**RPGGVHQ** 163	152 **HAAY**F**RPGGVHQD** 164	340 **FGVYLVSDG**TNK**P** 352
PD	169 L**HAAY**F**RPGGVHQ** 181	170 **HAAY**F**RPGGVHQD** 182	357 **FGVYLVADG**TNK**P** 369
EC	165 **MH**PAWF**RIGGV**AH 177	166 **H**PAWF**RIGGV**AH**D** 178	544 NSY**YLTSDGS**TMS 556

Table 10.3 The extent of conservation of the substituted amino acids (R228Q, P229Q and S413P) in several pro- and eukaryotes (PT = patient; WT = wildtype human; BT = *Bos taurus*; NC = *Neurospora crassa*; WT = *Wood tobacco*; MT = *Mycobacterium tuberculosis*; SM = *Sinorhizobium meliloti*; PD = *Paracoccus denitrificans*; EC = *Escherichia coli*).

In summary, we found missense mutations in the highly conserved *NDUFS2* mRNA in four isolated complex I deficient patients (three families). The identification of this gene as a possible host for mutations causing complex I deficiency increases our possibilities for genetic counselling and prenatal diagnostics. Future cellular studies with fibroblasts of these patients might increase our insight in the binding and reduction of ubiquinone by complex I as well as the hypothesised proton translocating function of the NDUFS2 subunit.

Electronic database information.

Genbank accession numbers *NDUFS2 / 49 kDa / NuoD:*
Homo sapiens NP_004541, O75306; *Bos taurus* P17694; *Neurospora crassa* P22142; *Wood tobacco* S57332; *Mycobacterium tuberculosis* CAB06291; *Sinorhizobium meliloti* CAB51623; *Paracoccus denitrificans* P29916; *Escherichia coli* P33600.

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MUTATIONAL ANALYSIS STUDIES OF FOUR NUCLEAR ENCODED SUBUNITS OF THE COMPLEX I IRON-SULPHUR PROTEIN (IP) FRACTION: WHICH GENES NEXT?

Submitted

Abstract.
Human complex I consists of at least 42 different proteins, of which 35 are encoded by nuclear genes With the use of chaotropic agents, complex I can be subdivided in a Flavoprotein (FP), Iron-Sulphur protein (IP), and Hydrophobic protein (HP) fraction The IP fraction is situated in the part of complex I, which protrudes into the mitochondrial matrix and contains together with the FP fraction almost all known redox groups
We performed mutational analysis studies on cDNA level of four IP subunits (NDUFS1, NDUFS3, NDUFS6 and NDUFA5) in 20 isolated complex I deficient patients, which revealed no pathological mutations With the results presented in this study, all 35 known nuclear encoded complex I subunits have been checked for mutations in our initial patient group Altogether these mutational analysis studies revealed mutations in genes of five different subunits (NDUFV1, NDUFS2, NDUFS4, NDUFS7, NDUFS8), in seven out of eighteen pedigrees (39%) Three of these subunits (four pedigrees) belong to the IP fraction

Introduction.

Isolated complex I deficiency is one of the main enzyme defects of the mitochondrial respiratory chain found in children clinically presenting with a mitochondrial encephalomyopathy (Loeffen et al 2000a) Various drugs and combinations of drugs have been supplied to complex I deficient patients such as riboflavine (precursor FAD), nicotinamide (precursor NAD) and succinate (complex II substrate) (Scholte et al 1995) These therapies have only been described to benefit individual or small number of patients Elucidation of the genetic cause(s) in complex I deficient patients, the main aim of our current investigations, may be worthwhile in this context for several reasons In the first place it will give us the opportunity to offer adequate genetic counselling and prenatal diagnostics Secondly, pathophysiological studies performed with tissues of patients in whom the genetic defect has been elucidated may enhance our understanding of the cell biological consequences of complex I deficiency and eventually may contribute to accurate disease models, necessary for developing adequate therapy strategies

Studies of the genetics of complex I deficiency are complicated by the fact that this complex is under double genomic control in eukaryotes (Walker 1992) Seven of the ~42 subunits of human complex I are encoded on the mitochondrial DNA (mtDNA, Anderson et al 1981) This implicates that the remaining 35 subunits are encoded by nuclear genes The last two years, we searched for mutations in the nuclear encoded subunits of complex I in a group of isolated complex I deficient patients Pre-screening for major rearrangements (long template PCR technique (Li et al 1995)) and for eleven common mtDNA mutations in these patients revealed no abnormalities (Loeffen et al 2000a)

The cDNA and amino acid sequence of unidentified human complex I subunits were elucidated using the high percentage of identity with bovine complex I subunits Recently the 35th nuclear encoded bovine complex I subunit has been identified (Skehel et al 1998) Equivalents of these 35 subunits have been found in humans (Loeffen et al 1998a, Triepels et al 2000) Strikingly, mutational analysis studies performed thus far in our patient group mainly revealed mutations in those subunits, which are highly conserved in evolution as illustrated by their presence in prokaryotic lifeforms (Loeffen et al 2000a)

In this study we focussed mutational analysis on four subunits of the Iron-Sulphur protein (IP) fraction, namely the NDUFS1, NDUFS3, NDUFS6 and NDUFA5 The IP fraction contains ~8 subunits and constitutes the main part of complex I which protrudes in the mitochondrial matrix (Galante & Hatefi 1978 and 1979, Belogrudov and Hatefi 1996) The largest subunit of the IP fraction is the NDUFS1 (Chow et al 1991) It contains one 2Fe 2S cluster binding site (cluster N1b) and probably two 4Fe-4S cluster binding sites (cluster N4 and N5, Ohnishi 1998) The second largest subunit is the NDUFS2, which is believed to influence proton transport and ubiquinone binding (Loeffen et al 2000b) Little is currently known of the function of the NDUFS3 subunit, but its high conservation during evolution implicates importance (Loeffen et al 1998b) The NDUFS4 subunit contains a cAMP regulated phosphorylation site, and probably plays a role in the regulation of complex I activity (Papa et al 1996) Another subunit, which is likely to contribute to the electron transport is the NDUFS8 This subunit contains highly conserved patterns for two 4Fe 4S cluster-binding

sites Of the other three subunits (NDUFS5, NDUFS6 and NDUFA5) is functionally little known (Pata et al 1997, Russell et al 1997, Loeffen et al 1998b, Loeffen et al 1999)
In this study we present the results of mutational analysis studies of four nuclear encoded IP subunits (NDUFS1, NDUFS3, NDUFS6 and NDUFA5) in a group of 20 isolated complex I deficient patients, which revealed no pathological mutations This study completes mutational analysis of the eight subunits present in the IP fraction With the results of this study, all currently known 35 nuclear encoded complex I subunits in our initial complex I deficient patient group have been checked for mutations

Patients, material and methods.

Patient group
Twenty isolated complex I deficient patients (age range 0-26 months, measured in all cases in cultured skin fibroblasts) were selected for mutational analysis studies of nuclear encoded complex I subunits Clinical characteristics of this patient group have recently been published (Loeffen et al 2000a)

Material
DNA Taq polymerase, superscript II RNAse H reverse transcriptase and oligonucleotide primers were purchased from Life Technologies Inc , gel purification systems from Qiagen, dRhodamine Dye Terminator Sequencing Kit from Perkin Elmer, poly A^+ RNA pool from Clontech and RNAzol from Biosolve BV

Tissue culture
Skin fibroblasts were cultured in a humidified atmosphere of 95% air, 5% CO_2 at 37°C in medium 199 with Earle's salts supplemented with 10%(v/v) foetal calf serum (FCS) and 100 IU penicillin/ml and 100 µG streptomycin/ml The cells were grown to confluence

Biochemical measurements
Fibroblasts were harvested according to standard procedures and suspended in a concentration of 1 10^7 cells/ml 1% Foetal Calf Serum in Phosphate Buffered Saline (FCS-PBS) A mitochondrial-enriched fraction was isolated from these fibroblasts as described by Bentlage and co-workers in 1996
All individual enzyme assays have been performed in mitochondrial enriched fractions Pyruvate dehydrogenase complex (PDHc) activity is estimated by measurement of the production rate of $^{14}CO_2$ by PDHc under basal and activated conditions, in presence of [1-^{14}C]-pyruvate, CoA and NAD^+ according to van Laack et al 1988 Citrate synthase activity is estimated by the extent of complex formation between CoA and 5',5'dithiobis-2 nitrobenzoate (DTNB) which is measured spectrophotometrically at 412 nm according to Srere in 1969 NADH O_2 oxidoreductase and NADH Q1 oxidoreductase are measured by following NADH oxidation spectrophotometrically at 340 nm in presence of O_2 or Q1 as electron acceptor in presence and absence of rotenone as described by Fischer et al 1986,

succinate:cytochrome *c* oxidoreductase is estimated by following the reduction of cytochrome *c* at 550 nm in presence of succinate, rotenone, antimycine A and KCN according to Fischer et al 1985. Cytochrome *c* oxidase activity is determined by measurement of the oxidation rate of completely reduced cytochrome *c* spectrophotometrically at 550 nm as described by Cooperstein and Lazarow 1951.

Mutational analysis.

Control RNA was obtained from commercially purchased pooled poly A^+ RNA from 12 healthy Caucasian males and females, age range 12-78 years. Patient RNA was extracted from cultured skin fibroblasts according to methods described by Chomczynski and Sacchi 1987. cDNA was made by reverse transcription of RNA by superscript II RNAse H^- reverse transcriptase, with oligo(dT) and random hexamer primers (Ploos van Amstel et al 1996).

The complete open reading frame (ORF) of the NDUFS1 subunit was divided in four overlapping fragments, which were separately amplified (F1+R2, F3+R4, F5+R6, F7+R8) in a volume of 50 µl, containing 2 µl cDNA template, 5 µl 10× PCR buffer, 100 ng forward and reverse primer, 0.25 mM $MgCl_2$, 0.25mM dNTPs and 1.0 unit Taq DNA polymerase. In case of the NDUFS3 (F1+R3), NDUFS6 (F1+R1) and NDUFA5 (F1+R1) subunit, one fragment containing the complete ORF was amplified in a volume of 25 µl, containing 1 µl cDNA template, 2.5 µl 10× PCR buffer, 50 ng forward and reverse primer, 0.25 mM $MgCl_2$, 0.25mM dNTPs and 0.5 unit Taq DNA polymerase. All reaction conditions and primer sequences used are listed in Table 11.1 and 11.2. PCR products were purified using the QIAquick PCR purification kit or GEL extraction kit.

Control and patient PCR fragments were sequenced with the ABI PRISM™ 377 using the sequence primers for each individual subunit, which are listed in table 11.1. Procedures were followed as recommended by the manufacturer.

Subunits		Forward oligonucleotides		Reverse oligonucleotides
NDUFS1	F1	CGGACAGTTTAGCAGAACAG	R1	GGCACAAGCAGCTACAACC
	F2	GGTTGTCTGTTGCTGGAAAC	R2	CCCAAATCATCTACTCCTGC
	F3	GTGGAAGACAAGAACATTGG	R3	CACTCTTCATTGATGTCCTC
	F4	GATGTAATGGATGCGGTTGG	R4	CAAATCTGTGCCAGCTCCTG
	F5	GTGGACTCTGACACCTTATG	R5	GAATTGCTGCTCCATCATTTC
	F6	GGTGGTTTTAGGCAGTTCTG	R6	CTCTACCCTCAGTGTTGAC
	F7	GCTGATGTTATTCTCCCAGG	R7	GAGGCTCTGCTAATCGAATC
	F8	CCCACTTGTTCCACCTCAG	R8	GGATCACTGCACTACAGTTG
NDUFS3	F1	CATCTGAGTAACATGGCGGC	R1	CTGTGCATTGGTGTGATCCC
	F2	GTTAGAGGTCTGTATCCATCC	R2	GGATCCTTCTTAGATCAGGG
	F3	AGCCAACTGGTATGAAAGGG	R3	GGACACTCAATCATAGATAAGG
NDUFS6	F1	AAATGGCGGCGGCGATGACC	R1	GCACAGAACCAGCGAGCTTCAC
NDUFA5	F1	GTCACCGAGTCGTTGGCG	R1	CAGTTTCCCATGAACACACC

Table 11.1 Sequence of primers used in the PCR reactions and of those used in sequence reactions (all primers).

	PCR conditions
NDUFS1F1+R2	92°C/1 min.; 92°C/1 min., 56 °C/1 min., 72 °C/1 min. (40 cycles); 72 °C/10 min.
NDUFS1F3+R4	92°C/1 min.; 92°C/1 min., 58 °C/1 min., 72 °C/1 min. (40 cycles); 72 °C/10 min.
NDUFS1F5+R6	92°C/1 min.; 92°C/1 min., 56 °C/1 min., 72 °C/1 min. (40 cycles); 72 °C/10 min.
NDUFS1F7+R8	92°C/1 min.; 92°C/1 min., 58 °C/1 min., 72 °C/1 min. (40 cycles); 72 °C/10 min.
NDUFS3F1+R3	94°C/1 min.; 94°C/1 min., 60 °C/1 min., 72 °C/1 min. (34 cycles); 72 °C/10 min.
NDUFS6F1+R1	94°C/1 min.; 94°C/1 min., 60 °C/1 min., 72 °C/1 min. (34 cycles); 72 °C/10 min.
NDUFA5F1+R1	94°C/1 min.; 94°C/1 min., 55 °C/1 min., 72 °C/1 min. (34 cycles); 72 °C/10 min.

Table 11.2 Conditions of the PCR reactions used in the amplification of each subunit.

Results and discussion.

Cycle sequencing of the wildtype amplified *NDUFS1* cDNA revealed an open reading frame (ORF) of 2184 base pairs (including stopcodon) coding for 727 amino acids. We found two mutations in the ORF of our wildtype and patient cDNA sequences compared to the published *NDUFS1* cDNA sequence (Chow et al 1991). It concerned a 1248A→T and 1249T→A, which change the published tryptophane residue at position 417 into an arginine (W417R). The NDUFS1 subunit has a molecular weight of 79.5 kDa deducted from its amino acid sequence. The bovine homologue (75-kDa subunit) contains a presequence of 23 amino acids (Runswick et al 1989) and is believed to contain at least two Iron-Sulphur cluster bindings sites (4Fe-4S (N4) and 2Fe-2S (N1b); Runswick et al 1989), but probably another 4Fe-4S binding site is present (Ohnishi 1998). The presence of these clusters implicates an important role for the NDUFS1 in the transport of electrons and is therefore one of the major candidate genes in isolated complex I deficiency. Belugrodov and co-workers showed by ligand blotting that the bovine 75 kDa subunit has a close interaction with the 51-kDa (NADH binding site, FMN, 4Fe-4S) and 24-kDa (2Fe-2S) subunit and therefore likely accepts the electrons from the Flavoprotein fraction (FP; Belogrudov and Hatefi 1996). Sequence analysis of patient *NDUFS1* cDNA of 20 isolated complex I deficient patients revealed however no pathological mutations. We did encounter several silent mutations, namely a 414T→C (D138D) heterozygously in one patient, a homozygous 966T→G (A322A) also in one patient (also heterozygously in the wildtype) and a 1251G→A (R417R) in several patients and wildtype (homozygous as well as heterozygous). Finally, we found a heterozygous 1291C→G mutation, which leads to the substitution of a leucine into a valine (L431V) in one patient.

The wildtype NDUFS3 subunit contains an ORF of 795 base pairs coding for 264 amino acids (Loeffen et al 1998b). Like the NDUFS1, this subunit is present in the prokaryotic complex I which is indicative for functional importance. Currently nothing is known about the function of this subunit. Mutational analysis studies revealed neither mutations nor polymorphisms.

The *NDUFS6* (ORF 375 base pairs) and *NDUFA5* (ORF 348 base pairs) wildtype cDNA sequence has been published by Loeffen et al in 1998b and Pata et al in 1997, respectively. Cycle sequencing of amplified cDNA of all patients again revealed neither mutations nor polymorphisms.

At this moment mutational analysis studies have been performed of all currently known 35

nuclear encoded subunits of complex I in an initial group of 20 isolated complex I deficient patients (18 pedigrees) In case of subunits present in the flavoprotein (FP) fraction these studies revealed compound heterozygous mutations leading to a nonsense mutation and two single amino acid substitutions in the NDUFV1 subunit (R59X and T423M in two brothers with an encephalomyopathy and a homozygous mutation resulting in an A341V in a girl with macrocephaly and leukodystrophy (Schuelke et al 1999)) Mutational analysis studies performed with the *NDUFV2* and *NDUFV3* cDNA revealed no pathological mutations (unpublished results) Similar studies performed with subunits of the IP fraction revealed amino acid substitutions in the NDUFS2 subunit in three pedigrees (R228Q, P229Q and S413P), however the patient in whom the P229Q substitution has been found did not belong to the initial patient group (Loeffen et al 2000b) In the *NDUFS4* gene a 5-bp duplication was found in a boy with Leigh-like syndrome Finally, the *NDUFS8* cDNA contained mutations in a boy with Leigh syndrome leading to a P79L and R102H amino acid substitution Screening of the remaining large group of the Hydrophobic Protein (HP) fraction (24 subunits) revealed an amino acid substitution (V122M) in two brothers with Leigh syndrome (Triepels et al 1999 and 2000) In summary, mutations were found in 7 of the initial 18 pedigrees (39%), all of which co-segregated within the family and were excluded in a large control population

This study completes the mutational analysis studies of the currently known nuclear encoded complex I subunits in our initial complex I deficient patient group (n=20) In contrary to cytochrome *c* oxidase deficiency where no mutations were found in the nuclear encoded subunits (Adams et al 1997, Jaksch et al 1998) mutations have been found in those of complex I Since the number of subunits is not definitely determined in cow (now 42 known, and one announced, Skehel et al 1998) there might be other mutations present in undetected nuclear encoded subunits Similar experiments to determine the number of subunits like those performed in cow (e.g electrospray mass spectrometry) still have to be performed in humans These studies will give the definite number of subunits of which human complex I is composed

The nuclear encoded complex I genes were clear candidates for mutational analysis studies in our complex I deficient patient group The elucidation of the gene defects in patients in whom no mutations were encountered in the cDNAs of the nuclear encoded complex I subunits will however be more complicated Several approaches can be followed to tackle this problem One can try to find homologues of human genes, which have been linked to the biogenesis of complex I in lower species and consequently perform mutational analysis studies Good candidates for this approach would be the CIA30 and CIA84 complex I chaperone/assembly proteins described in *Neurospora crassa* (Kuffner et al 1998) This method has been successfully applied by Papadopoulou and co-workers in 1999 for complex IV deficiency, but can lead to many candidates genes. The other more structural approach comprises the establishment of complementation groups as has been performed for complex IV deficiency (Tiranti et al 1995) This process can be followed by the combination of techniques like microcell-mediated chromosome transfer, deletion mapping and positional cloning which led to the solution of cytochrome *c* oxidase deficient patients with Leigh syndrome in whom mutations were found in the SURF1 gene (Zhu et al 1998, Tiranti et al 1998)

Mutations might also be present in one of the ND genes or tRNA genes encoded by the

mtDNA in our "unsolved" complex I deficient patients. We checked for major rearrangements and eleven common point mutations, but this does not cover the complete area At this moment it might be wise to exclude or prove mutations in the mtDNA by complementation studies with ρ^0 cells as described by King and Attardi in 1989

In summary, we checked for mutations in the cDNA of four Iron-Sulphur protein fraction subunits, namely the NDUFS1, NDUFS3, NDUFS6 and NDUFA5 in our isolated complex I deficient patient group This revealed no disease-causing mutations Currently all known 35 nuclear encoded complex I subunits have been analysed for mutations in a group of twenty isolated complex I deficient patients. This revealed mutations in seven of the eighteen pedigrees (39%) Techniques like microcell-mediated chromosome transfer and positional/functional cloning should elucidate the genetic defect in the remaining patients

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CHAPTER 12

CONSIDERATIONS, CONCLUSIONS AND FUTURE PERSPECTIVES

Human complex I: structural and functional aspects.

Over 50 years ago, mitochondria were for the first time identified to host oxidative phosphorylation (Kennedy and Lehninger 1948) Adequate oxidative phosphorylation requires synthesis of a few rRNAs, tRNAs and proteins by the mitochondrion itself (for a review see Taanman 1999) as well as importation of many proteins encoded by the nuclear DNA into these mitochondria (Koehler et al 1999) This dual genetic origin demands a delicately tuned nuclear and mitochondrial collaboration, which partly has been elucidated (Butow and Bahassi 1999)

Complex I or NADH ubiquinone oxidoreductase is the first, largest and most complicated complex of the OXPHOS system (Walker 1992, Smeitink et al 1998) Currently, its subunit composition has been elaborately studied in several prokaryotes and eukaryotes *Escherichia coli* (Leif et al 1993, Weidner et al 1993, Leif et al 1995, Friedrich et al 1995, Friedrich 1998), *Rhodobacter capsulatus* (Dupuis et al 1995), *Thermus thermophilus* (Yano et al 1997), *Paracoccus denitrificans* (Xu et al 1993), *Helicobacter pylori* (Finel 1998), *Neurospora crassa* (Videira 1998), *Bos taurus* (Walker et al 1992, Walker 1992) and now also *Homo sapiens* (this thesis, and references therein)

In general, the number of subunits seems to increase when species ascend the evolutionary ladder Prokaryotes are considered to have a fourteen subunit complex I (Leif et al 1993, however in some bacteria like *E coli*, NuoC en D are fused to NuoCD which leaves it with thirteen subunits (Braun et al 1998)) Sodium Dodecyl Sulphate (SDS) gel electrophoresis revealed that there are at least 35 subunits present in *N crassa* complex I (Weiss et al 1991), however at this moment they have not been all genetically characterised It is striking that several subunits present in complex I of *N crassa* do not seem to have a homologue in *B taurus* and *H sapiens* and vice versa It could be possible that the genes coding for these subunits diverged very fast, with the result that the interspecies homology is lost The core of fourteen subunits present in prokaryotes is however highly conserved in *N crassa* again illustrating the functional importance of this part The number of subunits of *B taurus* was believed to be 41 in 1992 (Walker 1992) Electrospray mass spectrometry experiments however, revealed that there are at least 43 subunits, which is likely to be the final number (Skehel et al 1998) The existence of at least one of these additional proteins in complex I has been confirmed with two-dimensional gel electrophoresis and has subsequently been characterised on cDNA and amino acid level (Skehel et al 1998) The amino acid sequence of the 43rd subunit has yet to be identified Similar experiments with human tissue specimens are not yet performed, so the final number of human complex I subunits remains to be elucidated

At this moment, all 42 amino acid sequences of complex I subunits that have been found in cow are also characterised in humans (Table 12 1), of which eighteen have been characterised by our research group Gene/protein names were assigned according to the database for human gene nomenclature (http //www gene ucl ac uk/nomenclature (White et al 1997)) At present, the chromosomal localisation of 30 nuclear encoded complex I subunits is known, which shows a random distribution across the chromosomes (Table 12 1, Spencer et al 1992, Duncan et al 1992, Ali et al 1993, Baens et al 1993, de Coo et al 1995, Hattori et al 1995, Hyslop et al 1996, Zhuchenko et al 1996, Russell et al 1997, de Coo et al 1997, Dunbar et al 1997, de Sury

et al 1998, Loeffen et al 1998a, Emahazion et al 1998, Triepels et al 1998, Procaccio et al 1998, Loeffen et al 1999)

Based on sequence similarity with homologues in lower species, functional aspects can be attributed to several human complex I subunits (Table 12 2) Yet, in the lion's share of the proteins, the function remains unknown for the time being Especially little is known about the additional subunits on top of the fourteen "minimal" subunits Several theories concerning these "plus" subunits have been postulated in literature Alignment of the structure of *E coli* and *N crassa* complex I revealed that most of the additional proteins are moulded around the conserved core (Friedrich 1998) It therefore seems that many of these additional proteins in eukaryotes function in stabilising the complex (Guenebaut et al 1997, Friedrich et al 1998) and/or shielding the redoxgroups from oxygen, which prevents these groups from premature reoxidation by oxygen

Known exceptions to this are the NDUFAB1 and the NDUFA9 proteins, which both are believed to have a biosynthetic function The first shows resemblance to a family of acylcarrier proteins (ACP) It contains a phosphopantetheine binding site which (when bound) can act as docking site for fatty acids and amino acids ACPs are therefore thought to participate in fatty acid and polypeptide biosynthesis (Cortes et al 1990, Runswick et al 1991) Disruption of the ACP gene in *N crassa* prevents the formation of the extramembranous arm (Schneider et al 1995) The NDUFA9 shows homology to a group of isomerases/reductases and is thought to have a nucleotide-binding site Furthermore it is proposed to participate in the synthesis of a recently discovered new redoxgroup (Schulte et al 1999)

Studies performed with specimens of LHON patients revealed interesting indications for possible functions of the ND subunits Lymphoblasts of LHON patients carrying the homoplasmic mtDNA 3460 (ND 1) mutation showed reduced electron transfer capacity in the presence of a ubiquinone analogue, but not in presence of ferricyanide as electron acceptor (Majander et al 1991) LHON patients with a defect in ND-4 (11778 mutation) did not have a defective electron transfer, yet did have reduced ATP synthesis when complex I substrates were used, but not after usage of complex II substrates (Majander et al 1991, Hofhaus & Attardi 1993) ND-2, ND-4 and ND-5 do have some degree of homology, which led to the speculation that these subunits participate in proton transport in some way If this assumption is correct, mutations in the genes coding for these latter subunits, can result in a decreased contribution of complex I to the proton-motive force (PMF), but the electron transport will be undisturbed Degli Esposti demonstrated that the 11778 mutation in ND 4 alters the affinity of complex I for ubiquinone and reduces the complex I inhibition capacity of rotenone (Degli Esposti et al 1994) Photoaffinity labelling studies with [^{3}H]dihydrorotenone indicated that ND 1 is probably also involved in the binding of rotenone (Earley et al 1987), while Friedrich postulated that the ND-1 subunit is involved in ubiquinone binding (Friedrich et al 1990) Based on the results of these studies one may speculate that the possibility exists that not all patients with a malfunctioning complex I will be detected with the used complex I enzyme assay Whenever complex I is capable of transporting the electrons from NADH to a ubiquinone analogue, the results of such assays will be normal, while proton transport may be seriously affected

Homo sapiens			*NCBI no. human subunit*	*Escherichia coli*		*Neurospora crassa*		*Bos taurus*	
Name	**kDa**	**Chr.**		**Name**	**kDa**	**Name**	**kDa**	**Name**	**kDa**
NDUFV1	51.0	11q13	NUBM_HUMAN	NuoF	49.3	51 kDa	54.3	51 kDa (FP)	50.7
NDUFV2	27.4	18p11.31-p11.2	NUHM_HUMAN	NuoE	18.6	24 kDa	28.8	24 kDa (FP)	27.3
NDUFV3	12.0	21q22.3	NUOM_HUMAN	-		-		10 kDa (FP)	11.9
NDUFS1	79.6	2q33-q34	NUAM_HUMAN	NuoG	100.3	78 kDa	81.7	75 kDa (IP)	79.4
NDUFS2	52.5	1q23	NUCM_HUMAN	NuoD	68.7[1]	49 kDa	54.0	49 kDa (IP)	49.2
NDUFS3	30.2	11p11.11	NUGM_HUMAN	NuoC	68.7[1]	31 kDa	32.3	30 kDa (IP)	30.3
NDUFS4	20.1	5q11.1	NUYM_HUMAN	-		21.3 kDa	24.5	AQDQ (IP)	19.8
NDUFS5	12.5	1p34.2-p33	NP_004543	-		-		15 kDa (IP)	12.7
NDUFS6	13.7	5pter-p15.33	NUMM_HUMAN	-		-		13 kDa (IP)	13.4
NDUFS7	18.0	19p13	NUKM_HUMAN	NuoB	25.1	19.3 kDa	25.0	PSST	23.8
NDUFS8	23.7	11q13.1-q13.3	NUIM_HUMAN	NuoI	20.5	23 kDa	24.9	TYKY (IP)	23.9
NDUFA1	8.1	Xq24	NP_004532	-		-		MWFE	8.1
NDUFA2	10.9	5q31.2	NP_002479	-		10.5 kDa	10.5	B8	11.1
NDUFA3	9.3	?	NP_004533	-				B9	9.3
NDUFA4	9.4	?	NP_002480	-		-		MLRQ	9.3
NDUFA5	13.5	7q32	NUFM_HUMAN	-		29.9 kDa	30.9	B13 (IP)	13.3
NDUFA6	15.1	22q13.2-q13.31	NP_002481	-		14.8 kDa	14.8	B14	15.1
NDUFA7	12.6	19p13.2	N4AM_HUMAN	-		-		B14.5a	12.7
NDUFA8	20.1	9q33.2-q34.11	AF044953_1	-		20.8 kDa	20.9	PGIV	20.1
NDUFA9	42.5	12p13.3	NUEM_HUMAN	-		40 kDa	43.0	39 kDa	42.8
NDUFA10	40.8	12p	NUDM_HUMAN	-		-		42 kDa	39.3
NDUFAB1	17.4	16p12.3-p12.1	NP_004994	-		9.6 kDa	14.5	SDAP	10.1
NDUFB1	7.0	14q31.3	NP_004536	-		-		MNLL	7.0
NDUFB2	12.1	7q34-q35	NP_004537	-		12 kDa	12.3	AGGG	12.3
NDUFB3	11.4	?	NP_002482	-		-		B12	11.1
NDUFB4	15.2	3[a]	NP_004538	-		-		B15	15.2
NDUFB5	21.8	?	NP_002483	-		-		SGDH	21.6
NDUFB6	15.5	9p13.2	NP_002484	-		-		B17	15.5
NDUFB7	16.4	19p13.12-13.11	AAF17188	-		-		B18	16.4
NDUFB8	21.8	10q23.2-q23.33	NP_004995	-		-		ASHI	21.7
NDUFB9	21.8	8q24-21	NP_004996	-		-		B22	21.8
NDUFB10	20.8	16p13.3	NP_004539	-		12.3 kDa	12.3	PDSW	21.0
NDUFC1	8.7	4q28.2-q28.3	NP_002485	-		-		KFYI	8.8
NDUFC2	14.2	11[b]	NP_004540	-		-		B14.5b	14.1
17.2HUM	17.1	?	AAF17196	-		-		B17.2	17.1
ND-1	35.7	mtDNA	ND1_12188	NuoH	36.2	ND-1	41.6	ND-1	35.7
ND-2	39.0	mtDNA	ND2_12188	NuoN	45.7	ND-2	65.8	ND-2	39.3
ND-3	13.2	mtDNA	ND3_12188	NuoA	16.5	ND-3	6.0	ND-3	13.1
ND-4	51.6	mtDNA	ND4_12188	NuoM	56.5	ND-4		ND-4	53.8
ND-4L	10.7	mtDNA	NULM_HUMAN	NuoK	10.8	ND-L4	9.8	ND-4L	10.8
ND5	67.0	mtDNA	ND5_12188	NuoL	66.4	ND-5	79.8	ND-5	68.3
ND-6	18.6	mtDNA	ND6_12188	NuoJ	19.9	ND-6		ND-6	19.2
-				-		17.8 kDa	20.1	-	
-				-		21 kDa	21.0	-	
-				-		12 kDa	12.3	-	
-				-		21.3 kDa	21.3	-	
-				-		9.5 kDa	9.3	-	
-				-		21.3 kDa	21.4	-	
-				-		18.3 kDa	21.8	-	

Table 12.1 Complex I subunit composition of *E. coli*, *N. crassa*, *B. taurus* and *H. sapiens.* [a] = STS G29346 [b] = STS G25213. Genbank accession numbers refer to human subunits. Nuo = NADH:ubiquinone oxidoreductase; Sequence similarities between species were found with use of the BLAST program provided by the National Centre for Biotechnology Information (Altschul et al 1990). [1] NuoC and D are fused in *Escherichia coli.*

Subunit	*Function/characteristic*	*Reference*
NDUFV1/51 kDa	NADH binding site FMN binding site 4Fe-4S cluster (N3)	Chen & Guillory 1981, Deng et al 1990 Patel et al 1991, Schuelke et al 1998 Ohnishi et al 1985, Ohnishi 1998
NDUFV2/24 kDa	2Fe-2S cluster (N1a) G-protein	Ohnishi et al 1985, Ohnishi 1998 Hegde 1998
NDUFS1/ 75 kDa	2Fe-2S cluster (N1b) 4Fe-4S cluster (N4) 4Fe-4S cluster (N5*)	Ohnishi 1998 Ohnishi 1998 Ohnishi 1998
NDUFS2/49 kDa	proton transport ubiquinone binding	Darrouzet et al 1998
NDUFS4/ 18 kDa	phosphorylation site for a cAMP dependent protein kinase	Papa et al 1996
NDUFS7/PSST	4Fe-4S cluster (N2)	Ohnishi 1998
NDUFS8/TYKY	2× 4Fe-4S cluster	Walker 1992
NDUFA9/39 kDa	Family of NAD(P)H reductases/isomerases	Schulte et al 1999
NDUFAB1/SDAP	acyl carrier protein	Runswick et al 1989
ND-1	binding site rotenone binding site ubiquinone	Earley et al 1987 Friedrich et al 1990
ND-4	binding site rotenone binding site ubiquinone	Degli Esposti et al 1994

Table 12.2 Functional aspects of human complex I subunits. Part of this data is based on homology with complex I subunits of lower evolutionary species, from which functional aspects have been studied.
* The presence of cluster N5 in the NDUFS1 subunit is subject of debate.

A piericidin A and rotenone resistant mutant of *Rhodobacter capsulatus* contained a V407M amino acid substitution in NuoD. Reintroduction of this amino acid substitution into the parental strain resulted in a similar resistance to piericidin A and rotenone as the original mutant (Darrouzet et al 1998). The authors propose a model in which NuoD forms the binding site for the polar head of piericidin A/ubiquinone and NuoH docks the hydrophobic isoprenalyl tail. Okun and colleagues tested members of the three groups of complex I inhibitors (piericidin A (class I/A type), rotenone (class II/B type) and capsaicin (C type), as proposed by Degli Esposti and Ghelli in 1994) for competition. They found that the rotenone site overlaps with the piericidin A site and the capsaicin site, but the latter two do not seem to overlap each other (Okun et al 1999). They suggest a fairly large binding site for ubiquinone of complex I, with different points of action for inhibitors.

Genetic counselling and prenatal diagnostics.

In our experience, patients suffering from isolated complex I deficiency have a very poor prognosis (Loeffen et al 2000a) and currently no widely applicable treatment besides supportive, exists. Eventually, in most patients early death will be inevitable. This leaves us at

the moment with little more to offer but the opportunity of genetic counselling. Several factors make this counselling extremely difficult. Firstly, the genetic cause of the disease can be situated in the mtDNA as well as the nDNA. The majority of mtDNA point mutations are heteroplasmic, which results in different amounts of mutated mtDNA between individual patients as well as between different tissues within the same patient. In addition, the phenotype often has a threshold dependency on the content of mutated mtDNA. Chinnery showed that the mutant load of the A3243G mutation present in muscle tissue corresponds with the severity of the symptoms, however the mutant load in blood did not (Chinnery et al 1997). The amount of mtDNA transmitted to the next generation can also be variable. According to the bottle neck theory a small number of mtDNA molecules are preferentially amplified to repopulate the oocyte (Hauswirth and Laipis 1982). These factors implicate that the percentage of mutated mtDNA can vary considerably within families and prediction of chance on disease presentation is usually not possible. Some exceptions to this seem to exist. Recently, White and colleagues reviewed 56 T8993G/C pedigrees in which they examined the relation between the severity of the clinical phenotype and mutant load and predicted clinical outcome of individual patients based on their percentage mutated mtDNA (White et al 1999). They found a strong positive relation between the mother's blood mutant load and amount of heteroplasmy of the 8993 mutation in her offspring. However, there is a wide 95% range of predicted probabilities, which indicates variation in mutant load in children of mother's with the T8993G/C mutation. Leber's hereditary optic neuropathy (LHON) is usually associated with a homoplasmic mutation within the mitochondrially encoded ND genes, which increases the accuracy of molecular-genetic prenatal diagnosis performed in chorionic villi. However, not all patients carrying an LHON mutation actually become ill. This implicates that additional (possibly nuclear) factors must contribute to the phenotype (Shoubridge 1998). Harding and colleagues estimated the recurrence risk of LHON to be 6-10% for female offspring and 30-46% for male progeny (Harding et al 1995). Many large deletions and duplications within the mtDNA are sporadic and are therefore not encountered in the mtDNA of relatives.

The majority of isolated complex I deficiencies seen in our centre seems to follow an autosomal recessive mode of inheritance (recurrence risk 25%). From the end of the eighties until now our centre has gained experience with the measurement of respiratory chain enzyme activities in chorionic villi (CV) and amniocytes. These measurements were for years the only opportunity for prenatal diagnostics in this patient group. Chorionic villi biopsies are usually performed between the 10-12th week of pregnancy. Enzyme assay procedures used in CV (slightly modified) are for NADH:ubiquinone oxidoreductase according to Fischer et al 1985 and 1986, cytochrome *c* oxidase according to Cooperstein and Lazarow 1951, citrate synthase as described by Srere in 1969 and PDHc is measured as described by Sperl et al in 1990. Studies performed in our centre in the early nineties, compared enzymatic findings in CV biopsies of 20 pregnancies (18 families). Three respiratory chain deficiencies were found (3× cytochrome *c* oxidase with Leigh syndrome). Respiratory chain enzyme assays performed in fibroblasts of these three infants confirmed the complex IV deficiency which was found in CV. None of the other pregnancies resulted in infants with an OXPHOS disorder (Ruitenbeek

et al 1992) The last few years however, we encountered some conflicting results when complex I activities of native CV are compared to values obtained from cultured CV of the same pregnancy In some cases enzyme measurements in cultured CV reveal a deficient complex I activity, while in native chorionic villi complex I activity is normal We have currently no explanation for this phenomenon In summary, it is possible to measure respiratory chain enzyme activities in CV However, for complex I activity, results are at this moment not always conclusive and demand additional investigation

DNA and RNA are easily extracted from cultured chorionic villi and therefore in abundance available It is of course necessary to check for maternal contamination The latter can be avoided by careful dissection of the maternal decidua from the chorionic and by the simultaneous amplification of a suitable polymorphism The interpretation of results obtained from molecular-genetic prenatal diagnostics is considerably easier than those obtained with biochemical enzyme measurements Consequently, the search for molecular-genetic causes of complex I deficiency is of utmost importance

Evidence pathogenicity mutations.

The complete characterisation of the human complex I subunits on cDNA level made mutational analysis studies possible. As described previously, our enzyme assay for complex I in fibroblasts is based on measurement of the conversion of NADH to NAD^+ in mitochondrial enriched fractions in presence of a ubiquinone analogue and in presence/absence of the selective complex I inhibitor rotenone Since the peripheral arm of complex I contains all known redox centres (Friedrich et al 1998, Skehel et al 1998), the FP and IP fraction remain the most important candidates for mutational analysis studies in our patient group. We focussed these studies on subunits present in the Iron-Sulphur protein fraction, which revealed different mutations in five pedigrees, which cosegregate within the family (chapter 8, 9 and 10) Except for the 5-bp duplication found in the NDUFS4 subunit, these mutations concerned subunits present in the minimal form of complex I as encountered in prokaryotes The point mutations resulted in the substitution of conserved amino acids while the 5-bp duplication resulted in a frameshift with destruction of the C-terminal part of the protein All mutations were excluded in a large control population In our patient group, mutations were also found in the NDUFV1 subunit present in the FP fraction in two pedigrees (Schuelke et al 1999), and in the NDUFS7 subunit of the HP fraction in one pedigree (Triepels et al 1999) At this moment all 42 complex I subunits have been checked for mutations on cDNA level in our patients (this thesis, Triepels et al 2000) So ultimately, we found mutations in 8 of 25 pedigrees described in chapter 2 (32%) The finding of these mutations offer the parents of these patients adequate prenatal counselling during subsequent pregnancies Currently, the cDNA of the subunits in which mutations have been encountered is screened for mutations by means of RT-PCR and direct DNA sequencing in new complex I deficient patients presented to our centre, in order to investigate the frequency of these and possibly other mutations within these subunits Recently, in two new patients frameshift mutations have been found in the NDUFS4 subunit (Budde et al 2000)

Although we believe that the observed mutations are certainly pathogenic, definite proof has

to come from complex I deficiency rescue experiments performed with fibroblasts of our index patients Rescue experiments were performed with fibroblasts of the patient described in chapter 9 We introduced a plasmid vector containing the *NDUFS4* cDNA construct into a portion of patient fibroblasts using a multi-component lipid-based transfection reagent However, the used transient transfection method failed to result in sufficient cells containing the plasmid for complex I measurement Important problems are therefore the large amount of transfected fibroblasts needed for our complex I and cytochrome c oxidase assay (about 10 million) and the low yield of fibroblasts containing the construct after transfection Currently we are immortalising our patient cell lines, which will be used in a permanent transfection procedure The group of Yagi restored complex I activity in two Chinese hamster cell lines, which both contained mutations in the NDUFA1 subunit (a deletion and a point mutation) by introducing the *NDUFA1* cDNA (Seo et al 1999) This procedure restored complex I activity to 100% in the cell line with the deletion, while the cell line with the point mutation only regained 40% It was suggested that the defective subunit still occupied its position in complex I and thereby inhibited complete restoration of the complex I activity This problem has to be taken into consideration when we perform our transfection experiments in the future Another method to rescue the complex I deficiency is introducing the wildtype chromosome on which the mutated gene is situated (or a more accurate reduced part of this chromosome) into our patient fibroblasts by microcell-mediated chromosome transfer (Cuthbert et al 1995, Zhu et al 1998) Since many complex I deficient fibroblast cell lines do not grow in media containing galactose instead of glucose (Robinson 1996), one might observe whether or not growth recovers in galactose containing media after introduction of the correct chromosome This may be considered as an indirect indication for rescue Other studies like e g normalisation of complex I subunit distribution visualised with Western blotting could also function as indication for rescue

An important step in order to obtain additional information concerning possible consequences of the mutations found in our patients will be investigating the steady state levels of the corresponding mutated transcripts by northern blot or RT-PCR Besides RNA studies, it is also possible to analyse consequences of mutations by use of specific antibodies against the mutated subunits Both of these approaches are currently investigated

The quest for new candidate genes.

Mutational analysis studies of all known 35 nuclear encoded complex I subunits revealed mutations in about 40% of our patients, which implicates that in ~60% of our patients the genetic cause remains unsolved One can consider the possible involvement of mtDNA mutations in this residual group As a screening of "unsolved" and new complex I deficient patients (but also combined respiratory chain deficiencies) we can investigate a mtDNA genetic cause by fusion of patient cell lines with ρ^0 cells (cells depleted of mtDNA by long term exposure to ethidium bromide (King and Attardi 1989)) Procaccio used ρ^0 cells, which needed uridine and pyruvate in the medium to grow and in addition, they were insensitive to bromodeoxyuridine (brdU) Cells of patients and controls do grow in uridine and pyruvate free medium but are sensitive to brdU After fusion of the ρ^0 cells with the patient and control

fibroblasts, they were able to select the fused cells by adding brdU to the medium and leaving uridine and pyruvate out (Procaccio et al 1999) This fusion of patient fibroblasts with ρ^0 cells should lead to rescue of the complex I deficiency in case of a nuclear cause, while nothing will happen in case the mutation is situated in the mtDNA Vice versa introduction of patient mitochondria by fusion of enucleated patient fibroblasts with ρ^0 cells results in complex I deficiency in case of mutations in the mtDNA, while activity remains normal in case of a nuclear cause (this latter strategy was used by Raha and colleagues with fibroblasts of a patient with hypertrophic cardiomyopathy and a mutation in the mitochondrially encoded $tRNA^{Gly}$ gene (Raha et al 1999)) After fusion of ρ^0 cells with the patient cell lines, several aspects like growth characteristics of cells, antibodies against mitochondrially encoded subunits and OXPHOS system enzyme measurements can be used to check whether or not complementation has occurred The use of ρ^0 cells has also been successfully applied in demonstrating the nuclear-genetic cause in a cytochrome *c* oxidase deficient patient (Tiranti et al 1995) Since the ND genes strongly contribute to the total amount of mtDNA mutations found in complex I deficient patients, it is also possible to search for mutations in these genes by means of PCR combined with direct DNA sequencing or Single Strand Conformation Polymorphisms (SSCP) Sequencing of all ND genes in eight complex I deficient patients carried out in our centre by de Vries and colleagues in 1994 however, did not reveal any mutations (de Vries, thesis)

One of the most direct methods to find nuclear encoded candidate genes is to search for human homologues of genes, which have been linked to the function of complex I in lower species These genes can code for a diversity of proteins involved in the functioning of complex I like transcription and translation factors of e g complex I subunits, proteins which participate in the targeting and importation of complex I subunits into mitochondria, proteins which assist in the assembly and incorporation of complex I into the inner mitochondrial membrane, proteins which have a function in the synthesis of factors associated to complex I like i e the flavin mononucleotide (FMN) and the iron sulphur clusters, but also nuclear genes controlling the perpetuation, propagation and expression of the mitochondrial genome The approach of candidate gene search in lower species has been successfully applied for complex I (Loeffen et al 1998b, van den Heuvel et al 1998, Schuelke et al 1999, Triepels et al 1999, Loeffen et al 2000b) and complex IV (Papadopoulou et al 1999) One of the major problems is choosing the right gene for mutational analysis in the large pool of candidate genes available by this approach For complex I, important future candidates are the human homologues of complex I intermediate associated proteins (CIA) 30 and 84, which have been associated with the large assembly intermediate of the membrane arm of complex I in *N crassa* (Kuffner et al 1998) Deletion of one of these genes in *N crassa* resulted in disruption of the assembly process (Kuffner et al 1998) Other important candidate genes involved in the biogenesis of complexes of the OXPHOS system obtained via homology searches with yeast proteins are BCS1, PET112, SCO1, COX15 and COX11 (Petruzzella et al 1998)

A very elegant step in elucidating the genetic cause in our remaining complex I deficient patients would be the investigation of existence of individual complementation groups as has been performed for cytochrome *c* oxidase deficiency by Munaro et al in 1997 First, the

existence of a nuclear cause should be proved in the patient group by use of ρ^0 cells as described before Subsequently two patient fibroblast cell lines can be fused with polyethylene glycol (PEG) Selection of hybrids can be obtained by making each of the two cell lines resistant to a different antibiotic before fusion by introducing a recombinant vector expressing a resistance-inducing gene (Double-antibiotic selection, Munaro et al 1997) These cells can then be cultivated in medium containing both antibiotics until an adequate amount of cells is available for measurement of the enzymes of the OXPHOS system If both patients are affected by mutations in the same gene, rescue of the complex I deficiency will not occur, while rescue will take place when both patients are affected by different genes

Several other methods are available to categorise our patients in different groups By using antibodies directed against different complex I subunits, we can analyse protein expression patterns in mitochondrial preparations by Western blotting Procaccio defined two different protein patterns in two complex I deficient patients by this approach, one expressing lowered amounts of the NDUFV1 and NDUFV2 subunits while the other tested subunits were expressed in normal amounts, and a patient who expressed reduced protein contents of all tested subunits (Procaccio et al 1999) Von Kleist-Retzow used this approach of immunoblot analysis with antibodies against nine complex IV subunits in 17 cytochrome *c* oxidase deficient patients This revealed mainly patterns with overall decreased protein levels, but in individual patients distinct patterns were also found (von Kleist-Retzow et al 1999) This approach can be expanded by using the technique of two-dimensional blue native gel electrophoresis (Nijtmans et al 1998) Coenen successfully showed with this technique that different two dimensional gel electrophoresis patterns exist for cytochrome *c* oxidase deficient patients with a mutation in the *SURF1* gene and those who do not (Coenen et al 1999) Specific patterns may well be present among complex I deficient patients

The finishing touch in elucidating the genetic cause in a complementation group of complex I deficient patients can be provided by the tremendous technique of microcell mediated chromosome transfer, successfully applied by Zhu and colleagues who found the link between the *SURF1* gene and cytochrome *c* oxidase deficient patients with Leigh syndrome (Zhu et al 1998) By fusing human rodent hybrid cell lines containing single human chromosomes (Cuthbert et al 1995), rescue of the enzyme deficiency will occur after introduction of the chromosome on which the gene defect in the patient is situated After establishment of the correct chromosome, one can reduce the size of the fragment on which the candidate gene is situated by analysing hybrids with deleted versions of this chromosome Zhu beautifully used the fact that introduced chromosomes delete and rearrange after fusion, by choosing informative markers in hybrids which compensate and clones which don't compensate to narrow the area of the gene of interest (deletion mapping, Zhu et al 1998) Finally positional/functional cloning might reduce the area containing the candidate gene to a "reasonable" size for direct DNA sequencing Preparations to introduce this technique in order to elucidate the genetic defects in our remaining complex I deficient patients are currently undertaken in collaboration with the researchgroup of Eric Shoubridge

Pathophysiological aspects of complex I deficiency.

A decreased production of ATP can be an important pathophysiological consequence of complex I deficiency Complex I contributes to the proton motif force (PMF) that is used by complex V to generate ATP When complex I malfunctions, this can lead to a decrease of the PMF and therefore to less available ATP MERRF and MELAS mutations lead to a generally decreased mitochondrial protein synthesis (Chomyn et al 1992, King et al 1992, Masucci et al 1995), often resulting in a combined complex I and IV deficiency In MERRF cells the aminoacylation capacity of $tRNA^{Lys}$ is decreased leading to an impairment of mitochondrial protein synthesis (Enriquez et al 1995) The specific pathophysiological mechanism occurring in cells harbouring the A3243G (MELAS) mutation remains unclear for the time being Theoretically, the decreased proton pumping by the respiratory chain can result in a decreased mitochondrial membrane potential ($\Delta\psi_m$) and PMF James et al used the lipophilic cation TPMP (which accumulation is related to the membrane potential) to estimate this mitochondrial membrane potential This revealed a clear decrease in membrane potential in MERFF as well as MELAS fibroblasts (James et al 1996) Moudy and colleagues used quantitative fluorescence imaging to measure the membrane potential of individual mitochondria of fibroblasts loaded with the dual emission dye JC-1 This revealed significantly reduced J aggregates in MELAS fibroblasts (JC-1 specifically labels mitochondria and expresses mitochondrial membrane potential as a function of the state of dye aggregation) indicative for reduced mitochondrial membrane potentials in these cells (Moudy et al 1995) Moudy also measured the ATP concentration in these cells, which was within the normal range James and colleagues hypothesised that once one increases the ATP turnover in quiescent fibroblasts, patient fibroblasts have less reserve capacity to replenish this ATP compared to control fibroblasts (James et al 1999) They measured oligomycin-sensitive ATP production by fibroblasts permeabilised with digitonine and found a decreased ability to synthesise ATP in cells derived from MERFF and MELAS patients However the amount of ATP in MERFF and MELAS fibroblasts (measured with the luciferase luciferin technique) was similar to that of controls, which also counts for the ATP/ADP ratio To increase the ATP turnover, gramicidin (Na^+ and K^+ ionophore) was added which indirectly increases the activity of the plasmamembrane Na^+/K^+ ATPase resulting in an increased ATP turnover They found that cells carrying the MELAS and MERRF mutations have problems to maintain their ATP/ADP ratio at gramicidin concentrations when control cells have no problems at all (James et al 1999)

Quiescent fibroblasts strongly depend on glycolysis instead of oxidative phosphorylation for their ATP production (Robinson 1993, Robinson 1996) James et al found that the culture medium of MELAS cells acidified faster compared to controls Measurement of lactate dehydrogenase (referred to citrate synthase activity) revealed increased activity pointing towards anaerobic respiration (James et al 1996) Fibroblasts cultured in glucose medium first convert glucose into pyruvate and lactate and when glucose is running out, the conversion of pyruvate into acetyl-CoA is accelerated which increases the contribution of the OXPHOS system to the production of ATP (Robinson 1996) This is further illustrated by the

experiment where in fibroblast culture medium glucose is exchanged for galactose in equimolar amounts The conversion of galactose to glucose-1-phosphate is limited so cells will increasingly depend on the OXPHOS system instead of glycolysis Complex I deficient fibroblasts will therefore grow poorly on galactose containing culture medium (Robinson 1996) This strong dependence on glycolysis could explain why resting ATP levels were found normal in MELAS and MERRF fibroblasts, since this part of the energy-providing system is not affected

Adequate ATP steady state and production measurements ideally should be performed in tissues, which often are affected in complex I deficient patients like brain, heart and skeletal muscle tissue A favourable improvement would be the applicability of myoblast cultures (Shoubridge et al 1996) in our OXPHOS enzyme assays, but also other pathophysiological studies One of the problems we encounter in culturing myoblasts is their rapid terminal differentiation into multinucleated myotubes This limits the number of culture passages and therefore the use of myoblasts in many pathophysiological experiments, including our OXPHOS enzyme assays Another major problem in culturing myoblasts is the contamination of these cultures with fibroblasts Myoblast cultures were successfully applied in the measurement of ATP and ADP of patients with the 3243A→G (MELAS) mutation, which both were found to be decreased (Rusanen et al 2000)

Last year, Barrientos and Moraes published studies in which they examined pathophysiological aspects of complex I deficiency using a model of human ape xenomitochondrial hybrids (HXC cells, 40% complex I deficiency) as well as a drug-induced-model (rotenone, 40% complex I deficiency) (Barrientos and Moraes 1999) These models showed similar results as described previously Cells used in both models grew poorly on galactose-containing medium compared to glucose-containing medium They also measured $\Delta\psi_m$ of individual mitochondria using the JC-1 probe, which revealed a mild decrease of $\Delta\psi_m$ in rotenone treated cells as well as in HXC cells

Another important damage inflicting mechanism possibly involved in complex I deficiency is abundant generation of reactive oxygen species (ROS) with as most prominent members superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH) This process probably results from interaction of oxygen with semiquinone molecules (Robinson 1998) and is therefore indirectly the result from the one electron reduction events taking place in complex I The first reactive oxygen compound likely to be generated is O_2^- by interaction of oxygen with semiquinone (in physiological as well as pathophysiological conditions) Superoxide dismutase (SOD) scavenges O_2^- by converting them two at a time to H_2O_2 and O_2, which is normally further processed by catalase in peroxisomes and glutathion peroxidase in mitochondria and cytosol When H_2O_2 has a close encounter with free iron or copper, it can be converted into the highly reactive OH This latter radical is believed to generate most of the damage attributed to biological macromolecules, like lipid peroxidation, protein aggregation and DNA hydroxylation One can speculate that malfunctioning of electron transport chain complexes leads to excessive escape of electrons from these complexes and therefore to a surplus of ROS Much data about involvement of excessive ROS formation in complex I deficiency has been generated by the research group of Robinson Pitkanen et al

showed with Southern blotting that mtDNA isolated from fibroblasts of complex I deficient patients with cataract and cardiomyopathy contains many multiple deletions in contrast to control fibroblasts (Pitkanen et al 1996), indicating free oxygen radical damage Furthermore, they showed with western blot analysis as well as with an enzymatic assay that the mitochondrial Mn-SOD levels were elevated in these patients Later this group used the luminometric probe lucigenin in order to measure the O_2 production on addition of NADH in isolated fibroblast mitochondrial membranes of complex I deficient patients Their results revealed a spectrum with on one side elevated O_2 levels combined with reduced Mn-SOD values (phenotype of cataract and developmental delay) while on the other side patients expressed reduced O_2 levels with increased values of Mn-SOD (phenotype of cataract and cardiomyopathy, Pitkanen and Robinson 1996) Measurement of O_2 production in control fibroblasts treated with rotenone revealed increased production rates The authors hypothesise that complex I deficiency increases the formation of ROS, though variable induction of Mn-SOD influences this rate Luo and colleagues demonstrated increased production of OH in complex I deficient patients expressing cataract and cardiomyopathy by using the aromatic hydroxylation assay with salicylate as a probe (Luo et al 1997) Furthermore Luo showed overproduction of aldehydes (indicative of lipid peroxidation) in fibroblasts of these patients Barrientos and Moraes used H_2DCF (converted to a fluorescent product after oxidation by H_2O_2) and found increased ROS formation in cells from the HXC as well as the rotenone model (Barrientos and Moraes 1999) Future studies must be performed to entangle the influence of excessive ROS formation in the pathophysiology of complex I deficiency

An important future topic in the study of the pathogenesis of complex I deficiency will be the role of calcium In normal cells, the cytoplasmic calcium concentration ($[Ca^{2+}]_c$) is kept strictly at 10^{-7} M Several factors influence cellular calcium homeostasis like Ca^{2+} pumping (e g $insP_3$ receptor in the ER membrane) and leaking mechanisms (Hofer et al 1998) Another organelle capable of calcium sequestration is the mitochondrion Despite the low affinity of mitochondria for calcium, increases of $[Ca^{2+}]_c$ are transmitted into mitochondria because of close contact between mitochondria and endoplasmic reticulum (ER) (Rizzuto et al 1998) This indicates the importance of the intracellular distribution of the mitochondrion, which can be disturbed in OXPHOS disorders The uptake of calcium by mitochondria is strongly dependent on the $\Delta\psi_m$, which can be reduced in complex I deficiency Moudy monitored the cytosolic calcium handling ($[Ca^{2+}]_c$) with confocal microscopy within single fibroblasts loaded with the fluorescent probe FURA-2 of patients with MELAS syndrome (Moudy et al 1995) They found that MELAS cells have an elevated resting $[Ca^{2+}]_c$ and in addition express a disturbed sequestration of $[Ca^{2+}]_c$ after inositol triphosphate (IP_3) mediated stimulation Padua and colleagues performed similar experiments with fibroblasts of a patient with a pyruvate dehydrogenase complex (PDHc) deficiency (Padua et al 1998) They found equal resting $[Ca^{2+}]_c$ between controls and patients, however bradykinine stimulated increase of $[Ca^{2+}]_c$ resulted in reduced $[Ca^{2+}]_m$ transients in PDHc deficient fibroblasts (~4% of controls) indicating that these mitochondria have decreased ability to sequester cytosolic calcium

We monitored the $[Ca^{2+}]_c$ handling of cultured skin fibroblasts suspensions of 5 complex I deficient patients with mutations in nuclear encoded complex I subunits, 3 SURF1 patients

and 5 controls, with the ratiometric Ca^{2+} probe FURA-2 Bradykinine stimulation of $[Ca^{2+}]_c$ in calcium-free medium and calcium containing medium revealed bound FURA-2/unbound FURA-2 (340 nm/380 nm) ratio peaks (averaged for each subgroup) which were highest in controls, followed by complex I deficient fibroblasts and were lowest in SURF1 cell suspensions (100%, 78%, 67%, respectively, unpublished results) The ascending and descending slope of the spike as well as the wide were comparable in all three groups Agonist stimulation of cells in calcium containing medium results in a higher 340/380 ratio after the peak, probably due to capacitative calcium entry (Berridge 1995) Strikingly, this effect was highest in controls, followed by complex I deficient cells and lowest in SURF1 fibroblasts (unpublished results) In order to study the calcium maintenance more specifically within these cells we used the inhibitor of the endoplasmic reticulum calcium ATPase (SERCA family) thapsigargin Subsequently, passive leakage of calcium out of the ER results in a temporary increase of $[Ca^{2+}]_c$ The 340/380 ratio showed a steeper slope in the ascending as well as descending part of the curve in controls compared to complex I and SURF1 fibroblasts This could be explained by decreased uptake capabilities for calcium of patient mitochondria To study this mechanism of different calcium kinetics after thapsigargin suppletion in controls and complex I and SURF1 deficient fibroblasts we want to target the ratiometric calcium probe MAG-FURA-2 (low affinity for calcium) into the ER after which the emptying of the ER can be studied (Hofer 1999) Landolfi showed in intact cells using this technique that uncoupling of mitochondria with CCCP immediately reduces the ER emptying (Landolfi et al 1998) The exact mechanism of this effect is unknown, however the decreased slope of the ascending part of the curve obtained after addition of thapsigargin to the medium in our complex I and SURF1 deficient fibroblasts could be caused by a similar phenomenon Hofer showed that leakage of calcium from the ER after blockage of the SERCA pump is specifically regulated by the ATP concentration (Hofer et al 1996) The leakage rate diminishes when the ATP concentration is lowered This could explain why the leak of calcium from the ER after similar SERCA blocking is reduced in our patient fibroblasts

Recently, Jouaville and colleagues described a powerful technique by which they can measure cytosolic and mitochondrial ATP levels with the specifically targeted ATP dependent protein luciferase (Jouaville et al 1999) Rizutto already showed that agonist stimulated calcium increase in the cytosol are followed by large calcium increases in the mitochondrial matrix (Rizzuto et al 1998) With the combined usage of aequorin (fluorescent protein which can visualise calcium fluctuations) and luciferase (reflects ATP content) which both can be targeted into mitochondria, Jouaville showed that following agonist stimulation, $[Ca^{2+}]_c$ increases, which is directly followed by an increase of mitochondrial calcium concentration ($[Ca^{2+}]_m$) In addition, this increase of $[Ca^{2+}]_m$ is followed by an increase of ATP production, which sustains after the calcium peak disappeared (Jouaville et al 1999) This phenomenon can partly be explained by the fact that several mitochondrial enzymes like PDHc, isocitrate dehydrogenase and α ketoglutarate dehydrogenase are regulated by calcium The results of these experiments (calcium peaks in mitochondria and subsequent ATP increases in mitochondria and cytosol) were more pronounced in myotubes compared to HeLa cells (in resting culture conditions) Brini and colleagues subsequently showed with this technique that

IP_3 stimulated $[Ca^{2+}]_c$ increases were equal between controls and 100% mutated MERRF fibroblasts, but the consequent rise in $[Ca^{2+}]_m$ is severely diminished in the MERRF fibroblasts (Brini et al 1999) They also saw, that ATP increase after IP_3 stimulation is diminished in MERRF fibroblasts

Several studies performed in the last few years have shown that excessive (slow) uptake of calcium by mitochondria leads to opening of the so-called mitochondrial permeability transition pore (MTP), probably leading to calcium induced cell death (Crompton 1999) Normally, the $[Ca^{2+}]_m$ is kept below 10 μM When calcium exceeds a limit of 200 μM in the presence of ADP, the pore opens Three components have been identified to participate in the MT pore formation, namely the Adenine Nucleotide Translocase (ANT), Cyclophilin-D (CyP-D) and the Voltage Dependent Anion Channel (VDAC) Several processes influence the opening and closing of the MT pore like the mitochondrial membrane potential, the redox state, matrix pH, reactive oxygen species (ROS) and adenine nucleotides (Fontaine et al 1998) *In vitro*, the opening of the MT pore leads to collaps of PMF, disruption of ionic homeostasis, mitochondrial swelling and ATP hydrolysis by complex V (Fontaine et al 1998) Eventually nuclear apoptosis is initiated Fontaine and co-workers summarised several mechanisms by which MTP opening can lead to cell damaging pathways like decreased levels of cellular ATP, increase of $[Ca^{2+}]_c$, release of apoptosis inducing factor (AIF) and release of cytochrome *c* Interestingly, they saw that the sensitivity of the MT pore for calcium as monitored by a decrease of the mitochondrial membrane potential after calcium loading was much higher when complex I substrates were used, compared to complex II substrates This increased sensitivity for calcium seemed to depend mainly on the rate of electron flux through complex I Barrientos and Moraes showed that rotenone induced cell death is strongly reduced by cyclosporine A and the phospholipase A_2 inhibitor ArA (both act synergistically in inhibiting MT pore opening) This indicates that some pathophysiological consequences of complex I deficiency eventually lead to MT pore opening and cell death (Barrientos and Moraes 1999)

Therapy for complex I deficiency: utopia or...?

Isolated complex I deficiency in children often leads to a devastating clinical course, usually resulting in death of the child Several therapeutic procedures have been tested in the past, usually with little effect Finding a cure for these patients should however be one of the main goals in future research Scholte and co-workers listed successful drugs which have been supplied to (mainly individual) patients with complex I deficiency Examples are riboflavin, succinate, nicotinamide, and menadione + succinate therapy (Scholte et al 1995) In general, these forms of therapy have only been described to work in individual cases Additionally, little is known about the natural cause of the diverse clinical phenotypes associated with complex I deficiency, so benefits are difficult to assign

A completely different approach has been developed by the research group of Shoubridge Many tRNA point mutations are distributed between tissues in a heteroplasmic way In muscle

tissue, high percentages of mutated mtDNA are often found in mature muscle fibres, while undifferentiated satellite cells contain undetectable amounts (Shoubridge et al 1997) MtDNA analysis of cultured muscle cells obtained from two biopsies taken from the biceps muscle of a patient (cytochrome c oxidase deficient) with a point mutation in the mitochondrially encoded $tRNA^{Leu}$ gene, showed that mature muscle fibres contained 95% mutated mtDNA, while this was nearly undetectable in simultaneously cultured satellite cells Fu and co-workers proposed that elimination of the mutated mtDNA could be achieved by stimulation of the regeneration of muscle tissue (Fu et al 1996) This patient was rebiopsied at the site of the original biopsy and regenerating muscle fibres were stained for cytochrome *c* oxidase activity which was almost invariably positive (Shoubridge et al 1997) Single fibre PCR analysis revealed that all regenerating muscle fibres had no detectable mutated mtDNA Clark and colleagues had similar results by inducing muscle fibre regeneration chemically in a patient with a point mutation in the $tRNA^{Leu}$ gene (Clark et al 1997) Taivassalo and co-workers expanded this approach by inducing a form of gene shifting therapy using concentric muscle training to promote muscle fibre regeneration (Taivassalo et al 1999) This approach led to a dramatic increase in the wildtype/mutated mtDNA ratio in regenerating muscle fibres

Taylor and colleagues also try to develop a therapy based on the heteroplasmy aspect of mtDNA Their goal is to develop a method to selectively inhibit the replication of mtDNA containing a mutation, which subsequently results in propagation of wildtype mtDNA To prove the possibility of this approach they synthesised peptide nucleic acids (PNAs) complementary to human mutated mtDNA templates (Taylor et al 1997) They showed that these antigenomic PNAs inhibited replication of mutated mtDNA but not of wildtype DNA, and that these PNAs indeed are taken up by myoblasts

Since these latter two approaches have their fundament in the principle of mtDNA heteroplasmy, it is not applicable in patients with mutations in nuclear DNA

A revolutionary approach has been performed by Seo and co-workers who successfully introduced the internal NADH quinone oxidoreductase of *Saccharomyces serevisiae* (rotenone-insensitive) into a complex I deficient hamster cell line In addition, they showed that the transfected cell lines had normal complex I activity, while the non-transfected remained deficient (Seo et al 1998) In later studies they proved that this enzyme is transcribed and translated in human cells, despite the presence of human complex I (Seo et al 1999) This tremendous approach might eventually lead to a successful therapy

Finally, I would to like to focus on one of the ultimate ways to study pathophysiological and therapeutical aspects of complex I deficiency, namely an animal model A mouse in which one of the nuclear genes coding for a complex I subunit has been knocked out or modified, will lead to enormous progress in our understanding of the consequences of complex I deficiency Of course this model can also figure as an important test model to investigate benefits and disadvantages of several therapeutic models In addition, such a model provides tissues, which are often affected in patients, yet difficult to obtain like heart, brain en liver tissue

Much is said by quoting one of the major energy providers in the world
complex I is on the move

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Samenvatting.

Introductie.

In de humane cel vinden zeer veel biochemische reacties plaats. Veel van deze reacties verlopen spontaan, omdat de toestand na de reactie energetisch gunstiger is dan voor de reactie. In dit laatste geval is de vrije energie (ΔG) van de reactie negatief. Veel biochemische reacties verlopen echter niet spontaan omdat ze energetisch ongunstig zijn (ΔG is positief). Een biochemisch energetisch ongunstig verlopende reactie kan toch verlopen wanneer deze gekoppeld wordt aan een energetisch gunstig verlopende reactie, zodat de som van beide reacties resulteert in een negatieve ΔG. In cellen worden reacties met een positieve ΔG vaak gekoppeld aan de hydrolyse van adenosine trifosfaat (ATP). De ΔG van deze laatste reactie is -7.3 kcal/mol. ATP wordt daarom wel de brandstof van de humane cel genoemd. De vrije energie in een ATP molecuul zit opgesloten in de fosfo-anhydride binding. Hydrolyse van deze binding resulteert in het vrijkomen van energie die ongebruikt vrijgegeven wordt in de vorm van warmte. Veel enzymen kunnen deze energie echter gebruiken en aldus energetisch ongunstige reacties toch laten verlopen.

ATP kan uit veel voedingsbestanddelen worden gevormd, met als belangrijkste glucose en vetzuren. Nadat glucose is opgenomen door de cel, wordt het in drie etappes afgebroken, waarbij het energierijke ATP wordt gevormd. Deze drie etappes zijn de glycolyse, de citroenzuurcyclus en de oxidatieve fosforylering. Vetzuren worden na opname in de cel omgezet in acyl-CoA, dat al of niet in de vorm van een carnitine ester naar de mitochondrien wordt getransporteerd en aldaar verder wordt geoxideerd via een proces genaamd β-oxidatie. De β-oxidatie levert uiteindelijk hetzelfde substraat op als glucose voor de citroenzuurcyclus, namelijk acetyl-CoA. De glycolyse vindt plaats in het cytosol van de cel, de citroenzuurcyclus en oxidatieve fosforylering vinden plaats in de mitochondrien. Tijdens de glycolyse, β-oxidatie en citroenzuurcyclus worden de substraten (NADH en $FADH_2$) voor de laatste etappe van de energieproductie gevormd: de oxidatieve fosforylering.

Het oxidatieve (OXPHOS) fosforyleringssysteem bestaat uit vijf enzymcomplexen en is gesitueerd in de binnenmembraan van mitochondrien. Complex I tot en met IV voorzien in het transport van elektronen van NADH en $FADH_2$ naar zuurstof. De energie die vrijkomt bij het transport van deze elektronen wordt geconserveerd door het gelijktijdige transport van protonen van de mitochondriele matrix naar de tussenmembraan ruimte. Dit transport resulteert in een elektrochemische gradient, die gebruikt wordt door het vijfde complex, ATP synthase om ATP te vormen.

Sommige kinderen worden geboren met een stoornis in een of meerdere enzymcomplex(en) van de oxidatieve fosforylering. Cellen van deze kinderen zijn dus niet in staat om in de behoefte aan ATP te voorzien. Dit uit zich onder meer in zeer ernstige multi-orgaan aandoeningen, waarbij organen die veel energie verbruiken als skeletspieren, hart en centraal zenuwstelsel het ernstigst zijn aangedaan. Het onderzoek dat beschreven staat in dit proefschrift, richt zich op kinderen met een stoornis in het eerste complex van de oxidatieve fosforylering: het NADH ubiquinon oxidoreductase (complex I).

Complex I voorziet in het transport van elektronen van NADH naar ubiquinon en gekoppeld

hieraan het transport van protonen over de mitochondriële binnenmembraan naar de tussenmembraanruimte. Hierdoor levert complex I een bijdrage aan de elektrochemische protongradiënt over de binnenmembraan van mitochondrien, welke gebruikt wordt door complex V om ATP te synthetiseren. Bij de mens bestaat complex I uit tenminste 42 verschillende eiwitten, waarvan er zeven door het mitochondriële DNA (mtDNA) gecodeerd worden, de rest door het kern DNA (nDNA).

Patiëntengroep.

Het is mogelijk om in diverse weefsels van patiënten verdacht van een stoornis in de energiestofwisseling (b.v. in gekweekte huidfibroblasten, skeletspier-, hartspier-, lever- en hersenweefsel), de overall capaciteit van het oxidatieve fosforyleringssysteem m.b.t. de productie van ATP uit diverse substraten, alsmede de oxidatiesnelheden van diverse substraten te meten. Daarnaast is het mogelijk om de enzymactiviteit van de individuele complexen van dit systeem te meten.

Voor onze studies selecteerden we 27 patiënten met een geïsoleerde complex I deficiëntie (in alle gevallen gemeten in gekweekte huidfibroblasten en in het merendeel van de patienten ook in skeletspierweefsel). De sekse verdeling liet een mannelijke meerderheid zien: 3,3 : 1. Klinische karakterisering van deze patiënten leverde een subclassificatie op in zes groepen. Een groot deel van de patienten (26%) leed aan het syndroom van Leigh. Een ongeveer even groot deel leed aan een syndroom dat sterke gelijkenis vertoont met het syndroom van Leigh (22%; Leigh-like), maar bij deze patiënten zijn postmortaal geen neuropathologische studies uitgevoerd die de diagnose Leigh syndroom hadden kunnen bevestigen. Verder vertoonde een deel van de patiënten bij de geboorte een neonatale cardiomyopathie en lactaat acidose (11%; NCLA) of fatale neonatale lactaat acidose (11%; FILA). Tenslotte vertoonde een klein deel van de patiënten macrocefalie en progressieve leukodystrofie (7%; MLD) en was het in een substantieel deel van de patiënten niet mogelijk een encefalomyopathie verder te specificeren (22%). Veel voorkomende symptomen in onze patiënten groep zijn: hypotonie; psychomotore retardatie; respiratoire stoornissen; epilepsie; voedingsproblemen; tekenen van ziekte aan de piramidebanen; nystagmus en cardiomyopathie. De gemiddelde leeftijd waarop symptomen zich manifesteerden was 4,6 maanden. De gemiddelde overlevingsduur na openbaring van de ziekte was 8,6 maanden. Op vijf kinderen na, zijn alle kinderen in de eerste vijf levensjaren overleden, 67% zelfs binnen de eerste twee levensjaren. De klinische kenmerken van de patiëntengroep staan beschreven in hoofdstuk 2 van dit proefschrift.

Aangezien er nauwelijks succesvolle therapeutische mogelijkheden voor deze kinderen beschikbaar zijn, is het van het grootste belang om de genetische oorzaken op te sporen, zodat adequate en betrouwbare genetische counseling en prenatale diagnostiek kan worden aangeboden. Het overervingpatroon lijkt op grond van het hoge percentage consanguiniteit (26%) en de verdeling van aangedane en niet aangedane kinderen binnen de families in deze patiëntengroep autosomaal-recessief te zijn. Verder blijkt uit de literatuur dat veel voorkomende mutaties in het mitochondriële DNA die aanleiding kunnen geven tot OXPHOS stoornissen, slechts in een klein percentage van kinderen met een dergelijke ziekte voorkomen (~5%). In onze patiëntengroep zijn grote mutaties in het mtDNA uitgesloten door middel van de "long template PCR" techniek. Verder zijn elf veel voorkomende pathogene puntmutaties

in het mtDNA eveneens uitgesloten door middel van polymerase kettingreactie (PCR) gecombineerd met restrictie enzym analyse Door dit alles lijken de genetische oorzaken van de complex I deficientie in deze patientengroep zich grotendeels te bevinden in het kern DNA en niet in het mitochondriele DNA Een belangrijke groep kandidaat genen is dan natuurlijk de kerngecodeerde subeenheden die daadwerkelijk onderdeel uitmaken van complex I Voordat mutatie analyse studies in deze patientengroep uitgevoerd konden worden, moesten eerst de kerngecodeerde subeenheden gekarakteriseerd worden

Karakterisatie van humane complex I subeenheden.

Het complex I van prokaryoten wordt gezien als de minimale functionele variant van dit enzym complex Het bestaat uit 14 subeenheden, welke tezamen zowel het elektronen als het protonentransport verzorgen Eukaryoten als b v *Neurospora crassa*, *Bos taurus* en *Homo sapiens* hebben een gecompliceerder complex I bestaande uit respectievelijk ≥35, ≥42 en nogmaals ≥42 subeenheden Op dit moment zijn alle 42 subeenheden van *Bos taurus* gekarakteriseerd op cDNA- en aminozuurniveau De sterke homologie tussen complex I eiwitten van de koe en de mens is gebruikt om de equivalenten van deze eiwitten bij de mens te karakteriseren Momenteel zijn alle 42 humane subeenheden gekarakteriseerd, waarvan 18 door onze onderzoeksgroep Van deze 18 subeenheden, staan er 14 in dit proefschrift beschreven (hoofdstuk 4, 5, 6, 7 en 8)

Nadat de cDNA structuur van deze kerngecodeerde complex I subeenheden opgehelderd was, werd een start gemaakt met mutatie analyse studies in onze patientengroep De biochemische enzym assay welke gebruikt werd om de complex I activiteit in fibroblasten te bepalen, is gebaseerd op meting van de omzetting van NADH naar NAD^+ in een geisoleerde mitochondriele eiwitfractie, in aanwezigheid van een ubiquinon-analoog in aan- en afwezigheid van de specifieke complex I remmer rotenon Dit impliceert dat een verlaagde activiteit van complex I vastgesteld met behulp van deze assay in wezen een stoornis in het transport van elektronen van NADH naar ubiquinon weerspiegelt Het flavoproteine (FP) deel en het ijzerzwavel proteine (IP) deel welke op een na alle bekende katalytische centra van complex I bevatten, werden geselecteerd voor mutatie analyse studies In dit proefschrift staan de resultaten van de op cDNA niveau uitgevoerde mutatie detectie studies, van subeenheden aanwezig in de IP fractie, beschreven

Mutatie analyse studies van subeenheden van de IP fractie van complex I.

Indien men complex I fragmenteert met chaotrope agentia, kan men drie subfracties onderscheiden de genoemde flavoproteine fractie en de ijzerzwavel proteine fractie, welke tezamen het deel van complex I vormen dat uitsteekt in de mitochondriele matrix, en de hydrofobe proteine fractie die het membraan deel van complex I vormt

De ijzerzwavel proteine fractie bestaat uit de volgende zeven of acht eiwitten de NDUFS1-6, de NDUFS8 en de NDUFA5 (de aanwezigheid van het NDUFS8 eiwit in deze fractie is onderwerp van discussie) Bekend is dat het NDUFS1 eiwit ten minste twee ijzerzwavel cluster bindingsplaatsen bevat, de NDUFS8 mogelijk ook twee, en de NDUFS2 zou betrokken zijn bij het binden van ubiquinon en rotenon Bij het rund is onlangs bewezen dat het NDUFS4 eiwit via een cAMP gereguleerde proteine kinase gefosforyleerd wordt Dit eiwit

bevat in het C-terminale deel een consensus fosforyleringsplaats die geconserveerd is in *N crassa* en de mens Deze fosforyleringsplaatsen hebben vaak een regulerende functie Van de overige eiwitten is in functioneel opzicht nog niets bekend Mutatie analyse studies d m v RT PCR gecombineerd met DNA sequentie-analyse van alle acht bovengenoemde subeenheden in onze patientengroep leverden de volgende resultaten op

Als eerste werd een homozygote duplicatie van vijf baseparen gevonden in het *NDUFS4* gen van een mannelijke patient die presenteerde met ernstig braken, groeiachterstand en spier hypotonie op een leeftijd van 8 maanden Dit beeld verslechterde progressief tot de dood volgde op een leeftijd van 16 maanden De NDUFS4 subeenheid is een eiwit bestaande uit 175 aminozuren waarvan studies gedaan met rund complex I hebben aangetoond dat deze een cAMP gereguleerde fosforyleringsplaats bevat De duplicatie bevindt zich direct na base 470 van het cDNA Het gevolg van deze duplicatie is dat door de ontstane "frameshift", de 18 C terminale aminozuren van het eiwit veranderen (met verlies van de consensus fosforyleringsplaats) Bovendien verdwijnt door de "frameshift" het reguliere stopcodon, zodat translatie verder gaat tot een volgend stopcodon, hetgeen resulteert in een eiwit dat 14 aminozuren langer is Met behulp van polyacrylamide gel electroforese werd de homozygote aanwezigheid van de duplicatie in het nDNA van de index patient bevestigd Beide ouders bleken heterozygoot te zijn voor de duplicatie en het niet aangedane broertje bleek de mutatie niet te hebben Dit is de eerste mutatie ooit gevonden in een kerngecodeerde subeenheid van complex I Details van deze studie staan beschreven in hoofdstuk 8 van dit proefschrift

De tweede mutatie in een kerngecodeerde subeenheid van complex I werd gevonden in het *NDUFS8* gen Het open reading frame van de *NDUFS8* bestaat uit 633 baseparen coderend voor 210 aminozuren Het betrof een patient lijdend aan het syndroom van Leigh, die presenteerde op een leeftijd van 5 weken met voedingsproblemen en perioden van apnoe en cyanose Ook deze patient vertoonde een progressief ziektebeloop en overleed op een leeftijd van 11 weken als gevolg van cardiorespiratoir falen Bij het syndroom van Leigh was eerder reeds vastgesteld dat een geïsoleerde complex I deficientie een van de belangrijkste oorzaken is, echter genetische oorzaken waren nooit aangetoond In de bovengenoemde patient werden twee puntmutaties in het *NDUFS8* cDNA aangetroffen Het betrof een C236T mutatie leidend tot aminozuursubstitutie van proline naar leucine op positie 79 en een G305A mutatie leidend tot substitutie van arginine naar histidine op positie 102 Restrictie-enzym analyse bevestigde dat beide mutaties bij deze patient in verschillende allelen voorkwamen De vader van dit kind bleek drager van de C236T mutatie evenals het oudste broertje, de moeder was draagster van de G305A mutatie evenals het jongere zusje Het jongste broertje bleek geen van beide mutaties te bezitten Deze mutaties zijn de eerste ooit gevonden in een kerngecodeerde subeenheid van complex I in een patient met het syndroom van Leigh De resultaten van deze studie staan beschreven in hoofdstuk 9 van dit proefschrift

Sequentie-analyse van het *NDUFS2* cDNA (open reading frame van 1392 baseparen coderend voor 463 aminozuren) in onze patientengroep leverde opnieuw mutaties op Het betrof hier drie families waarvan de ouders in twee families verwant waren De eerste familie bestond uit drie kinderen waarvan de laatste twee (een jongen en een meisje) leden aan een geïsoleerde complex I deficientie Het oudste kind is tot heden gezond In beide aangedane kinderen vonden we een homozygote G683A mutatie die leidt tot substitutie van arginine naar

glutamine op positie 228 Beide kinderen leden aan een progressieve encefalomyopathie met hypertrofische cardiomyopathie en opticus atrofie en overleden op een leeftijd van respectievelijk 24 en 18 maanden De mutatie werd bevestigd op nDNA niveau met behulp van restrictie-enzym analyse en was homozygoot aanwezig in beide index patienten, heterozygoot in beide ouders en afwezig in het gezonde oudste kind

In een tweede familie werd eveneens een mutatie gevonden in het *NDUFS2* gen Het betrof hier een patientje dat leed aan een neonatale hypertrofische cardiomyopathie en lactaat acidose Het kind overleed vier dagen na de geboorte als gevolg van cardiorespiratoir falen In het *NDUFS2* cDNA van deze patient werd een homozygote C686A mutatie gevonden leidend tot substitutie van proline in glutamine op positie 229 Restrictie-enzym analyse op nDNA niveau bevestigde dat de patient homozygoot is voor de mutatie en dat beide ouders heterozygoot zijn In het gezin zijn verder geen kinderen

De derde familie bestaat uit vier kinderen waarvan de eerste drie (allen jongens) leden aan een complex I deficientie Het vierde kind werd gezond geboren na eiceldonatie Alle drie de aangedane kinderen leden aan een beeld van progressieve encefalomyopathie en overleden twee tot drie jaar na de geboorte In het tweede kind werd bij sequentie-analyse van het *NDUFS2* cDNA een homozygote T1237C mutatie gevonden (leidend tot substitutie van serine in proline ter hoogte van aminozuurpositie 413) Beide ouders bleken heterozygoot te zijn voor de mutatie Helaas was van de andere kinderen geen DNA of RNA beschikbaar

Alle drie de mutaties resulteren in de substitutie van aminozuren die door de evolutie heen in vele soorten geconserveerd zijn, wat een indicatie is voor het functionele belang van deze aminozuren De resultaten van deze mutatie analyse studie staan beschreven in hoofdstuk 10 van dit proefschrift Alle gevonden mutaties (hoofdstuk 8, 9 en 10) zijn uitgesloten in een grote controle populatie

cDNA amplificatie gevolgd door sequentie-analyse van de overige subeenheden van de IP fractie, NDUFS1, NDUFS3, NDUFS5, NDUFS6 en NDUFA5, leverde geen ziekte veroorzakende mutaties op De resultaten van deze studies staan beschreven in hoofdstuk 5 en 11 van dit proefschrift

Mutatie analyse studies met *NDUFA1* en *NDUFB6* cDNA en chromosomale lokalisatie van het *NDUFS2*, *NDUFS3*, *NDUFS4* en *NDUFS5* gen.

Tenslotte hebben we nog het cDNA bestudeerd van twee subeenheden aanwezig in de hydrofobe fractie, namelijk NDUFA1 en NDUFB6 De NDUFA1 subeenheid is momenteel het enige complex I eiwit waarvan het bekend is dat het gen gelegen is op het X-chromosoom Het feit dat het merendeel van onze complex I patienten uit mannen bestaat, wijst op mogelijke betrokkenheid van een X-gekoppelde factor We hebben om die reden de chromosomale lokalisatie bepaald van 4 genen coderend voor complex I subeenheden beschreven in dit proefschrift, namelijk de *NDUFS2*, *NDUFS3*, *NDUFS4* en *NDUFS5* Op dit moment is de chromosomale lokalisatie van 30 van de 35 kerngecodeerde subeenheden van complex I bekend, wat een random verdeling over de chromosomen laat zien Mutatie analyse van het *NDUFA1* en *NDUFB6* cDNA leverde geen mutaties op in onze patienten De resultaten van deze studies staan beschreven in hoofdstuk 3 en 4 van dit proefschrift

Conclusies en toekomst.

Als eerste stap in de opheldering van de veronderstelde nucleair-genetische oorzaken bij kinderen met een geïsoleerde complex I deficiëntie hebben we alle onbekende nucleair gecodeerde eiwitten van complex I gekarakteriseerd Mutatie analyse studies van het cDNA van de complex I subeenheden aanwezig in de ijzerzwavel proteine fractie, leverde mutaties op in vijf families Alle mutaties zijn gelegen in genen coderend voor subeenheden die sterk geconserveerd zijn gedurende de evolutie wat het belang van deze subeenheden illustreert Verder cosegregeerden alle mutaties in de families en werden ze uitgesloten in een grote controlepopulatie, hetgeen populatiepolymorfismen onwaarschijnlijk maakt Toekomstige expressiestudies moeten de pathogeniciteit van de gevonden mutaties definitief bewijzen Eveneens moeten de frequentie van deze mutaties, alsmede de betrokkenheid van mogelijke andere mutaties binnen deze gemuteerde subeenheden in een grote groep complex I deficiente patienten bepaald worden Het vinden van genetische oorzaken leidt tot een meer betrouwbare genetische counseling in het algemeen en prenatale diagnostiek in het bijzonder

Op dit moment zijn alle 35 bekende kerngecodeerde subeenheden van complex I onderzocht op de aanwezigheid van mutaties in onze patientengroep Dit bracht in ±40% de genetische oorzaak aan het licht Hoe nu verder om in de overige patienten het genetische defect op te helderen? Een van de mogelijkheden is het creeren van complementatiegroepen waarbij patienten die tot een bepaalde complementatiegroep behoren het defect in hetzelfde gen hebben liggen De techniek van "microcell-mediated chromosome transfer" stelt ons in staat om in zo'n complementatiegroep "gezonde" chromosomen eén voor één in te brengen tot herstel van de complex I activiteit optreedt, waardoor vastgesteld wordt op welk chromosoom het gemuteerde gen ligt Een aantal technieken als "deletion mapping" en positioneel/functioneel cloneren stellen ons dan in staat om het gemuteerde gen op te sporen

Naast het ophelderen van het genetische defect in complex I deficiente patienten, zijn studies naar de pathofysiologische consequenties van deze mutaties van uitermate groot belang Factoren die een rol spelen bij de pathofysiologie van complex I deficientie zijn onder andere een verminderde ATP productie, een verlaging van de mitochondriele membraanpotentiaal, radikaalvorming en een gestoorde cellulaire calciumhuishouding Hopelijk leidt meer inzicht in deze processen uiteindelijk tot een rationele therapie waar patienten profijt van hebben Genezing van deze patientjes is immers het uiteindelijke doel

De conclusies en toekomstperspectieven staan beschreven in hoofdstuk 12 van dit proefschrift

Summary.

Introduction.

Many biochemical reactions take place within human cells Some of these reactions run spontaneously, because conditions after the reaction are energetically favourable with respect to conditions before In this case, the reaction's free energy (ΔG) is negative Other biochemical reactions do not run spontaneously because they are energetically unfavourable (ΔG is positive) A biochemical energetically unfavourable reaction can run when it is linked to a biochemical favourable reaction The sum of these reactions then results in a negative ΔG Within cells, reactions with a positive ΔG are often linked to the hydrolysis of adenosine triphosphate (ATP) The ΔG of this latter reaction is –7 3 kcal/mol Therefore ATP is often called the energy source of human cells The free energy of an ATP molecule is situated in its phosphoanhydride bond Hydrolysis of this bond results in the release of free energy, which (when unused) is released as heat However, many enzymes can use this energy to make energetically unfavourable reactions possible

ATP can be synthesised from many food components, with as main contributors glucose and fatty acids After glucose is taken up by the cell, it is degraded in three stages glycolysis, citric acid cycle and oxidative phosphorylation After entering the cell, fatty acids are converted into acyl-CoA and can be transported in the form of a carnitine ester into mitochondria, where they are further oxidised by a process which is called β-oxidation This β-oxidation eventually results in the same substrate as degradation of glucose does, namely acetyl CoA, which can enter the citric acid cycle Glycolysis takes place in the cytosol of the cell, the citric acid cycle and oxidative phosphorylation take place in mitochondria During glycolysis, β-oxidation and citric acid cycle, the substrates (NADH and $FADH_2$) are formed for the last stage of energy production oxidative phosphorylation

The oxidative phosphorylation (OXPHOS) system, which is localised in the inner mitochondrial membrane, is composed of five enzyme complexes Complex I to IV transport electrons from NADH and $FADH_2$ to oxygen The energy that is released by the transport of these electrons is conserved by the simultaneous transport of protons from the mitochondrial matrix into the intermembrane space This transport results in an electrochemical gradient, which is used by the fifth complex (ATP synthase) to generate ATP

Some children are born with a disturbance of one or more enzyme complex(es) of the OXPHOS system Cells of these children are therefore incapable to meet their need for ATP This expresses (amongst others) as serious multiple-organ disease, with most prominent involvement of organs with high-energy demands like heart, central nervous system and skeletal muscle The research described in this thesis, focuses on children with a disturbance in the first complex of the OXPHOS system NADH ubiquinone oxidoreductase (complex I) Complex I transports electrons from NADH to ubiquinone and simultaneously to this electron transport, protons are translocated across the inner mitochondrial membrane to the intermembrane space Because of this, complex I contributes to the electrochemical protongradient across the inner membrane of mitochondria, which is used by complex V to synthesise ATP In humans, complex I exists of at least 42 different proteins, of which seven

are encoded by the mitochondrial DNA (mtDNA). The remainder are encoded by nuclear DNA (nDNA).

Patientgroup.

The overall capacity of the oxidative phosphorylation system with regard to the production of ATP from several substrates, as well as the oxidation rates of substrates can be measured in several tissues (like cultured skin fibroblasts, skeletal muscle, brain, heart and liver tissue) of patients suspected to suffer from a disturbance in the cellular energy production. Furthermore, it is possible to measure the enzyme activity of the individual complexes of this system.

We selected 27 patients suffering from an isolated complex I deficiency (measured in all cases in cultured skin fibroblasts and in the majority of these patients also in skeletal muscle tissue). The sex distribution showed a strong male preponderance: 3.3 : 1. Clinical characterisation of these patients resulted in a classification into six subgroups. A large part of the patients suffered from Leigh syndrome (26%). A similar percentage suffered from a syndrome, which has strong resemblance to Leigh syndrome (22%; Leigh-like syndrome), however autopsy has not been performed in these patients, which could have confirmed the diagnosis Leigh syndrome. Furthermore, a part of our isolated complex I deficient patients suffered at birth from a hypertrophic cardiomyopathy and lactic acidosis (11%; NCLA) or fatal infantile lactic acidosis (11%; FILA). Finally, a small part of our patients suffers from macrocephaly and progressive leukodystrophy (7%; MLD). In a substantial part of our patients it was not possible to specify an encephalomyopathy (unspecified encephalomyopathy (UE); 22%). Frequent symptoms observed in our patient group are: hypotonia; psychomotor retardation; respiratory disturbances; epilepsy; feeding problems; pyramidal signs; nystagmus and cardiomyopathy. Mean age of disease presentation within our patientgroup was 4.6 months. The mean survival interval after disease presentation was 8.6 months. All but five children died within the first five years of life, 67% died within the first two years. The clinical characteristics of our patient group are described in chapter 2 of this thesis.

Since there are no successful therapeutic possibilities for these children available, it is of utmost importance to find the genetic causes, which will give us the opportunity to offer adequate genetic counselling and prenatal diagnostics. On the basis of the high percentage of consanguinity (26%) and the distribution of affected and non-affected children within families with isolated complex I deficient patients, the inheritance pattern seems to be autosomal-recessive. Furthermore, recent literature revealed that common mtDNA mutations, which can result in OXPHOS disturbances, are usually not present in children with such a disease. In our patientgroup, major rearrangements of mtDNA are excluded with the long mtDNA template polymerase chain reaction (PCR) technique. Furthermore, eleven common pathogenic mtDNA mutations have been excluded by means of PCR combined with restriction enzyme analysis. The factors mentioned in this paragraph concerning the possible genetic origin of isolated complex I deficiency in our patient group, make mutations in the nuclear DNA compared to mutations in the mtDNA, more likely. An important group candidate genes are therefore the nuclear genes, which encode subunits which are part of complex I. Before mutational analysis studies could be performed in our patientgroup, we had to elucidate the cDNA and amino acid sequence of these nuclear encoded subunits.

Characterisation of human complex I subunits.

Prokaryotic complex I is generally considered to be the minimal functional version of this enzyme complex It consists of fourteen subunits, which together take care of the electron- and protontransport Eukaryotes like *Neurospora crassa, Bos taurus* and *Homo sapiens* have a more complicated complex I, consisting of ≥35, ≥42 and again ≥42 subunits, respectively At this moment, all 42 subunits of Bos taurus have been characterised at cDNA and amino acid level The strong resemblance between subunits of cow and humans has been used to characterise the human equivalents of these bovine subunits Currently, all 42 human subunits have been characterised, of which 18 by our researchgroup Of these latter subunits, 14 are described in this thesis (chapter 4, 5, 6, 7 and 8)

When the cDNA structure of all nuclear encoded complex I subunits was elucidated, we started with mutational analysis studies within our patient group The biochemical enzyme assay we used to measure the complex I activity in fibroblasts, is based on the conversion of NADH to NAD^+ in an isolated mitochondrial protein fraction in the presence of a ubiquinone analogue and in presence or absence of the specific complex I inhibitor rotenone This implicates that a reduced complex I activity according to this assay actually reflects a disturbance in the transport of electrons from NADH to ubiquinone The flavoproteine (FP) fraction and the iron-sulphur protein (IP) fraction, which contain all but one known catalytic centres of complex I, were selected for mutational analysis studies In this thesis the results of these studies (at cDNA level) are described

Mutational analysis studies of subunits present in the IP fraction of complex I.

It is possible to fragment complex I with chaotropic agents, which results in three subfractions the previously mentioned flavoprotein (FP) and iron-sulphur protein fraction, which together constitute the part of complex I which protrudes into the mitochondrial matrix, and a hydrophobic protein fraction (HP), which largely forms the membranous part of complex I

The iron-sulphur protein fraction consists of seven or eight proteins NDUFS1-6, NDUFS8 and NDUFA5 (the presence of the NDUFS8 subunit in this fraction is subject of debate) It is known that the NDUFS1 subunit contains at least two iron-sulphur cluster bindings sites, the NDUFS8 possibly also two and the NDUFS2 is hypothesised to participate in ubiquinone and rotenone binding Recently, it was found that the NDUFS4 subunit of *Bos taurus* is phosphorylated by a cAMP dependent protein kinase The NDUFS4 contains a consensus phosphorylation site in the C-terminal part of the protein, which is conserved in *N crassa* and humans These phosphorylation sites often have a regulating function Currently, nothing is known of the function of the other proteins present in the iron-sulphur protein fraction Mutational analysis studies by means of RT-PCR and DNA sequence analysis of these eight subunits revealed the following results

The first mutation we found was a homozygous 5 bp duplication in the *NDUFS4* gene of a male patient who presented at the age of eight months with serious vomiting, failure to thrive and muscle hypotonia The clinical course was rapidly progressive, until the child's death at the age of 16 months The NDUFS4 protein consists of 175 amino acids As mentioned previously, the bovine equivalent of the NDUFS4 subunit contains a cAMP regulated

phosphorylationsite. The duplication is present directly after cDNA base 470. The results of this duplication (due to the frameshift) for the translation product are change of the 18 C-terminal amino acids (with destruction of the cAMP consensus phosphorylationsite) and destruction of the stopcodon, which theoretically results in elongation of the translation product until the next inframe stopcodon, which elongates the NDUFS4 in this patient with 14 amino acids. We confirmed the homozygous presence of the duplication in the nuclear DNA (nDNA) of the index patient with polyacrylamide gel electrophoresis. Both parents were heterozygous for the duplication while nDNA of the healthy sibling only contained wildtype alleles. This duplication was the first mutation found in a nuclear gene encoding a subunit of complex I. Details of this study are described in chapter 8 of this thesis.

The second mutation we found in a nuclear encoded subunit of complex I was located in the NDUFS8 subunit. The open reading frame (ORF) of the *NDUFS8* cDNA contains 633 base pairs, encoding 210 amino acids. It concerned a patient who suffered from Leigh syndrome, who presented at the age of 5 weeks with feeding difficulties and periods of apnoea and cyanosis. This patient expressed a progressive clinical course and died at the age of eleven weeks as a consequence of cardiorespiratory failure. Isolated complex I deficiency was previously recognised as a frequent cause of Leigh syndrome, however genetic links had never been found. We found two compound heterozygous mutations in the *NDUFS8* cDNA of the patient described in this paragraph. It concerned a C236T mutation leading to the substitution of proline into leucine at position 79 and a G305A mutation leading to the substitution of arginine to histidine at position 102. Restriction enzyme analysis performed with amplified *NDUFS8* cDNA of this patient confirmed that both mutations were located on different alleles. The father of this child is carrier of the C236T mutation just as the younger brother, while the mother is carrier of the G305A mutation just as the younger sister. The youngest brother does not contain either of the two mutations. These mutations were the first to be found in a nuclear encoded subunit of complex I in a patient with Leigh syndrome. The results of this study are described in chapter 9 of this thesis.

Sequence analysis of the *NDUFS2* cDNA (ORF of 1392 base pairs encoding 463 amino acids) in our patientgroup again revealed mutations. These mutations were encountered in three families, of which two were known with consanguinity. The first family consisted of three children, of whom the latter two suffered from isolated complex I deficiency. The oldest child is currently alive and healthy. Both complex I deficient children contained a homozygous base substitution in their *NDUFS2* cDNA (G683A) leading to the substitution of arginine into glutamine at position 228. Both children suffered from a progressive encephalomyopathy, hypertrophic cardiomyopathy and optic atrophy and died at the age of 24 and 18 months, respectively. The mutation was confirmed to be homozygously present in the nuclear DNA of both patients by means of restriction enzyme analysis, while both parents turned out to be heterozygous for the mutation, and the healthy child contained only wildtype alleles.

We found a homozygous C686A mutation leading to the substitution of proline into glutamine at position 229 in a complex I deficient child of a second family. The child suffered from a neonatal cardiomyopathy and lactic acidosis and died 4 days after birth of cardiorespiratory failure. Restriction enzyme analysis with amplified nDNA fragments confirmed the presence of the homozygous mutation in the patient, while both parents turned out to be heterozygous

carriers. There were no other siblings in this family.
The third family consisted of four children, of whom the oldest three (all boys) suffered from isolated complex I deficiency. The fourth child was born healthy after egg cell donation. All three affected children suffered from a clinical phenotype of progressive encephalomyopathy and died two to three years after birth. We found in the second affected child by means of sequence analysis with amplified *NDUFS2* cDNA a homozygous T1237C mutation leading to the substitution of serine into proline at amino acid position 413. This mutation was also homozygously present in the nDNA of this patient, while both parents are heterozygous carriers. Unfortunately, DNA and RNA of the other three children were unavailable.
All three mutations result in the substitution of amino acids, which are highly conserved in many species during evolution, which is indicative for the functional importance of these amino acids. The results of these mutational analysis studies are described in chapter 10 of this thesis. All found mutations (chapter 8, 9 and 10) have been excluded in a large control population.
cDNA amplification followed by sequence analysis of the other subunits of the IP fraction (NDUFS1, NDUFS3, NDUFS5, NDUFS6 and NDUFA5) did not reveal any disease causing mutations. The results of these studies are described in chapter 5 and 11 of this thesis.

Mutational analysis studies of *NDUFA1* and *NDUFB6* cDNA and chromosomal localisation of the *NDUFS2*, *NDUFS3*, *NDUFS4* and *NDUFS5* gene.
Finally we checked the cDNA for mutations of two subunits present in the hydrophobic subfraction, namely the NDUFA1 and NDUFB6 subunit. The NDUFA1 subunit is presently the only complex I protein, whose gene is located on the X-chromosome. The fact that the majority of our complex I deficient patients is male, points to the possible involvement of an X-linked factor. For this reason we performed the chromosomal localisation of four genes encoding complex I subunits, namely the *NDUFS2*, *NDUFS3*, *NDUFS4* and *NDUFS5*. At this moment the chromosomal localisation of 30 of the 35 nuclear encoded subunits of complex I is known, which shows a random distribution across the chromosomes. Mutational analysis studies of the *NDUFA1* and *NDUFB6* cDNA revealed no mutations within our patients. The results of these studies are described in chapter 3 and 4 of this thesis.

Conclusions and future perspectives.
As a first step in the elucidation of the hypothesised nuclear-genetic causes in children with an isolated complex I deficiency we characterised all unknown nuclear encoded subunits of complex I. Mutational analysis studies on cDNA level of the subunits present in the iron-sulphur protein fraction revealed mutations in five pedigrees. All mutations are present in subunits that are highly conserved during evolution. All mutations cosegregated within the families and were excluded in a large control population, which makes the mutations being rare population polymorphisms unlikely. Future expression studies should prove the pathogenicity of the found mutations. Likewise the frequency of these mutations in a large group of complex I deficient patients as well as the presence of new mutations in these mutated subunits has to be investigated. The elucidation of genetic causes of complex I deficiency leads to a more accurate genetic counselling and prenatal diagnostics.

At this moment, all 35 known nuclear encoded subunits of complex I have been checked for mutations in our initial group of 20 isolated complex I deficient patients This revealed the genetic cause in ~40% Which path should we follow next in order to elucidate the genetic cause in our remaining patients? One of the possibilities is the generation of complementation groups (patients belonging to the same complementation group carry the genetic defect in the same gene) The technique of microcell-mediated chromosome transfer will give us the opportunity to introduce "healthy" chromosomes one by one in cells of patients belonging to the same complementation group When the introduction of a specific chromosome results in recovery of the complex I activity, the mutated gene is located on that specific chromosome Techniques like deletion mapping and positional/functional cloning give us the opportunity to locate the mutated gene

Besides the elucidation of the genetic causes of complex I deficiency, studies of the pathophysiological consequences of this disease are very important Factors which play a role in the pathophysiology of complex I deficiency are amongst others diminished ATP production, reduction of the mitochondrial membrane potential, reactive oxygen species (ROS) formation and a disturbed cellular calcium maintenance Hopefully these studies lead to increased understanding of these processes, which may result in a rational therapy Cure of our patients is after all our final goal

The conclusions and future perspectives are described in chapter 12 of this thesis

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Jan.

Curriculum vitae.

Johannes Lambertus Christianus Maria Loeffen werd geboren op 6 mei 1970 te Oss Na het voltooien van de opleiding VWO-β aan het Titus Brandsma Lyceum te Oss werd wegens uitloting voor de studie Geneeskunde aangevangen met de studie Gezondheidswetenschappen te Nijmegen In de loop van het studiejaar deed de gelegenheid zich voor over te stappen naar de studie Geneeskunde te Utrecht Het doctoraal examen Geneeskunde werd in 1995 afgelegd wat gevolgd werd door het artsexamen in april 1997 Tijdens de opleiding tot arts participeerde hij in diverse wetenschappelijke projecten bij de afdeling Interne Geneeskunde (Diakonessenhuis te Utrecht), afdeling Kindergeneeskunde, subafdeling voedingsallergieen (Wilhelmina Kinderziekenhuis te Utrecht), afdeling Kindergeneeskunde, subafdeling Stofwisselingsziekten (Wilhelmina Kinderziekenhuis te Utrecht) en het laboratorium voor Kindergeneeskunde en Neurologie (Universitair Medisch Centrum St Radboud)
Van april 1997 tot en met december 1999 was hij als artsonderzoeker verbonden aan het Laboratorium voor Kindergeneeskunde en Neurologie (hoofd Prof dr ir JMF Trijbels) en de subafdeling Metabole Ziekten (hoofd Dr JAM Smeitink) van de afdeling Kindergeneeskunde (hoofd Prof dr RCA Sengers) van het Universitair Medisch Centrum St Radboud, alwaar het in dit proefschrift beschreven onderzoek is uitgevoerd Vanaf 1 april 2000 is hij teruggekeerd naar de klinische patientenzorg en werkt hij als arts bij de afdeling Kindergeneeskunde van het Universitair Medisch Centrum St Radboud te Nijmegen

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