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MITOCHONDRIAL DYSFUNCTION IN NEURODEGENERATION

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ABSTRACT

The mitochondrial respiratory chain (RC) is responsible for providing most of the cellular energy in the form of ATP, and is also one of the main sites of reactive oxygen species (ROS) formation. The RC, consisting of five enzyme complexes in the inner mitochondrial membrane, is unique in its bipartite dependence on both nuclear and mitochondrial DNA (mtDNA). One example of this is mitochondrial transcription factor A (TFAM), a nuclear encoded protein that is imported into mitochondria where it is essential for transcription and maintenance of mtDNA. We have studied the two aspects of TFAM function, mitochondrial transcription and mtDNA maintenance, in transgenic mice, and also used tissue-specific knockouts of TFAM to impair mitochondrial function in the mouse brain. An increasing amount of evidence suggest that mitochondrial dysfunction is of central importance in the pathogenesis in common neurodegenerative disorders, such as Parkinson's disease (PD), as well as in the normal aging process. Neurological symptoms are also prominent in a group of genetic disorders collectively called mitochondrial encephalomyopathies, which are caused by defects in the RC. We show here that human TFAM (hTFAM) is a poor activator of mouse mitochondrial *in vitro* transcription, despite its strong capacity for unspecific DNA binding. PAC-transgenic mice expressing hTFAM had elevated mtDNA copy number but no changes in levels of most mitochondrial transcripts or in RC function. We estimated the molar ratio of TFAM to mtDNA to 1 TFAM molecule per 15-20 bp of mtDNA. Thus TFAM is an abundant protein within mitochondria capable of regulating mtDNA copy number. The human *TFAM* transgene was furthermore unable to complement the loss of endogenous mouse TFAM (mTFAM) in homozygous knockout embryos, probably due to the reduced ability of hTFAM to activate transcription. We were however successful in rescuing tissue-specific knockout mice with mTFAM depletion in the heart by crossing them to PAC-TFAM mice. Interestingly, such rescued hearts displayed signs of altered transcription regulation in mitochondria, probably as a compensatory response to maintain energy production. Mice with tissue-specific knockout of TFAM in forebrain neurons (MILON mice) survived for a surprisingly long time. At 4 months of age many neurons were severely RC deficient, but the mice still appeared normal. RC deficient neurons in such presymptomatic animals did however display increased sensitivity to excitotoxic stress. MILON mice died from massive synchronized neurodegenerative events in neocortex and hippocampus, at 5-6 months of age, without showing any major up-regulation of oxidative defenses. We next generated mice with a dopamine (DA) neuron specific inactivation of the *Tfam* gene. These "Parkinson mice" developed progressive symptoms replicating many of the clinical features of PD, such as bradykinesia, rigidity and abnormal gait. Symptoms were transiently reversed by administration of normal PD medication (L-DOPA). In more advanced stages (>30 weeks of age) of the disease, similarly to PD, the efficiency of L-DOPA treatment declined and animals showed clear signs of dyskinetic movements. The behavioral disturbances correlated with a slow degeneration of the nigro-striatal DA pathway. Primarily DA neurons in substantia nigra pars compacta were lost, but in later stages also the ventral tegmental area neurons succumbed. Many surviving DA neurons displayed abnormal morphology, containing α-synuclein and ubiquitin immunoreactive inclusions, similar to Lewy bodies observed in PD.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I. **Ekstrand MI**, Falkenberg M, Rantanen A, Park CB, Gaspari M, Hultenby K, Rustin P, Gustafsson CM and Larsson NG (2004). Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum Mol Genet*, 13:935-944.

II. Freyer C, **Ekstrand MI**, Wibom R and Larsson NG (2005). Human TFAM can complement a heart specific knockout of mouse *Tfam* despite its reduced capacity to initiate mouse mtDNA transcription. *Manuscript*

III. Sorensen L, **Ekstrand M**, Silva JP, Lindqvist E, Xu B, Rustin P, Olson L, and Larsson NG (2001). Late-onset corticohippocampal neurodepletion attributable to catastrophic failure of oxidative phosphorylation in MILON mice. *J Neurosci*, 21:8082-8090.
L.S. and M.E. contributed equally to this work.

IV. **Ekstrand MI**, Terzioglu M, Lindqvist E, Zhu S, Trifunovic A, Mohammed AH, Olson L and Larsson NG (2005). Progressive degeneration and α-synuclein pathology in respiratory chain deficient nigro-striatal mouse dopamine neurons. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

Abf2p	ARS-binding factor 2
ANF	Atrial natriuretic factor
ANT1	Adenine nucleotide translocator
APP	Amyloid-β precursor protein
ATP	Adenosine triphosphate
CaMKII	Calmodulin kinase II
Ckmm	Muscle creatine kinase
COX	Cytochrome *c* oxidase
CSB	Conserved sequence block
DA	Dopamine
DAT	Dopamine transporter
D-loop	Displacement loop
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GPX	Glutathione peroxidase
HMG	High-mobility group
HSP	Heavy-strand promoter
KSS	Kearns-Sayre syndrome
LSP	Light-strand promoter
MELAS	Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes
MERFF	Myoclonic epilepsy with ragged red fibers
MILON	Mitochondrial late-onset neurodegeneration
MPTP	1-methyl-4-1,2,3,6-tetrahydropyridine
mtDNA	Mitochondrial DNA
ND	NADH dehydrogenase
OXPHOS	Oxidative phosphorylation
PAC	P1-artificial chromosome
PEO	Progressive external ophthalmoplegia
PINK1	PTEN-induced kinase I
POLRMT	Mitochondrial RNA polymerase
RC	Respiratory chain
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SNpc	Substantia nigra pars compacta
SOD	Superoxide dismutase
TAS	Termination associated sequences
TFAM	Mitochondrial transcription factor A
TFB1/2M	Mitochondria transcription factor B1 or B2
TH	Tyrosine hydroxylase
UCHL1	Ubiquitin carboxyl-terminal hydrolase I
VTA	Ventral tegmental area

INTRODUCTION

Mitochondria are cellular organelles of prokaryotic origin that are found in almost all eukaryotic cells. They produce most of the energy in eukaryotic cells but are also involved in several other processes such as: lipid metabolism, Krebs cycle, apoptosis, formation of reactive oxygen species and calcium buffering.

Mitochondria are believed to be of monophyletic origin – that is the result of a single endosymbiotic event in early eukaryote evolution. Most likely the common ancestor was related to what we today know as the rickettsial subdivision of the α-Proteobacteria, a group of eubacterial obligate intracellular parasites (Gray et al. 1999). In fact, the most mitochondria-like eubacterial genome found to date is that of the α-Proteobacteria *Rickettsia prowasekii* (Andersson et al. 1998). Conversely, the most bacteria-like mitochondrial genome found to date is that of the protozoon *Reclinomonas americana* (Lang et al. 1997).

Mitochondria are double membrane organelles that continuously divide and fuse, forming a dynamic network. Genetic screens in yeast have identified several fission and fusion genes that affect mitochondrial morphology (Yaffe 2003). The outer mitochondrial membrane is separated from the inner membrane by the intermembrane space and the inner membrane has a folded structure, supposedly to maximize surface area. The reason for this is that the site of mitochondrial energy production, the respiratory chain, is located in the inner mitochondrial membrane.

A patient with severe hypermetabolism and abnormal mitochondria in skeletal muscle was diagnosed as the first known case of a mitochondrial disease by Luft and colleagues in 1962 (Luft et al. 1962). Since then the mitochondrial genome has been identified (Nass and Nass 1963) and subsequently sequenced (Anderson et al. 1981; Bibb et al. 1981), and today we know of a range of diseases caused by defects in mitochondrial energy production. Neurological symptoms are the most common clinical manifestations of these disorders, suggesting that the nervous system is especially sensitive to mitochondrial dysfunction. Furthermore, an increasing amount of evidence indicates that mitochondrial dysfunction is also an important causative factor in more common forms of neurodegenerative disease, such as Parkinson's and Alzheimer's disease, and even in the normally occurring aging process (Trifunovic et al. 2004).

MITOCHONDRIAL ENERGY PRODUCTION

Most of the cellular energy in the form of adenosine triphosphate (ATP) is produced by mitochondria through a process known as oxidative phosphorylation (OXPHOS). This process is carried out by the mitochondrial respiratory chain (RC), a series of five enzyme complexes embedded in the inner mitochondrial membrane.

The respiratory chain *(Figure 1, page 40)*

Together the five enzyme complexes of the RC consist of ~90 subunits out of which only 13 are encoded in the mitochondrial genome (mtDNA). The remaining subunits are encoded in nuclear DNA, synthesized in the cytoplasm and imported into mitochondria where the complete enzyme complexes are assembled. All complexes except complex II contain subunits encoded in mtDNA, and are thus dependent on the integrity and correct expression of this genome.

Electrons are fed by NADH into complex I (NADH dehydrogenase) or by succinate into complex II (succinate dehydrogenase) and are then passed to a pool of coenzyme Q (ubiquinone). From reduced coenzyme Q (ubiquinol) electrons are passed on to complex III (cytochrome *c* oxidoreductase) and then to cytochrome *c* and finally to complex IV (cytochrome *c* oxidase). As the electrons leave complex IV they reduce molecular oxygen, the final electron acceptor, to water. The transport of electrons through complexes I, III and IV, creates the driving force for pumping protons over the inner mitochondrial membrane and into the intermembrane space. This results in an electrochemical gradient over the inner mitochondrial membrane that is used to generate ATP as the protons flow back through complex V (ATP synthase) (Wallace 1992; Larsson and Clayton 1995).

Generation of reactive oxygen species

The RC is not only responsible for generating energy to be used in different cellular processes. It is also one of the main sites of reactive oxygen species (ROS) formation. ROS are extremely reactive molecules and oxidative damage is believed to be involved in many neurodegenerative diseases as well as in normal aging (Shigenaga et al. 1994; Betarbet et al. 2000). Superoxide ($O_2^{\bullet -}$) is formed when electrons passing through the RC leak and react with molecular oxygen. $O_2^{\bullet -}$ is rapidly converted into hydrogen

peroxide (H_2O_2), another oxidant, by the mitochondrial superoxide dismutase (MnSOD). In addition, in the presence of reduced metals such as Fe^{2+}, H_2O_2 can be converted to hydroxyl radical ($OH^{\bullet}$). $O_2^{\bullet-}$, $OH^{\bullet}$ and H_2O_2 are collectively denoted ROS and can damage cellular macromolecules including DNA, proteins and lipids (Andersen 2004).

The formation of ROS is probably not only a destructive process, leading to disease and aging. An increasing amount of evidence indicates that ROS formation is tightly regulated and that radicals actively participate in a number of normal cellular processes. Thus, radical formation in the context of oxidative signaling may not be a harmful by-product of enzymatic activity, but rather, an indispensable factor required for specific biological processes. Indeed, a subset of protein targets within the cell can alter their function in response to redox changes (Finkel 2003).

THE MITOCHONDRIAL GENOME

Vertebrate mitochondrial genomes (mtDNA) are closed circular molecules present in multiple copies, normally 1.000-10.000 molecules per cell. While mtDNA in different vertebrate species range in sizes between 16 and 18 kb, mammalian mitochondrial genomes are approximately 16.5 kb in size (human - 16.6 kb; mouse - 16.3 kb). Only 13 proteins, all essential components of the mitochondrial RC, are encoded in mtDNA while all other components of the mitochondrial proteome are synthesized in the cytoplasm and subsequently imported into mitochondria (Shadel and Clayton 1997). Nucleotide substitutions (point mutations) are estimated to present at a 10-fold higher frequency in mtDNA than in nuclear DNA (Brown et al. 1979). An individual cell can contain more than one species of mtDNA molecules, *e.g.* wild-type and mutant molecules, a condition referred to as heteroplasmy. The opposite situation, *i.e.* cells only containing one species of mtDNA, is referred to as homoplasmy (Larsson and Clayton 1995). The inheritance of mammalian mtDNA is considered to be strictly maternal (Birky 2001). Although one case of paternal transmission was recently described in a patient with mitochondrial myopathy (Schwartz and Vissing 2002), later studies motivated by this surprising finding have not been able to demonstrate other cases of paternal mtDNA transmission in humans. If paternal transmission indeed occurs it is probably a very rare event and mtDNA inheritance is still considered to be uniparental (Filosto et al. 2003; Taylor et al. 2003; Schwartz and Vissing 2004). The

underlying mechanism of maternal inheritance of mtDNA could be simple dilution of paternal mtDNA molecules (the oocyte contains 1000 times as many mtDNA molecules as the sperm) or active destruction of paternal mtDNA (Birky 2001). It has been shown in interspecific mouse crosses that paternal mtDNA can be transmitted to the offspring if the parents are of different species. These experiments suggest that there is a species-specific mechanism in place for the destruction of paternal mtDNA (Gyllensten et al. 1991; Kaneda et al. 1995; Shitara et al. 1998).

Structure of mtDNA *(Figure 2, page 40)*

Mammalian mtDNA contains only 37 genes that encode 13 mRNAs (all translated to RC proteins) as well as 22 tRNAs and 2 rRNAs necessary for the translation of the mRNAs. All other components needed for the correct expression and maintenance of mtDNA are imported from the cytoplasm (Shadel and Clayton 1997). The mammalian mitochondrial genome is extremely compact as all mRNA genes are immediately flanked by tRNA genes and completely lack introns (Anderson et al. 1981; Bibb et al. 1981; Montoya et al. 1981). In fact, the only longer non-coding part of the mtDNA molecule is the ~1 kb long displacement loop (D-loop) region, which has evolved as a regulatory region important for both transcription and replication of mtDNA. The D-loop contains the origin of heavy (leading) strand replication (O_H) as well as both the light (L) and the heavy (H) strand promoters (LSP and HSP) for initiation of transcription. The origin of light (lagging) strand replication (O_L) is nested in a cluster of five tRNA genes and it is not activated until the replication of the H strand has proceeded two thirds around the circular mtDNA molecule (Larsson and Clayton 1995). The heavy and light strand terminology used in mitochondrial genetics corresponds to the leading and lagging strand of replication, respectively. The two strands can be separated on alkaline cesium chloride gradients due to their inherent difference in density and have consequently been dubbed heavy and light strand (Clayton 1991). A large subset of mtDNA molecules constantly maintains a short piece of nascent DNA strand, starting at the O_H and ending close to a conserved sequence element, the termination associated sequences (TAS), at the end of the D-loop. This short (7S) DNA displaces the parental H strand and forms a bubble structure, thus the name displacement loop (Larsson and Clayton 1995).

Replication of mtDNA

Replication of mammalian mtDNA is under relaxed control and there is no mechanism to ensure that each molecule is replicated once and only once during each cell cycle. A minimal mitochondrial replisome was recently reconstituted *in vitro* using recombinant purified proteins. Mitochondrial DNA polymerase (POLγ) together with the TWINKLE DNA helicase, forms a processive replication machinery, capable of using dsDNA as a template for synthesis of short stretches of ssDNA. Addition of mitochondrial ssDNA-binding protein (mtSSB) stimulates the reaction further, generating products of about the size of the mitochondrial genome (Korhonen et al. 2004). Mitochondrial replication *in vivo* requires both RNA synthesis and subsequent processing for proper initiation, indirectly coupling transcription to replication (Larsson and Clayton 1995). At present, the exact mechanism by which mammalian mtDNA is replicated is intensely debated. For many years the widely accepted model was the *strand-asynchronous* (asymmetric) model of mtDNA replication (Clayton 1982). This model has recently been challenged as a more conventional *strand-synchronous* (symmetric) model, mainly based on 2-D gel electrophoresis data, has been put forward (Holt et al. 2000).

Clayton model (asymmetric)

The strand-asynchronous model of mtDNA replication postulates that there are two distinct origins of replication: the O_H in the D-loop and the O_L nested in five tRNA genes well away from the D-loop. Unidirectional DNA synthesis initiates at O_H, proceeds through the D-loop and around the mtDNA molecule, at the same time displacing the parental H strand. Not until leading-strand synthesis has reached two thirds of the genome, and O_L is exposed as a single-stranded structure, does lagging-strand synthesis in the opposite direction initiate (Clayton 1982). Replication is coupled to transcription and the short primer needed to initiate replication at O_H is believed to be generated by transcription from the LSP. The switch from transcription to replication takes place near three conserved sequence blocks (CSBI-III) located between LSP and O_H and requires processing by the endoribonuclease RNase MRP (Chang and Clayton 1985; Shadel and Clayton 1997).

Holt model (symmetric)

The more recent strand-synchronous model of mtDNA replication has been added on to in several steps (Holt et al. 2000; Yang et al. 2002; Bowmaker et al. 2003). These studies have all used 2-D gel electrophoresis to demonstrate the presence of conventional replication forks (Y arcs on 2-D gels) in isolated mitochondria, indicative of coupled leading and lagging-strand synthesis. Coupled replication was first believed to initiate at or near O_H and then proceed unidirectionally around the mtDNA molecule (Holt et al. 2000). This model was later modified when replication was demonstrated to initiation at multiple sites across a broad zone in mtDNA molecules and subsequently proceed bidirectionally (Bowmaker et al. 2003). Large regions of RNA:DNA hybrid was furthermore shown to be present in mitochondrial replication intermediates. These ribonucleotides are believed to be a result of ribonucleotide incorporation on the L strand during coupled replication. If the ribonucleotide part of such a hybrid is lost during nucleic acid preparation, the remaining single-stranded DNA may resemble the displaced H strand described in the strand-asynchronous model of replication (Yang et al. 2002).

Transcription of mtDNA

The mitochondrial genome contains 37 genes that are distributed on both DNA strands and is expressed as three polycistronic transcription units. The mitochondrial heavy-strand promoter (HSP) and light-strand promoter (LSP) are located about 150 bp apart within the regulatory D-loop regulatory region. Two overlapping transcription units of different size are generated by transcription from the HSP, while the third transcription unit is synthesized from the LSP. The L-strand transcription unit contains only 1 mRNA and 8 tRNAs, but as mentioned above, LSP transcription is also involved in mtDNA replication according to the strand-asynchronous model of replication. The shorter of the two H-strand transcription units contains the 12S and 16S rRNA genes as well as 2 tRNAs. The other, almost genome-length, transcript contains the two rRNAs, 13 tRNAs and 12 mRNAs (Fernandez-Silva et al. 2003). Containing mainly the two rRNAs, the short H-strand transcription unit is generated at a higher frequency than the longer genome-length unit, probably as a way of differentially regulating the synthesis of rRNAs and H-strand mRNAs. The increased activity of this short transcription unit

is linked to a transcription termination event immediately downstream of the 16S rRNA gene inside the gene for $tRNA^{Leu}$ (Gelfand and Attardi 1981; Montoya et al. 1983). This termination event is mediated by the mitochondrial termination factor (mTERF), binding to a tridecamer sequence in the tRNA Leu^{UUR} gene (Christianson and Clayton 1988; Kruse et al. 1989). Another level of H-strand transcription regulation may be the suggested additional H-strand promoter (HSP-2), located within the $tRNA^{Phe}$ gene just upstream of the 12S rRNA gene. According to this model, transcription from the main HSP in the D-loop always terminate after the 16S rRNA gene, while the 20 times less frequent initiation at HSP-2 continues past the termination point, synthesizing near genome-length H-strand transcripts (Montoya et al. 1982; Montoya et al. 1983). Thus both transcription initiation and transcription termination events may contribute to determining the relative abundance of the two H-strand transcription units.

For a long time, only two components of the basal human mitochondrial transcription machinery had been identified: mitochondrial RNA polymerase (POLRMT) and mitochondrial transcription factor A (TFAM). Studies showed that a partly purified POLRMT fraction, together with pure TFAM, was adequate for correct transcription initiation *in vitro* (Fisher and Clayton 1985; Fisher and Clayton 1988). Attempts to use pure POLRMT in these assays were unsuccessful, suggesting that an additional factor was present in the partly purified POLRMT fraction. Indeed, an additional specificity factor necessary for correct mitochondrial transcription has been identified in the yeast *S. cerevisiae* (Schinkel et al. 1987) as well as in the vertebrate (frog) *X. laevis* (Antoshechkin and Bogenhagen 1995; Bogenhagen 1996). Recently, two homologous human proteins: mitochondrial transcription factors B1 and B2 (TFB1M and TFB2M) were identified. This resulted in the successful reconstitution of the basal human mitochondrial transcription machinery in a pure *in vitro* transcription system. In addition to the previously known factors POLRMT and TFAM, correct transcription in this system required TFB1M or TFB2M. Both proteins interact directly with POLRMT, but TFB2M is at least one order of magnitude more active in promoting transcription than TFB1M (Falkenberg et al. 2002).

MITOCHONDRIAL TRANSCRIPTION FACTOR A (TFAM)

TFAM (previously mtTFA, mtTF1, TCF6 and TCF6L2) is a ubiquitously expressed nuclear encoded transcriptional activator that is imported into mitochondria, where it is essential for mitochondrial transcription and mtDNA maintenance.

Early *in vitro* transcription studies dissecting the human mitochondrial transcription machinery, showed that in addition to mitochondrial RNA polymerase, an additional factor of unknown identity was necessary for specific transcription initiation from mitochondrial promoters (Fisher and Clayton 1985). This factor (TFAM) was later identified as a protein of about 25 kDa that showed specific binding affinity to sequences immediately upstream of both HSP and LSP, although it also displayed considerable binding to random DNA sequences. Interestingly, transcription activation from the LSP was clearly stronger and responded to a much wider TFAM concentration range, compared to HSP transcription (Fisher et al. 1987; Fisher and Clayton 1988). The function of TFAM seemed to be well conserved in mammals as human and mouse TFAM proteins were shown to be functionally interchangeable. Each protein could bind the heterologous LSP and activate transcription, despite the sequence identity at the binding sites being only 50% (Fisher et al. 1989). When human TFAM was finally cloned and sequenced it was evident that it shared strong similarities with the high-mobility group (HMG)-box group of proteins. The general protein structure contained two HMG boxes separated by a short linker region, followed by a carboxyl-terminal tail (Parisi and Clayton 1991). Consistent with the function of other HMG-box proteins, TFAM was shown to be capable of wrapping and binding random DNA sequences (Fisher et al. 1992). Later mutational analyses revealed that the carboxyl-terminal tail was essential for the function of TFAM as a transcriptional activator (Dairaghi et al. 1995). As the mouse *Tfam* gene was cloned and characterized by Larsson and colleagues in 1996, mouse and human TFAM peptide sequences were shown to share 81% similarity and 61% identity. Thus TFAM protein structure seemed to be conserved in mammals, consistent with the fact that mouse and human proteins had earlier been shown to be functionally interchangeable (Fisher et al. 1989; Larsson et al. 1996).

Homozygous knockout of *Tfam* in the mouse is embryonic lethal as embryos die in mid-gestation between embryonic day (E) 8.5 and E10.5. Knockout embryos have a

mutant phenotype with delayed neural development, indistinct somites, lack of optic discs and no heart structures. They also contain abnormal enlarged mitochondria, similar to those observed in patients with mitochondrial disease, and completely lack mtDNA. Heterozygous knockouts are viable and develop normally but have reduced levels of mtDNA in all tissues and a mild RC deficiency in the heart (Larsson et al. 1998). Several conditional tissue-specific *Tfam* knockouts have been described and they all manifest as a mitochondrial disease in the affected tissue, resulting from a loss of mtDNA and mitochondrial transcripts and a severe RC deficiency (Wang et al. 1999; Li et al. 2000; Silva et al. 2000; Sorensen et al. 2001; Wredenberg et al. 2002; Hansson et al. 2004).

The fact that TFAM is essential for correct transcription initiation, and that it specifically recognizes and binds mitochondrial promoters, is in full agreement with its function as a transcription factor (Gaspari et al. 2004). The unspecific DNA binding activity of the protein as well as the apparent dramatic effect on mtDNA copy number in *Tfam* knockouts, are however not expected of a typical transcription factor. Furthermore, nuclear transcription factors are normally present only at minute amounts while TFAM seems to be quite an abundant protein. Estimates of the molar ration between TFAM and mtDNA in mammals vary considerably from as low as 15 TFAM molecules per mtDNA (Fisher and Clayton 1988) up to 1700 (Alam et al. 2003). The latter high estimate suggests that TFAM indeed binds the entire length of the mtDNA molecule, perhaps as a stabilizing factor in mitochondrial chromatin. In fact, several studies have lately indicated that mtDNA is organized in a chromatin-like higher-order structure and that TFAM may be an integral part of that structure (Spelbrink et al. 2001; Alam et al. 2003; Garrido et al. 2003).

Several clues to an additional function of TFAM can be had from studies of the yeast *S. cerevisiae* TFAM homologue Abf2p (ARS-binding factor 2). Abf2p completely lacks the carboxyl-terminal tail present in TFAM and has only a minor effect on yeast mitochondrial transcription (Xu and Clayton 1992). Furthermore, Abf2p levels have been shown to regulate mtDNA copy number (Zelenaya-Troitskaya et al. 1998) and disruption of the *ABF2* gene leads to complete loss of mtDNA in yeast grown on a fermentable carbon source (Diffley and Stillman 1991). These data indicate that the main role of the yeast TFAM homologue, Abf2p, is not in transcription but rather in mtDNA stability and maintenance. Interestingly, human TFAM has been shown to be

able to complement the loss of Abf2p in yeast cells with a disrupted *ABF2* gene (Parisi et al. 1993). Abf2p can conversely be converted into a functional transcriptional activator in mammals by only adding the human TFAM carboxyl-terminal tail to the protein (Dairaghi et al. 1995).

In conclusion, TFAM is without doubt a *bona fide* transcription factor in mammalian mitochondria. It specifically recognizes sequences upstream of both mitochondrial promoters and is absolutely essential for transcription initiation. However, the high abundance of TFAM as well as its strong unspecific DNA binding activity together with the fact that TFAM is necessary for mtDNA maintenance suggests that it is has additional functions. One likely explanation would be that TFAM binds and protects mtDNA in chromatin-like structure, similar to the situation in yeast mitochondria, where Abf2p has a role in mtDNA stability and maintenance.

MITOCHONDRIA IN NEURODEGENERATIVE DISEASE

Neurological symptoms such as ataxia, epilepsy, paresis and dementia are common features of mitochondrial disorders. The central and peripheral nervous systems as well as the visual and auditory sensory systems are often affected, perhaps because of inherent high energy demand in those tissues. Mitochondrial dysfunction is also increasingly recognized as a possible causative factor in more common neurodegenerative disorders, such as Parkinson’s disease and Alzheimer’s disease.

Mitochondrial disorders

More than 40 years ago, a patient presenting with hypermetabolism whose skeletal muscle contained large numbers of abnormal mitochondria, was diagnosed as the first known case of a mitochondrial disorder (Luft et al. 1962). An impressive achievement especially considering that the existence of mitochondrial DNA was not demonstrated until the following year (Nass and Nass 1963). Although the pathogenesis of many different disorders could be said to involve some aspect of mitochondrial dysfunction, the term “mitochondrial disorder” is mostly used to describe clinical syndromes related to impaired mitochondrial energy production through oxidative phosphorylation (OXPHOS). Clinical manifestations of mitochondrial disorders vary tremendously. They range from specific lesions in a single tissue to widespread multisystem syndromes. However, the visual and auditory systems, as well as the central and

peripheral nervous systems seem to be the tissues most sensitive to disruption of OXPHOS, as neurological symptoms are the most common clinical manifestations of mitochondrial disorders. The genetic origin of mitochondrial disorders can be both mtDNA mutations and mutations in nuclear genes. Some syndromes, such as the early onset encephalopathy Leigh syndrome can be caused by both defects in mtDNA and nuclear DNA (Zeviani and Di Donato 2004).

Mitochondrial DNA mutations

Defects in mtDNA can be either point mutations or rearrangements, such as deletions or duplications. Point mutations are most often maternally inherited while large-scale rearrangements normally are sporadic. The human mitochondrial genome was sequenced in 1981 (Anderson et al. 1981), leading to the first descriptions of disease causing point mutations (Wallace et al. 1988) and mtDNA deletions (Holt et al. 1988).

“Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes” (MELAS) and “myoclonic epilepsy with ragged red fibers” (MERFF) are two maternally inherited disorders caused by heteroplasmic point mutations in mtDNA. Their clinical manifestations contain clear neurological components such as stroke-like episodes due to focal brain lesions (MELAS), epilepsy and cerebellar ataxia (MERFF), as well as dementia (both). Two disorders caused by heteroplasmic mtDNA rearrangements, that also display neurological symptoms, are Kearns-Sayre syndrome (KSS) and sporadic progressive external ophthalmoplegia (PEO) (Zeviani and Di Donato 2004).

Mutations in nuclear genes

Mutations in a wide variety of nuclear genes can directly or indirectly affect mitochondrial energy production. Patients with autosomal dominant progressive external ophthalmoplegia (adPEO) accumulate multiple mtDNA deletions due to mutations in nuclear genes (Zeviani et al. 1989). Mutations have been identified in three genes: *ANT1*, encoding the adenine nucleotide translocator (Kaukonen et al. 2000); *TWINKLE*, a mitochondrial helicase (Spelbrink et al. 2001); *POLG1*, encoding the catalytic subunit of mitochondrial DNA polymerase (Van Goethem et al. 2001). These are all proteins involved in mtDNA maintenance thus indirectly affecting

OXPHOS function. Several mutations in nuclear DNA encoded complex I and complex II subunits cause neurological disorders (Bourgeron et al. 1995; Morris et al. 1996; Parfait et al. 2000). Furthermore, mutations in the *SURF1, SCO1, SCO2* or *COX10* genes prevent proper assembly of complex IV and are also a common cause of mitochondrial encephalopathies (Papadopoulou et al. 1999; Tiranti et al. 1999; Valnot et al. 2000a; Valnot et al. 2000b). Thus mutations in RC subunits, directly affecting OXPHOS, can cause similar symptoms as mutations in proteins necessary for assembly of RC complexes.

Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by selective loss of dopaminergic (DA) neurons in substantia nigra pars compacta (SNpc). The vast majority (>90%) of cases are sporadic but several rare familial forms, often with an early age of onset, have been described. Clinical manifestations of PD include bradykinesia, resting tremor, rigidity and impaired balance. A pathological hallmark of PD is the presence of Lewy bodies, round esosinophilic intracellular inclusions, in surviving neurons of affected areas.

The first mutation shown to cause one of the rare familial forms of PD was found in the α-synuclein gene (Polymeropoulos et al. 1997). Interestingly, α-synuclein was later shown to be a major component of the Lewy bodies observed in post-mortem PD patient brains, also in sporadic cases without any mutation in the α-synuclein gene (Spillantini et al. 1997). Overexpression of wild-type α-synuclein has also been shown to cause PD in humans (Singleton et al. 2003). Two additional mutations causing PD affects ubiquitin carboxyl-terminal hydrolase I (*UCHL1*) and the ubiquitin E3 ligase *parkin*. Both are components of the ubiquitin-proteasome system that degrades damaged proteins (Kitada et al. 1998; Leroy et al. 1998). Pathogenic mutations have also been identified in DJ-1, a protein that has a role in the oxidative stress response (Bonifati et al. 2003). The most recently identified mutation in familial PD is in the putative mitochondrial protein kinase called PTEN-induced kinase I (*PINK1*) (Valente et al. 2004). Thus the genetics of familial PD indicate that aberrant protein handling, oxidative stress and mitochondrial dysfunction, play an important role in the pathogenesis of the disease.

Several other observations strengthens the idea that mitochondrial dysfunction is an important player in PD neurodegeneration. **(1)** A decreased activity of complex I of the RC has been found in brains as well as in platelets of PD patients (Mizuno et al. 1989; Parker et al. 1989; Schapira et al. 1989). **(2)** Certain mitochondrial DNA (mtDNA) polymorphisms reduce the risk of PD (van der Walt et al. 2003). **(3)** MPP+, the toxic metabolite of 1-methyl-4-1,2,3,6-tetrahydropyridine (MPTP), inhibits complex I of the RC and causes parkinsonism after selective uptake in DA neurons (Langston et al. 1983; Mizuno et al. 1987). **(4)** The pesticide rotenone is a strong inhibitor of complex I and long term exposure leads to loss of DA neurons in certain strains of rats (Betarbet et al. 2000). **(5)** The herbicide paraquat inhibits complex I and can in combination with other agents cause selective degeneration of DA neurons (Thiruchelvam et al. 2000).

Animal models of Parkinson's disease

The use of animal models is crucial for our understanding of neurodegenerative disease. In this paragraph I will briefly go through the most commonly used animal models of PD today. The focus will be on rodent models, as these constitute the bulk of the models used in labs over the world, although primates, flies and other animals are also used.

An ideal genetic model for PD would have the following characteristics (Beal 2001): **(1)** Normal number of dopaminergic (DA) neurons at birth with a gradual loss during adulthood. **(2)** Easily detectable motor deficits including bradykinesia, rigidity and resting tremor and transient effective restoration of motor ability in response to L-DOPA. **(3)** Histology showing typical α-synuclein pathology including formation of the intracellular inclusions known as Lewy bodies. **(4)** Changes in DA levels and DA metabolism paralleling human PD. **(5)** Robust genetics allowing easy propagation of the genotype. **(6)** A progressive disease course of just a few months for rapid testing of different therapeutic strategies.

6-Hydroxydopamine (6-OHDA) was the first chemical discovered that had specific neurotoxic effects on catecholaminergic pathways. Since 6-OHDA is unable to cross the blood-brain barrier, the toxin is injected stereotactically into the substantia nigra or the striatum. Injections into the substantia nigra result in a rapid depletion of striatal

dopamine (2-3 days) while injections into the striatum result in a slower retrograde degeneration over a period of weeks. One advantage with this model is that usually injections are unilateral, with the untreated hemisphere serving as an internal control. Unilateral injections furthermore lead to asymmetric circling motor behavior after administration of dopaminergic drugs. This stereotypic turning behavior can easily be quantified and correlated with the degree of lesion. However, the acute nature of the model differs from the progressive nature of PD and several key clinical and pathological features of PD, such as presence of Lewy bodies, are not mimicked.

Unintentional injection of MPTP caused clinical symptoms very similar to PD in humans. After systemic administration, MPTP passes the blood-brain barrier and is converted in astrocytes to its active metabolite MPP+. MPP+ is taken up into DA neurons by the dopamine transporter, and is thus selectively toxic, through inhibition of complex I, to this neuronal population. Administration of MPTP results in nigro-striatal dopaminergic degeneration in a number of species, but susceptibility to the toxin varies between species and strains of animals. Rats are very resistant to MPTP toxicity and mouse strains vary extensively in their sensitivity to the toxin. Thus the MPTP model of PD has most of its merits in primate systems where administration of the toxin has been shown to mimic several behavioral characteristics of PD (Hantraye et al. 1993) as well as α-synuclein aggregation (Kowall et al. 2000). Administration of MPTP is mostly acute and the outcome variable, hence, also this model is less than ideal for modeling a progressive disorder such as PD.

Systemic low-dose administration of the pesticide and potent inhibitor of complex I, rotenone, has been demonstrated to induce specific degeneration of the nigrostriatal pathway in certain strains of rats. The central difference to the MPTP model is that rotenone causes a moderate RC deficiency in all tissues, as there is no specific uptake into DA neurons. The rotenone model is also considerably more progressive than the 6-OHDA or MPTP models, and reproduces several behavioral characteristics of PD as well as α-synuclein aggregates similar to Lewy bodies. There is, however, large inter-animal variability as well as substantial morbidity and mortality, probably due to systemic effects of the drug (Betarbet et al. 2000).

In a very recent study McNaught and colleagues demonstrated that repeated injections of proteasome inhibitors cause selective degeneration of the nigrostriatal pathway as well as of other brain areas in rats. Injected rats progressively develop a parkinsonian

condition and post-mortem analyses showed presence of α-synuclein/ubiquitin containing inclusions (McNaught et al. 2004).

Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia, characterized by the formation and accumulation in the brain of extracellular plaques together with intraneuronal neurofibrillary tangles. One of the main components of these plaques is amyloid-β (Aβ), a 39-43 amino acid peptide generated through processing of the Aβ precursor protein (APP). All known mutations associated with familial AD are in the genes for APP or the presenilins, enzymes involved in APP processing (Canevari et al. 2004). Indeed, accumulation of Aβ seems to be sufficient to cause dementia, even though formation of actual plaques may not be necessary. In AD, it seems to be the total load of Aβ in the brain, rather than the plaque load, that correlates with the degree of cognitive impairment (Naslund et al. 2000).

Several observations suggest that mitochondrial dysfunction plays an important role in the pathogenesis of AD. A reduction in glucose metabolism has been observed in AD brains (Ibanez et al. 1998) and reduced mitochondrial mass as well as reduced mtDNA levels have been shown to be early pathological signs in AD (de la Monte et al. 2000; Hirai et al. 2001). The activities of several key mitochondrial enzymes, especially complex IV, have been shown to be decreased in AD brains (Mutisya et al. 1994; Chandrasekaran et al. 1996). Decreased complex IV activity has even been demonstrated in platelets of AD patients, indicating that a systemic decrease in mitochondrial function may cause AD (Parker et al. 1994). Cytoplasmic hybrid cell lines (cybrids), containing mitochondria from sporadic AD patients, also display several characteristics similar to the disease. Increased accumulation of Aβ, decreased mitochondrial membrane potential, oxidative stress and decreased complex IV activity have been demonstrated in such cell lines (Khan et al. 2000). However, no convincing evidence that identifies specific mtDNA mutations as directly causative factors in AD exists to date. Recently, a study demonstrated that full-length APP is partly targeted to mitochondria, where it may block the mitochondrial import machinery, thus causing mitochondrial dysfunction (Anandatheerthavarada et al. 2003).

Huntington's disease

Huntington's disease (HD) is a progressive and lethal neurological disorder first described by George Huntington in 1872 (Huntington 2003). The major symptoms in HD are chorea, dystonia, psychiatric impairment and decreased cognitive functions. The most prominent neuropathological change in HD is the progressive loss of GABA-ergic (γ-aminobutyric acid) neurons in the caudate putamen. The genetic basis of HD is an expansion of a trinucleotide CAG repeat in the *Huntingtin* gene, a gene encoding a widely expressed cytoplasmic protein of unknown function. How mutant huntingtin is damaging to the cell or how it causes brain region-specific damage is not known. One prominent characteristic of HD pathogenesis is altered energy metabolism, as evidenced by reduced glucose metabolism in affected areas as well as elevated lactate levels. Studies have also shown decreased activities of RC complexes II-IV in symptomatic HD patients. Furthermore, mitochondrial toxins that inhibit complex II (3-NP and malonate) induce striatal-specific lesions in humans and rodents that resemble those seen in HD (Browne and Beal 2004).

SPECIFIC AIMS

The general aim of this thesis was to study the effects of mitochondrial dysfunction in different neuronal populations. To accomplish this we used tissue-specific knockouts of TFAM and the two first papers address different aspects of TFAM function.

The specific aim of each paper was to:

Paper I: Study if human *TFAM* functionally can complement a germ-line knockout of mouse *Tfam* and to study if TFAM protein levels regulate mtDNA copy number *in vivo.*

Paper II: Study if human TFAM functional can complement tissue-specific knockout of mouse *Tfam* in a differentiated tissue such as heart

Paper III: Study the effects of mitochondrial dysfunction and RC deficiency in forebrain neurons.

Paper IV: Test the hypothesis that mitochondrial dysfunction has a critical role in the pathogenesis of Parkinson's disease by knocking out TFAM selectively in dopamine neurons.

RESULTS AND DISCUSSION

EXPRESSING HUMAN TFAM IN THE MOUSE (PAPER I)

We know from numerous knockout studies in the mouse that loss of TFAM inevitably leads to loss of both mtDNA and mitochondrial transcripts (Larsson et al. 1998; Wang et al. 1999; Li et al. 2000; Silva et al. 2000; Sorensen et al. 2001; Wredenberg et al. 2002; Hansson et al. 2004). Although TFAM is a *bona fide* transcription factor that is absolutely required for transcription initiation, it is also generally accepted that TFAM has a role in mtDNA maintenance in mammals. It is however not clear if TFAM directly regulates mtDNA copy number. TFAM dependent mtDNA maintenance may be related to the RNA primer needed for correct replication initiation at O_H. However, the unspecific DNA-binding capacity of TFAM raises the question if TFAM also play a role as a stabilizing protein in mitochondrial chromatin. In this study we expressed a transcriptionally impaired form of TFAM in the mouse to distinguish between these two different aspects of TFAM function.

Human TFAM is a poor activator of mouse transcription *in vitro*

We developed a mouse mitochondrial *in vitro* transcription system using purified recombinant histidine-tagged proteins. As in a previously described human system (Falkenberg et al. 2002), a combination of the three components: POLRMT, TFB2M and TFAM was necessary for transcription initiation from templates containing the L or H strand mitochondrial promoters. We substituted the mouse TFAM protein (mTFAM) in the transcription assay with recombinant human TFAM (hTFAM) to study the ability of this protein to initiate transcription in the mouse system. It was evident from this experiment that, compared to endogenous mTFAM, the human protein was a very poor activator of transcription in the mouse system, especially when initiating transcription from the HSP.

The observed difference in transcription initiation capacity could, theoretically, be due to differences in unspecific DNA binding capabilities. To compare the unspecific DNA binding capabilities of human and mouse TFAM we performed gel retardation analyses with plasmid DNA. The human and mouse TFAM proteins gave identical

results, and thus the reduced capacity of hTFAM to activate transcription in mouse assays could not be explained by differences in unspecific DNA binding capabilities.

Knowing that hTFAM is a poor activator of mouse mitochondrial transcription, while retaining a DNA binding capacity similar to that of the mouse protein, prompted us to generate a transgenic mouse line expressing hTFAM. Such a mouse would give us the opportunity to distinguish, *in vivo*, between the two roles of TFAM, i.e. the activity in specific transcription initiation *vs*. the unspecific DNA binding ability of the protein. It would at the same time enable us to study the effects of generally increased TFAM levels in mammalian tissues.

PAC-TFAM transgenic mice have a net overexpression of TFAM protein

We made P1-artificial chromosome (PAC) transgenic mice using large stretches (120-170 kb) of human genomic DNA containing the full *TFAM* gene. PAC-transgenes are advantageous in that they contain important regulatory sequences (genomic DNA), and that, to a certain extent, their size diminish the positional effects normally seen with conventional cDNA-transgenes. To control for TFAM-independent effects of neighboring genes also present in the large PACs, we generated mice from three different PAC-clones (PAC2, PAC9 and PAC19), centered around the *TFAM* gene but with different flanking sequences.

Southern blot genotyping of the three PAC-TFAM transgenic lines showed that all of them contained only a few copies of their respective transgenes. The PAC9 and PAC19 lines had a slightly higher copy number than the PAC2 line. Western blot analyses showed that hTFAM was expressed at roughly the same level as endogenous mTFAM in all three lines and in all studied tissues. Importantly, we could not see any signs of decreased mTFAM protein levels in PAC-TFAM transgenic mice. This clearly showed that the addition of hTFAM to the mouse mitochondrial genetic system was not compensated for by down-regulation of endogenous mTFAM.

Mitochondrial DNA copy number is proportional to TFAM protein levels

To see if the net TFAM overexpression resulted in increased mtDNA copy number we used Southern blot analyses to measure mtDNA levels in heart, kidney and skeletal muscle of all three transgenic lines. These experiments clearly demonstrated

not only that these tissues contained significantly elevated mtDNA levels, but also that the increase correlated with *TFAM* transgene copy number. PAC2 mice generally showed a 30-40% increase in mtDNA copy number while the increase in PAC9 and PAC19 mice was 40-70%, depending on the tissue studied.

To estimate the molar TFAM:mtDNA ratio, we used a combination of Southern and western blot analyses on DNA and proteins, respectively, from isolated kidney mitochondria. These experiments gave us an estimate of one TFAM molecule per 15-20 bp of mtDNA for both PAC19 and wild-type mice, well in agreement with other estimates (Takamatsu et al. 2002; Alam et al. 2003). Taken together these results show that mtDNA copy number is directly proportional to TFAM protein levels in these mice and that TFAM alone can regulate mtDNA copy number in a variety of mammalian tissues. The fact that TFAM is very abundant within mitochondria, much more abundant than would be expected for a classical transcription factor, suggests that TFAM is indeed an integral part of the mitochondrial chromosome.

Mitochondrial DNA levels can be dissociated from mtDNA expression

Next, we probed northern blots with strand-specific riboprobes to measure steady-state levels of LSP and HSP transcripts in kidneys from PAC-TFAM transgenic mice. We could not detect any changes in cytochrome *c* oxidase subunit I (COXI) or NADH dehydrogenase subunit 4 (ND4) mRNAs, both generated by transcription from the HSP. In contrast, both PAC9 and PAC19 animals contained 50-60% increased levels of the LSP transcript NADH dehydrogenase subunit 6 (ND6). These results are consistent with the *in vitro* studies where hTFAM was a poor activator of mouse transcription, in particular from the HSP. No corresponding increase could be demonstrated in PAC2 animals, but as described above they have a lower transgene copy number and a less significant increase in mtDNA levels.

12 of the 13 RC subunits encoded by mtDNA are generated by transcription from the HSP. Thus we did not expect to see an increase in RC activity in PAC-TFAM transgenic animals. We measured the activity of complex II and IV of the RC in kidneys, but as expected, we could not detect any changes. Citrate synthase activity, a commonly used marker for mitochondrial mass, was also unchanged in these samples. Furthermore, when measuring the volume density of mitochondria on electron micrographs, we failed to see any changes in PAC-TFAM transgenic mice.

The human *TFAM* gene cannot rescue homozygous *Tfam* knockout embryos

In a final experiment we attempted to complement the loss of endogenous mTFAM in *Tfam* knockout animals with hTFAM. Homozygous knockout of *Tfam* is embryonic lethal and *Tfam*$^{-/-}$ embryos die during mid-gestation between embryonic day (E) 8.5 and E10.5. We used a two-step breeding strategy in an attempt to generate homozygous *Tfam* knockout animals also carrying a *PAC-TFAM* transgene (*Tfam*$^{-/-}$*; PAC-TFAM*). Unfortunately, we were unable to obtain such mice using any of the three *PAC-TFAM* transgenes. However, when we isolated embryos at E8.5-E9.5 we found *Tfam*$^{-/-}$*; PAC-TFAM* embryos at the expected Mendelian frequency. Interestingly, Southern blot quantification showed that such embryos contained ~30% of normal mtDNA levels while *Tfam*$^{-/-}$ embryos had hardly detectable levels. These "partially rescued" embryos still displayed signs of abnormal development; were smaller in size compared to wild-type embryos and were unable to survive. Northern blot analyses of steady-state transcript levels demonstrated slightly reduced levels of LSP transcripts (ND6) but very low levels of HSP (COXI) transcripts, as compared to wild-type embryos. These results combined suggest that hTFAM is unable to initiate a sufficient amount of HSP transcription in the mouse, thus inferring embryonic lethality due to depleted mitochondrial transcripts.

In summary, PAC-TFAM transgenic mice showed increased mtDNA copy number and increased LSP transcript levels, but no corresponding increase in RC activity or mitochondrial mass. Thus elevated mtDNA levels alone, are not sufficient to increase expression of mitochondrial genes. This provides evidence that it is indeed possible to dissociate regulation of mtDNA copy number from mitochondrial function, and that TFAM plays an important role in the former.

RESCUING TISSUE-SPECIFIC *TFAM* KNOCKOUT MICE (PAPER II)

In **paper I** we demonstrated that human TFAM is a poor activator of mouse mitochondrial transcription and is subsequently unable to rescue homozygous *Tfam* knockout embryos. The load on the mitochondrial genetic system is probably very high during embryonic development because of extensive cell proliferation. We hypothesized that the transcriptional activity of hTFAM could be sufficient to support

mitochondrial function in a differentiated adult tissue such as cardiac muscle. In this study we attempted to rescue or improve the cardiomyopathy phenotype of tissue-specific knockout mice, with TFAM depletion in the heart, by crossing them to PAC19 hTFAM expressing animals.

Human TFAM fully rescues the cardiac hypertrophy in *Tfam* knockout hearts

In a previous study we used the cre-loxP recombination system to generate heart- and muscle-specific *Tfam* knockout mice. We used a mouse strain expressing cre recombinase from the muscle creatine kinase (*Ckmm*) promoter to induce tissue-specific inactivation of a loxP-flanked *Tfam* locus ($Tfam^{loxP}$). Tissue-specific knockout mice develop a dilated cardiomyopathy and die at around 12 weeks of age because of severe depletion of mitochondrial DNA and mitochondrial transcripts in the heart. Knockout mice suffer from a cardiac hypertrophy as evidenced by an increased heart weight/body weight (HW/BW) ratio (Hansson et al. 2004). In several breeding steps we generated tissue-specific *Tfam* knockout mice also carrying the PAC19 transgene expressing the human *TFAM* gene. Such mice developed normally and still appeared healthy when their knockout littermates started to display symptoms of disease. We followed them for up to one year of age and observed no abnormalities during this time. In contrast to knockout animals the HW/BW ratio of “rescued” animals was completely normal at both 12 weeks and 12 months of age. This clearly showed that hTFAM could functionally replace mTFAM in a fully differentiated heart, despite its reduced capabilities as a transcriptional activator.

Reduced HSP transcript levels in rescued hearts associated with a moderate respiratory chain deficiency

We used Southern and northern blot analyses to measure levels of mtDNA and mitochondrial transcripts in hearts of rescued mice. While we were not able to detect changes in mtDNA or LSP transcript levels, transcripts generated from the HSP appeared to be reduced by ~20-30% in rescued hearts.

To investigate the possible effects of decreased HSP transcription, we directly measured the activity of different RC complexes as well as mitochondrial ATP-production rate in hearts of 1 year old mice. These measurements showed that hearts of

rescued mice were negatively affected by the slight reduction in HSP transcript levels. Complex VI (cytochrome *c* oxidase) activity was significantly reduced by 25%, paralleled by a decrease in ATP production when feeding substrate through complexes containing subunits encoded in mtDNA (all except complex II). The general decrease in ATP production was ~15% with an even greater reduction of ~25% when feeding substrate only through complex IV. Surprisingly, complex I (NADH coenzyme Q reductase) activity showed a small but significant up-regulation of ~10%.

To determine if this moderate complex IV deficiency and decreased ATP production affected heart function, we used northern blots to measure transcript levels of atrial natriuretic factor (ANF). ANF is a well known and commonly used marker for heart failure that is released into the circulation in response to atrial dilatation or increased intravascular fluid volume (Seidman et al. 1984; de Bold et al. 1996). ANF transcript levels were about 5-fold higher in rescued compared to normal hearts, strongly suggesting that these hearts responded to the moderate RC deficiency.

Rescued hearts contain high levels of an HSP transcript of unknown function

Given the results from **paper I** we were initially surprised to see that transcript levels were normal (LSP) or only slightly reduced (HSP) in hearts of rescued animals. We used northern blot analyses to study possible modifications in transcription in the D-loop regulatory region of mtDNA. We found high levels of a novel 1 kb transcript, approximately covering the non-coding D-loop regulatory region, in hearts from rescued mice. This transcript was not present or present at very low levels in kidneys of rescued mice or in hearts of wild type, knockout or PAC-TFAM transgenic mice. In order to determine if this transcript was generated from the heavy strand (HSP) or light strand (LSP) promoter we probed the northern blots with strand-specific riboprobes. This clearly showed that the transcript originated from the HSP. This is surprising since HSP transcription is believed to normally terminate before the D-loop region and reinitiate at the promoter for a new round of transcription of the circular mtDNA molecule. A similar transcript has been described by Selwood and colleagues in a study where they inhibited mitochondrial protein synthesis in cell lines (Selwood et al. 2001). One explanation for this could be that mitochondria under stress, requiring greater

amounts of transcripts, allow the RNA polymerase to read through the D-loop region when transcribing from the HSP.

THE MILON MOUSE – MODELING MITOCHONDRIAL NEURO-DEGENERATION (PAPER III)

Deficient function of the mitochondrial RC is increasingly recognized as an important cause of neurodegenerative disease. Mitochondrial dysfunction has been implicated in the pathogenesis of Parkinson's and Alzheimer's diseases, as well as in normal aging. Furthermore, dementia is a common symptom in mitochondrial disease suggesting that neurons are sensitive to impaired mitochondrial function. We used a forebrain specific conditional knockout of *Tfam* to study the effects of RC failure in neurons of cerebral cortex and hippocampus.

Mice survive for several months with *Tfam* knockout in forebrain neurons

By crossing $Tfam^{loxP}$ mice to a forebrain-specific cre mouse strain, we generated mice with a RC deficiency restricted to forebrain neurons. Targeted cre expression was achieved by using the CaMKII-cre mouse previously described by Xu et al., a transgenic strain expressing cre mainly in neurons of cerebral cortex and hippocampus (Xu et al. 2000). The CaMKII-cre mouse has a peak cre expression, and subsequently recombination of *loxP*-sites, around postnatal day (P) 14.

Western blot analysis of neocortical homogenates showed that TFAM levels were reduced from 2 months of age and throughout the life of knockout mice. Despite this, the mice appeared normal and showed no overt signs of disease or behavioral disturbances for several months. Not until they reached the age of 5-6 months did they start to develop symptoms and display signs of a deteriorating physical condition. This progressed rapidly and usually the animals had to be euthanized within 1-2 weeks after symptom onset. Thus the knockout mice survived for a surprisingly long time after TFAM levels were reduced (3-4 months). This delay in symptom onset inspired us to dub them Mitochondrial Late-Onset Neurodegeneration (MILON) mice.

Southern and northern blot analyses of neocortex from MILON mice at different ages showed that levels of mtDNA and mitochondrial transcripts were significantly reduced after 2 and 4 months of age, respectively. Also at 4 months of age we could see a reduced activity of mtDNA-dependent RC complexes and COX/SDH staining revealed COX-deficient cells in all affected brain regions. At this time point, the most concentrated population of COX negative cells was seen in the CA3 region of the hippocampus. However, many COX-deficient cells were also seen in the CA1, the dentate gyrus as well as in many neocortical areas. Note that even though MILON brains at this time point (4 months) contained large amounts of RC deficient neurons, symptom onset was still at least one month away.

Severe cortico-hippocampal neurodegeneration in MILON mice

No TUNEL-positive nuclei could be detected, nor could any evidence of cell loss be seen, in 2-, 3-, or 4-month-old presymptomatic MILON mice. This supports the notion that TFAM deficient neurons survive for several months. Partially due to the mixed genetic background of MILON mice the time of disease onset varied. We still defined two time points to study symptomatic animals: *early-stage* (5-5.5 months; around symptom onset) and *end-stage* (5.5-6 months; when the mice had to be euthanized). Early-stage and end-stage mice displayed a progressive marked nerve cell loss in neocortex and hippocampus. There was substantial degeneration in neocortex and a severe disruption of cortical organization in end-stage MILON mice as determined by cresyl violet staining. Early-stage symptomatic MILON mice displayed a substantial nerve cell loss, as well as a cellular infiltration indicative of an inflammatory response in the CA1 region of hippocampus. Other hippocampal areas appeared normal in these animals. In end-stage animals, the pyramidal cell layer of the medial part of CA1 was completely absent, CA2 was intact, and CA3 only slightly affected. In early- and end-stage symptomatic animals (age 5-6 months), TUNEL-positive nuclei were seen in all areas suffering from cell loss.

Immunohistochemistry further supported the presence of neurodegeneration and an inflammatory response in end-stage MILON mice. Neurofilament (NF-10) immunohistochemistry showed extensive axonal degeneration, as evidenced by increased axonal beading and fragmentation in both early- and end-stage symptomatic MILON mice. We demonstrated the presence of gliosis by using GFAP antibodies to

detect reactive astrocytes. Large amounts of reactive astrocytes could be seen in corpus callosum, with many cells having processes directed into the degenerating neocortex. In all hippocampal regions, we observed massive gliosis, corresponding fully to the increased cellularity noted with cresyl violet staining. Pathological vascularization, as indicated by large abnormal vessels, particularly in neocortical areas, could also be seen using antibodies against the von Willebrand factor.

Apoptosis and reactive oxygen species in MILON mice

Because TUNEL staining may detect necrotic as well as apoptotic cell death, we used complementary methods to detect apoptosis. Using a combination of northern blot analyses and immunohistochemistry we were unable to demonstrate strong evidence of ongoing apoptosis in MILON brains. By using a sensitive PCR assay, we did however detect faint DNA ladders in cortical and hippocampal samples of MILON mice at the age of 5-6 months but not at the age of 4 months.

Reactive oxygen species (ROS) have been implicated as a major causative factor in mitochondrial nerve cell death. We next evaluated whether RC deficiency in MILON neurons affect ROS production or ROS defense mechanisms. Northern blot analyses of RNA samples from neocortex of MILON mice showed moderately increased transcript levels of two antioxidant enzymes: glutathione peroxidase (GPX) and mitochondrial superoxide dismutase (SOD2). Unfortunately, no corresponding increase in enzyme activities could be demonstrated. Western blot analysis and immunohistochemistry to detect protein nitrosylation, a marker for oxidative stress did not show any difference between 4-to 6-month-old MILON and control animals. These results suggest unexpectedly low levels of ROS production in the MILON mice.

MILON neurons do not up-regulate glycolysis to generate more ATP

In a previously published heart-specific *Tfam* knockout, high levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were detected in cardiomyocytes. GAPDH is a glycolytic enzyme and the up-regulation was interpreted as a compensatory response where cardiomyocytes switch from oxidative phosphorylation to glycolysis in order to support energy production (Wang et al. 2001). Interestingly, MILON mice did not display such an up-regulation of GAPDH

transcripts, suggesting that neurons were unable to switch to glycolysis to maintain ATP production. One speculative conclusion to draw from this could be that the brain, in contrast to the heart, is never exposed to similar hypoxic conditions as experienced by heart and skeletal muscle during heavy exercise, and thus consequently lacks such a mechanism.

Excitotoxic stress causes nerve cell death in MILON mice

As described above, there were no signs of neurodegeneration in 4-month-old MILON mice with widespread and profound RC deficiency in forebrain neurons. We therefore challenged MILON mice with injections of the epileptogenic drug kainic acid to determine whether they would be more susceptible to stress-induced neuronal death. There was no significant difference in the probability to develop high-level seizures after kainic acid injections between MILON and control mice at the age of 4 months. However, MILON mice with high grade seizures displayed significantly larger numbers of TUNEL-positive cells in the CA3 region of hippocampus 24 hours after seizure onset than did controls. This clearly showed that MILON neurons were more sensitive to excitotoxic stress than were wild-type neurons.

A GENETIC MOUSE MODEL OF PARKINSON'S DISEASE (PAPER IV)

There is strong evidence that mitochondrial dysfunction is at the center of the pathogenesis in many cases of Parkinson's disease (REF). However, at present no reliable genetic rodent model that addresses the role of mitochondria as a causative factor in PD is available. We showed in **paper III** that knockout of *Tfam* in forebrain neurons leads to a RC deficiency associated with delayed neuronal cell death. In this study we used the same system to impair RC function in the dopaminergic neurons that are lost in PD.

Cre expression in DAT-cre mice restricted to dopamine neurons

The primary pathological change seen in Parkinson's disease (PD) is loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) of the ventral midbrain. We used the cre-loxP system, as previously in the MILON mouse, to impair RC function specifically in DA neurons. Targeting cre expression to DA neurons required a mouse strain with a DA cell specific expression of cre. We utilized

homologous recombination to generate a knockin mouse strain with a cre-cassette inserted into the dopamine transporter (DAT) locus. Such DAT-cre mice displayed a restricted expression of cre recombinase transcripts in DA neurons of the ventral tegmental area (VTA) and SNpc as evidenced by *in situ* hybridization. To assess cre activity we also crossed DAT-cre mice with reporter mice that upon cre-mediated excision of an upstream stop site start to express the lacZ gene from the ROSA26 locus (Soriano 1999). Fluorescence immunohistochemistry with β-gal antibodies resulted in a highly specific labeling confined to the DA neurons in the VTA and SNpc, corresponding well with the pattern observed with *in situ* hybridization.

Parkinson mice display a progressive movement disorder

DAT-cre mice were crossed with *Tfam*^{loxP} mice and in two breeding steps we generated DA neuron specific homozygous knockout animals (*DAT-cre; Tfam*^{loxP}*/Tfam*^{loxP}). These “Parkinson mice” were born at the expected Mendelian frequency, appeared normal at birth and developed normally up to ~15 weeks of age. At this stage they started to display reduced activity as seen by simply observing them together with control littermates in their home cage. In some cases a slightly abnormal gait, especially affecting the hind legs, could be seen in Parkinson mice. Apart from these minor symptoms they appeared healthy with normal weight and well groomed fur. After 20 weeks of age the condition of Parkinson mice worsened, they clearly displayed additional symptoms such as tremor, involuntary twitching and rigid limbs, and eventually they started to loose weight. The weight loss progressed rapidly and most Parkinson mice had to be euthanized shortly after reaching 30 weeks of age. However, by supplying the mice with moist food on the cage floor the weight loss was noticeably reduced and the general physical condition of the mice improved, allowing them to survive for up to 45 weeks. The dramatic improvement in physical condition achieved by giving moist food on the cage floor indicated that the progressive movement disorder of Parkinson mice made it difficult for them to reach food and water on the cage grid above them.

Substantia nigra DA nerve cell loss associated with α-synuclein reactive intra-cellular inclusions

To assess pathological changes in the DA systems we performed immuno-histochemistry on Parkinson mice 5 weeks after symptom onset (20-22 weeks of age). Tyrosine hydroxylase (TH) antibodies, a marker protein for catecholamine neurons, showed an almost complete loss of dopamine terminals in dorsal and lateral areas of the striatum. Since TH is present in both DA and noradrenergic neurons, the few TH-positive terminals left in dorsolateral striatum could well be noradrenergic. Consistent with the changes observed with immunohistochemistry dopamine was almost absent in dorsal striatum as evidenced by HPLC analysis. Dopamine levels in ventral striatum were slightly higher although still much reduced compared to controls. Dorsolateral striatum is the target area where SNpc neurons project, and thus the selective loss of DA terminals indicated that primarily SNpc neurons were affected by the *Tfam* knockout. At the level of the dopamine cell bodies of the substantia nigra we did indeed observe a corresponding pattern of cell loss. There was a clear reduction of cell number in SNpc while the VTA appeared almost unaffected. Again, HPLC analysis was consistent with immunohistochemistry as there was a decrease in DA at the level of the cell bodies of SNpc and VTA.

Many of the remaining TH positive cells in SNpc appeared abnormal with TH-negative cytoplasmic inclusions and morphological malformations. We hypothesized that the TH-negative inclusions might be similar to the Lewy bodies observed in most cases of PD. Presence of intra-cellular inclusions containing high levels of α-synuclein, *i.e.* Lewy bodies, in neurons of affected areas is a pathological hallmark of PD. Immunohistochemistry with α-synuclein antibodies showed a massive increase of α-synuclein immunoreactivity, not only in the SNpc but also in the VTA of Parkinson mice. Double staining clearly showed that much of the α-synuclein reactivity was located inside TH positive cells, in many cases co-localizing with TH negative inclusions.

These observations provide strong genetic evidence that DA neurons in SNpc are more sensitive to mitochondrial dysfunction than those in VTA. Furthermore, the strong α-synuclein reactivity observed in SNpc and VTA, prior to and during cell loss,

demonstrates that RC dysfunction can cause typical parkinsonian pathology in DA nerve cells, including formation of intra-cellular inclusions similar to Lewy bodies.

Lack of TUNEL positive cells and gliosis in Parkinson mice

In the MILON mouse (**paper III**) neuronal cell death was paralleled by large amounts of TUNEL positive cells and a dramatic inflammatory response as evidenced by gliosis and small cell infiltration. In the Parkinson mice, however, TUNEL labeling to visualize ongoing cell death did not reveal apoptotic dopamine neurons in the mesencephalon of parkinsonian animals. No significant increase in glial fibrillary acidic protein (GFAP) immunoreactivity could be seen in striatum and only a slight increase in immunoreactivity was observed in SNpc. These results suggested that DA cell death in Parkinson mice was slow and progressive as opposed to fast and synchronized and not associated with a major inflammatory response.

Parkinson mice display decreased activity, reduced motoric function and respond well to L-DOPA treatment

Decreased activity was apparent in Parkinson mice from around 15 weeks of age. Next, we used established methods to properly quantify the activity of the mice at the ages of 10 and 14 weeks. Locomotion (horizontal movement) and rearing (vertical movement) were quantified in open field activity boxes in 10 minute intervals over 1 hour. At 10 weeks of age no significant behavioral changes could be seen in Parkinson mice. At 14 weeks of age a significant decrease was seen for both locomotion and rearing in Parkinson mice.

To study motoric performance and motor learning in the Parkinson mice we also performed a Rota-Rod test. The Rota-Rod is a horizontal rotating rubber-coated rod that increases in speed from 4-40 rpm over 300 seconds and the time until the mouse falls off is measured. With the Rota-Rod test we saw a non-significant trend toward decreased performance of 10 week old Parkinson mice. At 14 weeks of age, the performance of Parkinson mice was similar to controls during the first trial, but during later trials they were unable to improve as the controls easily did.

Administration of L-DOPA ameliorates many of the symptoms associated with PD and is the only known treatment for the disease. We treated 20 and 30 week old Parkinson

mice with Madopark (20 mg/kg; *i.p.*), a commercially available L-DOPA drug, and measured the behavioral response in activity cages. When mice were left untreated in the cages for 1 hour to record spontaneous behavior, both age groups of Parkinson mice again displayed significantly lower locomotion and rearing counts than controls did. The difference was more pronounced than in the younger group (14 weeks) as spontaneous activity clearly decreased with disease progression. After Madopark injection, both 20 and 30 week old Parkinson mice showed an immediate and robust increase in mean locomotion as well as a more delayed but dramatic increase in mean rearing. Interestingly, the effect of Madopark was age dependent as the 20 week old group showed a strong and long lasting (>1 hour) increase in locomotion while the response in locomotion of the 30 week old group was smaller and started to decay already after 10 minutes.

The difference in response to Madopark we saw in 20 and 30 week old animals resembles the on-off effects described in Parkinson patients. In later stages of PD, after several years of medication, ~50% of patients start to show a reduced and shortened response to L-DOPA. It is unknown if this phenomenon is a consequence of the disease progression, the pharmacokinetics of L-DOPA or a combination of both (Dewey 2004). Our results show that, at least in this animal model of PD, on-off like effects can appear without long-term treatment with L-DOPA, suggesting that they are inherent to the disease progression. An additional general observation was that even though Madopark convincingly increased the activity of Parkinson mice, they did not appear completely normal after treatment. Irregular movements or abnormal gait could easily be seen, especially in the older group (30 weeks) of animals.

CONCLUDING REMARKS

This thesis work has focused on studies of the *in vivo* role of TFAM (**paper I and II**) and on studies of the effects of mitochondrial dysfunction in different CNS neuronal populations (**paper III and IV**).

Main conclusions from **paper I** were:

- Human TFAM is a poor activator of mouse mitochondrial *in vitro* transcription despite its high capacity for unspecific DNA binding.
- TFAM can directly regulate mtDNA copy number *in vivo* in various mouse tissues.
- TFAM is a very abundant protein in mammalian mitochondria that is likely to bind and package mtDNA.
- Regulation of mtDNA copy number can be dissociated from regulation of mtDNA expression. Regulation of mitochondrial transcription is not simply mediated by the number of available mtDNA molecules.
- Human TFAM cannot complement the loss of endogenous mouse TFAM in knockout embryos.

These results are in agreement with several other studies indicating that TFAM has a role in mtDNA maintenance that is separate from its function in transcription initiation. In addition to specific interaction with mitochondrial promoter sequences, TFAM is likely an integral part of mitochondrial chromatin where it binds, stabilizes and protects mtDNA.

Main conclusions from **paper II** were:

- Human TFAM can rescue a tissue-specific knockout of *Tfam* in mouse heart, despite its poor capacity to activate transcription of mouse mtDNA.
- Compensatory mechanisms alter mtDNA expression regulation in rescued cardiomyocytes, so that the oxidative phosphorylation capacity can be reasonably well maintained.

Similar evidence of altered transcriptional regulation has been described in cell lines with blocked mitochondrial protein synthesis. Together with our data, this indicates that mitochondria have intrinsic mechanisms to increase mtDNA expression by modifying key elements of transcriptional regulation. In the future, as we identify more factors involved in mtDNA transcription and maintenance, these mechanisms will hopefully be understood in more detail.

Main conclusions from **paper III** were:

- Neurons are perhaps not as dependent on mitochondrial energy production as previously believed, as they can cope with a severe RC deficiency for several months.
- The expression of ROS scavenging enzymes is unaltered or only minimally increased in MILON mice, suggesting that ROS is not a major factor in the cell death process in respiratory chain deficient cortical neurons.
- RC deficient hippocampal neurons have an increased sensitivity to excitotoxic stress.

The most surprising finding in MILON mice is the length of time these neurons can survive after developing a RC dysfunction. Resistance to glutamate-induced excitotoxic damage is energy dependent, thus it is not surprising that MILON hippocampal neurons display a decreased resistance. Increased ROS production has been implicated in many neurodegenerative diseases linked to mitochondrial dysfunction. The fact that we do not see a corresponding effect in MILON mice could be due to the nature of the deficiency. Loss of all mtDNA encoded RC subunits will probably lead to complete RC failure, and it is likely that such a condition will not generate increased amounts of ROS. Thus the neuronal cell death in MILON mice is probably directly related to ATP depletion.

Main conclusions from **paper IV** were:

- Mitochondrial dysfunction in DA neurons causes PD-like pathology with appearance of α-synuclein inclusions prior to the onset or cell death and clinical symptoms.
- SNpc neurons are more sensitive than other DA neurons to mitochondrial dysfunction.
- The progressive degeneration of nigro-striatal DA pathways in Parkinson mice leads to PD-like behavioral disturbances, such as bradykinesia, rigidity and abnormal gait.
- The symptoms observed in Parkinson mice can be reversed by L-DOPA administration.
- The effect of L-DOPA decreases with disease progression and also becomes more transient.

The presence of inclusions similar to Lewy bodies has been described in toxin-based PD models. This, however, is the first direct genetic evidence that mitochondrial dysfunction can lead to α-synuclein and ubiquitin reactive inclusions in DA cells. The decreased efficiency of L-DOPA to ameliorate symptoms in advanced stages of both PD and this animal model is striking. Not only is the increase in locomotion after L-DOPA treatment less pronounced in older animals, dyskinetic movements are also much more apparent. The minimal amount of inter-animal variability and the faithful reproduction of several behavioral and pathological characteristics of PD make this novel animal model extremely valuable.

FIGURES

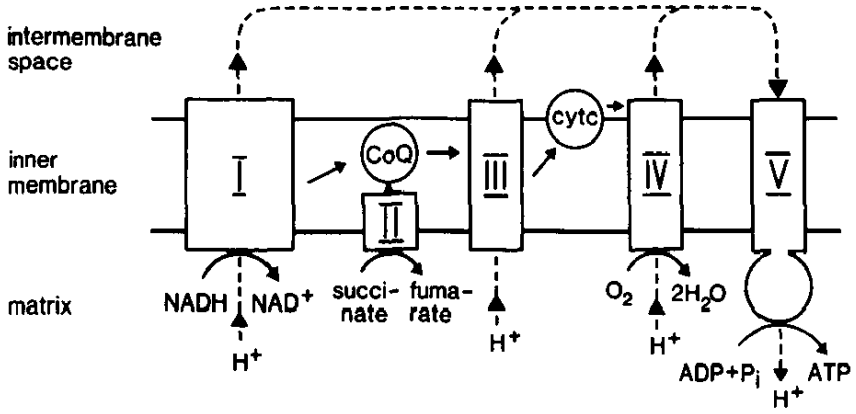

Figure 1. Schematic illustration of the structure and function of the respiratory chain. The respiratory chain consists of five different enzyme complexes (complexes I-V), coenzyme Q (CoQ) and cytochrome *c* (cytc). NADH and succinate are oxidized by complexes I and II, the electrons are transferred to coenzyme Q, complex III, cytochrome *c*, complex IV, and finally to molecular oxygen (O_2), which is reduced to water (H_2O). Protons (H^+) are pumped out of the mitochondrial matrix by complexes I, III, and IV and a proton gradient is formed across the inner membrane of the mitochondrion. The protons reenter the matrix through complex V (ATP synthase) and the energy of the proton gradient is used to synthesize ATP. From Larsson & Clayton, 1995

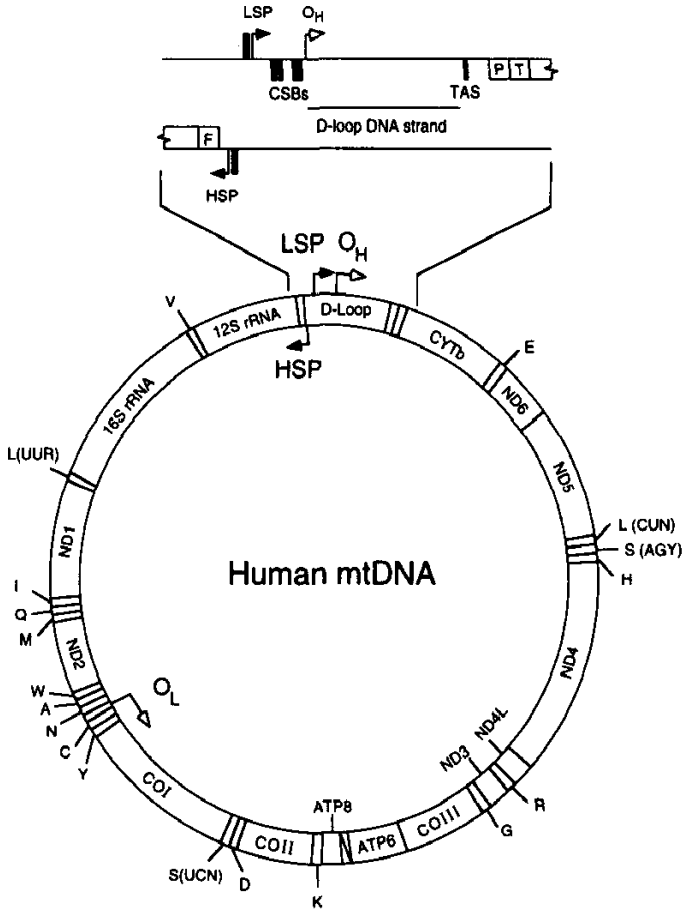

Figure 2. Map of human mtDNA. The following genes are indicated: NADH dehydrogenase subunits (ND1-ND6 and ND4L), cytochrome b (CYTb), cytochrome c oxidase subunits (COI-III), ATP synthase subunits (ATP8 and 6), rRNAs (12S and 16S), and tRNAs (indicated by the one-letter amino acid symbol). The arrows indicate the promoters for transcription of the heavy (HSP) and light (LSP) strand of mtDNA and the origins of heavy (O_H) and light (O_L) strand replication. Enlarged region shows the displacement loop (D-loop) with the nascent D-loop DNA strand, the conserved sequence blocks (CSBs), and the termination associated sequence (TAS). From Larsson & Clayton, 1995

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