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Regulation of mitochondrial transcription and mtDNA copy number in mammals

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To my Family

Abstract

Functional mitochondria are essential for wellbeing of the cell and the whole organism. Gene expression from the mitochondrial genome (mtDNA) is indispensable for oxidative phosphorylation, but also for the replication of mtDNA, as the replication primers are processed from mtDNA transcripts. Mitochondrial transcription factor A (TFAM) is a key transcriptional activator that is also necessary for the maintenance of mtDNA. In this thesis we have focused on characterizing the relationship between TFAM protein levels and mtDNA copy number and expression in mammals.

mtDNA copy number seems to correlate with TFAM protein levels, which is strikingly apparent in human and mouse testis. We characterized the *Tfam* gene and its expression pattern in rat and could identify a set of conserved features concerning mtDNA and TFAM expression during mammalian spermatogenesis. These features include expression of alternate *TFAM/Tfam* transcripts and downregulation of TFAM protein levels and mtDNA copy number. These features most likely constitute one of the mechanisms involved in preventing paternal transmission of mtDNA.

We studied the gene-dosage effects of TFAM *in vivo* by creating transgenic mice carrying the whole human *TFAM* gene and various amounts of flanking DNA. Enhanced TFAM protein levels led to increased mtDNA copy number. However, only L-strand transcription was affected with 50-60 % higher transcript levels, while there was no effect on H-strand transcript levels. Also respiratory chain enzyme activities were unaffected. Complementation analyses demonstrated that human TFAM protein cannot functionally replace the endogenous mouse TFAM protein. Nevertheless, the *Tfam* null embryos carrying the human *TFAM* gene contain mtDNA, whereas the homozygous *Tfam* knockout embryos do not. These results suggest the human TFAM protein is able to activate L-strand transcription and support mtDNA maintenance in mouse.

In this thesis we have also sought to identify "missing" factors required for mitochondrial transcription. With the help of profile-based BLAST searches and Mtf1p peptide sequence, we were able to identify two novel mammalian mitochondrial transcription factors called TFB1M and TFB2M. Using pure

recombinant mitochondrial RNA polymerase, TFAM and TFB1M or TFB2M, and a template containing the LSP and HSP, we could for the first time reconstitute faithful mitochondrial transcription *in vitro*.

In yeast, there is only one mitochondrial RNA polymerase specificity factor, Mtf1p. The existence of two such factors in human, mouse and *Drosophila* prompted us to study their origin and evolutionary conservation. Cluster analyses indicate that the TFBMs most likely originate from a common ancestral gene that duplicated early in metazoan evolution.

Keywords: mtDNA, transcription factor, evolution, transmission, inheritance, expression, mitochondria, transgenic, mapping, testis-specific

List of publications

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

I **Rantanen A**, Jansson M, Oldfors A and Larsson NG (2001). Downregulation of Tfam and mtDNA copy number during mammalian spermatogenesis. *Mamm Genome* 12, 787-792

II Ekstrand M, **Rantanen A**, Park CB, Rustin P & Larsson NG (2003) Expression of human TFAM in the mouse increases mtDNA copy number without affecting respiratory chain function. *Manuscript*

III Falkenberg M, Gaspari M, **Rantanen A**, Trifunovic A, Larsson NG and Gustafsson CM (2002). Mitochondrial transcription factors B1 and B2 activate human mtDNA transcription. *Nature Genet* 31, 289-294

IV **Rantanen A**, Gaspari M, Falkenberg M, Gustafsson CM & Larsson NG (2003). Characterization of the mouse genes for mitochondrial transcription factors B1 and B2. *Mamm Genome* 14, 1-6

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Abbreviations

aa	amino acids
bp	base pair
cDNA	complementary DNA
COX	cytochrome c oxidase, complex IV
CSB	conserved sequence block
D-loop	displacement loop
E	embryonic day
GFP	green fluorescent protein
HeLa cells	human epithelial cells from a uterine cancer
HMG	high mobility group
HSP	heavy strand promoter
kb	kilobase
kDa	kiloDalton
LSP	light strand promoter
mtDNA	mitochondrial DNA
mtRNA	mitochondrial RNA
O_H	origin of heavy strand replication
O_L	origin of light strand replication
OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
RRF	ragged-red fibers
RT	reverse transcription
rRNA	ribosomal RNA
SAM	S-adenosyl methionine
SDS	sodium dodecyl sulphate
TAS	termination associated sequences
tRNA	transfer RNA
UTR	untranslated region

Introduction

Origin of mitochondria

Mitochondria are cellular organelles of prokaryotic origin that are found in almost all eukaryotic cells. They produce energy in a process called oxidative phosphorylation (OXPHOS), but are also known to play a central role in apoptosis, cellular stress responses and various genetically inherited diseases.

Modern mitochondrial proteome is a product of both reductive and expansive processes. It is a combination of proteins with eukaryotic and prokaryotic origin: several mitochondrial proteins encoded by nuclear genes do not have bacterial homologues indicating that they originated in the eukaryotic host after the endosymbiosis was established (Andersson *et al.* 2003). The mitochondrial genome (mtDNA) usually encodes a limited number of RNAs and proteins essential, although not sufficient, for formation of a functional mitochondrion. The arrangement and expression of the genes varies markedly between species, and the size of mtDNA ranges from < 6 kb in *Plasmodium* to almost 400 kb in *Arabidopsis*.

The mitochondrial origin has been studied by analyzing mtDNA sequences from different species. Around 200 complete mtDNA sequences are available, and research on the gene order and single gene phylogenies strongly suggest that mitochondria have a monophyletic origin (Gray *et al.* 1999). Although endosymbiotic events must have occurred several times, the modern mitochondria appear to have arisen only once in a common ancestor of all extant eukaryotes.

The ancestral endosymbiont (protomitochondrion) was most likely an a-proteobacterium (Gray *et al.* 1999). The a-proteobacterium *Rickettsia prowazekii* genome of 1.1 Mb is the most mtDNA-like bacterial genome, while the mtDNA of 69 kb of *Reclinomonas americana* is the most bacteria-like modern mitochondrial genome. Evolution of the protomitochondrial genome was rapid as genes for different biosynthetic pathways that could be compensated by the host were lost at an early stage. Genes encoding proteins involved in different metabolic pathways, mitochondrial proliferation, mtDNA replication and expression were transferred to the host nucleus where they are expressed. The metazoan evolution spans a period ranging between 800 and 1000 million years ago, during which their mtDNA remained practically unchanged. In animals, changes in the mitochondrial genetic

code have effectively blocked gene transfer from mitochondria to nucleus. Modern animal cell mtDNAs are 15-17 kb in size, contain no introns and short (or no) intergenic sequences and there is usually only one major non-coding region (Saccone *et al.* 2002).

Mitochondrial diseases

It was discovered in the 1960's that mitochondria might have a pathogenic role in human disease (Luft *et al.* 1962). Sequences of the human and mouse mtDNAs were released in 1981 (Anderson *et al.* 1981, Bibb *et al.* 1981), which led to the identification of the first pathogenic mtDNA mutations (Holt *et al.* 1988, Wallace *et al.* 1988). The first nuclear genes involved in mitochondrial disorders were discovered a few years later (Zeviani *et al.* 1989, Suomalainen *et al.* 1995).

Mitochondrial diseases include an array of disorders with varying phenotypes. They are characterized by deficient OXPHOS caused by mutations in mtDNA or nuclear genes (nDNA) for mitochondrial proteins. The overall incidence of mitochondrial disorders is ~1 X 10^{-4} in adults, out of which roughly two thirds are caused by mtDNA mutations, while mutations in nDNA are responsible for the remaining one third. Mitochondrial disorders typically affect tissues with great energy demands, such as the skeletal muscle, central nervous system, heart, pancreas, eye and kidney. The clinical symptoms can start at any age, and the disease can progress gradually or fast. A typical finding in mitochondrial myopathies is ragged-red fibers (RRF), a sign of pathological increase of abnormal mitochondria. The increased mitochondrial mass is a compensatory response stimulated by poor respiration (Moraes *et al.* 1992). Defective OXPHOS is known to affect several cellular processes, such as the redox status of the cell, production of ATP, reactive oxygen species (ROS) formation and induction of apoptosis.

Genetic control of mitochondrial morphology

Mitochondria are not distinct particles but rather form a dynamic network. Genetic screens in yeast have identified several mutations that change mitochondrial morphology by affecting mitochondrial fission and fusion (Shaw & Nunnari 2002). The mitochondrial shapes range from multiple small compartments to complex tubular networks (Fig 1). Mitochondria grow, divide and fuse continuously, and the equilibrium between fission and fusion events determines organelle morphology at a given time.

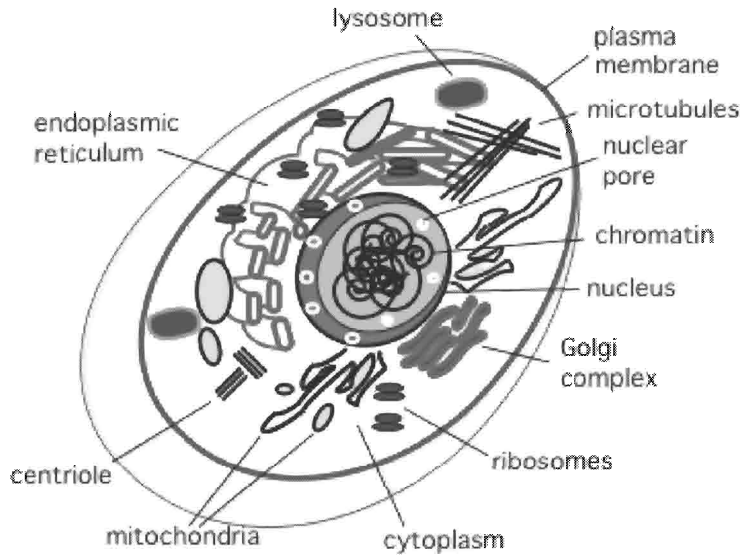

Figure 1 Mitochondria take many shapes in a cell.

Mitochondrial compartment remodeling has been studied most in the baker's yeast *Saccharomyces cerevisiae*. Fission always takes place within a mitochondrial tubule or at a branchpoint in the mitochondrial network, and is regulated by the Dnm1p, a dynamin-related GTPase. Dynamin-related proteins (DRP1, DLP1) have also been identified in humans, where they have been implicated in controlling mitochondrial morphology.

Mitochondrial fusion regularly occurs between two tips or between a tip and the side of a tubule and is controlled by the Fzo1p GTPase. The Fzo (fuzzy onion) protein was originally characterized in *Drosophila* and two human homologues; mitofusins 1 and 2 have been identified. They have been shown to associate with mitochondria and alter their morphology (Santel & Fuller 2000). A third factor important for maintaining the mitochondrial morphology is Mgm1p. Its exact function is not clear, but it is known to localize to the mitochondrial intermembrane space (Shaw & Nunnari 2002). The Mgm1p homologue in humans is called OPA1. Mutations in OPA1 are linked to a dominant optic atrophy that leads to childhood blindness (Alexander *et al*. 2000, Delettre *et al*. 2000).

Nuclear genes regulating mitochondrial mass

The mitochondrial mass is controlled by physiological stimuli, and the expression of both nuclear and mitochondrial genes is required. The mitochondrial mass increases in response to endurance training (in skeletal muscles) or during adaptive thermogenesis (in brown adipose tissue). Also thyroid hormone induces mitochondriogenesis in a variety of tissues. Since mitochondria are not generated *de novo*, they replicate prior to cell division and are passed on to the daughter cells. The mammalian mitochondrial genome only encodes 13 respiratory chain subunits, whereas nuclear genes encode the remaining ~80 respiratory chain subunits, all proteins needed for mitochondrial translation and all protein factors required for the transcription and replication of mtDNA.

The expression of nucleus-encoded respiratory genes is controlled by a set of nuclear transcription factors and cofactors for nuclear receptors. A handful of these trans-activate several genes that contribute to the respiratory chain. The nuclear respiratory factor family (NRF1, NRF2) have been shown to have positive effects on several respiratory gene promoters (Evans & Scarpulla 1990; Chau *et al.* 1992), e.g. mitochondrial transcription factor A (*TFAM*) in humans. Disruption of the *Nrf1* gene in mouse leads to death at embryonic day (E) 3.5-6.5. NRF1 also has a role in mtDNA maintenance, since the *Nrf1* knockout embryos have a reduced mtDNA copy number (Huo & Scarpulla 2001). Another member of the NRF family, NRF2, seems to be a sensor for cellular conditions. For example high levels of reactive oxygen species (ROS) may have a negative effect on NRF2 DNA-binding activity (Martin *et al.* 1996). Also, NRF2 subunit localization in the cell may be regulated according to physiological conditions, enhancing the nuclear localization of the subunits upon e.g. stimulation of neuronal activity (Zhang & Wong-Riley 2000).

A third factor commonly known to bind to respiratory gene promoters is the transcription factor Sp1. It has been shown to be involved in both activating and inhibiting the expression of the genes for e.g. adenine nucleotide translocator 2 (*ANT2*) (Li *et al.* 1996) and ATP synthase β-subunit (Zaid *et al.* 1999). However, there is no evidence showing that Sp1 would directly regulate the expression of respiratory genes. It may act synergistically with NRF1, although a direct interaction has not been demonstrated (Virbasius & Scarpulla 1994). *Sp1* knockout embryos die at E9.5 and their growth is severely retarded. Still, many genes with Sp1 binding sites in their

promoters are normally expressed in the knockout embryos, suggesting a possible redundancy or compensation by other transcription factors (Philipsen & Suske 1999).

A family of key regulatory factors controlling mitochondrial respiration and mass are the peroxisome proliferator-activated receptor γ (PPAR γ) coactivators (PGCs). The family includes three members: PGC1α, PGC1β, and the PGC1α-related coactivator (PRC). The members of the PGC1 family are expressed in a variety of tissues and are activated upon different physiological stimuli e.g. cold, fasting, thyroid hormone or growth signals. They are known to heterodimerize with and function through nuclear transcription factors, such as PPARα, PPARγ, NF4α, host cell factor (HCF) and NRF1 (Puigserver *et al.* 1998, Lin *et al.* 2002). They promote the expression of several respiratory genes as well as genes of the mitochondrial fatty acid oxidation pathway (Vega *et al.* 2000). Interestingly, PGC1β interaction with HCF promotes transcription of all three members of the PGC1 family, along with other target genes. In addition, PGC1α and PGC1β have been shown to control the expression of gluconeogenic enzymes during fasting and thus be involved in the regulation of blood glucose levels (Yoon *et al.* 2001).

HCF has been shown to induce expression of factors involved in mediating cell cycle progression (Wilson *et al.* 1997, Goto *et al.* 1997). It interacts with NRF2 (Vogel & Kristie 2000) and PGC1β, and is also believed to interact with other members of the PGC1 family of nuclear coactivators. HCF could thus function as a coactivator of NRF2-dependent gene expression and integrate the transcriptional activities of NRF2 and the PGC1 family.

The mitochondrial genome

The mammalian mitochondrial genome is an approximately 16.6 kb circular molecule of double-stranded DNA (Fig 2). It is histone-free and has been considered naked, although some recent evidence suggests that it may be fully coated by TFAM. The mtDNA is present in thousands of copies per cell, but normally only represents less than 1% of the total cellular DNA (Clayton 1982).

Animal mtDNAs contain a handful of genes that code for 13 proteins required for the respiratory chain plus 2 rRNAs and 22 tRNAs necessary for their expression. The mRNA genes are flanked by tRNA genes and contain no introns.

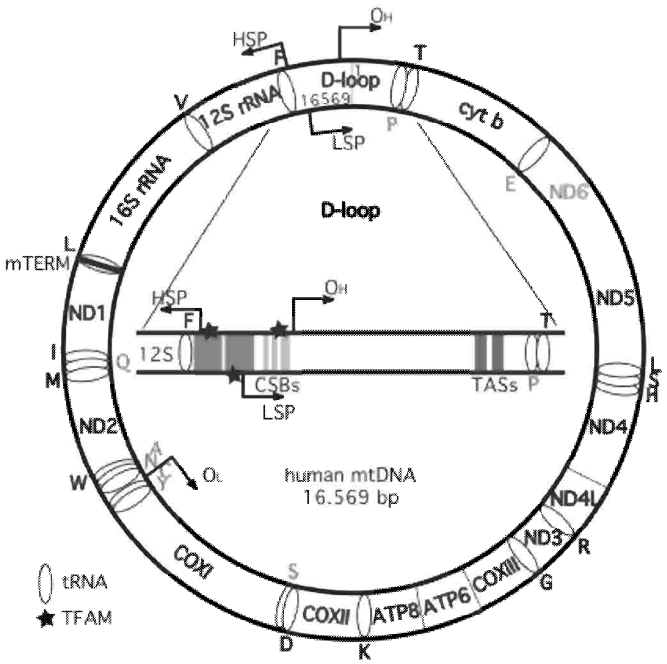

Figure 2 The gene organization of the human mitochondrial genome. L-strand transcripts are shown with grey color. A closer look at the D-loop region with the conserved regulatory elements is presented in the middle. For simplicity, the D-loop is drawn without the nascent 7S DNA strand.

The only longer non-coding region of the mammalian mtDNA is a 1.1 kb segment called the displacement loop (D-loop). It is a three-stranded structure that has evolved as the control site for both mtDNA transcription and replication. Besides the origin of heavy strand replication (O_H) and the mitochondrial promoters, the D-loop contains two conserved elements called the termination-associated sequences (TASs), where the 3'ends of the 7S DNA (D-loop DNA) strands map. Upstream of the TASs there are regions with extreme sequence conservation called conserved sequence blocks (CSBs). This is where the transition from RNA to DNA synthesis in mtDNA replication takes place.

The animal mtDNA coding sequence has been virtually unchanged for the 800 million years, while the evolution of the non-conserved parts of the D-loop is rapid.

mtDNA replication in mammals

Nuclear genes encode all factors required for the transcription and replication of mtDNA. Unlike replication of nuclear DNA, replication of the mammalian mitochondrial genome is independent of the cell cycle. Two modes of replication have been suggested: the asymmetric model (Clayton 1982) and the strand-synchronous model (Holt *et al.* 2000).

According to the Clayton model mtDNA replication is strand-asynchronous and asymmetric (Clayton 1982). There are two origins of replication, one for each mtDNA strand: the origin of heavy strand synthesis (O_H) located downstream of the light strand promoter (LSP) within the D-loop region, and the origin of light strand synthesis (O_L) nested within a cluster of five tRNA genes about two thirds of the genome away (Fig 2). mtDNA synthesis starts at the O_H and the strand is synthesized for the entire length of the genome. L-strand elongation is initiated after O_L is exposed as a single-stranded template. Synthesis of both the leading and lagging strands occurs continuously. According to the Holt model replication of mtDNA is unidirectional and the replication of the heavy- and light-strand are coupled (Holt *et al.* 2000). In this model, the synthesis of the leading (heavy) strand is continuous and the lagging (light) strand is synthesized discontinuously (in Okazaki fragments). Elongation of both strands starts at or near the O_H and proceeds in one direction.

Replication of mtDNA is dependent on mitochondrial transcription, since primers necessary for the initiation of mtDNA replication are produced by processing of L-strand transcripts. A specialized endoribonuclease called the RNase MRP (Mitochondrial RNA Processing) is involved in this process (Chang & Clayton 1987). It cleaves the "R-loops" at a site between the conserved sequence blocks (CSBs) II and III (Chang & Clayton 1985; Chang *et al.* 1985) thus creating proper 3'-hydroxyl groups for the extension by the polymerase g (Lee & Clayton 1997).

Transcription of mammalian mtDNA

The mtDNA transcriptome of 37 genes is expressed as three polycistronic transcription units. They are generated from the major mtDNA promoters, called the heavy strand promoter (HSP) and the light strand promoter (LSP). The promoters are ~50 bp in length and separated by 150 bp in the D-loop (Fig 2).

Two of the mitochondrial transcription units are produced from the heavy (H) strand and the third from the light (L) strand. The L-strand encodes one mRNA and eight tRNAs. RNA-primers required in mtDNA replication are also generated from LSP. The first H-strand transcript contains the 12S and 16S rRNA genes and the second, almost genome-length transcript contains the two rRNAs plus 12 mRNAs and 14 tRNAs. The transcript containing the rRNAs alone is about 20-50 times more abundant than the full-length H-strand transcript (Gelfand & Attardi 1981). H-strand transcription is probably regulated by transcription termination, where the mitochondrial termination factor (mTERF) plays an important role (Kruse *et al.* 1989). It binds a tridecamer sequence (mTERM) in the $tRNA^{Leu(UUR)}$ gene located after the 16S rRNA gene. In addition, two independent H-strand promoters, one for each H-strand transcription unit, have been described. Thus both transcription initiation and transcription termination events may determine the relative abundance of transcripts of the two H-strand transcription units.

Until recently, only two proteins of the mammalian basal mitochondrial transcription machinery had been identified: the mitochondrial RNA polymerase (POLRMT) and a transcriptional activator, TFAM (Fisher & Clayton 1985). TFAM (previously mtTFA, mtTF1, TCF6 or TCF6L2) binds DNA with relaxed specificity, but has high affinity for the mitochondrial core promoters. Binding of TFAM to the mitochondrial promoters is essential for the initiation of mtDNA transcription. Early mitochondrial *in vitro* transcription assays demonstrated that pure TFAM protein and a partly purified POLRMT fraction were adequate for faithful mitochondrial transcription initiation *in vitro* (Fisher *et al.* 1989). However, trials to reconstitute mitochondrial transcription *in vitro* using pure TFAM protein together with pure POLRMT were not successful, suggesting that additional factors (may) exist in the POLRMT fraction.

POLRMT is a 150 kDa core RNA polymerase of viral origin. Especially the C-terminal part shares similarity with the bacteriophage T7, T3 and SP6 RNA polymerases. These viral RNA polymerases recognize their promoters faithfully in the absence of accessory factors. However, the yeast *S. cerevisiae* mtRNA polymerase (Rpo41p), also homologous to the viral RNA polymerases, requires a specificity factor, Mtf1p. The basal mitochondrial transcription machinery in *S. cerevisiae* thus consists of two factors: Rpo41p and the specificity factor Mtf1p (Schinkel *et al.* 1987, 1988). Mtf1p is required for the formation of an active mtRNA polymerase

holoenzyme, and it also stabilizes Rpo41p by heterodimerizing with it. A specificity factor for the mitochondrial RNA polymerase has been characterized in another lower eukaryote, the frog *Xenopus laevis* (Antoshechkin & Bogenhagen, 1995; Bogenhagen 1996). It shares the same properties with the yeast Mtf1p and functions as a cofactor required for the formation of an active mtRNA polymerase holoenzyme.

Mitochondrial transcription factor A (TFAM)

TFAM is a ubiquitously expressed transcriptional activator of about 25 kDa. It is encoded by a nuclear gene, translated in cytoplasm and imported into mitochondria, where the aminoterminal mitochondrial leader peptide is cleaved off. The TFAM protein structure is highly conserved in mammals (Larsson *et al.* 1996): the human and mouse TFAM peptide sequences share 67 % identity and 81 % similarity. There are two high mobility group (HMG) -boxes separated by a linker and followed by a carboxyl (C) -terminal activation domain (Fig 3). The C-terminal tail is essential for the activation of mitochondrial transcription (Dairaghi *et al*. 1995) and is believed to interact with the transcription machinery. As said, TFAM has high affinity for the mitochondrial promoters, and binds at sites 12-39 bp upstream of the transcription initiation sites as demonstrated by footprinting experiments. Like other HMG-box proteins, TFAM can also bend and wrap DNA in an unspecific fashion suitable for packing mtDNA within the organelle. Chemical cross-linking experiments in *Xenopus* showed that TFAM binds mtDNA as a tetramer (Antoshechkin *et al.* 1997).

The *S. cerevisiae* TFAM homologue Abf2p (ARS-binding factor 2) lacks the C-terminal transcriptional activation domain (Fig 3). Consequently, Abf2p has only been shown to have a minor effect on mitochondrial transcription (Xu & Clayton 1992). It contains the two HMG-boxes separated by a short linker, and has the same unspecific DNA binding properties as TFAM.

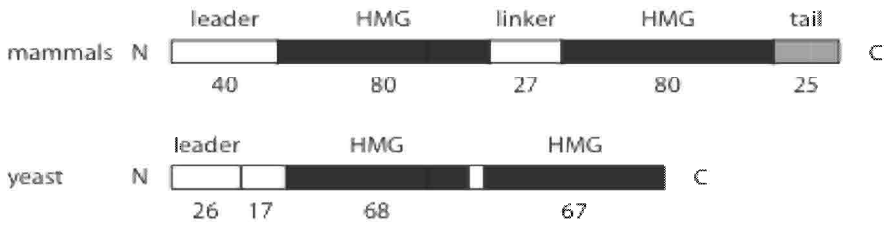

Figure 3 Comparison of the mammalian TFAM and yeast Abf2p proteins.

Changes in the Abf2p level alter the mtDNA copy number (Zelenaya-Troitskaya *et al.* 1998) and disruption of the *ABF2* gene leads to complete loss of mtDNA (Diffley & Stillman 1991). Furthermore, the replication of mtDNA in yeast is preferably driven by recombination. The fact that Abf2p has been shown to be critical for efficient mtDNA recombination *in vivo* (MacAlpine *et al.* 1998) may explain its effects on mtDNA copy number and stability. The general function of Abf2p thus seems to be in mtDNA stabilization and/or packaging.

There is also data from higher eukaryotes suggesting that TFAM may be dispensable for mitochondrial transcription. In a recent study, an RNA interference (RNAi) approach was used to deplete *Drosophila* Kc167 cells of *d*-TFAM protein to less than 5 % of the normal levels (Goto *et al.* 2001). The mtDNA copy number decreased by 60 %, while the levels of mitochondrial mRNAs remained unaffected. In contrast, the amount of mitochondrial rRNAs decreased. This may mean that when low levels of TFAM are available, the transcription of full-length transcripts from HSP is favored.

An average mammalian nuclear transcription factor is only present in minute amounts, while TFAM is abundantly expressed. The estimated molar ratio between TFAM and mtDNA in human cells varies from as low as 15 TFAM molecules per mtDNA (Fisher & Clayton 1988) up to 1700 (Alam *et al.* 2003). In *Xenopus* oocytes there is an average of 200 TFAM molecules per mtDNA (Shen & Bogenhagen 2001). The high ratio of TFAM protein to mtDNA suggest that TFAM may have other functions besides transcriptional activation.

Similar to the yeast Abf2p, there is a correlation between mtDNA copy number and TFAM protein levels in mammals. Patients with mitochondrial myopathies have increased levels of TFAM and mtDNA in ragged-red fibers, while low levels of TFAM are found in patients with depletion of mtDNA. Disruption of the mouse *Tfam* gene causes embryonic lethality between E8.5 and 10.5 (Larsson *et al.* 1998). Homozygous *Tfam* knockout embryos are small and have a mutant phenotype with delayed neural development, indistinct somites, lack of optic discs and no heart structures. Furthermore, they have abnormal mitocondria and completely lack mtDNA. Heterozygous *Tfam* knockout mice are healthy, but have decreased mtDNA levels. Conditional *Tfam* knockout in the mouse leads to loss of mtDNA and mtRNA. This causes defective OXPHOS and is manifested as mitochondrial

disease in the affected tissue (Wang *et al.* 1999; Li *et al.* 2000, Silva *et al.* 2000; Sörensen *et al.* 2001, Wredenberg *et al.* 2002).

Gene expression during mammalian spermatogenesis

Expression of isozymes or testicular variants for housekeeping proteins is common during spermatogenesis (Hecht 1995). Alternate promoters are frequently used for generating testis-specific transcripts or protein isoforms of ubiquitously expressed genes. This may be required because of chromatin changes as the germ cells differentiate. Testis-specific isoforms have been found for e.g. phosphoglycerate kinase, enolase hexokinase, alcohol dehydrogenase, glyceraldehyde 3 phosphate dehydrogenase and cytochrome c. Also genes of the oxidative phosphorylation pathway express alternate isozymes and protein isoforms in testis. For instance cytochrome c is expressed from two separate genes: the testis-specific gene (cyt c_T) and the somatic gene (cyt c_S), that only differ in 14 amino acids out of 104. In addition, the somatic cyt c_S gene is expressed from a testis-specific promoter producing a different mRNA with an extended 5' untranslated region (UTR). The ~1 kb long 5'UTR strongly affects translation, thus contributing to the downregulation of cyt c_S and upregulation of cyt c_T during meiosis (Hake & Hecht 1993).

Testis-specific variants of histones are also expressed and may be needed for structural changes in the nuclei of male germ cells. During meiosis, the somatic histones are replaced by testis-specific histone isoforms. Other testis-specific histones and basic transition proteins sequentially replace these during spermiogenesis. Finally at the end of spermiogenesis the transition proteins are replaced by protamines.

Most of the transcripts expressed during spermatogenesis are probably produced during meiosis. Transcription continues from the haploid genome, and ceases as the nuclear genome condensation begins in the middle of spermiogenesis (Schäfer *et al.* 1995). Proteins needed by the post-meiotic cells are either synthesized and stabilized prior to that, or the mRNAs are stored and translated later. Transcripts and proteins generated before meiosis are distributed in the cytoplasm thus rendering the haploid stages of spermatogenesis physiologically diploid. Also post-meiotic transcripts from the haploid genome are shared through cytoplasmic bridges between spermatids in the same syncytium (Braun *et al.* 1989).

TFAM expression in mammalian testis

Testicular variants of *TFAM/Tfam* are expressed in humans and mice. The testis-specific transcripts contain testis-specific exons and are generated from alternative transcription start sites thus producing long 5'UTRs. As shown in figure 4, the *TFAM/Tfam* transcript levels are very high, while the levels of TFAM protein are low (Larsson *et al.* 1996, 1997). The structure of the testis-specific *TFAM/Tfam* mRNAs suggests they are not translated. Similar to the testis-specific transcript isoforms of cyt c_S, these transcripts may serve to prevent translation of the "somatic" mRNAs, thus downregulating the TFAM protein levels in testis. Interestingly, in parallel to the declining TFAM protein levels the amount of mtDNA decreases 5-7 –fold (Fig 4), to roughly 700 copies per cell (Diéz-Sánchez *et al.* 2002).

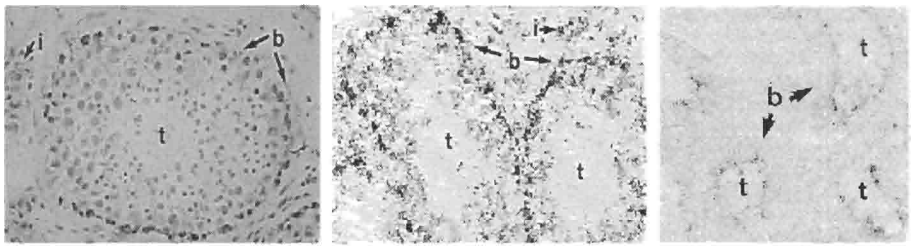

Figure 4 Localization of TFAM protein, mtDNA and testis-specific *TFAM* mRNA on human testis sections (Larsson *et al.* 1997). Mitochondrial TFAM protein and mtDNA are seen in interstitial Leydig cells (i) and in diploid basal cells (b), but not in later stages of spermatogenesis close to the lumen (t). Testis-specific *TFAM* mRNAs are only expressed at the end of spermiogenesis.

However, downregulation of TFAM protein and mtDNA copy number have no effect on the mtDNA transcript (mtRNA) levels, which remain stable throughout spermatogenesis. This is most likely to ensure a maximal OXPHOS capacity required by spermatozoa (De Martino *et al.* 1979).

Surprisingly, in mouse elongated spermatids some of the testis-specific *Tfam* transcripts are translated into an abundant nuclear protein with an unknown function (Larsson *et al.* 1996). It is attractive to consider that it functions as a transcriptional activator for the nuclear genome as well. It might also help transition proteins and protamines in packing the haploid genome. However, absence of the nuclear TFAM protein from human testis suggests it does not have a critical role in mammalian spermiogenesis.

Inheritance of mitochondria and mtDNA in mammals

Most sexual organisms transmit their mtDNA asexually, through one parent only. The advantage of the uniparental mode of inheritance is that the spread of cytoplasmic parasites and selfish organelle genes decreases. A negative consequence is accumulation of deleterious mutations known as the Müller's ratchet (Müller 1964, Felsenstein 1974). In mammals the inheritance of mtDNA is strictly maternal: in humans, transmission of paternal mtDNA has only been reported once in a patient with severe exercise intolerance (Schwartz & Vissing 2002). Paternal mtDNA has also been shown to leak to the progeny in interspecific mouse crosses (Gyllensten *et al.* 1991; Kaneda *et al.* 1995; Shitara *et al.* 1998).

The mechanism of uniparental inheritance differs between species: transmission of mtDNA from the other parent may be blocked via prezygotic or zygotic mechanisms, or during fertilization (Birky 1995). In mammals, prezygotic mechanisms dominate and are also reflected in the germ cell volume. The tiny paternal gamete (sperm) loses over 80% of its mtDNA, while the mtDNA in the large maternal gamete (oocyte) is amplified to comprise about 30 % of total cellular DNA (Pikó & Matsumoto 1976). Until the blastocyst stage (~100 cells), there is no mtDNA replication, and the early embryo is sustained with the ~100 000 maternally inherited copies (Pikó &Taylor 1987). The paternal mitochondria enter the oocyte upon fertilization and can be detected up to third or fourth cell division, after which they are degraded (Sutovsky *et al.* 1999). There is data suggesting that paternal mitochondria are tagged with ubiquitin (Sutovsky *et al.* 2000), which is recognized by the proteasome and rapidly eliminated in the oocyte.

Aims of this study

The differences between *TFAM* expression pattern in mouse and human testis raise questions about the mechanisms of mtDNA downregulation during spermatogenesis and the function and necessity of the nuclear TFAM protein isoform.
Characterization of the mitochondrial basal transcription machinery is of utmost importance due to the impact of mitochondrial transcription on mtDNA copy number and cellular energy production.
In this series of studies we wished to determine the following:

- Is the nuclear TFAM protein isoform expressed in rat testis?
- Does TFAM directly regulate mtDNA copy number?
- How does overexpression of (human) TFAM affect mtDNA transcription and copy number in mouse?
- Are there other mitochondrial transcription factors in mammals?

Comments on methods

All methods used in this study have been described in the original publications that are included in the end of this thesis. Central methods that have not been described or are only referred to in the original publications will be described in this section. Basic procedures such as preparing DNA, RNA or proteins will not be described.

Western analyses

For western blots, commercially available gels (Criterion, BIORAD) or standard sodium dodecyl sulphate (SDS) gels were used. The protein samples (10-50 mg) were boiled for 10 min, loaded on the gel and electrophoresed for 2-3 h at 100-120 V (commercial gels) or overnight at 30 mA (SDS gels). The gels were then blotted using a semi-dry blotting device according to the manual. After overnight blocking in phosphate buffered saline (PBS) with 5 % milk at 4°C, the membranes were probed with polyclonal antibodies raised against the mouse or human TFAM (1:1000), cytochrome c oxidase subunit 2 (1:5000) or actin (1:500) diluted in 5% milk-PBS for 2-4 h at room temperature (RT). The membranes were then washed for 30 min in three changes of PBS at RT and probed with a secondary antibody (TFAM, COXII: horseradish peroxidase –conjugated goat anti-rabbit Ig; actin: donkey anti-sheep/goat IgG) for 1 h at RT. After washing the membranes for 30 min in three changes of PBS at RT, the proteins were detected by enhanced chemoluminescence (ECL) and visualized by autoradiography.

Biochemistry

Biochemical methods used in this study are extensively described by Rustin *et al.* 1994 and Chretien *et al.* 1994.

Immunohistochemistry to detect TFAM protein

Cryostat sections of fresh frozen rat testis tissue were incubated in 80 mM K-PIPES (pH 6.5), 5 mM EGTA and 2 mM $MgCl_2$ for 5 s at 37°C and then fixed in the same solution with the addition of 0.3% glutaraldehyde (GA) and 0.1% Triton X-100 for 10 min at RT. The sections were rinsed in PBS, incubated in PBS with 1 mg/ml of $NaBH_4$ and rinsed in PBS again. The sections were incubated with 1% bovine serum albumine (BSA) in TBS for 10 min and with the rabbit a-mTFAM antiserum or preimmune serum diluted 1:1000-2000 in TBS with 1% BSA overnight at 4°C. After rinsing with TBS the sections were incubated with biotinylated swine anti-rabbit

immunoglobulin (Dako) diluted 1:300 in TBS with 1% BSA for 30 min. The sections were rinsed in TBS and the immunoreactive material was visualized using the avidin-biotin-peroxidase complex method (Dakopatts) with diaminobenzidine as fluorochrome.

In situ hybridizations to detect mtDNA and mtRNA

Eight-micrometer cryostat sections of rat testis were mounted on poly-L-lysine coated slides. For detection of mtRNA, the sections were fixed in 2 % GA in 0.1 M sodium cacodylate buffer for 2 min, briefly rinsed in distilled water and then in PBS for 10 min. The sections were then digested with pronase (100 mg/ml) for 5 min, rinsed in distilled water and incubated in 25 mM HCl for 10 min and postfixed in 2 % GA in buffer for 2 min. Control sections were treated with RNase A (50 mg/ml) at 37°C for 30 min. After rinsing in distilled water, prehybridization was performed in a solution with 0.1 % Ficoll, 1.25 % glycine, 0.1 % polyvinylpyrrolidone (PVP), 0.1 % BSA, 10 mM dithiothreitol (DTT), 60 % formamide, 5 X SSC, denatured salmon sperm DNA (0.1 mg/ml) in 62 mM sodium phosphate buffer at pH 6.8. Prehybridization was performed for 4 h at 42°C. The hybridization solution consisted of a modified prehybridization solution (modified concentrations: 0.02 % Ficoll, 0.02 % PVP, 0.02 % BSA, 50 % formamide and no glycine) with the addition of the probe and 10 % dextran sulfate. The hybridization solution (30 ml/ section) was added to the sections, which were covered with cover slips and sealed with contact cement. Hybridization was performed overnight at 42°C, after which the sections were rinsed in a series of ethanol (30 %, 60 %, 90 %) with 0.25 M ammonium acetate and dried in air.

For *in situ* hybridization of mtDNA, the sections were fixed in freshly prepared 4 % paraformaldehyde (PFA) for 2 min, rinsed in PBS containing 5 mM $MgCl_2$ and treated with proteinase K (5 mg/ml) for 10 min at RT, washed in PBS and incubated with 0.1 M glycine in 0.2 M Tris-HCl, pH 7.5, for 10 min. After rinsing in PBS the sections were treated with RNase A (50 mg/ml) at 37°C for 30 min. Prehybridization was performed for 1 h in 10 mM Tris-HCl, pH 7.5 with 0.6 M NaCl, 1 mM EDTA, 0.12 % PVP, 0.6 % BSA, 0.12 % Ficoll, 50 % formamide, denatured salmon sperm DNA (200 mg/ml), yeast total RNA (0.5 mg/ml) and yeast tRNA (50 mg/ml). After prehybridization, the sections were incubated for 5 min in 70 % formamide in 2 X SSC at 75°C. Hybridization was performed overnight at 42°C in a solution consisiting of the prehybridization solution with the addition of probe, 10 % dextran sulfate and

10 mM DTT. Control sections were incubated without subjecting them to DNA-denaturing conditions. The sections were rinsed in 2 X SSC for 1 h at RT and in 0.1 X SSC for 3 h at 50°C and dried in air.

All sections were dipped in film emulsion (NTB2, Kodak) and exposed for 2 weeks and developed (D19, Kodak).

DNA probes for *in situ* hybridizations to detect mtRNA and mtDNA

Fourteen different fragments covering most of the human mtDNA molecule (nt14956-16053, nt15591-16569, nt1-740, nt41-2578, nt2578-4122, nt4122-5274, nt5275-6204, nt6204-7441, nt7441-8287, nt8287-8597, nt8592-9648, nt8729-10254, nt10254-11922 and nt11922-12640) were used as probes. The fragments were purified and radiolabeled with [a-^{35}S]dCTP by the random priming method.

In situ hybridizations to detect *Tfam* mRNAs

Three-micrometer sections of formalin-fixed and paraffin-embedded tissue were mounted on Superfrost/Plus slides, deparaffinated and incubated in 25 mM HCl for 10 min. The sections were rinsed in PBS and incubated in 0.3% Triton X-100 in PBS for 15 min, then rinsed in PBS and treated with pronase E (Sigma), 0.1 mg/ml in 0.05 M Tris-HCl, 50 mM EDTA, pH 7.5 for 5 min. The sections were rinsed in PBS again and incubated for 5 min in 0.1 M glycine in PBS and post-fixed in 4 % PFA in PBS for 5 min. After rinsing in PBS the sections were acetylated by treatment with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min. After rinsing in water the sections were dried in air and pre-hybridizaton was performed in 5 X SSPE, 50% formamide, 1% PVP, 10% dextran sulfate, 5 X Denhardt's solution, 0.5% SDS, denatured salmon sperm DNA (0.1 mg/ml), 0.1 M DTT for 2 h at 42°C. Hybridization was performed overnight at 55°C in the same solution with the addition of ^{35}S-labeled (10^7 cpm/ml) RNA probes. After hybridization the sections were washed in 4 X SSC with 10 mM DTT for 1 h at 55°C and then for 30 min at 50°C. The sections were then washed in 2 X SSC with 50% formamide and 10 mM DTT for 20 min at 65°C. After rinsing in 10 mM Tris-HCl, 1 mM EDTA, 0.5 M NaCl (pH 8.0) the sections were treated with RNase A (20 mg/ml) in the same buffer for 30 min at 37°C. After rinsing in RNase buffer and then in 0.1 X SSC, the sections

were dehydrated and dried in air. Autoradiography was performed with LM-1 emulsion (Amersham) and D19 developer (Kodak).

RNA probes for *in situ* hybridization to detect *Tfam* transcripts

In vitro transcription with the pBluescript (SK-) containing the longest rat *Tfam* cDNA obtained in the testis cDNA library screenings and [a-^{35}S]UTP was performed to obtain antisense probes corresponding to exons II-VII of *Tfam*. Transcripts were subjected to partial alkaline hydrolysis to obtain RNA fragments with an estimated average length of 0.3 kb prior to hybridization.

Results and Discussion

Characterization of the rat *Tfam* gene (paper I)

The special expression pattern of *TFAM/Tfam* in human and mouse testis prompted the characterization of the gene and its expression pattern in rat (**paper I**). To compare the *Tfam* gene structures between different mammals, we screened a rat testis cDNA library and a rat liver genomic library using mouse or rat *Tfam* cDNAs as probes. The rat *Tfam* cDNAs isolated from the testis cDNA library were all truncated and lacked exon I (or sequences upstream of regions corresponding to exons II through VII of the mouse *Tfam* cDNA). The sequence of the full-length cDNA (~1.5 kb) was obtained in database searches. Rat *Tfam* encodes a protein of 245 amino acids (aa) with an aminoterminal 42 aa cleavable leader peptide. After import to mitochondria the leader peptide is cleaved, leaving a mature protein of 203 aa or ~25 kDa. Comparisons of rat, mouse and human TFAM peptide sequence revealed a high degree of conservation with ~90 % similarity between rat and mouse, ~62 % between rat and human and ~81 % between human and mouse TFAM amino acid sequences. Characterization of the genomic clones containing the whole rat *Tfam* gene showed that its division in exons and introns is conserved in mammals. Mapping of the rat *Tfam* gene by fluorescence *in situ* hybridization (FISH) indicated a locus on Chr 20p11. The Chr 20p11 region has conserved linkage synteny with the mouse Chr 10B5 and human Chr 10q21 where the mouse and human *Tfam/TFAM* genes have been mapped.

Expression of the rat *Tfam* is ubiquitous as determined by Northern and Western blots and corresponds well to the expression pattern seen in mice and humans. The proximal promoter regions of the mammalian *Tfam/TFAM* genes are conserved and contain binding sites for the transcription factors NRF2 and Sp1. A consensus binding site for NRF1 is only found in the human *TFAM* promoter. Also the 3'UTR was found to be well-conserved and may contain important regulatory elements.

TFAM expression pattern in mammalian testis (paper I)

The TFAM protein levels decrease as rat spermatogenesis commences. This we observed on Western blots, where we detected low levels of TFAM protein in testis. Consistent with the TFAM expression pattern in human testis, we only observed a single TFAM protein isoform in rat testis (Fig 5). In mouse a nuclear TFAM isoform is expressed in elongating spermatids.

We performed *in situ* hybridizations and immunohistochemistry to determine the distribution of mtDNA, mtRNA and TFAM protein in rat testis. Similar to mouse and human testis, mtRNA levels remained unchanged, while mtDNA and TFAM protein levels decreased in parallel as spermatogenesis progressed. No TFAM protein, mitochondrial or nuclear, was detected in the haploid rat sperm cells (Fig 5). The fact that the nuclear TFAM protein isoform only appears in mouse elongating spermatids suggests that it is dispensable for mammalian spermatogenesis.

Different from other mammals, only low levels of *Tfam* mRNAs were detected in rat testis by *in situ* hybridizations and Northern blots (Fig 5). Approximately 30 % of the *Tfam* mRNAs in testis lacked exon I, suggesting the existence of a testis-specific transcription start site in intron I. The use of alternate *Tfam* transcription start sites in rat testis was demonstrated by primer extension experiments. In liver, a single product of 380 bp was observed, whereas several products of 600-750 bp were seen in testis. The existence of testis-specific *Tfam* mRNAs was also verified by an RT-PCR assay.

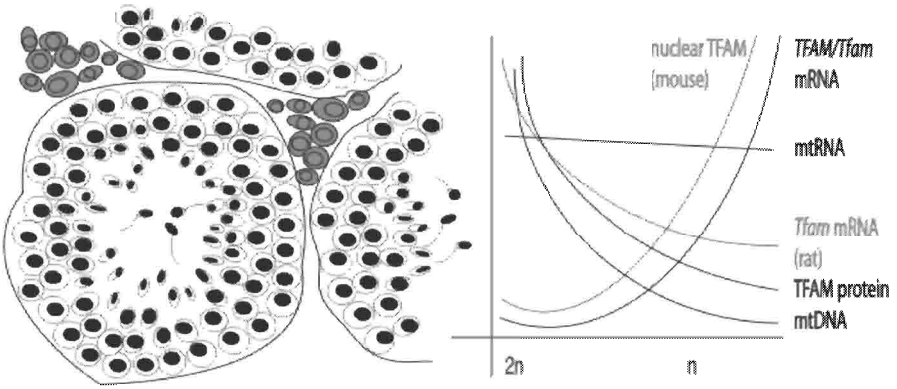

Figure 5 Schematic view over mammalian spermatogenic tubuli. The graph shows TFAM and mtDNA levels and expression patterns during spermatogenesis.

As discussed before, the testis-specific *Tfam/TFAM* transcripts may serve to specifically downregulate TFAM protein levels and thus the mtDNA copy number in spermatid mitochondria. The decreased mtDNA level is most likely an important factor for ensuring uniparental transmission of mtDNA.

Manipulation of TFAM protein levels in mouse testis (unpublished)

To study the mechanisms preventing transmission of paternal mtDNA and the importance of the nuclear TFAM protein isoform, we studied transgenic mice (unpublished data).

We used the *cre*-loxP system to create mice with a testis-specific *Tfam* knockout in testis. Mice homozygous for a floxed (loxP flanked) *Tfam* allele (Larsson *et al.* 1998) were crossed with mice carrying the *cre* recombinase driven by the protamine 1 (Prm1) promoter that is active at the end of spermiogenesis.

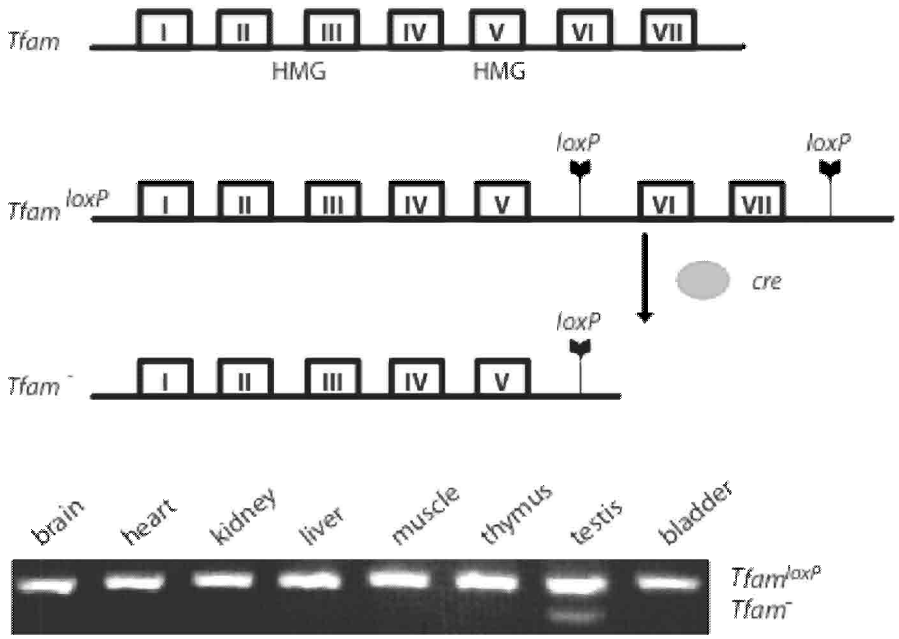

Figure 6 The *cre*-loxP recombination system used for the conditional *Tfam* knockout. The PCR genotype below reveals the recombination specificity and efficiency in multiple tissues of the Prm1-*cre*; *Tfam*$^{loxP/loxP}$ mice.

Recombination detected by PCR was testis-specific, but not complete (Fig 6). Despite the low recombination efficiency an almost complete loss of both nuclear and mitochondrial TFAM protein isoforms from testis of homozygous mutant mice

(Prm1-*cre*; *Tfam*$^{loxP/loxP}$) was seen on Western blots (Fig 7). The mice were fertile, strongly supporting the idea that the nuclear TFAM protein isoform is dispensable for mammalian spermatogenesis (Larsson *et al*. 1996).

We also tried to manipulate the mtDNA copy number in mouse sperm by introducing extra copies of the somatic (mitochondrial) form of *Tfam* cDNA into the mouse genome (unpublished data). The extra copies of *Tfam* were controlled by the Prm1 promoter (Prm1-*Tfam*), and thus only expressed in haploid sperm cells. Even with the increased TFAM protein levels in sperm mitochondria (Fig 7), there was no clear change in mtDNA copy number, and the mice were fertile.

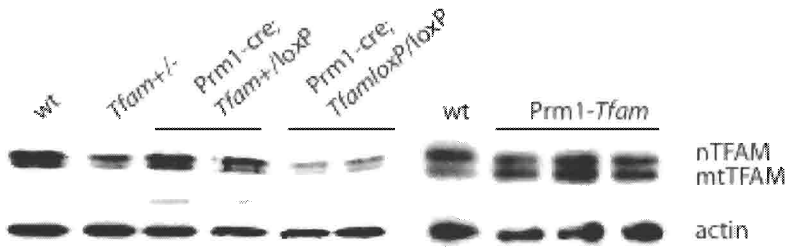

Figure 7 Western blot showing TFAM protein levels in the testis of wild type (wt), heterozygous *Tfam* knockouts (*Tfam+/-*) and transgenic mice (Prm1-*cre*; *Tfam*$^{loxP/loxP}$, Prm1-*cre*; *Tfam*$^{+/loxP}$ and Prm1-*Tfam*). nTFAM, nuclear TFAM protein, mtTFAM, mitochondrial TFAM protein, actin is a loading control.

These results would thus suggest that manipulating TFAM expression and protein levels in testis does not affect fertility and sperm function in mouse. Some matters are, however, worth considering. It is not known exactly in which stage of spermatogenesis the testis-specific *Tfam* transcripts are expressed. The *Prm1* promoter is activated at the end of spermiogenesis, which might be too late to make a difference for the appearance of the testis-specific transcripts and the success of spermatogenesis. Our failure to detect significant changes in the testis mtDNA content may be due to the fact that testis consists of a mixture of different cells types. Sperm cells only comprise a fraction of all testicular cells, and the other cell types contain higher amounts of mtDNA. Also, sperm cells are known to share gene products through cytoplasmic bridges. Low recombination efficiency in a single spermatid may thus leave one copy of the *Tfam* gene intact, which can then be

expressed, and the gene products shared with other sperm cells in the same syncytium.
The common and highly conserved pattern in mammalian testis thus seems to include downregulation of TFAM protein levels and mtDNA copy number and the appearance of testis-specific *Tfam* transcripts (Fig 5). These transcripts may serve to directly downregulate TFAM protein levels and thus indirectly, the mtDNA copy number. This conserved pattern may be one mechanism preventing the transmission of paternal mtDNA, and thus guarantee the inheritance of exclusively maternal mtDNA.

Mice overexpressing human TFAM (paper II)

Previous *Tfam* knockout studies in mouse have shown that TFAM is required for the maintenance of mtDNA and mtRNA. However, it is not clear whether TFAM only functions in mitochondrial transcription, or if it also plays a role in mtDNA packaging as its yeast homologue, Abf2p. To study this we introduced a copy of the whole human *TFAM* gene (~10 kb) into the mouse genome in a 120-170 kb fragment of human genomic DNA from a PAC library (**paper II**). According to the NCBI sequence database the ~400 kb surrounding the *TFAM* gene only contain a few loci, e.g. inositol polyphosphate multikinase gene (*IMPK*), a protein expressed in hematopoietic stem or progenitor cells (*MDSO29*), a ubiquitin-conjugating enzyme gene (*UBE2D1*) plus a few predicted loci with no determined function (LOC222351, LOC222350). To control for these genes, we selected three PAC clones (called PAC2, PAC9, PAC19) containing the *TFAM* gene with different flanking regions.

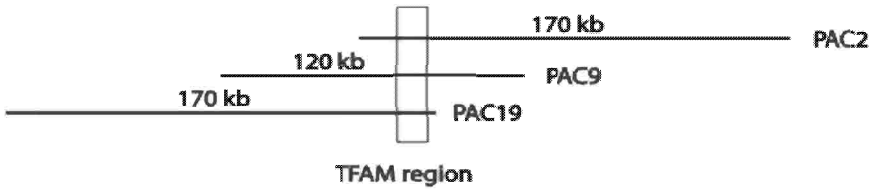

Figure 8 A schematic view of the PAC clones introduced into the mouse genome.

Expression of the *TFAM* transgene was physiologically regulated, as seen on Western blots, and seemed to be highest in PAC19 and lowest in PAC2. As expected, overexpression of TFAM resulted in roughly 50 % increase in mtDNA copy number as demonstrated by Southern blots.

We then performed Northern and Western blots to measure the levels of mtDNA expression. Surprisingly, the enhanced TFAM levels had a strand-specific effect on mtDNA transcription. The level of the L-strand encoded NADH dehydrogenase subunit 6 (ND6) transcript was increased by 50-60 %, whereas levels of the H-strand encoded transcripts ND4 and cytochrome c oxidase subunit 1 (COXI) were unaffected. Also Western blots showed that there was no change in the levels of H-strand encoded cytochrome c oxidase subunit 2 (COXII). Furthermore, biochemical measurements revealed that the activities of the respiratory chain complexes II and IV were unaffected in the transgenic mice and there was no change in their mitochondrial mass, as estimated by the citrate synthase activity. A plausible explanation is that the 81% similarity between mouse and human TFAM proteins is not enough for the human protein to convey full transcriptional activation in mouse.

In fact, one of our initial questions while making the TFAM overexpressor mice was whether the human TFAM protein could complement for the loss of the endogenous mouse *Tfam* gene. We approached this question by mating the PAC-*TFAM* transgenic mice with heterozygous *Tfam* knockout mice (*Tfam*$^{+/-}$) and then backcrossing the heterozygous knockout mice carrying the PAC-*TFAM* transgene (*Tfam*$^{+/-}$; PAC-*TFAM*) to the heterozygous *Tfam* knockout mice (Fig 9).

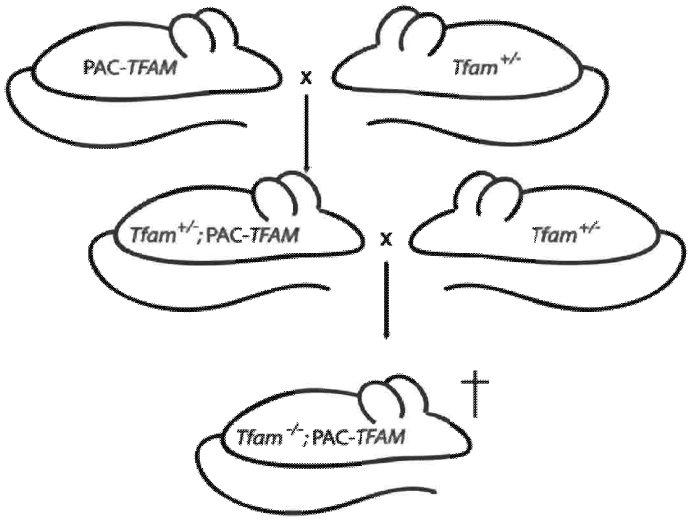

Figure 9 Mating scheme used to produce the *Tfam*$^{-/-}$; PAC-*TFAM* mice. No such mice were born, indicating embryonic lethality and inability of the human TFAM to rescue *Tfam* knockout.

Despite its significant conservation, the human TFAM could not rescue the lack of endogenous mouse TFAM protein. However, *Tfam*$^{-/-}$; PAC-*TFAM* embryos contain mtDNA, whereas the homozygous *Tfam* knockout embryos do not. Furthermore, the *Tfam*$^{-/-}$; PAC-*TFAM* embryos have increased levels of L-strand transcripts compared to the homozygous *Tfam* knockout embryos, while there is no change in the H-strand transcript levels. These findings are in line with our previous results from the PAC-*TFAM* transgenic mice and support the existence of a link between the L-strand transcription and mtDNA replication. An explanation for the enhanced mtDNA copy number may thus be that the increased L-strand transcription results in higher amounts of replication primers, and allows mtDNA replication to take place more frequently. Alternatively, the elevated mtDNA levels may be due to improved mtDNA stability.

It is possible that human TFAM is not compatible with the mouse mitochondrial transcription machinery. However, since human TFAM could activate transcription from the LSP, it is likely that there is a problem in recognizing the HSP. A classical study by Dairaghi *et al.* in 1995 demonstrated that addition of the C-terminal tail of human TFAM to yeast Abf2p transforms a simple DNA-binding protein (Abf2p) into a transcriptional activator *in vitro*. In their study they used a template containing the human LSP. Additional studies are required to find out if the fusion protein can activate transcription from the HSP as well. Similarly, introduction of mutated forms of human TFAM protein in mouse should answer the question whether it is possible to dissociate its function as a transcriptional activator from its function in mtDNA maintenance and copy number control.

Mitochondrial transcription factors B1 and B2 (papers III & IV)

Early trials to reconstitute mammalian mitochondrial transcription *in vitro* only succeeded using pure TFAM protein with a partially purified POLRMT fraction. This suggested that another, yet unknown factor from the POLRMT fraction was required and a mammalian homologue for the yeast mitochondrial RNA polymerase specificity factor Mtf1p was an obvious candidate. We thus performed profile-based BLAST searches against the NCBI sequence bank with the Mtf1p peptide sequence from *Schizosaccharomyces pombe*. This resulted in the identification of a human peptide sequence we named the mitochondrial transcription factor B1 (TFB1M) and another homologous human peptide, which we called TFB2M (**paper III**). Both of the novel peptides, especially TFB1M, were similar to bacterial rRNA dimethyl transferases. We also identified and characterized the homologous genes in mouse (*Tfb1m* and *Tfb2m*; previously, *Hkp1*) (**paper IV**).

Most bioinformatics tools predicted a mitochondrial localization for both human and mouse TFBMs. We verified this by targeting HeLa cells with plasmids where either mouse *Tfb1m* or *Tfb2m* cDNA was fused to the green fluorescent protein (GFP) coding sequence. Confocal microscopy of the HeLa cells revealed a GFP pattern indicative of mitochondrial localization.

We studied expression of the human and mouse *TFBM/Tfbm* genes by Northern blots and found ubiquitous expression similar to that of genes encoding other proteins of the mitochondrial transcription machinery. Interestingly, there was a clear difference in the expression level between the two factors in testis: the *TFB1M/Tfb1m* transcript levels were high, whereas the *TFB2M/Tfb2m* transcript levels were relatively low. The promoter regions of both human and mouse *TFBM/Tfbm* genes contain several binding sites for the transcription factors NRF2 and Sp1, but no NRF1 consensus elements were found in any of the promoters (**paper IV**).

We demonstrated by *in vitro* transcription assays (**paper III**) that the minimal set of factors required for mitochondrial transcription initiation in mammals comprise the POLRMT, TFAM and either TFB1M or TFB2M (Fig 10). The stimulatory effect of both TFB1M and TFB2M was only seen on promoter-dependent transcription initiation, not on tailed templates. Our *in vitro* experiments also suggested that

TFB2M is an at least 10 times more effective transcription initiation factor than TFB1M.

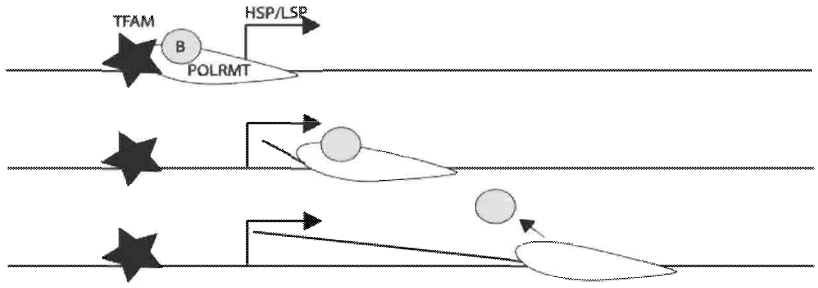

Figure 10 An illustration showing the mammalian basal mitochondrial transcription machinery. TFAM binds upstream of LSP/HSP and most likely interacts with the POLRMT-TFBM heterodimer thus forming a functional holoenzyme complex. As transcription proceeds, the TFBM most likely dissociates from the POLRMT

Several lines of evidence suggest that the TFBMs form heterodimers with the POLRMT. First, the maximal transcriptional activity is seen when TFB2M is present in 1:1 molar ratio with the POLRMT. Second, purification of the recombinant POLRMT is only possible when coexpressed with either TFB1M or TFB2M. The third and strongest piece of evidence was demonstrated in immunodepletion experiments, where adding recombinant TFB2M (or TFB1M) alone to TFB2M-immunodepleted mitochondrial extracts was not enough to reconstitute mitochondrial transcription. The addition of POLRMT together with TFB2M (or TFB1M) was required for transcription initiation to take place.

TFAM is absolutely required for mtDNA maintenance, as previously shown by knockout studies (Larsson *et al*. 1998, Wang *et al*. 1999, Li *et al*. 2000, Silva *et al*. 2000, Sörensen *et al*. 2001, Wredenberg *et al*. 2002). Having the mitochondrial *in vitro* transcription system in our hands we were able to test the effect of different TFAM concentrations on the efficiency of mtDNA transcription. We found that while L-strand transcription was activated at low TFAM levels, and stayed relatively high at a broad range of TFAM concentrations, activation of the H-strand transcription required relatively high levels of TFAM. Consistently, it has been shown that TFAM has higher affinity for LSP than HSP.

Evolution of the mitochondrial transcription factor B (paper IV)

In search of the mammalian Mtf1p homologues from the NCBI sequence database we identified not one, but two homologous proteins in several metazoan species. The Mtf1p crystal structure revealed structural homology to bacterial rRNA methyltransferases, especially the ErmC' from *Bacillus subtilis* (Schubot *et al.* 2001). Also TFBMs, especially the TFB1Ms, shared structural homology with bacterial rRNA methyltransferases. TFB1M was in fact shown to bind S-adenosyl methionine, a methyl donor (McCulloch *et al.* 2002), although no methyltransferase activity has been demonstrated so far. The conserved methyltransferase domain may thus be just a memory of an ancient function the protein had before acquiring new functions in mitochondrial transcription.

To find out more about the TFBM function and origin, we studied their conservation (**paper IV**). Comparison of TFBM peptide sequences from different species in a cluster analysis divided them in three groups. The first group contained the single yeast TFBM homologues (called Mtf1p) from *S. pombe* and *S. cerevisiae*. The second group consisted of TFB1M homologues from human, mouse and *Drosophila* plus the single *Caenorhabditis elegans* TFBM. The third cluster included peptides homologous to TFB2M from human, mouse and *Drosophila*.

To study the TFB1M and TFB2M peptides in more detail, we characterized the mouse genes *Tfb1m* and *Tfb2m*. We used The Jackson Laboratory BSB backcross panel to map the loci.

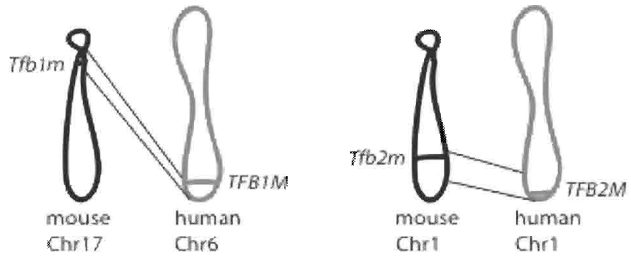

Figure 11 The *TFBM/Tfbm* loci and regions of conserved linkage synteny shown on mouse and human chromosomes.

The *Tfb1m* gene was found to map to the distal end of Chr 17 in a region of conserved linkage synteny with the human *TFB1M* on Chr 6q25. The *Tfb2m* locus in the proximal part of Chr 1 is also in a region of conserved linkage synteny with the human *TFB2M* on Chr 1q44 (Fig 11).

BLAST searches against the Celera genome databases demonstrated that also the gene structures are well conserved. The mouse *Tfb1m* gene is divided in seven exons and spans 38.5 kb. The *Tfb2m* locus consists of eight exons and spans 18.2 kb. Interestingly, search with the *Tfb2m* cDNA gave two significant hits: the *Tfb2m* locus on Chr 1, and another sequence of 1.9 kb on Chr 12. This second locus encodes an open reading frame of 347 aa starting from aa 50 of the mouse TFB2M peptide sequence. As the locus contains no introns it is most likely a processed pseudogene. Similarly, there is a human *TFB2M* processed pseudogene on human Chr 6. We only detected single *Tfb2m* transcripts in Northern analyses indicating that no messages are produced from the *Tfb2m/TFB2M* putative pseudogenes (**papers III and IV**).

The human and mouse TFB1M peptide sequences are 86 % identical in the first 322 aa, while the TFB2M peptides are 53 % identical. Interestingly, the human TFB1M and TFB2M are only 25 % similar, and the mouse TFBMs are even less so, only 21 %. These figures suggest that a gene duplication event took place early in metazoan evolution, and the two modern *Tfbm/TFBM* genes most likely originate from a single ancestral gene.

Concluding remarks

During this series of studies several central aspects about mitochondrial transcription and TFAM have been uncovered.

We defined the basal mammalian mitochondrial transcription machinery in Paper III. The three proteins required for faithful, promoter-specific initiation of mtDNA transcription are POLRMT, TFAM and TFB1M or TFB2M. The B factors stimulate transcription by forming heterodimers with POLRMT, thus stabilizing it. TFAM is required for the activation of mtDNA transcription.

We concluded in paper I that the conserved features of TFAM expression during mammalian spermatogenesis include downregulation of TFAM protein levels and mtDNA copy number and the expression of alternative *TFAM/Tfam* transcripts. This pattern is likely to have an important role in preventing the transmission of paternal mtDNA.

Further conclusions form papers I, III and IV are that the expression and gene structures of the mitochondrial transcription factors A, B1 and B2 have been well conserved during mammalian evolution. They also map to regions of conserved linkage synteny. All these evidence suggest the genes encoding proteins of the mitochondrial transcription machinery are under strict evolutionary control.

In paper II we observed that the human TFAM protein has strand-specific effects on mitochondrial transcription when expressed in mouse. Furthermore, human TFAM protein cannot rescue the *Tfam* knockout, but can enhance the levels of mtDNA in homozygous *Tfam* null embryos. This suggests that human TFAM is not fully functional in mouse, but can improve mtDNA stability or increase replication of mtDNA. The strand-specific effects could be studied in a mouse mitochondrial *in vitro* transcription system, where human TFAM replaces the mouse TFAM protein.

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