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LINKING TRANSCRIPTION TO DNA REPLICATION IN MAMMALIAN MITOCHONDRIA

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ABSTRACT

Most of the adenosine phosphate (ATP) used to drive cellular processes is produced through oxidative phosphorylation (OXPHOS). At the heart of this process is the electron transport chain, which contains a number of protein complexes located in the inner membrane of mitochondria. In addition to about 90 nuclear encoded gene products, the electron transport chain also contains 13 subunits encoded by mitochondrial DNA (mtDNA). This double-stranded, circular DNA genome is therefore essential for proper OXPHOS function; mutations, deletion, or depletion of mtDNA can cause mitochondrial dysfunction and human disease.

The mtDNA molecule is replicated and transcribed by molecular machineries that are distinct from those present in the nucleus. Many of the components are related to proteins identified in bacteria and bacteriophages, in keeping with the prokaryotic origin of the mitochondrion. The transcription machinery in mammalian mitochondria contains three essential factors: mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM), and mitochondrial transcription factor B (TFB2M). Recently, the role of TFAM as a core initiation factor has been questioned. In this thesis, we demonstrate that both TFAM and TFB2M are required for mitochondrial transcription. We also demonstrate that TFB1M, a TFB2M paralog, does not function as a *bona fide* transcription factor. Even if TFAM is an essential factor for transcription initiation *in vitro,* the absolute requirement of TFAM can be circumvented by experimental conditions that stimulate DNA breathing at the promoter. We suggest a possible explanation for this effect, by demonstrating that TFAM can introduce negative supercoils and cause conformational changes in mtDNA that facilitates promoter recognition and transcription initiation by POLRMT and TFB2M.

Our studies also demonstrate that a substantial fraction of the transcription events initiated from the light strand promoter (LSP) are prematurely terminated at a conserved sequence element, CSBII. The 3′-ends of these transcripts correspond to prominent RNA-DNA transition sites mapped *in vivo*, strongly suggesting that they are used to prime mitochondrial DNA synthesis.

The mitochondrial transcription termination factor (MTERF1) was originally identified as a regulator of rRNA transcription. MTERF1 binds to a 28-bp mtDNA sequence downstream of the 16S rRNA gene. MTERF1 supposedly terminates transcription at this location to produce more rRNAs than the mRNAs species encoded by down stream sequences on the same strand. In this thesis, we explore an alternative role for MTERF1 as a regulator of mtDNA replication. We demonstrate that binding of MTERF1 causes a pause in the progression of the mitochondrial replication fork in one direction, but not in the other. The effect is explained by MTERF1's ability to counteract the DNA unwinding activity of the mitochondrial DNA helicase, TWINKLE. Based on our findings, we speculate that MTERF1 can coordinate synthesis of the two mtDNA strands.

LIST OF PUBLICATIONS

I. Human mitochondrial transcription revisited: only TFAM and TFB2M are required for transcription of the mitochondrial genes in vitro.

Litonin D, Sologub M, **Shi Y**, Savkina M, Anikin M, Falkenberg M, Gustafsson CM, Temiakov D.

J Biol Chem. 2010 Jun 11;285(24):18129-33.

II. Mammalian transcription factor A is a core component of the mitochondrial transcription machinery.

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Proc Natl Acad Sci U S A. 2012 Oct 9;109(41):16510-5.

III. Conserved sequence box II directs transcription termination and primer formation in mitochondria.

Pham XH, Farge G, **Shi Y**, Gaspari M, Gustafsson CM, Falkenberg M.

J Biol Chem. 2006 Aug 25;281(34):24647-52.

IV. MTERF1 is a contra-helicase that stimulates DNA replication fork pausing *in vitro* and *in vivo*.

Shi Y, Zhu X, Falkenberg M, Gustafsson CM.

Manuscript, 2013

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CSB	Conserved sequence block
C-terminal	Carboxy terminal
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
FRET	Fluorescence resonance energy transfer
HMG	High mobility group
HSP	Heavy strand promoter
kb	Kilo base
kDA	Kilo dalton
LSP	Light strand promoter
mRNA	Messenger ribonucleic acid
MRP	Mitochondrial RNA processing
mtDNA	Mitochondrial DNA
mtSSB	Mitochondrial single-stranded DNA binding protein
mTERF	Mitochondrial transcription termination factor
nt	Nucleotides
N-terminal	Amino terminal
O_H	Origin of heavy strand replication
O_L	Origin of light strand replication
OXPHOS	Oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PEO	Progressive external opthalmaplegia
POLγ	Mitochondrial DNA polymerase gamma
POLγA	Catalytic subunit of POLγ
POLγB	Accessory subunit of POLγ
POLRMT	Mitochondrial RNA polymerase
PPR	Pentatricopeptide repeat
RITOLS	Ribonucleotide incorporation throughout the lagging strand
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
ssDNA	Single-stranded DNA
TAS	Termination associated sequence
TFB1M	Mitochondrial transcription factor B1
TFB2M	Mitochondrial transcription factor B2
TFAM	Mitochondrial transcription factor A
tRNA	Transfer RNA

tRNA Leu	Transfer RNA for Leucine
TWINKLE	T7 gp4-like protein with intramitochondrial nucleoid localization (mitochondrial helicase)

1 INTRODUCTION

Mitochondria are eukaryotic cellular organelles that generate most of the cellular energy, in the form of ATP, through the oxidative phosphorylation system (OXPHOS). In addition to their function as cellular power-houses, mitochondria also play a role in many metabolic pathways, e.g. regulation of cytosolic calcium concentration, control of apoptotic cell death, signaling, and cellular differentiation (Carafoli et al. 1978, Brookes et al. 2002, Ott et al. 2007, Chen et al. 2012).

Mitochondria have a double membrane; the outer membrane encloses the whole organelle and separates it from the cytosol, whereas the inner membrane folds inwards, forming the cristae. The intermembrane space is located between the two membranes and the space inside the inner membrane is termed the matrix (Fig. 1). Mitochondria are unique among mammalian organelles as they contain their own genome, mitochondrial DNA (mtDNA), which is physically separated from the nuclear genome. The mtDNA differs from nuclear DNA in several aspects. The genetic code used in mitochondria deviates from the standard genetic code, e.g. "UGA" that is a stop codon in most organisms, codes for tryptophan in vertebrate mitochondria. In addition, mtDNA is exclusively maternally inherited and the mutation rate is much higher than that of nuclear DNA. However, mitochondrial function and biogenesis are still heavily dependent on nuclear DNA, since most of the mitochondrial proteins are encoded in the nucleus, synthesized in the cytoplasm and imported into mitochondria. According to current estimations, there are about 1,500 mitochondrial proteins and only 13 of these are encoded by the mtDNA.

There are many examples of mutations in mtDNA or mitochondrial protein-encoding genes in nuclear DNA that lead to mitochondrial dysfunction. Dysfunction of mitochondria has been implicated to a wide range of human diseases and conditions (Wallace 1999), such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, as well as aging and cancer (Blanchard et al. 1993, Shoffner et al. 1993).

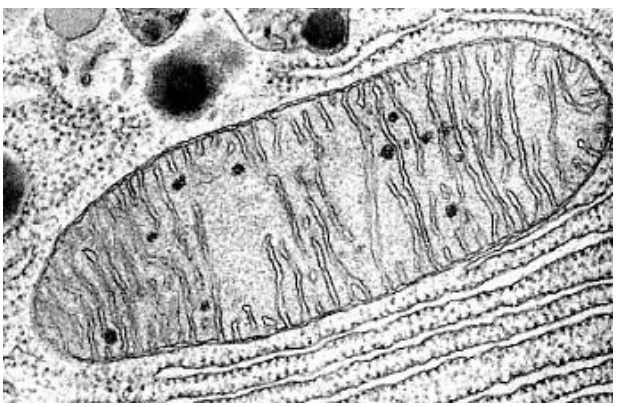

Figure 1. Electron microscope image of a mitochondrion. The double membrane structure and the cristae are clearly visible. (Micrograph by K.R.Porter)

1.1 ORIGIN OF MITOCHONDRIA

According to the generally accepted endosymbiotic theory, mitochondria have evolved from an alpha-proteobacterium that was taken up as an endosymbiont by an ancestral eukaryotic cell about two billion years ago (Martin et al. 1998). During evolution, a large number of ancestral bacterial genes have either been lost or transferred from the endosymbiont to the nuclear genome. Today, only a small part of the genes derived from the endosymbiont are found in the mitochondrial genome and most of the mitochondrial proteins are encoded in the nucleus.

The mitochondrion has evolved from an endosymbiont to an organelle, but why does it still retain a small, separate DNA genome? There are several hypotheses on the permanence of the mitochondrial genome. First, the remaining mtDNA-encoded polypeptides may be too hydrophobic to be translated in the cytoplasm and imported into mitochondria (Claros et al. 1995). This hypothesis is based on the observation that nuclear-encoded mitochondrial proteins have lower hydrophobicity score than those encoded by mtDNA. Mitochondria-encoded proteins, for example cytochrome b (Cyt b) and cytochrome C oxidase subunit I (cox I) which have 8 and 12 transmembrane domains respectively, are among the most hydrophobic proteins in the eukaryotic cell. Protein import experiments demonstrate that the more hydrophobic domains a protein contains, the more difficult it is to import the protein into mitochondria. Hybrid Cytb protein with more than 4 transmembrane domains could not be imported into mitochondria (Claros et al. 1995). However, there are also experimental data showing that the *ATP8* gene can be introduced to nuclear genome and the expressed protein transported into mitochondria (Oca-Cossio et al. 2003).

The second hypothesis states that the preserved mitochondrial protein coding-genes are difficult to transfer since they have distinct genetic codes, which is different from the code used in nuclear DNA. This makes mitochondrial genes un-interpretable in the nucleus (Andersson et al. 1991), e.g. AGG and AGA encode arginine in the nucleus, but corresponds to stop codons in mitochondria, whereas UGA codes for tryptophan in mitochondria, but is a stop codon in the nucleus.

Another hypothesis suggests that the expression of mtDNA is regulated in response to the energy requirement of the eukaryotic cell and thus plays an important role in metabolic control. Mitochondrial gene transcription and mtDNA replication could e.g. be directly influenced by the redox state or the activity of the electron transport chain. An analogous model exists in chloroplasts, where there is a direct redox control of transcription (Allen 1993). There may also be an evolutionary aspect of this regulation, since the small size and high mutation rate of the mitochondrial genome makes it easier to respond rapidly to changes in the environment (Wallace 2007).

1.2 THE MITOCHONDRIAL GENOME

Mitochondrial DNA was discovered in the 1960s by M. Nass and S. Nass when they studied chick embryo mitochondria with electron microscope (Nass et al. 1963). The mammalian mitochondrial genome is a closed circular double-stranded molecule with a size of about 15,000 - 18,000 base pairs (Fig. 2). There are 2 - 10 copies of mtDNA per mitochondrion and many hundreds to thousands of mitochondria per cell. The amount of mitochondria varies among different cell types; energy-consuming cells like those in muscle, brain and liver contain more copies of mtDNA. The nucleotide content of the two human mtDNA strands differs and the strands can be separated by alkaline cesium chloride gradient centrifugation. The guanine rich strand with higher density is termed the heavy strand, and the opposing strand is termed the light strand.

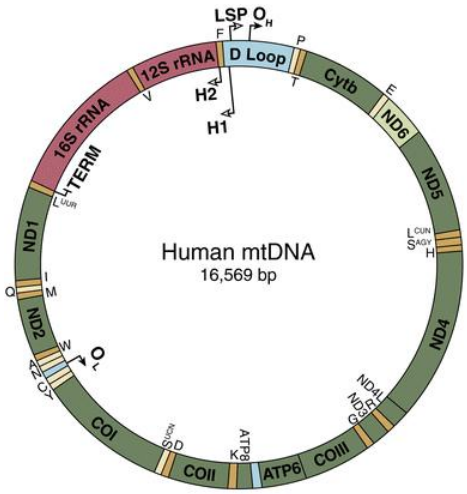

Figure 2. Map of the human mitochondrial genome. The origins of H-strand (O_H) and L-strand (O_L) replication and the direction of DNA synthesis are indicated by solid arrows; transcription promoters (LSP and HSP) are denoted by arrows. The 22 tRNA genes encoded on the two strands are indicated with the standard one-letter symbols for amino acids. The genes coding for the two rRNA species (12S and 16S) and the 13 protein coding genes are depicted by shaded boxes (Adapted from Falkenberg M, et al. 2007 Annu. Rev.Biochem.76: 679-99).

Mammalian mtDNA is organized in a highly compact manner with all the coding sequences very close to each other without introns; some of the protein-coding genes even overlap. The only longer noncoding region in the genome harbors the control elements for transcription and DNA replication. Located in this region are the two major transcription promoters, the heavy strand promoter (HSP1) and the light strand promoter (LSP), which are responsible for polycistronic transcription of the two DNA strands. Each of the two DNA strands also contains a separate origin of DNA replication, the origin of heavy strand replication (O_H) and the origin of light strand replication (O_L). O_H is located in the control region, about 100 bp downstream of LSP, whereas O_L is localized to the opposite side of the genome, in a short non-coding region of about 30 nts (Fig. 2).

An interesting feature of mammalian mtDNA is the existence of a triple-stranded DNA structure termed the displacement loop (D-loop). The D-loop is located in the control region and consists of a short heavy strand DNA product (500 – 700 nts

long) that remains annealed to the parental L-strand. The D-loop structure is formed by abortive DNA synthesis initiated from O_H and it is terminated at a region named the termination associated sequence (TAS).

In contrast to the compact organization of mtDNA in mammals, the yeast *Saccharomyces cerevisiae* mitochondrial genome contains abundant non-coding sequences (Bernardi et al. 1970, Bernardi 1976). The genome size is about 75 kb (Faugeron-Fonty et al. 1979), which makes yeast mtDNA almost five times larger than that of the mitochondrial genome in animals.

Although the size of mtDNA differs slightly among mammalian species (15,000 to 18,000 bp) (Saccone et al. 1999), the structure, genetic content and organization are quite conserved. Human mitochondrial DNA is 16,569 bp and encodes 37 genes including 13 protein-coding genes, 2 ribosome RNAs (12S and 16S) and 22 transfer RNAs (see Fig. 2). The 13 peptides encoded are all essential subunits of the respiratory complexes I, III, IV and V. The rRNA and tRNA molecules are necessary for the translation of the respiratory subunits within mitochondria.

1.3 MITOCHONDRIAL TRANSCRIPTION

Each strand of human mtDNA harbors a single promoter region for transcription initiation: HSP and LSP. There are also reports of a second heavy strand promoter, HSP2, located about 80 bp downstream of HSP1 (Montoya et al. 1983, Martin et al. 2005) (Fig. 3). Transcription from the mtDNA promoters leads to the production of polycistronic precursor RNAs that contain the genetic information encoded by the individual strands. The primary transcripts are subsequently processed to generate the individual tRNA, rRNA and mRNA molecules.

1.3.1 Mitochondrial Transcription Factors

Mitochondrial RNA polymerase

Mitochondrial DNA expression is dependent on the single-subunit mitochondrial RNA polymerase (mtRNAP). The first identified and characterized mtRNAP was the one from budding yeast *S. cerevisiae,* which is encoded by the *RPO41* gene (Greenleaf et al. 1986). In 1997, the mammalian mtRNAP (POLRMT) was identified (Tiranti et al. 1997). Both RPO41 and POLRMT are homologous to the T7 bacteriophage RNA polymerase (T7 RNAP) (Masters et al. 1987, Tiranti et al. 1997). Unlike the T7 enzyme, POLRMT itself cannot initiate promoter-specific transcription, but requires the assistance of transcription factor A (TFAM) and the mitochondrial transcription factor B2 (TFB2M, also denoted mtTFB2) (Falkenberg et al, 2002). The basic transcriptional machinery in *S. cerevisiae* is less complicated and contains only Rpo41 and the TFB2M homologue Mtf1 (Schinkel et al. 1987), which together forms a heterodimer that recognizes promoters and initiates transcription.

MtRNAP proteins comprise three different domains: a C-terminal polymerase domain (CTD), an N-terminal domain (NTD), and an N-terminal extension (NTE)

domain (Nayak et al. 2009, Ringel et al. 2011). Structural and sequential comparison of mtRNAPs to T7 RNAP (Deshpande et al. 2012) showed that the C-terminal domain is relatively conserved and forms a structure resembling a human right hand with palm, thumb and finger subdomains. The C-terminal domain of mtRNAP contains elements required for fundamental polymerase activities, i.e. DNA template binding, substrate binding, and NTP incorporation. The N-terminal domain is more flexible and displays only vague sequence similarity to the N-terminal domain of T7 RNAP. There are two important elements in the N-terminal domain: the AT-rich recognition loop and the intercalating hairpin, which both are believed to interact with the promoter and help with promoter melting during transcription initiation (Ringel et al. 2011). The N-terminal extension part in mtRNAP shows high variation across species, and it is missing from T7 RNAP. The NTE region contains the mitochondrial targeting sequence, which is cleaved off when the protein is imported into mitochondria. Studies with the truncated forms of Rpo41 demonstrated that deletions in the N-terminal of Rpo41 weakened interactions with Mtf1 (Paratkar et al. 2011), suggesting that the NTE region may in some way regulate Rpo41-Mtf1 interactions. There are also reports suggesting that the NTE is involved in coupling transcription with translation. Mutations in the NTE result in a reduction of mitochondrial translation and cause an increased sensitivity to the mitochondrial translation inhibitor erythromycin. In addition, the NTE interacts directly with Nam1, a yeast protein involved in RNA processing and translation in mitochondria (Rodeheffer et al. 2001, Rodeheffer et al. 2003).

Human POLRMT also contains a pentatricopeptide repeat (PPR) domain between the N-terminal domain and the NTE region (Ringel et al. 2011). PPR domain containing proteins were first identified in plant organelles and many function in RNA editing and processing (Delannoy et al. 2007, Saha et al. 2007). The function of the PPR domain in POLRMT is not characterized yet, but one possibility is that the domain interacts with the newly synthesized RNA as it exists from the catalytic core of the enzyme.

Mitochondrial transcription factor A (TFAM)

TFAM was the first identified mammalian mitochondrial transcription factor (Fisher et al. 1985, Fisher et al. 1987, Fisher et al. 1988, Larsson et al. 1997). As most of the mitochondrial proteins, TFAM is encoded in the nuclear genome, synthesized in the cytoplasm, and transferred into the mitochondria. The premature form of TFAM consists of 246 amino acids. The first 42 amino acids code for the mitochondrial targeting sequence, which is removed after the protein is imported into mitochondria (Parisi et al. 1991). The mature form of TFAM has a molecular weight of about 24 kDa.

TFAM is a member of the high mobility group (HMG) box family of proteins, which can bind, unwind, and bend DNA (Parisi et al. 1991, Gangelhoff et al. 2009). These features are related to the multiple functions that this family of proteins play in e.g. transcription, replication, and DNA repair. TFAM contains two HMG motif domains (HMG Box A and B) separated by a 27 amino acid linker region, and a 25 amino acid C-terminal tail. Structural analyses revealed that TFAM BoxA is a

canonical HMG domain, which can efficiently bind to double-stranded DNA, whereas BoxB has only weak affinity to dsDNA (Gangelhoff et al. 2009). TFAM is a multifunctional protein, which is important for mtDNA transcription initiation, replication and maintenance (Chang et al. 1985, Fisher et al. 1988, Kang et al. 2007).

The C-terminal tail of TFAM is required for promoter-specific initiation of mtDNA transcription (Dairaghi et al. 1995). Abf2p, the TFAM homolog in yeast, lacks the C-terminal tail part, and it cannot stimulate transcription. However, a hybrid protein of Abf2, with the C-terminal tail of human TFAM, exhibited transcriptional activation function at the human LSP (Dairaghi et al. 1995). TFAM has also been shown to interact directly with the other components of the mitochondrial transcription machinery to stimulate transcription initiation (McCulloch et al. 2003).

TFAM binds in a sequence specific manner to the upstream of both HSP1 and LSP to allow initiation of transcription (Fisher et al. 1987). The exact distance between these TFAM high-affinity binding sequences and the transcription initiation sites is of critical importance for promoter activity (Dairaghi et al. 1995), further supporting the idea that TFAM interacts directly with the POLRMT-TFB2M heterodimer to stimulate transcription initiation.

Besides its ability to recognize specific high-affinity sites close to the mitochondrial promoter, TFAM also displays a strong, non-sequence-specific DNA-binding activity. In yeast, the abundant Abf2 protein covers the whole mitochondrial genome (Diffley et al. 1991). Abf2 does not function in mitochondrial transcription initiation, but instead packages the yeast mtDNA, forming a protein-DNA complex termed the nucleoid (Friddle, Klare et al. 2004). Nucleoids are also present in mammalian cells and dozens of proteins have been claimed to be components of these structures, including mtSSB, Twinkle, POLγ, and TFAM (Spelbrink et al. 2001, Wang et al. 2006, Kang et al. 2007, Ruhanen et al. 2010).

According to previous estimates, each mitochondrial nucleoid contains 2 - 8 copies of mtDNA (Legros et al. 2004). However, recent studies using high resolution STED microscopy demonstrated that the majority of mitochondrial nucleoids only contains a single copy of mtDNA covered with about 1000 molecules of TFAM (Kukat et al. 2011), corresponding to one TFAM molecule for every 10 – 20 bp of mtDNA. The quantification of TFAM is consistent with the results published before (Schultz et al. 1969, Alam et al. 2003, Wang et al. 2006, He et al. 2007) and demonstrates that TFAM, similar to the situation in yeast, plays a key role in mtDNA packaging, which is consistent with its function in mtDNA maintenance. The mtDNA abundance in somatic tissues is proportional to the amount of TFAM. Mouse genetics have also demonstrated that TFAM is a key regulator of mtDNA copy number. Loss of TFAM leads to depletion of mtDNA and causes a lethal phenotype in mice. This regulation is independent of TFAM's function in transcription initiation and rather suggests that copy number control is dependent on TFAM's ability to package mtDNA into nucleoids (Ekstrand et al. 2004).

Mitochondrial transcription factor B2

TFB2M was first identified in 2002 and like its counterpart in yeast, Mtf1, the

protein shares sequence similarity with rRNA methyltransferases (Falkenberg et al. 2002). Originally, a second transcription factor termed TFB1M was also identified. It was hypothesized that the presence of two proteins that could interact with mammalian POLRMT would allow for flexible regulation of mtDNA gene transcription in response to changing metabolic requirements (Falkenberg et al. 2002, McCulloch et al. 2002). Both TFB1M and TFB2M are similar to a family of rRNA methyltransferases, which dimethylates 12S rRNA during ribosome biogenesis, and phylogenetic analyses have suggested that the proteins are derived from the rRNA dimethyltransferase of the mitochondrial endosymbiont. In agreement with this notion, TFB1M can bind S-adenosylmethionine and methylate rRNA at a stem-loop structure. However, this function does not seem to be required for transcription activity (Seidel-Rogol et al. 2003).

Recent studies suggest that the two TFBM factors are functionally distinct and have identified TFB2M as the *bona fide* mitochondrial transcription factor in mammalian cells. Knockdown of TFB1M or TFB2M in Drosophila cultured cells showed that decrease of TFB2M resulted in lower levels of mtDNA transcription, whereas decrease of TFB1M led to lower levels of mitochondrial translation (Matsushima et al. 2004, Matsushima et al. 2005). Similar results were reported in mice, where deletion of the TFB1M did not directly affect *in vivo* mitochondrial transcription. Instead the translation levels were dramatically reduced, an effect, which could be linked to TFB1M's activity as a dimethyltransferase in mitochondria, regulating 12S rRNA methylation (Metodiev et al. 2009). These data demonstrate that the B factor isoforms (TFB1M and TFB2M) are not functionally redundant. The factors probably both originated from one single gene, but in the long evolutionary process and following gene duplication, they specialized into distinct functions. TFB1M functions as a mitochondrial translational regulation factor; TFB2M is essential for mitochondrial transcription.

1.3.2 Mitochondrial Transcription Initiation

In budding yeast, *S. cerevisiae*, only Mtf1 (also denoted sc-mtTFB) and Rpo41 are necessary and sufficient to initiate transcription from the mitochondrial promoter *in vitro*. Similar to the situation in yeast, mammalian TFB2M interacts directly with POLRMT and forms a heterodimer, which binds to transcription initiation sites and initiates promoter-specific transcription *in vitro.* In contrast to the yeast system, initiation of mammalian mitochondrial transcription requires the presence of an additional factor, TFAM. Site-specific protein-DNA photo-cross-linking studies in yeast showed that when Rpo41 and Mtf1 bind to the promoter region, Mtf1 traps the non-template strand to facilitate DNA melting (Paratkar et al. 2009). Studies of the human mitochondrial transcription initiation complex demonstrate that TFB2M interacts with the bases at position +1 to +3 in the template strand. In addition, TFB2M also interacts with the priming substrate through its N-terminal domain (Sologub et al. 2009). These activities of TFB2M might help to unwind the promoter and stabilize the open complex. Neither TFB2M nor Mtf1 is required for the transcription elongation stage and the factors are released when the mtRNAP enters the transcription elongation mode (Mangus et al. 1994, Sologub et al. 2009).

The T7 RNAP recognizes promoter sequences via a "specificity loop", which is inserted into the DNA major groove at position -8 to -12 relative the transcription start site. POLRMT also contains a specificity loop and the POLRMT-TFB2M heterodimer footprints to a region surrounding the transcription start site (+10 to -4 region) of LSP (Gaspari et al. 2004). Footprinting data also reveal that the human TFAM binds to the upstream region of the promoter, covering position -35 to -17 (Fisher et al. 1987, Gaspari et al. 2004). Binding of TFAM introduces a U-turn in the promoter DNA, which brings the C-terminus of TFAM close to the initiation site; a conformation that could facilitate recruitment and direct interactions with POLRMT or TFB2M (Ngo et al. 2011, Rubio-Cosials et al. 2011). The essential role of TFAM in transcription initiation has recently been questioned. *In vitro* experiments demonstrated transcription initiation from HSP1 and LSP in the absence of TFAM (Shutt et al. 2010). The relevance of these findings is discussed in one of the publications presented in this thesis (Shi et al. 2012).

The C-terminal tail of TFAM interacts directly with TFB2M in GST-pulldown experiments, which led to the idea that TFAM may recruit the basal transcription machinery to the mitochondrial promoters via TFB2M interactions (McCulloch et al. 2003). The relevance of this finding has been questioned, since direct interactions were also observed with the translation factor TFB1M and the TFAM-TFB2M interaction could not be verified with alternative experimental approaches (M. Falkenberg, unpublished observations). It appears however likely TFAM interacts directly with TFB2M or POLRMT, in order to help recruit the basal transcription machinery to promoters, but additional studies are required to elucidate the nature of these interactions in molecular detail.

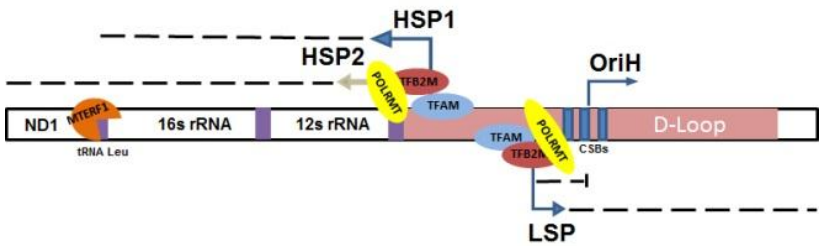

Figure 3. Schematic model of mammalian mtDNA transcription. Mitochondrial transcription initiates from two main promoters (HSP1 and LSP) in the D-loop region. Three core factors (POLRMT, TFAM and TFB2M) are required for transcription initiation. Transcription initiated from LSP generates full-length transcripts or pre-terminates at the CSBII region to form primers for heavy strand mtDNA replication initated at O_H. The location of the MTERF1 binding site just downstream of 16S rRNA is indicated.

Besides the full-length transcripts, transcription from LSP also produces prominent shorter products that are terminated at a conserved DNA sequence element (Conserved Sequence Block II: CSBII) located about 100 bp downstream of the

promoter. The terminated RNAs at CSBII are likely used by the mtDNA polymerase (POLγ) as primers to initiate heavy strand mtDNA synthesis at O_H (Lee et al. 1998, Pham et al. 2006).

A G-rich sequence element on the non-template strand of the CSBII region is required for the ability of this element to promote sequence-specific termination of transcription. During transcription of this region, a G-quadruplex structure is formed in the nascent RNA, which in turn stimulates transcription termination (Wanrooij et al. 2010). The mechanism is similar to Rho-independent transcription termination in prokaryotes, in which a hairpin loop is formed in bacterial mRNA during transcription of a terminator sequence. As will be demonstrated in this thesis, the premature transcription termination sites at CSBII coincide with the major RNA-DNA transition points at the O_H. This conclusion led to the idea that similar to T7 bacteriophage DNA replication initiation, primer processing is not required to initiate DNA replication in mitochondria (Pham et al. 2006). This observation was in contrast to previous reports, which had identified a RNA processing enzyme, RNase MRP, that was supposed to digest the primary transcript to generate a mature primer (Lee et al. 1998). However the RNase MRP enzyme localizes mainly in nucleus, and it is almost undetectable in mitochondria (Kiss et al. 1992).

The pre-terminated transcripts at CSBII remain attached to the control region, forming a stable and persistent RNA-DNA hybrid termed the R-Loop (Xu et al. 1995, Xu et al. 1996). We have recently demonstrated that extraordinary stability of this RNA-DNA hybrid is dependent on the CSBII element, which stimulates the formation of a G-quadruplex structure between the nascent transcript and the non-template DNA strand (Wanrooij et al. 2012). Since the structure involves the non-template strand, the RNA must dissociate from the template DNA strand. In theory this dissociation should prevent POLγ from using the preterminated RNA transcripts as primers for heavy strand DNA replication. In agreement with this notion, even if CSBII dependent transcription termination can be reconstituted *in vitro*, the primers formed cannot be directly used by POLγ to initiate DNA synthesis (unpublished observation). The RNA molecules must probably be released from the G-quadruplex structure before they can be used as primers for DNA synthesis. Primer utilization may therefore be an important regulatory step during the initiation of the mtDNA replication and additional factors are likely required to resolve the RNA-DNA hybrid.

1.3.3 Mitochondrial Transcription Termination

Besides initiation and elongation, transcription termination may constitute another important step to control the steady-state levels of mitochondrial transcripts. According to previously published reports, H-strand transcription involves two, partially overlapping transcription units. The steady state level of mitochondrial rRNAs is about 50 times higher than that of mRNAs, leading to the idea that the HSP transcription units were under separate control. In support of this notion, the human mitochondrial transcription factor (MTERF1), a 39-kDa protein, was identified as sequence-specific DNA binding protein interacting with elements

immediately downstream of the 16S rRNA, within the $tRNA^{Leu(UUR)}$ gene (Kruse et al. 1989). The finding of this protein led to the speculation that transcription from HSP1 is terminated at the MTERF1 binding site to mainly synthesize the two rRNAs: 12S and 16S; whereas transcription initiated at HSP2 leads to the formation of a polycistronic RNA that covers the entire genome. (Kruse et al. 1989, Daga et al. 1993, Martin et al. 2005). Even if MTERF1 can terminate transcription *in vitro*, there is no *in vivo* experimental evidence showing MTERF1 dependent transcription termination from HSP1 and regulation of the relative levels of rRNA and mRNA. Instead, the protein may function to block antisense transcription of the ribosomal transcription unit. In support of this notion, the termination activity of MTERF1 displays a distinct polarity, termination of transcripts from the LSP direction being much more efficient than termination of transcription events coming from HSP (Asin-Cayuela et al. 2005). In addition, knockdown or overexpression of MTERF1 in cultured human cells exhibited little effect on 16S and mRNA gene transcription, but a clear effect was seen on the antisense transcripts in the rDNA region (Hyvarinen et al. 2010). As discussed below, there may be another role for MTERF1, as a regulator of mtDNA replication.

The MTERF family is evolutionary conserved and members have been identified in metazoa and plants, but not in fungi. Sequence alignment analysis of MTERF1 identified three additional MTERF proteins in vertebrates: MTERF2, MTERF3, and MTERF4. All four proteins localize to mitochondria (Kruse et al. 1989, Linder et al. 2005, Park et al. 2007, Pellegrini et al. 2009, Camara et al. 2011). Interestingly, the functions of these newly discovered MTERF proteins appear to be distinct from that of MTERF1. MTERF2 has been hypothesized to function as a nucleoid-packaging factor (Pellegrini et al. 2009). Other reports suggest that MTERF2 plays a role in mitochondrial transcription and replication. MTERF2 was shown to interact with MTERF3 and DNA sequences in the control region, in order to regulate transcription initiation (Wenz et al. 2009). Another study reported that MTERF2 binds all over the mtDNA to regulate transcription (Huang et al. 2011).
The MTERF3 protein is the most conserved member of the MTERF family and it has been shown essential to mouse embryonic development. Chromatin immunoprecipitation demonstrated that MTERF3 interacts with the mtDNA promoter region and represses initiation of mitochondrial transcription on both mtDNA strands (Park et al. 2007). In support of this notion, depletion of MTERF3 in mouse tissue causes a dramatic increase in *de novo* transcription. However, the mechanism for MTERF3-dependent regulation remains obscure. Even if MTERF3 is associated with the promoter region, there is no evidence for direct interactions with mtDNA in this region. An alternative role for MTERF3 has been suggested from studies of MTERF3 in Drosophila. Knockdown of MTERF3 by RNAi showed decreased level of mitochondrial protein synthesis, but transcription levels remained unaffected (Roberti et al. 2006). These data partially conflict with a recent study of Drosophila MTERF3, which demonstrated that lower expression levels of MTERF3 leads to increased mitochondrial transcription as well as a failure to assemble the large mitochondrial ribosomal subunit (Wredenberg et al. 2013). These data indicate that beside its function in mitochondrial transcription, MTERF3 also plays a role in mitochondrial translation.

Like MTERF3, MTERF4 is also essential for embryonic development in the mouse. MTERF4 functions in mitochondrial ribosomal biogenesis, and it forms a complex with the rRNA methyltransferase NSUN4 and recruit it to the large ribosomal subunit (Camara et al. 2011, Spahr et al. 2012, Yakubovskaya et al. 2012). Loss of MTERF4 leads to impairment of ribosomal assembly and a drop in translation.

1.4 MITOCHONDRIAL DNA REPLICATION

Even if mitochondria have developed from an ancient alpha-proteobacteria, both the transcription and DNA replication machineries are more similar to those observed in T-odd bacteriophage. POLRMT, POLγ, and the replicative DNA helicase TWINKLE are all similar to their counterparts in the T7 bacteriophage. A detailed knowledge of the T7 replication machinery and its mechanisms therefore facilitates studies of the mitochondrial transcription and DNA replication.

1.4.1 Replication of T7 Phage

Bacteriophage T7 harbors a linear double stranded DNA genome of about 40 kb. T7 DNA replication is a well characterized process performed by only a few factors. The basic DNA replication system includes the T7 RNA polymerase (T7 RNAP or gp1), which produces RNA primers for initiation of leading strand synthesis; a monomeric DNA polymerase (gp5) responsible for DNA synthesis; a primase-helicase (gp4), which synthesizs the RNA primers required for initiation of lagging strand DNA synthesis and unwinds the dsDNA in the 5' to 3' direction to create a single-stranded DNA template; a single-stranded DNA binding protein (gp2.5) that binds transiently to ssDNA; and finally thioredoxin, which is encoded by the bacterial host and functions as accessory factor for the DNA polymerase (Fig. 4).

Bacteriophage T7 DNA replication is initiated with the binding of the T7 RNAP to the origin site and the production of a short RNA molecule that can function as a primer. Next, the gp5 DNA polymerase displaces RNAP and adds dNTPs to the 3′-OH end of the primer to initiate leading strand DNA synthesis. DNA polymerase gp5 activity is strongly stimulated by the accessory factor thioredoxin. Thioredoxin stimulates the processivity of gp5 by stabilizing its binding to template ssDNA. The hexameric gp4 helicase domain unwinds the double-stranded DNA and produces single-stranded template for DNA synthesis. Lagging-strand synthesis requires the production of primers at the moving DNA replication fork, which can be used to initiate synthesis of Okazaki fragment. The primase domain of the gp4 protein initiates primer synthesis at specific primase recognition sites on the DNA template, which is an AT-rich region at which RNA primer synthesis is initiated during unwinding of dsDNA. During the DNA replication process SSB binds to the exposed ssDNA, thereby dramatically increasing the activity of both DNA polymerase and the helicase-primase.

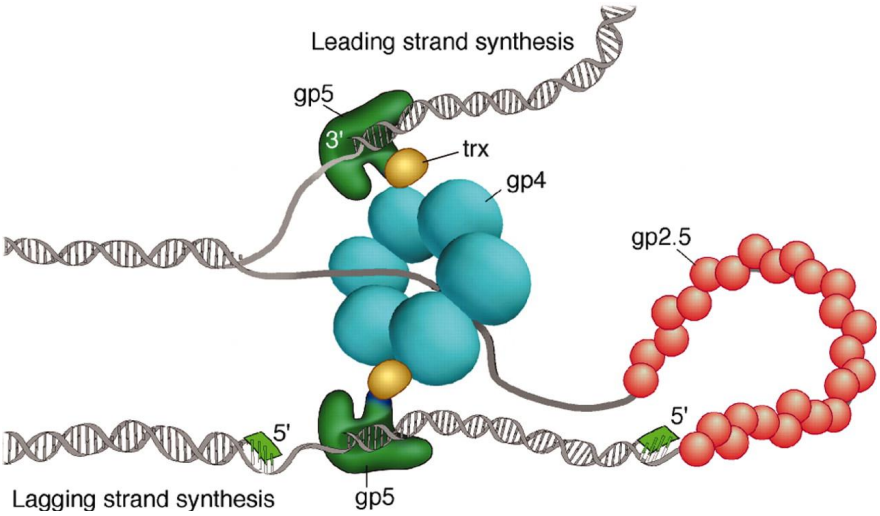

Figure 4. Model of the bacteriophage T7 replication fork. The gp5/trx complex synthesizes the leading strand continuously as the helicase unwinds the duplex. The lagging strand is synthesized as short Okazaki fragments. Synthesis of the lagging strand is initiated from RNA primers (green) catalyzed by the primase domain of primase-helicase gp4. A loop is formed on the lagging strand to align both gp5/trx complexes. The single-stranded DNA binding protein gp2.5 coats the ssDNA regions of the lagging strand that are generated as the helicase unwinds the DNA. (Adapted from (Johnson DE, Takahashi M, 2007, PNAS))

1.4.2 Replication in Mitochondria

MtDNA replication occurs in the mitochondrial matrix, and is largely independent of the cell cycle and nuclear DNA replication (Bogenhagen et al. 1977). MtDNA replication, however, is heavily dependent on the nucleus, since all of the mtDNA replication factors are encoded by the nuclear genome. The basic mtDNA replication machinery includes DNA polymerase γ (POLγ), a DNA helicase (TWINKLE), a single-strand DNA binding protein (mtSSB), and POLRMT, which produces the primers required for initiation of DNA synthesis.

1.4.2.1 MtDNA Replication Models

The exact mechanisms of mtDNA replication are still under debate. There are mainly two mtDNA replication models (Fig. 5). The first model "the strand-displacement model" was proposed many years ago based on studies of replicative intermediates using electron microscope. A series of later biochemical studies have

verified the principles of this model and explained the molecular mechanisms of the individual steps. According to the strand-displacement model mtDNA replication is asynchronous. First, H-strand DNA synthesis is initiated from O_H in the D-loop region. H-strand DNA synthesis proceeds for a distance of about almost two thirds of the mitochondrial genome before it reaches O_L. Strand displacement leads to the presentation of O_L in its single stranded conformation and causes the origin to adopt a stem–loop structure. L-strand DNA synthesis is initiated from the activated O_L and DNA synthesis of both strands continues until they reach full circle. In this model, replication of both strands is unidirectional (Martens et al. 1979, Tapper et al. 1981). Consistent with the strand-displacement model, POLRMT can use the stem-loop structure formed at O_L to synthesis short RNA products, and these short RNAs can be used as primers by POLγ to initiate the light strand DNA synthesis. Non-specific RNA synthesis at other sites is blocked by the binding of mtSSB (Wanrooij et al. 2008, Fuste et al. 2010) (Fig6).

An alternative model for mtDNA replication, "the strand-coupled model" has been suggested based mainly on studies of replicative intermediates with Neutral/neutral two-dimensional agarose gel electrophoresis (2-D-AGE) (Holt et al. 2000, Yasukawa et al. 2005). This model states that mtDNA replication proceeds symmetrically, with leading- and lagging-strand DNA synthesis initiating from multiple, bidirectional replication forks within a broad area. However, later 2D-AGE studies concluded that these replication intermediates were RNaseH-sensitive (Yang et al. 2002), leading to the idea that this class of replication intermediates derives from ribonucleotide-rich DNA synthesis initiated unidirectionally from the non-coding region (Yasukawa et al. 2005).

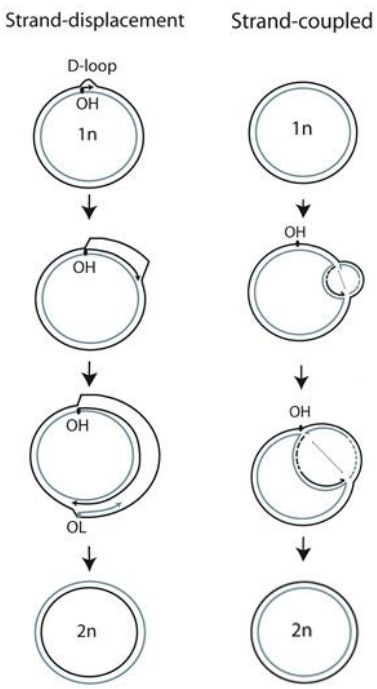

Figure 5. The asymmetric and strand-coupled models of mtDNA replication.
The displacement-model of replication is shown on the *left* and proceeds with single-stranded replication of the H-strand with further expansion and displacement of the D-loop. This proceeds until the L-strand origin (O_L) is exposed, with subsequent synthesis of the new L-strand in the opposite direction. The strand-coupled model of replication is shown on the *right*. In this model, there is thought to be a zone of replication initiation within a broad area beyond the simple D-loop. Within this zone, both strands are synthesized bidirectionally as the double-stranded replication forks proceed through the length of the mtDNA (Adapted from (Brown TA, Cecconi C, 2005))

Based on these observations, a third model was later proposed, the RITOLS (ribonucleotide incorporation throughout the lagging strand) model, which predicted that the lagging-strand is initially laid down as RNA, before being converted to DNA. Later, this model has been further modified and today it resembles the strand-displacement model, but claims that the lagging strand is not single-stranded, but instead covered with RNA prior to L-strand DNA synthesis (Yasukawa et al. 2006). Today, the strand-displacement and RITOLS models still lack conclusive, biochemical support.

1.4.2.2 MtDNA Replication Factors

Mitochondrial DNA polymerase

POLγ, the only known DNA polymerase in mitochondria, is involved in both mtDNA replication and repair processes. POLγ in vertebrates is a heterotrimeric complex, with one copy of the larger catalytic subunit termed POLγA, and two copies of the accessory subunit named POLγB. The catalytic subunit POLγA is about 140 kDa in size; it possesses DNA polymerase activity for mtDNA synthesis and a 3′ to 5′ exonuclease activity for proofreading. POLγA belongs to the family A group of DNA polymerases. Other members of this family are the T7 DNA polymerase and the *E. coli* DNA polymerase I.

POLγB has a size of 55 kDa and acts to increase the affinity of POLγA to DNA and stimulate DNA synthesis. The X-ray structure of POLγ suggests that POLγB changes the conformation of POLγA so that it interacts with a longer stretch of template DNA and thereby increases processivity (Lee et al. 2009).

More than 160 pathogenic mutations have been identified in *POLG1* and *POLG2*, the genes encoding POLγA and POLγB respectively (Walter et al. 2010). Most of the disease-related *POLG* mutations affect POLγA and may lead to mtDNA deletions, mutations, or depletion. Diseases associated with *POLG* mutations include progressive external ophthalmoplegia (PEO), childhood myocerebrohepatopathy spectrum (MCHS), Alpers Syndrome, and myoclonic epilepsy myopathy sensory ataxia (MEMSA) (Chan et al. 2005, Ferrari et al. 2005, Chan et al. 2009).

Mitochondrial DNA helicase

The gene encoding the mitochondrial DNA helicase (TWINKLE) was first identified in a search for pathogenic mutations that cause progressive PEO, a human disorder associated with multiple mtDNA deletions (Spelbrink et al. 2001). Biochemical characterization of the protein revealed a DNA helicase activity with 5' to 3' directionality. DNA unwinding was specifically stimulated by mtSSB. To initiate unwinding, TWINKLE required a substrate with a 5'-loading site and a short 3'-tail (Korhonen et al. 2003). When combined with mtSSB and POLγ, TWINKLE could support processive DNA synthesis on a double-stranded template

in vitro, strongly suggesting that TWINKLE was the replicative helicase in mammalian mitochondria (Korhonen et al. 2004).

TWINKLE shares structural similarity with gp4, the primase/helicase in bacteriophage T7. The similarities are especially pronounced in the C-terminal helicase part, whereas they are less pronounced in the N-terminal primase domain. Initially, it was speculated that TWINKLE could also function as a primase for lagging-strand DNA synthesis (Shutt et al. 2006), but so far there are no experimental evidence for a primase activity associated with mammalian TWINKLE. TWINKLE is a hexameric DNA helicase that can load onto a closed circular double-strand DNA and initiate DNA unwinding without the assistance of a helicase loading factor (Jemt et al. 2011). Mutations in the TWINKLE-encoding gene (*PEO1*) cause disorders similar to those associated with POLγ. The molecular phenotypes observed for TWINKLE mutations include deletions and depletion of mtDNA, which e.g. may cause mitochondrial diseases such as PEO (Spelbrink et al. 2001, Copeland 2008), sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (SANDO) (Hudson et al. 2005).

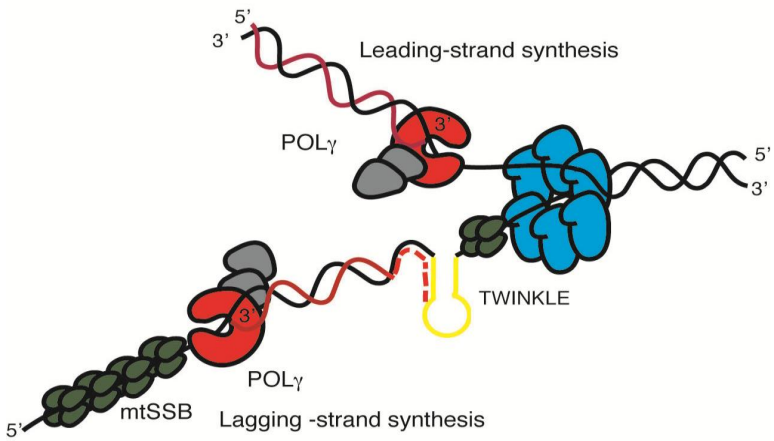

Figure 6. The mtDNA replisome. The TWINKLE helicase (blue) moves in the 5' to 3' direction while unwinding dsDNA. MtSSB (dark green) stabilizes unwound, single-stranded DNA and stimulates POLγ (red (A) and gray (B)). POLRMT synthesizes the RNA primer (yellow line) needed for lagging strand DNA synthesis. (Adapted from (Wanrooij S, Falkenberg M, 2010))

Mitochondrial single-strand DNA binding protein

Single-stranded DNA-binding proteins play essential roles in DNA replication, repair and recombination. MtSSB has been found in all eukaryotes studied. In contrast to e.g. POLγ and TWINKLE, the protein is not related to the T7 bacteriophage SSB protein, but instead shares high levels of structural similarity with bacterial SSBs (Webster et al. 1997). MtSSB is required to maintain the stability of the mitochondrial genome (Ruhanen et al. 2010). Human mtSSB is about 16 kDa in size, forming a tetramer with 2 dimers interacting head to head (Yang et

al. 1997). When mtSSB interacts with DNA, the ssDNA wraps around the tetrameric protein structure through electropositive channels.

According to the strand displacement replication model, the heavy strand DNA will be exposed as ssDNA until replication from O_L is initiated and proceeds to make the second strand. During this process, the binding of mtSSB protects the ssDNA from degradation by chemicals or enzymes, and it also greatly enhances the unwinding activity of dsDNA helicase and the processing activity of mitochondrial DNA polymerase (Farr et al. 1999, Oliveira et al. 2010). The need to cover long stretches of ssDNA during mtDNA replication may explain why mtSSB is such an abundant protein, with about 2800 mtSSB molecules per mitochondrial genome (Takamatsu et al. 2002). MtSSB has also been reported to regulate the stability of the triple-stranded D-Loop structure (Takamatsu et al. 2002). Studies in *Drosophila* demonstrated that lower levels of mtSSB expression might lead to mtDNA depletion and growth defects (Farr et al. 2004), which further shows that mtSSB plays a key role in mtDNA maintenance.

The mitochondrial lagging-strand primase

Primers required for initiation of DNA synthesis at OH are produced by POLRMT dependent transcription initiated at LSP. As reported in this thesis, we have suggested a model for primer formation at OH directed by premature transcription termination at a (CSB II) (Pham et al. 2006).

RNA primers are also needed to initiate L-strand DNA synthesis. We have in recent years presented a series of observations which demonstrate that POLRMT also functions as the lagging-strand primase in mammalian cells. When the replication machinery reaches O_L, the origin becomes single stranded and folds into a stem-loop structure. POLRMT binds to the O_L structure and initiates primer synthesis from the loop region. The short RNA primers synthesized by POLRMT are then used by the POLγ to initiate DNA synthesis (Wanrooij et al. 2008, Fuste et al. 2010). *In vivo* saturation mutagenesis in the mouse strongly supports this idea, since only O_L variants that can support wild type levels of primer synthesis by POLRMT *in vitro* are retained *in vivo*.

Mitochondrial topoisomerase I

DNA topoisomerases regulate topology of DNA by cleaving and rejoining of DNA strands. There are two types of topoisomerases, type I proteins cut only one strand of DNA at a time in an ATP-independent manner, whereas type II cleaves both DNA strands in an ATP-dependent way (Champoux 2001). Type I topoisomerases play important roles in DNA replication (Brill et al. 1987) and transcription (Zhang et al. 1988). Topoisomerase activity has also been found in mitochondria (Fairfield et al. 1979). Human mitochondrial topoisomerase (Top1mt) belong to type I, and shares high similarity with its nuclear homologous, Top1. Top1mt is critical for mitochondrial integrity and energy metabolism (Douarre et al. , Douarre et al. 2012), but the vitality of Top1mt knockout mice (Zhang et al. 2007) indicates that a compensatory system exists, which awaits to be identified.

1.5 MITOCHONDRIAL DYNAMIC

Mitochondrial morphology is not static; mitochondria undergo continuous fusion and division. The dynamics plays an important role in maintenance of mitochondrial morphology and imbalance between fission and fusion causes structural changes. Mitochondria normally form a tubular network, and inhibition of fusion results in short rods or spheres (Chen et al. 2005); Decreased levels of fission leads to elongated, interconnected tubules (Stojanovski et al. 2004).

Mfn1, Mfn2 and OPA1 are factors known to regulate mammalian mitochondrial fusion (Chen et al. 2003, Cipolat et al. 2004), whereas Fis1 and dynamin-related protein 1 (Drp1) are required for fission (Smirnova et al. 2001, Yoon et al. 2003). Mutations in Mfn2 are pathogenic, causing Charcot–Marie–Tooth type 2A and autosomal dominant optic atrophy. Blocking mitochondrial fusion by regulating the levels of Mfn1, Mfn2, or OPA1 leads to decreased mitochondrial membrane potential, poor cell growth, and sometimes even cell death. Inhibition of mitochondrial fission affects apoptotic pathways (Chan 2006, Ferree et al. 2012). These data show that mitochondrial morphology is closely linked to its function, but how changes in the shape affect the cellular function of mitochondria is still not clear.

1.6 MITOCHONDRIAL DISORDERS

Mitochondrial diseases refer to those disorders that are caused by mitochondrial dysfunction. Mitochondrial dysfunction can be caused by point mutations in mtDNA, mtDNA deletion or depletion, or mutations in nuclear genes that encode mitochondrial factors. The first identified mitochondrial disease that was caused by a mtDNA mutation was Leber's hereditary optic neuropathy (LHON), reported in 1988 (Wallace et al. 1988). Since then more than 300 pathogenic mtDNA mutations have been found (Mitomap, 2012). Mitochondrial defects can cause a wide range of degenerative diseases, aging, and cancer. The symptoms of mitochondrial diseases often include a slow growth rate, muscle weakness, loss of hearing, visual impairment, and heart problems. The symptoms usually are clearer in tissues with higher energy requirements, such as heart, muscle and the central nervous system.

Each cell can contain 1000 - 10000 copies of mtDNA and a disease causing mutation can either be present in all copies (homoplasmy) or just a certain fraction of the mtDNA copies (heteroplasmy). There is no mechanism ensuring that each mtDNA molecule is replicated only once during the cell cycle and that the mtDNA molecules are evenly distributed to daughter cells. Therefore, there will be an uneven segregation of heteroplasmic mtDNA mutations, which explains why mutated mtDNA displays a mosaic distribution in humans. Patients with mitochondrial disease caused by heteroplasmic mtDNA mutations even display varying levels of mutated mtDNA in different cells of a single organ. The level of heteroplasmy may also affect the disease phenotype, since pathogenic mtDNA

must be present in a certain fraction to reach the threshold level where respiratory chain deficiency can be observed (Larsson 2010).

Transmission of mtDNA is maternal and the level of heteroplasmy may vary a great deal between offsprings. This is explained by the so called bottleneck phenomenon, which is caused by the fact that only a handful of mtDNA molecules are used as templates to synthesize the nearly 100,000 copies of mtDNA that are present in the mammalian oocyte (Shoubridge et al. 2007). The molecular mechanisms underlying the selection of certain mtDNA molecules for replication are not yet understood.

2. AIMS OF THE THESIS

The mitochondrial genome was discovered 50 years ago. Since then, scientists have identified the factors that govern mtDNA replication and transcription, and elucidated many aspects of their function in molecular detail. Even if we have come a long way in our understanding of mtDNA, many questions still remain. During the last 10 years there has been an intense debate about the exact mechanism of mtDNA replication and, the relevance of the strand displacement model was questioned. Just when this issue appears to be fading away, a new debate has been initiated about transcription and the definition of the basal transcription machinery in mammalian mitochondria.

In this thesis, we have used a biochemical approach to address some of the fundamental questions that still remain about mtDNA replication and transcription. The role of TFAM as a basal transcription factor has been questioned and there have been reports suggesting that this factor functions as an activator of mtDNA transcription rather than a basal component of the transcription system. The existence of two separate H-strand promoters, HSP1 and HSP2, has also been under debate and even if we have not arrived at a final conclusion, we addressed this issue *in vitro* and in mitochondrial extracts. In connection to this, we have also made attempts to reexamine the role of MTERF1. According to a series of publication, this factor regulates the relative levels of the HSP1 and HSP2 transcription units. In this thesis, we have examined the possibility that MTERF1 may have alternative functions, i.e. in the regulation of mtDNA replication.

Transcription and DNA replication are linked events in mammalian mitochondria. Initiation of transcription at LSP may either produce polycistronic transcripts covering the entire L-strand or form short RNA products that are used for initiation of mtDNA synthesis at O_H. The mechanism underlying the switch between primer formation and full-length transcription elongation is of fundamental importance, since it may decide if a mtDNA molecule should be transcribed or replicated. In this thesis, we have studied the process of primer formation at O_H and the mtDNA sequence elements involved.

The specific aims of the individual manuscripts have been to:

Paper I: To define the basal human mitochondrial transcription machinery and the promoter elements required to initiate transcription *in vitro*.

Paper II: To address the functional importance of TFAM for initiation of mitochondrial transcription.

Paper III: To study the mechanisms and sequence elements that govern primer formation at O_H.

Paper IV: To investigate a possible function for MTERF1 in the regulation of mtDNA replication.

3. RESULTS AND DISCUSSION

Paper I. Human mitochondrial transcription revisited: only TFAM and TFB2M are required for transcription of the mitochondrial genes in vitro.

The basic human mitochondrial transcription system was established ten years ago (Falkenberg et al. 2002). Besides POLRMT, there are two other core factors, TFAM and TFB2M required, for the initiation of mitochondrial transcription from HSP and LSP. However, a number of published observations preclude a comprehensive view of gene transcription and its regulation in mitochondria (Shutt et al. 2010, Zollo et al. 2012). In this article we verified the essential roles of TFAM and TFB2M in mitochondrial transcription, and excluded some factors, like TFB1M, MRPL12, from being required for transcription in mitochondria. We also investigated the *in vitro* activity of HSP2.

We performed *in vitro* transcription with synthetic templates containing the LSP and HSP1 promoters, and found that efficient transcription initiation from LSP and HSP1 required both TFAM and TFB2M. Addition of TFB1M to the in vitro transcription reaction did not stimulate transcription activity on either LSP or HSP1. This result, together with reported *in vivo* data showing that loss of TFB1M primarily affects ribosome maturation, demonstrated that TFB1M is not a mitochondrial transcription factor. Since MRPL12, a mitochondrial ribosomal protein, has been reported to stimulate mitochondrial transcription (Surovtseva et al. 2011), we next tested the activities of this protein in *in vitro* transcription assays, both using our highly purified recombinant system and mitochondrial extracts. No stimulation of transcription was observed when increasing amounts of recombinant MRPL12 were added. Our results question the relevance of a previous report suggesting that MRPL12 is involved in mitochondrial transcription. We also investigated if HSP2 can promote transcription initiation *in vitro*. We observed no transcription, neither with recombinant proteins nor with mitochondrial extract. Our biochemical analysis therefore failed to support the existence of HSP2.

Paper II. Mammalian transcription factor A is a core component of the mitochondrial transcription machinery.

TFAM is required for both transcription and packaging of mtDNA (Chang et al. 1985, Fisher et al. 1988, Kang et al. 2007). TFAM can bind dsDNA non-specifically, an activity that is related to its role in mtDNA maintenance. It can also bind in a sequence-specific manner to the upstream regions of promoters (Fisher et al. 1987, Gaspari et al. 2004). TFAM binding to the upstream of LSP induces a U-turn in mtDNA and helps to recruit POLRMT and TFB2M to the promoter. Since its discovery about 30 years ago, TFAM has been considered as a core factor in mitochondrial transcription initiation. But some recently published data claimed that promoter-specific transcription initiation, especially from the heavy strand promoter (HSP), is not TFAM-dependent. These observations led to the conclusion that the mitochondrial transcription machinery is a two-factor system, which only needs POLRMT and TFB2M (Shutt et al. 2010, Zollo et al. 2012).

In our study, we could demonstrate that TFAM is essential for transcription initiation. We used antibodies to immuno-deplete TFAM from the mitochondrial extract and found that loss of TFAM abolishes transcription activity at both LSP and HSP. We also performed *in vitro* transcription assays using mutant DNA templates to show that the high-affinity binding sites for TFAM upstream of mitochondrial promoters are required for transcription activity *in vitro*.

We noted that the reported experiments demonstrating TFAM-independent transcription had been performed at very low ionic strength. We therefore tested if low salt conditions could relax the absolute requirement of TFAM. Indeed, at salt concentrations below 12 mM, we could observe weak, but promoter specific transcription in the absence of TFAM. At these conditions, we also observed abundant transcription initiation from other, non-specific locations on the DNA template used. When salt concentrations were increased above 50 mM, TFAM-independent transcription completely disappeared. In the presence of TFAM, transcription levels were dramatically stronger and remained unchanged at relatively high concentrations of salt.

Low salt causes DNA breathing and thus helps to unwind the double-stranded nature of the promoter. We reasoned that DNA breathing may explain TFAM independent transcription at low-salt conditions. An alternative way to promote breathing is negative supercoiling. We therefore investigated if negative supercoiling could relax the absolute requirement of TFAM for basal transcription. On a linearized template, TFAM was required at both LSP and HSP1 for transcription initiation *in vitro*. On a negatively supercoiled template, no LSP transcription was observed in the absence of TFAM, whereas HSP1 was active also in the absence of TFAM. Interestingly, a topoisomerase assay demonstrated that TFAM could introduce negative supercoiling in circular DNA. In addition, FRET experiments showed that TFAM could cause DNA conformational changes, consistent with DNA breathing. Based on our findings we suggested that one important aspect of TFAM function might be to promote DNA breathing at the promoter and thus facilitate promoter opening and transcription initiation by POLRMT and TFB2M.

Paper III. Conserved sequence box II directs transcription termination and primer formation in mitochondria.

In human cells, mitochondrial transcription from the light strand promoter (LSP) generates not only the full-length transcript, but also short products terminated in the conserved sequence boxes (CSBs) region. These shorter transcripts can be used as RNA primers needed for initiation of heavy strand DNA synthesis (Chang et al. 1985). How the CSBs may contribute to primer formation has however not been clarified. In this report we demonstrate that the conserved sequence box II is a sequence-dependent transcription termination element. We used *in vitro* transcription assays and demonstrated the existence of prominent, prematurely terminated transcripts just downstream of CSBII. We mapped the pre-termination

sites at positions 300-282 in the mitochondrial genome, which coincide with the major RNA-DNA transition region in the D-loop of human mitochondria.

We next prepared a series of mutant DNA templates in which the sequences of the individual CSB boxes (CSBI, CSBII, and CSBII) had been changed. We found that CSBII is required to generate the short, preterminated transcripts. We also investigated the RNA-DNA transition sites *in vivo*. To this end we prepared mtDNA from Hela cells and thrombocytes, and performed primer extension after RNase H1 treatment. The experiments demonstrated that the RNA-DNA transition sites *in vivo* were located within a region immediately downstream of CSB II, with a maximum at positions 301–299 and 292–289 of the human mtDNA. These transitions therefore coincide with the 300–282 region in which we had observed premature termination of transcription. Previous studies had suggested that primer formation involved RNase MRP digestion of the LSP transcripts (Lee et al. 1998). Our findings demonstrated that RNase MRP is not necessary for primer formation *in vitro* and that the pre-terminated transcripts generated by LSP transcription might be used directly by POLγ to initiate mtDNA synthesis at O_H.

Paper IV. MTERF1 is a contra-helicase that stimulates DNA replication fork pausing *in vitro* and *in vivo*.

MTERF1 was originally identified as a regulator of ribosomal transcription in mammalian mitochondria (Daga et al. 1993). It was proposed that transcription from HSP1 is terminated downstream of 16S rRNA by the binding of MTERF1 to the tRNA-Leu gene, whereas transcription from HSP2 is responsible for formation of long polycistronic transcripts, covering the entire L-strand (Martin et al. 2005). *In vitro* transcription assay showed that MTERF1 could terminate transcription bidirectionally, but that the termination effect was most efficient for transcription events coming from the LSP direction (Asin-Cayuela et al. 2005). Later studies revealed that overexpression of the protein also stimulated pausing of the mitochondrial DNA replication machinery *in vivo* (Hyvarinen et al. 2007). Furthermore, knockdown of MTERF1 in human cells did not lead to changes in the relative levels rRNA genes and downstream mRNA transcripts (Hyvarinen et al. 2010).

In this manuscript we demonstrate that MTERF1 can function as an contra-helicase that forces TWINKLE, the replicative DNA helicase in mammalian cells, to pause during unwinding of double-stranded DNA. The contra-helicase activity affects the movement of the entire mitochondrial replisome. The pausing effect displays a distinct polarity; MTERF1 only pauses the replisome when it travels from the O_H (LSP) direction. In the opposite direction, MTERF1 does not affect replisome movement.

We performed helicase assays using fork-like DNA templates with a MTERF1 binding site in the double stranded region. The MTERF1 binding site was positioned in the forward or reverser direction. Our experiments demonstrated that MTERF1 is a contra-helicase with distinct polarity. In the absence of MTERF1, the TWINKLE

helicase could unwind the DNA substrates, but addition of increasing amounts of MTERF1 blocked DNA unwinding when the MTERF1 binding sequence is oriented as would be the case when a replication fork approaches from the O_H (LSP) direction. MTERF1 oriented in the opposite direction did not block helicase unwinding. The contra-helicase activity of MTERF is not specific to TWINKLE, since when we tested the T7 helicase gp4 protein and observed the same effect. Mutations in the MTERF1 binding site abolished the blockage effect, which verified that the effect is caused by MTERF1 binding. The blockage causes pausing of unwinding and not termination, since more unwinding activity was seen with longer incubation times. Chromatin immunoprecipitation assays demonstrated that the contra-helicase activity of MTERF1 also exists *in vivo*, since TWINKLE accumulates upstream of the MTERF1 binding site in the tRNA-Leu gene, and overexpression of MTERF1 increases this TWINKLE accumulation.

In vitro rolling circle replication assay showed that MTERF1 pauses mtDNA replication in an orientation-dependent manner. According to the strand displacement model of mtDNA replication, replication of the heavy strand starts much earlier than the light strand. Based on our observations we suggest that MTERF1 is a functional homologue of the *E. coli* Tus protein and that MTERF1 helps to coordinate synthesis of the two DNA strands in mammalian mitochondria.

4. CONCLUDING REMARKS AND PERSPECTIVES

Mitochondrial transcription has been studied for nearly 40 years. During this time scientists have identified many of the key factors, e.g. POLRMT, TFAM, and TFB2M, and explored the molecular mechanisms by which they function. A major step in the biochemical characterization of these processes was the development in 2002 of a reconstituted *in vitro* system for human mitochondrial transcription system. This system allows for detailed mechanistic studies under strictly controlled conditions and has been used in all the reports presented in this thesis.

As described here, many questions still remain unanswered about mtDNA transcription and replication. One key point that must be addressed is the formation of RNA primers for mtDNA replication at O_H. We have still not been able to reconstitute initiation of H-strand DNA synthesis *in vitro* and it appears likely that additional factors are required to perform this crucial step in mtDNA biogenesis. To identify such factors and to describe how they functionally interact with the transcription and mtDNA replication machineries in the control region will therefore be an important task for the future.

The mechanism of mammalian mtDNA replication has been under intensive debate during the past decade. Although we cannot exclude the existence of the strand-coupled model under certain conditions or in certain cell types, it seems more and more likely that the strand displacement model is correct. However there are some problems with the strand displacement model. Since the leading and lagging strands are not replicated simultaneously, the replication of the leading strand starts much earlier than the lagging strand. There must therefore exist regulatory systems that can coordinate replication and ensure that both strands are synthesized. Our studies of MTERF1 in this thesis may be a first step in this direction.

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