# Initiation of mammalian mitochondrial DNA replication

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Cover illustration: Electron microscopy image of the mitochondrial helicase TWINKLE taken by Stefan Bäckström. Image edited by Jonas Carlsten.

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## ABSTRACT

Mitochondria produce most of the adenosine triphosphate required in a eukaryotic cell and they contain their own genome. The mitochondrial DNA (mtDNA) is a double stranded circular molecule that codes for proteins required for cellular respiration and RNA molecules involved in translation of these proteins. Replication of the mtDNA is therefore essential for cell viability and the aim of this thesis has been to understand the molecular mechanisms of mtDNA replication.

In general, initiation of DNA replication involves a series of steps including recognition of an origin of replication, loading of replicative helicases, and synthesis of an RNA primer that can be used by DNA polymerases to initiate DNA synthesis. We have studied this process in mammalian mitochondria and demonstrate that the mitochondrial RNA polymerase (POLRMT) synthesizes the RNA primer required for initiation of lagging strand replication at the origin of light strand ($O_L$). We have reconstituted, and in detail characterized, $O_L$-dependent initiation of lagging strand replication *in vitro* using purified POLRMT and core factors of the mitochondrial replisome.

We have also addressed how the TWINKLE helicase is loaded during initiation of leading strand replication. TWINKLE is a ring-shaped helicase and must be opened up to accommodate DNA in its central channel. Many helicases require specialized loading factor to assemble onto DNA, but we find that TWINKLE can function without such a factor. In the presence of the other components of the mitochondrial replisome, we show that TWINKLE can assemble on a DNA template resembling the mtDNA *in vivo* and support primer dependent initiation of DNA synthesis.

Most mtDNA replication initiation events are prematurely terminated and do not result in duplication of the entire mtDNA molecule. We address the mechanisms responsible for this termination event and identify a highly conserved sequence with palindromic character located immediately downstream of the premature mtDNA replication termination site. Interestingly, transcription initiated at the heavy strand promoter (HSP) is also terminated at this region, suggesting that the termination sequence functions in a bidirectional manner. Based on the results of *in vitro* biochemistry and cell culture experiments, we propose that a *trans*-acting factor binds to the palindromic sequence and simultaneously directs termination of both mtDNA transcription and replication.

MTERF1 binds specifically to an mtDNA sequence just downstream of the ribosomal RNA transcription unit. The function of MTERF1 has been debated and to elucidate its functional role *in vivo*, we here characterize an Mterf1 knock-out mouse model. We find that MTERF1 is non-essential and that the protein acts to prevent the transcription machinery from interfering with the downstream light strand promoter (LSP), an incidence that may disturb expression of coding genes, but also the formation of primers required for initiation of mtDNA replication.

**Keywords**: mitochondria, mtDNA, DNA replication

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# SAMMANFATTNING PÅ SVENSKA

Mitokondrierna är cellens egna kraftverk. Där skapas medparten av de ”energipaket”, ATP, som används för att driva olika cellulära processer så som produktion av enzymer, olika enzymatiska reaktioner, celldelning m.m. Mitokondrierna har sitt eget DNA som är skilt från kärnans DNA, d.v.s. DNA som vi i dagligt tal kallar för kromosomer och oftast diskuterar när vi pratar om gener. Kärnans DNA är stort och linjärt medan mitokondriens DNA är litet och cirkulärt. Mitokondriens DNA kodar för proteiner som behövs för att producera ”energipaketen”, ATP. Skador i mitokondriens DNA kan leda till nedsatt energiproduktion i cellen med sjuksom som följd. Symtomen för dessa sjuksomar inkluderar muskelförtvining, demens och utvecklingsstörningar. En anledning till att skador uppkommer i mitokondriens DNA är mutationer i de enzymer som är involverade i kopieringen av det mitokondriella DNA:t. Vår forsking har som mål att på detaljnivå försöka förstå hur mitokondriens DNA kopieras. Denna grundläggande kunskap kommer förhoppningsvis på längre sikt kunna hjälpa patienter med mitokondriella sjukdomar.

Många av de faktorer som är involverade i kopieringen av mitokondriens DNA är identifierade men vi förstår fortfarande inte alla delar av denna komplicerade process. I den här avhandlingen har vi studerat hur kopiering av mitokondriens DNA initieras bl.a. med hjälp av biokemiska metoder. Vi har visat att ett enzym, det mitokondriella RNA polymeraset, behövs för denna process samt undersökt hur ett annat enzym, TWINKLE, laddas på DNA:t för att kunna starta kopiering. Vi har även försökt förstå den mekanism som bestämmer när mitokondriens DNA ska kopieras. Vi har i cellextrakt kunna påvisa existensen av en faktor som vi tror är involverad i denna reglering och har även förslagit en möjlig mekanism.

# LIST OF PAPERS

**I.** **Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication.**
Fusté JM, Wanrooij S, **Jemt E**, Granycome CE, Cluett TJ, Shi Y, Atanassova N, Holt IJ, Gustafsson CM, Falkenberg M.
*Mol Cell*. 2010 Jan 15; 37(1): 67-78

**II.** **The mitochondrial DNA helicase TWINKLE can assemble on a closed circular template and support initiation of DNA synthesis.**
**Jemt E***, Farge G*, Bäckström S, Holmlund T, Gustafsson CM, Falkenberg M.
*Nucleic Acids Res*. 2011 Nov; 39(21): 9238-49.
* Shared first authorship

**III.** **MTERF1 binds mtDNA to prevent transcriptional interference at the light-strand promoter but is dispensable for rRNA gene transcription regulation.**
Terzioglu M, Ruzzenente B, Harmel J, Mourier A, **Jemt E**, López MD, Kukat C, Stewart JB, Wibom R, Meharg C, Habermann B, Falkenberg M, Gustafsson CM, Park CB, Larsson NG.
*Cell Metab*. 2013 Apr 2; 17(4): 618-26

**IV.** **A conserved sequence element is involved in termination of mitochondrial DNA replication and transcription.**
**Jemt E**, Persson Ö, Mehmedovic M, López M, Shi Y, Freyer C, Samuelsson T, Falkenberg M
*Manuscript*

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine diphosphate |
| ATP | adenosine triphosphate |
| bp | base pairs |
| CSB | Conserved Sequnece Block |
| C-terminal | carboxyl terminal |
| D-loop | displacment loop |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside triphosphate |
| dsDNA | double strand DNA |
| FAD | flavin adenine dinucleotide |
| GTP | guanosine triphosphate |
| HSP | heavy strand promoter |
| H-strand | heavy strand |
| kb | kilo bases |
| kD | kilo Dalton |
| KSS | Kearns-Sayre Syndrome |
| LSP | light strand promoter |
| L-strand | light strand |
| MDS | MtDNA Depletion Syndrome |
| MELAS | Myopathy Encephalopathy Lactic Acidosis and Stroke-like episodes |
| MNGIE | Mitochondrial NeuroGastroIntestinal Encephalomyophathy |
| mRNA | messenger RNA |
| mtDNA | mitochondrial DNA |
| $NAD^+$ | nicotinamide adenine dinucleotide |
| N-terminal | amino terminal |
| NTP | nucleoside triphosphate |

| | |
|---|---|
| $O_H$ | origin of heavy strand replication |
| $O_L$ | origin of light strand replication |
| OXPHOS | oxidative phosphorylation |
| PEO | Progressive External Ophtalmoplegia |
| Pi | inorganic phosphate |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| ssDNA | single strand DNA |
| TAS | Termination Associated Sequence |
| TCA | tricarboxylic acid cycle, citric acid cycle |
| tRNA | transfer RNA |

# 1 INTRODUCTION

## 1.1 Mitochondria

Mitochondria are subcellular organelles found in most eukaryotic cells. These structures are often referred to as the ”powerhouses” of the cell since they produce most of the adensosine triphosphate (ATP) required in the cell. To this end, mitochondria contain essential enzyme systems involved in energy metabolism, such as the respiratory chain, the citric acid cycle, and the enzymes required for β-oxidation. Mitochondria also contain their own genome present in multiple copies. The mitochondrial DNA (mtDNA) is a small circular molecule that codes for some proteins in the respiratory chain and RNA molecules involved in translation of these proteins inside mitochondria. All other components required for mitochondrial function are encoded by the nuclear genome and include factors needed for mtDNA replication. Mitochondrial function is thus dependent on two different genomes and the crosstalk between them.

The importance of proper mitochondrial function is highlighted by the large number of human disorders due to mitochondrial dysfunction. Many of these disorders are a result of defective mtDNA maintenence, often caused by genetic defects in proteins involved in mtDNA replication. In my thesis work, I have investigated the molecular mechanisms of mtDNA replication, with a special focus on how this process is initiated. I hope my work has contributed to a deeper understanding of mtDNA maintenance and that it will be of relevance for future studies on human disorders affecting this process.

### 1.1.1 Origin and structure of mitochondria

Evolution of the eukaryotic cell involved the development of an energy producing organelle, the mitochondrion. The observation that mitochondria showed similarities to bacteria together with the discovery that mitochondria contain their own genome (Nass and Nass, 1963) lead scientists to propose the hypothesis of the endosymbiotic theory (Margulis, 1981).

The endosymbiotic theory suggests that around 2 billion years ago an ancient α-proteobacterium fused with an archeabacterium (methanogen) (Gray et al., 1999; Lang et al., 1999). The conversion of the endosymbiont into a mitochondrion is a key step in the development of a eukaryotic cell and

eukaryotic complexity. Furthermore, the evolution of multicellular organisms was highly dependent on mitochondrial capacity to produce energy.

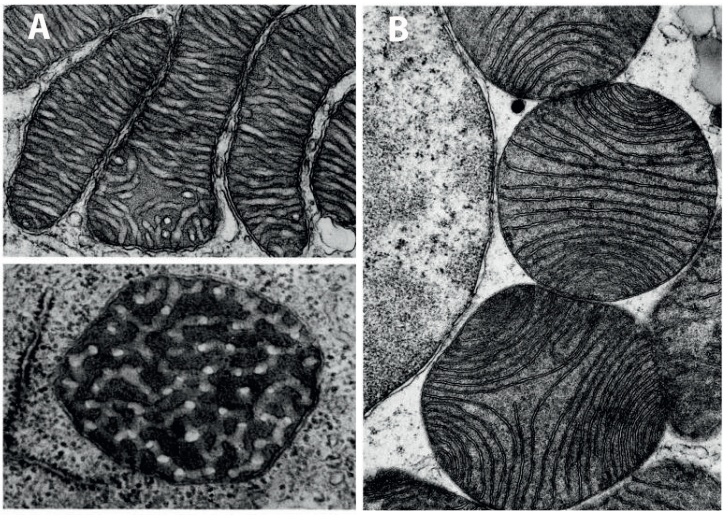


**Figure 1. Transmission electron microscopy images on mitochondria**
**A.** Tubular-shaped cristae from hamster adrenal cortex (upper) and from the Singh amoeba (lower) **B.** Mitochondria from adipose cells from *Myotis lucifugus* (little brown bat). The cristae are seen in transverse. (Picture adapted from "The Cell, 2nd Edition " by Don W. Fawcett M.D, Cell Image Library 11434 (A) and 11428 (B)).

When studying the mitochondrion using electron microscopy it became evident that the mitochondrion has two membranes: one outer membrane and one inner membrane that was convoluted and folded into *cristae* (Figure 1). The number and morphology of the *cristae* has been suggested to reflect the energy demand of the cell since highly folded *cristae* are found in cell types with high respiration such as muscle cells and neurons. The larger inner mitochondrial membrane surface of these cells might contribute to more respiratory chain complexes that in turn can result in higher respiration capacity. The morphology of the mitochondrion varies between different cell types and organisms. In some cells mitochondria have the shape of a bean whereas in other cells they form elongated tubules. In hepatocytes and

fibroblasts the mitochondrion has a typical sausage-like shape with the dimensions of 3-4 μm (length) and around 1 μm (diameter). The number of mitochondria per cell varies between different cell types and estimation from serial sections of different cells has given values from a few hundred to a few thousand per cell (Scheffler, 2008).

The mitochondrial genome (mtDNA) encodes a subset of the components of the respiratory chain and RNA molecules necessary for translation of these components inside the mitochondria (Falkenberg et al., 2007). Interestingly, genes coding for the remaining subunits of the respiratory chain, proteins needed for transcription and translation inside the mitochondira as well as proteins involved in mtDNA maintenance have been transferred to the nuclear genome. This raises the question; why have not all genes been transfered to the nucleus? Scientists have been trying to answer this question, and a general hypothesis is that the mtDNA usually seems to code for key subunits of the respiratory chain. It seems favourable to control and maintain the redox balance by synthesizing these key subunits when and where they are needed, i.e close to the mitochondrial membranes (Allen, 1993, 2003; Lane and Martin, 2010; Race et al., 1999). Another hypothesis is that the proteins encoded by the mitochondrial genome cannot be imported through the mitochondrial membranes because of their highly hydrophobic nature (von Heijne, 1986).

### 1.1.2 Mitochondrial dynamics

It is clear when observing live cells that their shape is not static. In fact, the organelles constantly change their shape by processes called fission and fusion (Shaw and Nunnari, 2002; Youle and van der Bliek, 2012).

Fission and fusion of mitochondria are processes that are essential for proper cell function. Fission of mitochondria is a necessity since cell division must generate cells containing mitochondria. It has also been proposed that fission protects cells from damaged mitochondria since damaged mitochondria can selectiviely be removed by autophagy if the damaged parts are distrubuted asymmetriclly during fission (Twig et al., 2008; Youle and van der Bliek, 2012). Moreover, damaged mitochondria lose their fusion machineries, suggesting that the absence of the fusion machinery is a mechanism that protects healthy mitochondria from contamination with damaged

mitochondrial parts. Fusion of mitochondria has also been suggested to allow distribution of metabolites, proteins and different mtDNA molecules that could rescue and complement damaged mitochondria. However, fusion with damaged mitochondria probably only takes place as long as the stress and damage is below a critical threshold (Twig et al., 2008; Youle and van der Bliek, 2012). In summary, fission seems to eliminate damaged mitochondria while fusion may compensate for damage and together these two mechanisms guarantee mitochondrial integrity within the cell.

The proteins involved in mitochondrial fission and fusion are guanosine triphospatases (GTPases) proteins, which are conserved between yeast, flies, and mammals. The master protein involved in fission Drp1 (Dnm1 in yeast) is located in the cytosol. During the fission process Drp1 is recruited to the mitochondrial outer membrane. The Drp1 proteins oligomerize and induce constriction of the mitochondrial membranes (Otera et al., 2013; Youle and van der Bliek, 2012). It is clear that Drp1 needs additional proteins to assemble on the mitochondrial outer membrane, and, in yeast, one of these proteins is named Fis1. However, depletion of human Fis1 does not affect mitochondrial recruitment of Drp1. Instead, another protein Mff has been proposed for this function in humans (Otera et al., 2013).

Fusion of mitochondria involves fusion of the outer membrane followed by fusion of the inner membrane. The outer membrane fusion machinery in yeast involves the GTPase Fzo1, and the mammalian counterpart includes the mitofusins MFN1 and MFN2. The mitofusins are located throughout the mitochondrial membrane, and they are anchored to the membrane by two transmembrane domains (Escobar-Henriques and Anton, 2013). The proteins are believed to tether adjacent mitochondria and fuse the two lipid bilayers together (Westermann, 2010). Mfn1 or Mfn2 kock-out mice are embryonic lethal, demonstrating the importance of mitochondrial fusion (Youle and van der Bliek, 2012).

The key protein involved in inner membrane fusion is called Mgm1 in yeast, and the mammalian counterpart is OPA1. Loss of Mgm1 results in respiratory incompetence as a consequence of mtDNA deletion. Knocking-out Opa1 in mice results in embryonic lethality. Interestingly, mutations in OPA1 have also been associated with multiple deletions in mtDNA, but the

mechanism behind this is still unknown (Escobar-Henriques and Anton, 2013).

## 1.2 Mitochondrial Metabolism

### 1.2.1 Oxidative phosphorylation

Mitochondria are often called the powerhouses of the cell since these organelles produce most of the ATP required in a eukaryotic cell. The mechanism by which it does so is called oxidative phosphorylation (OXPHOS) and refers to the process in which electron are transferred via electron carriers to $O_2$ and the subsequent production of ATP. The source of electrons comes from the reduced molecules NADH and $FADH_2$, produced by the citric acid cycle (also called tricarboxylic acid cycle or Krebs cycle). NADH and $FADH_2$ contain electrons with high transfer potential and the electrons donated by these molecules are transferred to the electron transport chain located in the mitochondrial inner membrane, which results in movement of protons from the mitochondrial matrix to the intermembrane space (Figure 2). This phenomenon creates a proton gradient, also referred to as a proton-motive force, which drives the flow of protons back to the matrix via ATP synthase (Complex V). At this step ATP is produced from ADP and Pi.

Both Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) accept electrons from NADH and $FADH_2$ respectively and transfer them to ubiquinone (coenzyme Q). The transfer via Complex I results in pumping of protons to the intermembrane space, whereas Complex II only contributes to electron movement. In the final step the electrons reach Complex IV (Cytochrome c oxidase), which uses them to reduce $O_2$ into water. During this process, protons are taken from the mitochondrial matrix to reduce $O_2$, but in addition protons are pumped through the complex to the intermembrane space. Both of these events contribute to the increase in the proton gradient over the mitochondrial inner membrane.

When Complex V (ATP synthase) uses the proton gradient to synthesize ATP, protons moves from the intermembrane space back to the matrix. The adenine nucleotide translocase (ANT) exports ATP from the mitochondrial matrix to the cytosol in exchange for one ADP. The most important factor

that controls the rate of OXPHOS is the concentration of ADP, which is coupled to the utilization of ATP. The importance of ADP levels is demonstrated by the fact that inhibition of ANT leads to inhibition of cellular respiration (Berg, 2002; Saraste, 1999).

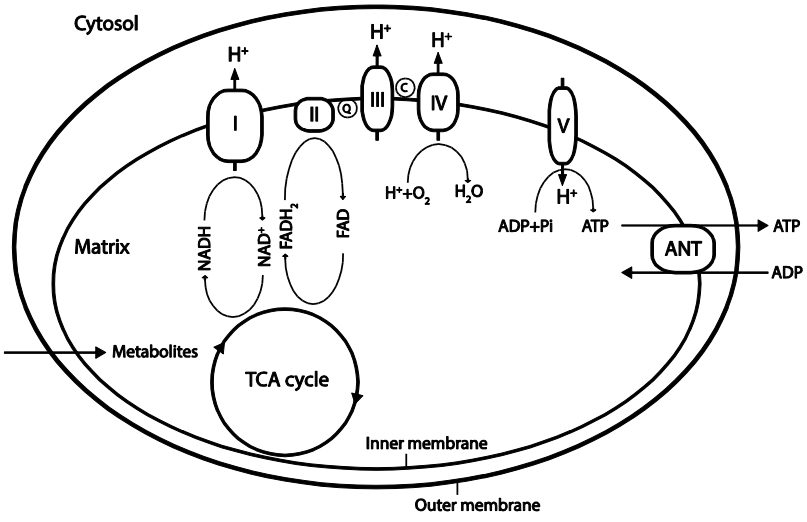


**Figure 2. Major metabolic pathways inside the mitochondria**
Schematic demonstration of the citric acid cycle (TCA cycle) and the OXPHOS pathway with the five different complexes (I-V). ANT (adenine nucleotide translocase), Q (ubiquinone), C (cytochrome c).

### 1.2.2 The citric acid cycle and other metabolic pathways

Mitochondria also harbor other important metabolic pathways in addition to oxidative phosphorylation. One of them is the citric acid cycle (Figure 2). The citric acid cycle completes the oxidation of glucose into $CO_2$. Pyruvate, produced by glycolysis, is transported into mitochondria and converted to Acetyl CoA. Acetyl CoA is the fuel for the citric acid cycle, which at different enzymatic steps produces $CO_2$, GTP and the electron carriers NADH and $FADH_2$. Moreover, the intermediates of the citric acid cycle are building blocks for biomolecules such as nucleotide bases and amino acids.

The mitochondrial matrix is also the site for degradation of fatty acids. Fatty acids are activated before being transported to the mitochondria and degraded by a mechanism called β-oxidation. β-oxidation produces the molecules NADH and $FADH_2$ that directly transfer electrons to the electron transport chain, but also Acetyl CoA that enters the citric acid cycle and contributes to production of more reducing agents (Berg, 2002).

## 1.3 The mitochondrial genome and the nucleoid

The mitochondrial genomes of plants and animals have evolved differently. Plant mitochondrial genomes are relatively large, ranging from 200 to 2400 kb, and exhibit introns, pseudogenes and pieces of foreign DNA of chloroplast and nuclear origin. In contrast, animal mitochondrial genomes are small with a size around 14-20 kb. They have genes lacking introns and the genes are tightly packed with an order that is well conserved (Lang et al., 1999). The mitochondrial genomes of fungi are, on the other hand, on average three to four times larger than animal genomes, but much smaller than plant genomes (Scheffler, 2008).

The human mitochondrial genome is a closed circular molecule that is 16 569 bp long (Figur 3) (Anderson et al., 1981). The genome codes for 13 of the about 90 subunits present in the respiratory chain, two ribosomal RNAs (rRNA) (12S and 16S), and 22 transfer RNAs (tRNA). The two strands differ in their base composition and can be separated on a cesium chloride gradient. For this reason one of the strands is named heavy strand (H-strand) and is G-rich, whereas the other strand is named light strand (L-strand) and is G-poor. The H-strand is the template for transcription of the majority of genes, whereas the L-strand is the template for ND6 mRNA and eight tRNAs (Falkenberg et al., 2007). Mitochondria use a genetic code that differs from the "standard" genetic code. As an example UGA, which is a stop codon in most organisms, codes for tryphtophan in vertebrate mitochondria.

Human mtDNA contains only one longer region, which is non-coding, and it is called the non-coding region or the control region. This region contains the regulatory elements the origin of H-strand replication ($O_H$) and the transcriptional promoters for both the L- and H-strand (LSP and HSP respectively). The length and sequence of the non-coding region varies between different animals except for some conserved elements that incude

the Conserved Sequence Blocks (CSBI-III) and a sequence named Termination Associated Sequences (TAS) (Figur 3). Most mtDNA molecules also contain a triple-stranded region called the D-loop that is located within the non-coding region. The D-loop spans the region from $O_H$ to the sequence around the TAS region and is believed to be a result of premature termination of DNA replication. The cause of the premature termination event is still unknown and will be discussed later in more detail. The origin of L-strand replication ($O_L$) is located around two-thirds away from $O_H$ (Falkenberg et al., 2007; Scheffler, 2008).

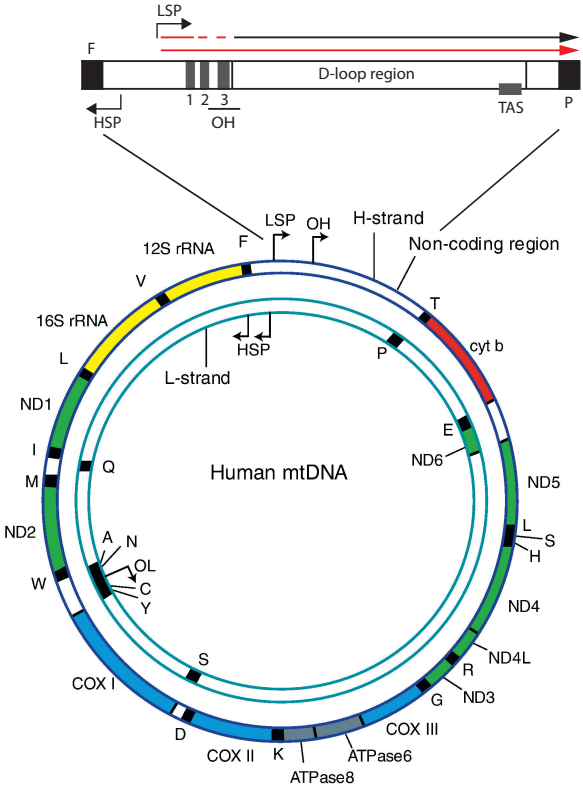


**Figure 3. The human mitochondrial genome**

Schematic presentation of human mtDNA (strands are shown separately) and a description of the non-coding region. Complex I genes in green; Complex III genes in red; Complex IV in blue; Complex V in grey. tRNAs are shown in black and rRNAs in yellow. Light strand promoter (LSP), heavy strand promoter (HSP), origin of heavy strand ($O_H$), origin of light strand ($O_L$). The non-coding region is situated between the tRNAs F ($tRNA^{Phe}$) and P ($tRNA^{Pro}$), Conserved Sequence Blocks (CSB) 1, 2, and 3, Termination Associated Sequence (TAS), RNA (red line), DNA (black line). (MtDNA from (Wanrooij and Falkenberg, 2010) with permission)

A somatic cell contains 1000-10 000 copies of mtDNA (Falkenberg et al., 2007). In contrast to nuclear DNA, mtDNA seems to be replicated over the entire cell cycle. It has been shown that the expression of mitochondrial genes is proportional to the levels of mtDNA, and that the mtDNA copy number, as well as the mitochondrial RNA level, is higher in tissues with high oxidative capacity (Williams, 1986). In other words, it seems that oxidative capacity and mtDNA copy number are tightly controlled with respect to each other.

The mitochondrial genome is packed into protein-DNA complexes called mitochondrial nucleoids (Alam et al., 2003; Chen and Butow, 2005). The number of mtDNA molecules per nucleoid has been calculated to be an average of 1.4 mtDNA/nucleoid (Kukat et al., 2011). One key protein of the mtDNA nucleoid is the mitochondrial transcription factor A (TFAM). TFAM was first identified as a transcription factor and was shown to be essential for initiation of mitochondrial transcription (Parisi and Clayton, 1991). TFAM contains two high-mobility-group (HMG) box domains. Similar to other proteins in the HMG-box family, TFAM can bind, unwind, and bend DNA in a non-sequence specific manner (Fisher et al., 1992). TFAM is highly abundant inside mitochondria, and measurements of TFAM levels in human fibroblasts estimated that there is one TFAM molecule per 10-20 bp of mtDNA (Chen and Butow, 2005; Kukat et al., 2011). In fact, TFAM has recently been shown to cover the entire mtDNA (Wang et al., 2013).

Depletion of TFAM in mice leads to loss of mtDNA and is embryonic lethal (Larsson et al., 1998). Overexpression of TFAM, on the other hand, results in increased mtDNA copy number (Ekstrand et al., 2004). Taken together, this suggests that TFAM, besides a role in transcription, stabilizes mtDNA.

Additional proteins, besides TFAM, that have been shown to associate with mitochondrial nucleoids are the DNA replication proteins (mtSSB, POLγ and TWINKLE) (Garrido et al., 2003; Wang and Bogenhagen, 2006) and some metabolic proteins of the citric acid cycle (Bogenhagen et al., 2003; Wang and Bogenhagen, 2006). However, the function of the citric acid cycle proteins in nucleoids is still not known.

# 1.4 Mitochondrial DNA transcription

## 1.4.1 The core mitochondrial transcription machinery

A mitochondrial RNA polymerase was first reported in yeast (Rpo41) (Greenleaf et al., 1986; Kelly and Lehman, 1986) and it was later found in human cells (POLRMT) (Tiranti et al., 1997). The C-terminal of both yeast and human mitochondrial RNA polymerases shows high sequence similarity to the C-terminal of RNA polymerases encoded by the T-odd lineage of bacteriophages such as T7 and T3 (Masters et al., 1987; Tiranti et al., 1997). However, the N-terminal part of the mitochondrial RNA polymerase is unique and the human protein harbors two pentatricopeptide repeat (PPR) motifs with hitherto unknown function (Asin-Cayuela and Gustafsson, 2007). Proteins containing PPR motifs have been reported to bind RNA and to facilitate interactions between RNA and proteins involved in RNA metabolism. Whether this is the function of the PPR motifs of mitochondrial RNA polymerase has to be further investigated.

POLRMT has also been shown to possess primase activity and can synthesize RNA 20-100 nucleotides in length using ssDNA as a template (Wanrooij et al., 2008). The primase activity of POLRMT will be discussed in more detail under section 1.6.2 (POLRMT - the mitochondrial RNA polymerase and primase).

In contrast to T7 RNA polymerase, POLRMT needs additional factors for transcriptional initiation at promoters. The additional factors are TFAM and the mitochondrial transcription factor 2B, TFB2M (Falkenberg et al., 2002). However, in budding yeast the core mitochondrial transcriptional machinery only consists of RNA polymerase and the TFB2M homologue, Mtf1 (Cliften et al., 1997; Mangus et al., 1994).

As discussed above, TFAM binds non-specifically to DNA (Fisher et al., 1992), but it has also been shown that TFAM binds sequence-specifically to regions upstream of the transcriptional start sites of the two promoters in mammalian cells (Fisher et al., 1987; Gaspari et al., 2004). This indicates that the role of TFAM in transcription initiation could be to recruit POLRMT or a POLRMT-protein complex to the promoters. In agreement with this suggestion, disrupting the distance between the specific TFAM binding site

and the transcriptional start site (LSP) leads to a decreased in transcriptional initiation efficiency (Dairaghi et al., 1995). Recent data also show that in the presence of TFAM, POLRMT binds DNA upstream of the TFAM binding site, suggesting that TFAM is important for recruitment of POLRMT to the promoters (Morozov et al., 2014; Posse et al., 2014).

The role of TFAM as a transcription factor for promoter-specific initiation was questioned in a study where the mitochondrial transcription machinery was reconstituted and analyzed *in vitro* (Shutt et al., 2010). The authors claimed that POLRMT and TFB2M alone could initiate transcription and proposed a two-component system similar to that in budding yeast. However, it could later be shown that the requirement for TFAM could be overcome only if certain *in vitro* conditions that promoted promoter breathing, e.g low salt concentrations, were chosen (Shi et al., 2012). Therefore, the overall conclusion is that TFAM is a transcription factor required for transcriptional initiation in mitochondria.

TFB2M is related to a family of rRNA methyltransferases, which methylates bases of the small subunit of rRNA. Phylogenetic analysis suggests that this factor originates from the rRNA methyltransferase of the mitochondrial endosymbiont (Cotney and Shadel, 2006). In fact, an additional paralogue of TFB2M named TFB1M can be found in mitochondria (Falkenberg et al., 2002). Both TFB1M and TFB2M exhibit rRNA methyltransferase activity but TFB2M is a less efficient enzyme than TFB1M (Cotney and Shadel, 2006; Seidel-Rogol et al., 2003). Therefore, it has been suggested that TFB2M has evolved into a mitochondrial transcription factor whereas TFB1M functions as a bona fide rRNA methyltransferase. The role of TFB2M in transcription seems to be to assist POLRMT in promoter melting, since the requirements of TFB2M can be circumvented when a pre-melted promoter DNA template is used (Sologub et al., 2009).

### 1.4.2 Transcription of mtDNA

Except for the non-coding region both strands of mtDNA contain coding sequences distributed over the entire genome. The non-coding region harbors the promoters for both the L- and H-strand, named LSP and the HSP respectively (Figure 3) (Falkenberg et al., 2007). Both promoters produce polycistronic transcripts of almost full genome-length (Aloni and Attardi,

1971). However, when analyzing mitochondrial RNA, the RNAs are found to be shorter molecules, which is probably a result of endonucleolytic cleavage that punctuates the polycistronic RNA, in most cases, right before and after a tRNA (Ojala et al., 1980; Ojala et al., 1981). This processing mechanism is called the "tRNA punctuation model". After RNA processing, the mitochondrial poly(A) polymerase (mtPAP) (Sarkar et al., 2005; Tomecki et al., 2004) adds a 50 nucleotide polyA tail onto the mRNAs (Chang and Tong, 2012). In fact, for some mRNAs the polyadenylation is needed to generate UAA stop codons that are not encoded in the mtDNA. Also, tRNA maturation may occur at the 3´termini of some tRNAs (Yokobori and Paabo, 1997).

It has also been proposed that an additional H-strand promoter exists, named HSP2, which is located around 80 bp downstream of HSP and excluds the $tRNA^{Phe}$ (Martin et al., 2005; Montoya et al., 1982; Montoya et al., 1983). HSP2 was proposed to transcribe the entire mtDNA while transcription starting from HSP (also called HSP1) was believed to terminate just downstream of the 16S rRNA gene (Asin-Cayuela and Gustafsson, 2007). *In vitro* transcription from both HSP1 and HSP2 has been reported (Bogenhagen et al., 1984; Falkenberg et al., 2002; Lodeiro et al., 2012; Walberg and Clayton, 1983), however the activity of HSP2 is inhibited in the presence of TFAM and the existence of HSP2 *in vivo* has therefore been questioned (Litonin et al., 2010; Lodeiro et al., 2012).

### 1.4.3 Transcription termination in mitochondria

The two observations that the steady-state levels of the mitochondrial rRNAs are about 50 times higher than the mRNAs (Montoya et al., 1982; Montoya et al., 1983) and that a the protein MTERF1 (mitochondrial termination factor 1) bound sequence-specifically within the $tRNA^{Leu}$ immediately downstream of 16S rRNA, lead to the idea of a separate transcriptional unit for the rRNAs that started at HSP and terminated at the MTERF1 binding site (Figure 3) (Kruse et al., 1989). In addition, MTERF1 terminated transcription *in vitro* at the MTERF1 binding site on an HSP template (Asin-Cayuela et al., 2005; Kruse et al., 1989).

Interestingly, a mutation in the MTERF1-binding site, and therefore also in the $tRNA^{Leu}$, which is associated with the human disease MELAS (myopathy,

encephalopathy, lactic acidosis and stroke-like episodes), resulted in less binding of MTERF1 in *in vitro* studies, but was shown to not affect the steady-state levels of the rRNAs *in vivo* (Chomyn et al., 1992). The observations suggested that a definite role of MTERF1 had still not been found. Actually, MTERF1 seemed to more effectively terminate transcription from the opposite direction, i.e. transcription initiated at LSP (Asin-Cayuela et al., 2005; Shang and Clayton, 1994). In addition, other studies have suggested alternative functions for MTERF1 including that MTERF1 binds close to HSP and stimulates transcription (Martin, Cho et al. 2005) or that MTERF1 causes DNA replication pausing at the MTERF1-binding site (Hyvarinen et al., 2007).

Evidently, a clear function of the MTERF1 protein has not yet been established. In order to elucidate the function of MTERF1 we created an Mterf1 knock-out mouse and found that MTERF1 is non-essential (Paper III in this thesis)(Terzioglu et al., 2013). Furthermore, we could not find any evidence that MTERF1 has a role in rRNA formation or stability. In fact, the only clear function that could be attributed to MTERF1 is that it terminates transcription initated at LSP, but not transcription initiated at HSP.

Termination of HSP transcription has instead been reported to take place within two different regions located at the 3´end of the D-loop (Freyer et al., 2010; Sbisa et al., 1990) and around HSP, which corresponds to transcription of the entire mtDNA (Freyer et al., 2010; Selwood et al., 2001; Vijayasarathy et al., 1995). The termination at HSP reveals that transcription within the D-loop region occurs. Interestingly, two mouse proteins have been shown to associate with this region and mediate transcription termination, but the identities of the proteins are still unknown (Camasamudram et al., 2003).

As discussed above, transcription initiated at LSP seems to terminate at the MTERF1 binding site (Paper III)(Terzioglu et al., 2013). However, several studies have identified a highly abundant (35.5% of polyadenylated LSP transcripts) polyadenylated LSP transcript with its 3´end mapping to the CSB1 motif (Chang and Clayton, 1985; Mercer et al., 2011). The function of this transcript is still unclear, but since the termination sites at CSB1 coinside with the location of $O_H$ and RNA has been found covalently attached to the newly synthesized DNA at CSB1, the proposition that this transcript is involved in primer formation at $O_H$ was made (Chang et al., 1985). However,

it is still not clear if this transcript is the result of processing of a longer transcript or the result of transcription termination.

### 1.4.4 Other factors involved in mitochondrial transcription

Three other factors related to MTERF1 have been identified in vertebrates and found to localize to mitochondria, MTERF2, MTERF3, and MTERF4 (Camara et al., 2011; Chen et al., 2005; Hyvarinen et al., 2011; Linder et al., 2005; Park et al., 2007; Pellegrini et al., 2009). MTERF2 seems to work as a positive regulator of mitochondrial transcription (Wenz et al., 2009) whereas MTERF3 appears to be a negative regulator of transcription initiation (Park et al., 2007). MTERF4 has been shown to be involved in the translation process rather than the transcription process (Camara et al., 2011).

Another protein named transcription elongation factor of mitochondria, TEFM, was recently identified. TEFM was shown to interact directly with POLRMT and to stimulate transcription elongation in mitochondria (Minczuk et al., 2011). Further studies of these factors are needed to sort out their precise roles inside mitochondria.

## 1.5 DNA replication - an introduction

Replication of duplex DNA molecules requires several specialized factors that assemble into replisomes and cooperate to replicate DNA. Replication of DNA involves assembly of proteins on DNA, unwinding of duplex DNA, synthesis of RNA primers, and template-mediated polymerization of nucleotides (Hamdan and Richardson, 2009).

DNA synthesis is initiated at specific sites on the chromosome called origins of replication. In bacteria two classes of elements are required at the origins, conserved repeats that are recognized by specific proteins and an AT-rich region that can easily melt and form a region of ssDNA. At the unwound DNA site replicative helicases can load and the DNA replication process can initiate. In *E. coli* the protein DnaA recognizes the repeated sequences. Binding of several DnaA molecules to the repeats result in melting of the AT-rich region and the subsequent loading of the replicative helicase at the newly formed ssDNA region (Costa et al., 2013).

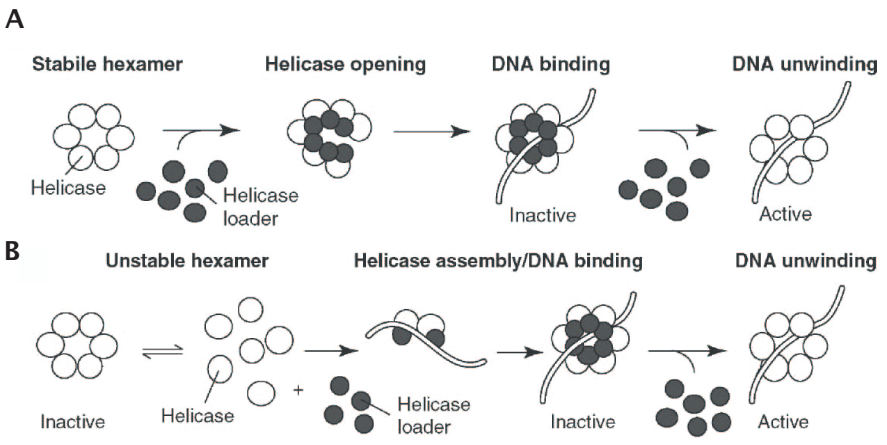


**Figure 4. Strategies for loading of replicative helicases**

**A.** Stable hexameric helicases need additional helicase loaders called "ring breakers" for loading onto DNA **B.** Unstable hexameric helicases need helicase loaders called "ring makers" for loading onto DNA. (From (Davey and O'Donnell, 2003) with permission.)

Unwinding of the duplex DNA during DNA replication is mediated by replicative helicases. These are ring-shaped and bind the ssDNA through their central channel and break the hydrogen bonds of the duplex DNA in front of the DNA polymerase (Davey and O'Donnell, 2003; Hamdan and Richardson, 2009). This process requires energy in the form of NTPs or dNTPs. *In vivo* the usual case is that there are no free DNA ends that can be threaded through the hole of the helicase ring structure and the helicases need assistance during loading onto DNA. Therefore, specialized factors or subdomains are needed for this task. They can be divided into two different classes where one of them is called the "ring breakers" (Figure 4A). This class of factors opens ring-shaped helicases to allow DNA to enter the central channel. One example of a "ring breaker" is the *E. coli* loading factor DnaC, which opens up the helicase DnaB ring structure for efficient loading of the helicase. The other class is called the "ring makers" and assembles helicase subunits around the ssDNA (Figure 4B). One example of a "ring maker" is DnaI, which is required for assembling the replicative helicase BsDnaC on ssDNA in *B. subtilis* (Davey and O'Donnell, 2003).

After loading the helicase, the whole replisome assembles on DNA and forms a replication fork (Figure 5). The newly synthesized DNA is produced by replicative DNA polymerases that incorporate nucleotides in a 5´ to 3´ direction and use ssDNA as a template. Due to the strict directionality of DNA replication one of the DNA strands is produced in a continuous manner (leading strand) while the other strand has to be synthesized in a discontinuous manner (lagging strand). Many DNA polymerases need a processivity factor, which interacts with both the DNA and the polymerase. A DNA polymerase without its processivity factor can only synthesize short DNA chains. Furthermore, replicative DNA polymerases have a proofreading exonuclease activity, which removes an incorrectly incorporated nucleotide. The proofreading mechanism enhances the fidelity of DNA polymerases by more than 100-fold (Hamdan and Richardson, 2009).

To initiate DNA synthesis, most DNA polymerases need a free 3´-hydroxyl group base-paired to the template strand. The free 3´end is usually RNA synthesized by a primase, which is able to initiate polymerization of ribonucleotides *de novo* (Berg, 2002). DNA primases synthesize 4-15 nucleotides long RNA primers, which are held by the primase until DNA polymerases utilize them to start DNA synthesis. Primases are required at the origin of replication to initiate DNA replication on the leading strand, but they are also needed repeatedly on the lagging strand for initiation of DNA synthesis. On the lagging strand the DNA is synthesized in short pieces of DNA (100-200 nucleotides long in eukaryotes and 1000-2000 nucleotides in prokaryotes) called Okazaki fragments. The RNA primer on Okazaki fragments and at the origin will eventually be removed and the resulting gap will be replaced with DNA. As a final step, the Okazaki fragments are ligated with each other. To coordinate lagging strand replication with leading strand replication it has been proposed that the lagging strand makes a loop so that its associated DNA polymerase replicates in parallel with the leading strand polymerase. To ensure that RNA primers are produced, DNA primases synthesize primers on most ssDNA. However, many primases produce primers much more frequently when exposed to specific DNA sequences, named primase recognition sequences. As an example, the primase DnaG in *E. coli* prefers the sequence 5´CTG3`.

During replisome progression, ssDNA is formed and the ssDNA is rapidly coated by single strand binding proteins to prevent re-annealing of the two

parental strands and to inhibit hairpin formation of the ssDNA. Single strand binding proteins are critical for replisome function and often stimulate the activities of DNA polymerases, helicases and primases (Benkovic et al., 2001; Frick and Richardson, 2001).

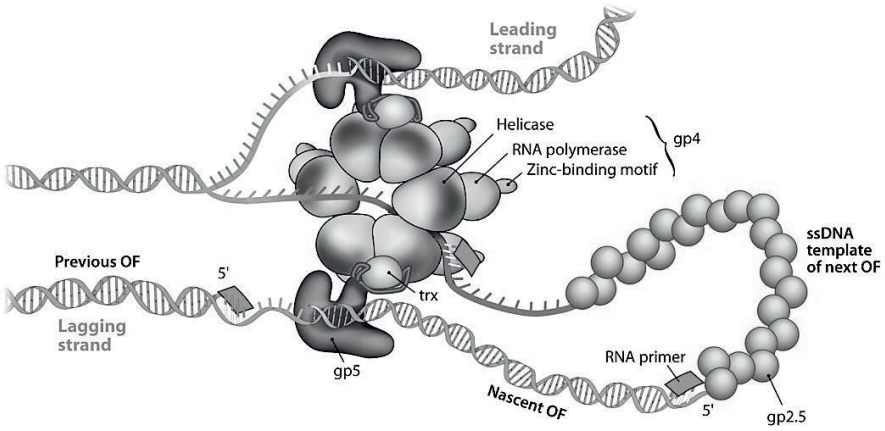


**Figure 5. The T7 bacteriophage replisome**
The T7 proteins gene 5 (gp5) DNA polymerase, gene 4 (gp4) helicase-primase, gene 2.5 (gp2.5) single strand binding protein, and the *E. coli* processivity factor thioredoxin (trx). Okazaki fragment (OF). (From (Hamdan and Richardson, 2009) with permission.)

### 1.5.1 T7 DNA Replication

Three key components of the mtDNA replication machinery, POLRMT, TWINKLE, and POLγA show significant sequence similarities with the T-odd lineage of bacteriophages (T3 and T7 bacteriophages and their close relatives). It has been speculated that these genes originate from an infected T-odd phage that infected the cell (Shutt and Gray, 2006a). The T7 DNA replication machinery is relatively simple. Here, some important characteristics of this highly explored system will be discussed.

T7 DNA replication can be reconstituted *in vitro* with the four proteins gene 5 DNA polymerase (gp5), *E. coli* thioredoxin (trx) processivity factor, gene 4 helicase-primase (gp4), and gene 2.5 single strand binding protein (gp2.5)

(Figure 5)(Shutt and Gray, 2006a). Gp5 forms a highly stable complex with trx, which increases the processivity of gp5 from a few nucleotides up to 1 kb. The gp4 helicase-primase forms both hexamers and heptamers, and it does not require any additional factors for loading onto ssDNA (Crampton et al., 2006). Instead, it has been proposed that the primase-domain makes the initial contact with the DNA and triggers the opening of the ring (Ahnert et al., 2000). Once loaded, gp4 interacts with gp5 and together they can synthesise DNA up to 17 kb with a leading strand replication rate of 165 bases/s (Hamdan and Richardson, 2009). The primase domain of gp4 specifically recognizes the trinucleotide sequence 5′GTC3′ on the lagging strand and synthesizes tetraribonucleotides that are used by gp5 to produce Okazaki fragments. The lagging strand forms a replication loop, which allows for coordinated leading- and lagging strand synthesis. During DNA replication, the replication loop is released when one Okazaki fragment is completed (Lee and Richardson, 2011).

The ssDNA on the lagging strand is coated by gp2.5 and it serves to inhibit hairpin formation and re-annealing of the unwound DNA. In addition, gp2.5 also increases the frequency and efficiency of primase initiation up to a 10-fold (Benkovic et al., 2001). Physical interaction between gp2.5 and gp5/trx and gp4 have been observed and gp2.5 seems to stimulate the activities of these proteins (Hamdan and Richardson, 2009). The importance of protein-protein interactions was demonstrated by the observation that truncation of the gp2.5 C-terminal, which restores ssDNA binding capacity of the protein but not the physical interaction with gp5, resulted in the failure to stimulate DNA polymerization *in vitro* (Kim and Richardson, 1994; Kim et al., 1992).

### 1.5.2 Termination/pausing of DNA replication

In prokaryotes the typical genome is circular and DNA replication initiates at one origin and proceeds bi-directionally until the replication forks approach specific Ter sites situated opposite to the origin where replication is terminated. In *E. coli* there are ten 23 bp sequences named TerA-J, which are arranged in two groups of five with the directionality opposite to each other at the termination region. Each Ter site binds the termination protein Tus. Tus has been shown to exhibit anti-helicase activity and is believed to block fork progression through this activity. The Ter-Tus complex only terminates DNA replication when it is approached from one direction but not the other. In other words, the Ter-Tus complex has one permissive face where the

replication fork passes, and one non-permissive face that terminates DNA replication (Duggin et al., 2008). The proposed explanation for this polarity is that when the fork reaches the Ter-Tus complex from the non-permissive face a strictly conserved DNA base (C6) locks Tus onto the DNA, resulting in removal of the helicase and termination of DNA synthesis (Mulcair et al., 2006). This does not occur at the permissive face, and, instead, Tus dissociates from DNA and the replication fork can progress.

The *B. subtilis* termination system is similar to the system seen in *E. coli*. Interestingly, the factor RTP that binds the Ter sites is completely unrelated to Tus, demonstrating that these two proteins have developed independently from each other but with equivalent functions nonetheless. The mechanism describing how DNA replication is terminated is not completely understood, but it has been suggested to involve anti-helicase activity (Duggin et al., 2008).

In eukaryotes replication termination/pausing has been described to occur at replication fork barriers situated e.g around the highly transcribed rDNA genes in a wide range of species including yeast, plants, mouse, and human. Replication fork barriers are believed to prevent the head-on collisions between the RNA polymerase and the replisome. The factor TTF1 binds specifically to a motif named the Sal box, which is repeated between the rDNA genes. Replication fork barriers have been observed around these Sal boxes and *in vitro* studies have shown that three different *cis*-acting elements (including the Sal boxes) and TTF1 are needed for full activity. Moreover, one of the *cis*-acting elements has been suggested to form a secondary structure that could potentially act as a barrier for the replisome (Duggin et al., 2008; Gerber et al., 1997). The mechanism responsible for the termination/pausing of DNA replication by TTF1 has been proposed to involve anti-helicase activity where the activity seems to operate in a polar manner (Putter and Grummt, 2002).

# 1.6 Mitochondrial DNA replication

## 1.6.1 Models of mtDNA replication

### The strand-displacement model

The first mechanism explaining how mtDNA is replicated was developed from electron microscopy images in which replication intermediates were shown to contain long stretches of single stranded H-strand (Kasamatsu and Vinograd, 1973; Robberson et al., 1972). The model proposed for mtDNA replication was called the strand-displacement model and refers to the displaced H-strand (Figure 6A). The mechanism was later established in more detail when the mtDNA origins were found by mapping the 5´ends of mtDNA replication intermediates (Tapper and Clayton, 1981). Accordingly, mtDNA replication initiated at $O_H$ continues unidirectionally, displacing the parental H-strand. About two-thirds away from $O_H$ another origin $O_L$ becomes activated on the displaced H-strand and replication of the displaced strand is initiated (Clayton, 1982). In this model both strands are replicated continuously but asynchronously.

The RNA primer that primes $O_H$-replication is believed to be produced by the transcription machinery initiating transcription at LSP (Gillum and Clayton, 1979). In human cells several free DNA 5´ends have been mapped where most 5´ends are located around nucleotides 110, 150, 170 and 190 (Chang and Clayton, 1985; Kang et al., 1997; Pham et al., 2006; Tapper and Clayton, 1981; Walberg and Clayton, 1981). One of the most frequently occurring 5´end corresponds to nucleotide 191 and is located close to CSB1. The observation that there is a transcript spanning from LSP to CSB1, the site where the DNA 5´end is situated, was one reason why LSP transcription was suggested to prime mtDNA replication at $O_H$ (Figure 3)(Chang and Clayton, 1985). However, some studies have shown that the RNA to DNA transition takes place around CSB2 (Kang et al., 1997; Pham et al., 2006). Priming of DNA replication requires that the RNA remains hybridized to the DNA forming a stable RNA-DNA hybrid, also called an R-loop. It was first suggested in yeast, and later in humans, that the GC-rich CSB2 element was necessary to form an R-loop *in vitro* proposing that CSB2 is involved in primer formation (Xu and Clayton, 1995, 1996). Moreover, the

endoribonuclease RNase MRP was shown to cleave the R-loop *in vitro* at sites that overlapped with the mapped DNA 5´ends and to produce primers that could be used for DNA synthesis (Lee and Clayton, 1997, 1998). However, RNase MRP is probably not the enzyme responsible for primer formation at $O_H$ since this protein was shown to localize the nucleus and not to mitochondria (Kiss and Filipowicz, 1992).

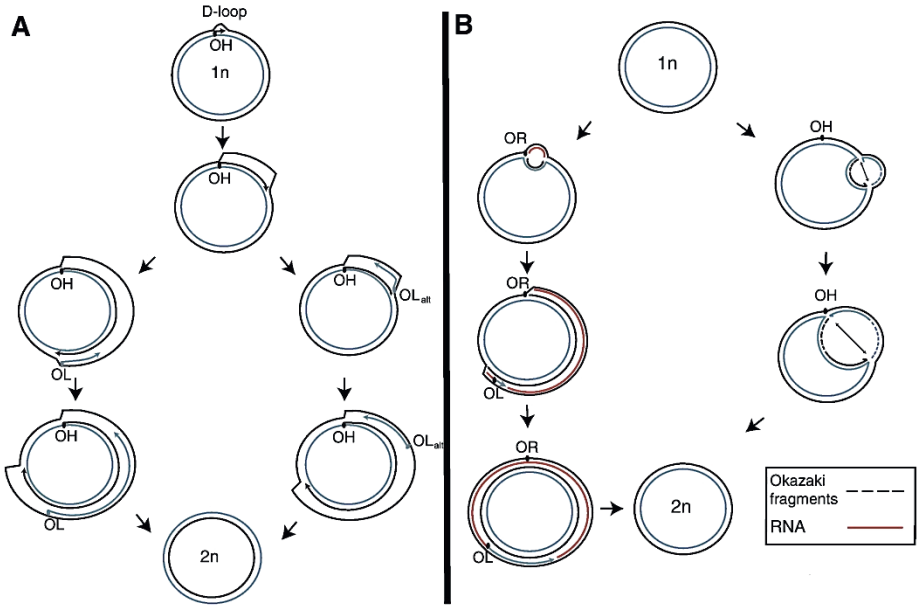


**Figure 6. Models of mtDNA replication**

**A.** The strand-displacement model. Replication is initated at $O_H$ and continues unidirectionally. Lagging strand replication is initiated at $O_L$ (left) or at $O_L$ alternative ($OL_{alt}$) (right) **B.** RITOLS model with RNA (red) annealed to the displaced strand (left) and the strand-coupled model with Okazaki fragments (right). (From (Wanrooij and Falkenberg, 2010) with permission.)

In 2006, Pham et al. demonstrated that *in vitro* transcription using an LSP template resulted in pre-termination of transcription at CSB2 (Pham et al., 2006). The pre-termination appeared to overlap with the RNA to DNA transitions sites and it was suggested that this pre-termination event was important for primer formation at $O_H$ (Kang et al., 1997; Pham et al., 2006). Later, it was shown that the G-rich CSB2 was able to form a G4 structure between the RNA and non-template DNA resulting in transcription termination and R-loop formation *in vitro* (Wanrooij et al., 2012a; Wanrooij et al., 2010). Whether a G4 structure is formed at CSB2 *in vivo* and its role in R-loop formation has to be further investigated.

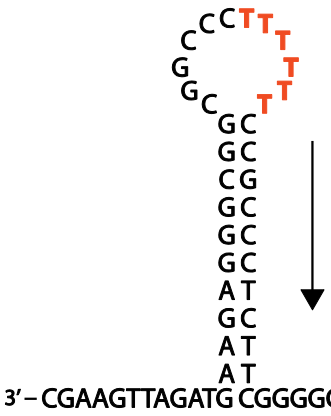


**Figure 7. The $O_L$ stem loop structure**
11 nucleotides form the stem whereas 12 nucleotides form the loop region of the $O_L$ stem loop. The RNA primer is initated at the poly-dT stretch (red Ts).

Based on electron microscopy images another mitochondrial origin, $O_L$ was proposed to exist about two-thirds away from $O_H$ (Robberson et al., 1972). The DNA 5´end at $O_L$ was later mapped downstream a potential stem loop structure, which in humans consists of a 34-nucleotide stem loop where 11 nucleotides comprise the stem (Figure 7) (Martens and Clayton, 1979; Tapper and Clayton, 1981). The stem loop structure is located in a tRNA cluster, and it is believed to adopt a secondary structure when $O_L$ becomes single stranded; i.e after the replication fork initiated at $O_H$ has reached $O_L$. The RNA, which primes DNA synthesis on the displaced H-strand, has been mapped to initiate at a poly-dT stretch in the single stranded loop region (Kang et al., 1997; Martens and Clayton, 1979; Tapper and Clayton, 1982; Wong and Clayton, 1985a). Although primase activity at $O_L$ had been observed (Wong and Clayton, 1985a, b) the identity of the primase remained

unknown over a long period of time. Paper I in this thesis shows that in a pure *in vitro* system, POLRMT specifically recognizes the poly-dT stretch in the loop region of $O_L$ and synthesizes RNA primers that can be used by POLγ to replicate DNA (Fuste et al., 2010). POLRMT is therefore not only the mitochondrial RNA polymerase but also a mitochondrial primase. Moreover, alternative origins on the displaced H-strand have been found by studying replicating mtDNA molecules by atomic force microscopy (Brown et al., 2005). However, $O_L$ seems to be essential since this stem loop is conserved throughout vertebrates and *in vivo* mutational saturation using proofreading-deficient mice (Trifunovic et al., 2004) shows that mutations in $O_L$ were selected against (Wanrooij et al., 2012b).

### Other mtDNA replication models

In year 2000 the strand-displacement model of mitochondrial DNA synthesis was challenged by another model suggesting that mtDNA is replicated by a unidirectional coupled leading- and lagging strand mode (Holt et al., 2000). In this study, two-dimensional agarose gel electrophoresis was used for analyzing the mtDNA, and it was found that some mtDNA molecules were resistant to single strand nuclease digestion. This observation was not in agreement with the strand-displacement model. Later, it was concluded that the DNA resistant to cleavage by single strand nucleases was annealed to RNA and hence sensitive to RNase H (Pohjoismaki et al., 2010; Yang et al., 2002; Yasukawa et al., 2006). The RNA-DNA duplex observed by two-dimensional gel electrophoresis was later found to be formed only on the displaced DNA H-strand (lagging strand) and DNA replication of this strand was suggested to initiate near or at $O_L$ (Yasukawa et al., 2006). This model was called RITOLS (ribonucleotide incorporation throughout the lagging strand). The initiation of mtDNA synthesis was mapped to or near $O_H$ and to a lesser extent somewhere within the non-coding region (Figure 6B). The strand-displacement model and RITOLS resemble each other since DNA replication in both models is believed to be unidirectional DNA synthesis initiated at $O_H$ (or within the non-coding region) and both models predicts a delay between leading- and lagging strand replication. However, in one aspect these two models are completely different. In the strand-displacement model, the displaced H-strand (lagging strand) is covered with mtSSB, whereas in the RITOLS model, the displaced strand is covered with RNA.

Another study of mtDNA replication intermediates in mammalian tissue suggested that initiation of mtDNA takes place in a broad zone including cyt b and the NADH dehydrogenase subunits 5 and 6 (Figure 6B)(Bowmaker et al., 2003). DNA synthesis initiated in this zone seems to be bidirectional coupled leading- and lagging strand synthesis until one of the replication forks reaches $O_H$ and terminates, giving rise to a persistent 5´end at $O_H$. The other fork progressing in the opposite direction proceeds unidirectionally to complete synthesis of the genome (Bowmaker et al., 2003). However, the coupled leading- and lagging strand replication observed at the origin in this study has later been suggested to be the result of maturation of RITOLS RNA-DNA duplexes into DNA-DNA duplexes (Holt and Reyes, 2012).

## 1.6.2 The core mitochondrial DNA replication machinery

Many of the core factors involved in mtDNA replication have been identified and characterized. They include the mitochondrial DNA polymerase POLγ, the mitochondrial replicative helicase TWINKLE, the mitochondrial single strand binding protein mtSSB, and the bi-functional mitochondrial RNA polymerase POLRMT (Figure 8). In 2004 the minimal mtDNA replisome, mimicking a leading strand replication fork, was reconstituted *in vitro* containing the recombinant forms of human POLγ, TWINKLE and mtSSB and a dsDNA template (Korhonen et al., 2004). Together, POLγ and TWINKLE were able to use this dsDNA template and synthesize ssDNA up to about 2 kb in length. However, addition of mtSSB stimulated DNA synthesis and resulted in ssDNA products of around 16 kb, the size of the human mitochondrial genome. For the first time, this paper provided biochemical evidence that the newly found helicase TWINKLE could function together with POLγ and mtSSB and that TWINKLE was the helicase at the leading strand replication fork in mitochondria. Work presented in this thesis (Paper I) demonstrate reconstitution of both leading- and lagging strand DNA synthesis *in vitro* by using an $O_L$-containing DNA template, the mitochondrial replisome and POLRMT (Fuste et al., 2010).

### POLγ - the mitochondrial DNA polymerase

The only replicative DNA polymerase found in mammalian mitochondria so far is the heterotrimeric protein POLγ. The human POLγ holoenzyme consists of one 140 kD catalytic subunit, POLγA, and two subunits of the 53 kD POLγB (Carrodeguas et al., 2001; Fan et al., 2006; Kaguni, 2004;

Yakubovskaya et al., 2006). The catalytic subunit POLγA has three known enzymatic activities: DNA polymerase activity, 3´ to 5´ exonuclease activity, and 5´-deoxyribose phosphate (dRP) lyase activity, which is involved in base excision repair (Foury and Vanderstraeten, 1992; Longley et al., 1998; Vanderstraeten et al., 1998). Amino acid sequence analysis of POLγA showed that POLγA shares conservation of the three DNA polymerase motifs and three 3´ to 5´ exonuclease active site motifs with *E. coli* DNA pol I, placing POLγA in the family A group of DNA polymerases (Kaguni, 2004). However, POLγA contains a long spacer region that connects the exonuclease domain with the polymerase domain that is not found in other polymerases within the family A group (Kaguni, 2004).

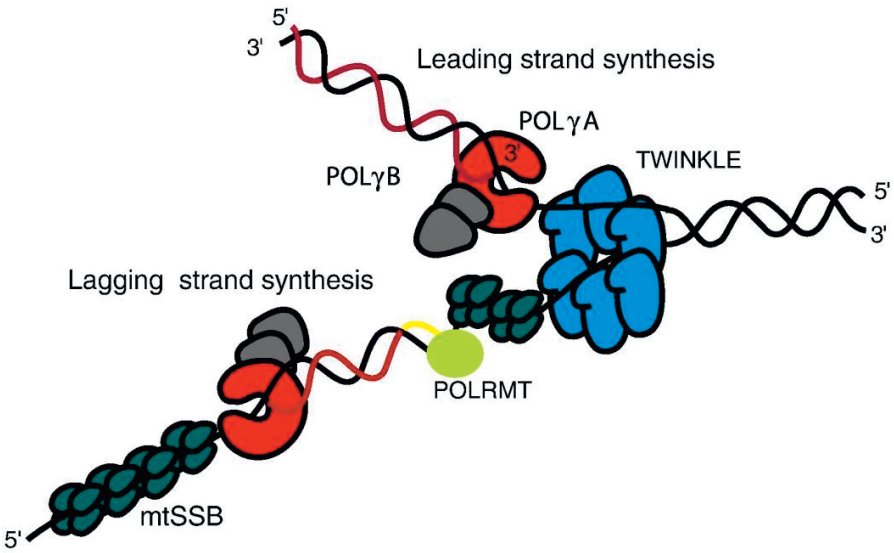


**Figure 8. The mitochondrial replisome**
TWINKLE, POLγ, and mtSSB is needed for mtDNA leading strand replication. Lagging strand DNA replication is initiated by POLRMT and the RNA primer (yellow) is used by POLγ to initiate DNA synthesis. (From (Wanrooij and Falkenberg, 2010) with permission.)

The processivity subunit POLγB is present in many eukaryotes but not in yeast (Carrodeguas et al., 1999; Lim et al., 1999; Wang et al., 1997). This protein shares structural homology with aminoacyl-tRNA synthetases, enzymes containing domains involved in tRNA binding. POLγB has

therefore been suggested to be involved in RNA primer recognition during initiation of DNA synthesis (Fan et al., 1999). PolγB contributes to tighter DNA- and nucleotide binding of the polymerase holoenzyme as well as an increased DNA polymerization rate (Carrodeguas et al., 1999; Fan et al., 2006; Johnson et al., 2000; Lim et al., 1999). Moreover, the base excision repair rate of POLγA seems to be enhanced by the POLγB subunits (Pinz and Bogenhagen, 2006). The dsDNA binding activity of POLγB was shown to be required for proper replication by POLγ on dsDNA templates but not on ssDNA templates (Farge et al., 2007). The observation suggests that the dsDNA binding activity of POLγB is required for coordination of POLγ and TWINKLE helicase activities at the mtDNA replication fork.

### TWINKLE - the mitochondrial helicase

The gene coding for the mitochondrial replicative helicase TWINKLE was first found during analysis of mutations causing the disease adPEO (autosomal dominant progressive external ophtalmoplegia), a neuromuscular disorder associated with multiple mtDNA deletions (Spelbrink et al., 2001). TWINKLE was shown to have high amino acid sequence similarities with the bacteriophage T7 gene 4 protein (gp4), which is the bi-functional primase-helicase required at the replication fork of bacteriophage T7 DNA. Gp4 can be divided into two functionally and structurally distinct regions. The N-terminal part of the protein harbors a primase domain and the C-terminal part possesses DNA helicase activity. A 26-amino acid linker region required for hexamer and hepatamer formation separates the two functional domains (Lee and Richardson, 2004). Similar to gp4 protein, TWINKLE forms hexameric and heptameric structures in solution and can be divided into three regions: an N-terminal primase-like domain, a short linker domain and a C-terminal helicase domain (Farge et al., 2008; Ilyina et al., 1992; Spelbrink et al., 2001; Ziebarth et al., 2010). Bioinformatic analyses suggest that TWINKLE might possess primase activity in many eukaryotes. However, the activity is lost in human and metazoans since an essential $Zn^{2+}$-finger motif, which in gp4 is involved in nucleotide sequence recognition and in transferring the primer to the DNA polymerase, is missing (Farge et al., 2008; Shutt and Gray, 2006b). Instead POLRMT acts as the mitochondrial primase in humans and probably in most metazoas.

TWINKLE has 5′ to 3′ helicase activity and can on its own unwind 20 bp streches of dsDNA when added to a DNA fork structure (Korhonen et al., 2003). However, as discussed above, together with the other replication factors POLγ and mtSSB, TWINKLE is an efficient helicase able to unwind 16 kb of dsDNA (Korhonen et al., 2004).

TWINKLE is an essential protein and conditional TWINKLE knock-out mice show severe mtDNA depletion and a complete lack of 7S DNA (Milenkovic et al., 2013). Furthermore, in Paper IV in this thesis we have been able to demonstrate that TWINKLE is enriched within the D-loop region and possibly loaded at $O_H$. These results indicate that no other replicative helicase in mitochondria can replace TWINKLE.

As discussed above, replicative helicases usually accommodate one of the DNA strands inside the ring structure and since there are no free DNA ends that can be threaded through the hole of the helicase ring structure, additional factors or subdomains may be needed for this purpose (Davey and O'Donnell, 2003). The primase domain of the gp4 protein has been suggested to act as a helicase loader, contributing to the initial contact with the DNA. A conformational change in the gp4 protein induces opening of the ring structure and facilitates entrance of the ssDNA into the central channel (Ahnert et al., 2000). In Paper II in this thesis we address the question whether the TWINKLE helicase needs an additional loading factor to load onto circular ssDNA. This study shows that TWINKLE, like the primase/helicase gp4, is able to load onto DNA without assistance from any additional factor (Jemt et al., 2011).

### mtSSB - the mitochondrial single strand binding protein

The mitochondrial single strand binding protein mtSSB is a 13-15 kD protein that forms tetramers in solution (Li and Williams, 1997; Thommes et al., 1995; Yang et al., 1997). MtSSB and *E. coli* SSB show high degree of sequence homology. However, a 60 amino acid C-terminal residue is missing in the mitochondrial version (Van Dyck et al., 1992; Yang et al., 1997). The protein binds ssDNA with high affinity (Thommes et al., 1995). It has been proposed that ssDNA wraps around the tetramer in electropositive channels guided by flexible loops (Yang et al., 1997). Mutation of the mtSSB gene in

*D. melanogaster* resulted in loss of mtDNA and subsequent death prior to eclosion, demonstrating the importance of this protein (Maier et al., 2001).

MtSSB has been shown to stimulate the activity of TWINKLE and POLγ as well as improving primer recognition and binding of POLγ to primer-templates (Farr et al., 1999; Korhonen et al., 2003; Korhonen et al., 2004; Thommes et al., 1995). In the strand-displacement model of mtDNA replication, mtSSB plays a very significant role since it is suggested to bind to the displaced strand. Paper I in this thesis shows that mtSSB represses RNA primer formation by POLRMT on ssDNA *in vitro* except for DNA containing the $O_L$ stem loop sequence (Fuste et al., 2010).

### POLRMT – the mitochondrial RNA polymerase and primase

As mentioned earlier, transcription initiated at LSP produces the RNA primer needed for initiation of DNA replication at $O_H$. However, primase activity required for lagging strand mtDNA synthesis had been observed long before the identity of the protein was determined (Wong and Clayton, 1985a, b, 1986). One study observed a primase activity in nuclear extract that was released upon infection by Herpes simplex virus type 1 in Vero cells (Tsurumi and Lehman, 1990). Based on biochemical characteristics, this protein was postulated to be POLRMT and the authors believed that this RNA polymerase was released from mitochondria by herpes virus-induced disruption of the mitochondrial membrane. However, the protein was never directly identified.

The primase activity of POLRMT was first described in 2008 using highly purified recombinant human POLRMT (Wanrooij et al., 2008). POLRMT was show to be less processive on ssDNA than on dsDNA, producing RNA of 25-75 nucleotides in length *in vitro*. Furthermore, the short RNA primers could be utilized by POLγ to initiate DNA replication. Later, it was shown that POLRMT specifically synthesized RNA primers of about 25 nucleotides at $O_L$ in the presence of mtSSB *in vitro* and that POLγ could use the primers for initiation of DNA synthesis (data presented in Paper I in this thesis (Fuste et al., 2010)). The 5´ends of the RNA primers were mapped the poly-dT stretch in the loop region and the 3´ends of the primer to the region just downstream of the stem. The results were in nice agreement with previous 5' and 3' end mapping of the RNA at $O_L$ *in vivo* (Kang et al., 1997; Wong and

Clayton, 1985a). The length and stability of the $O_L$-stem as well as the poly-dT stretch in the loop region seems to be important for proper recoginztion and function of POLRMT (Fuste et al., 2010; Wanrooij et al., 2012b).

### 1.6.3 Additional factors involved in mtDNA replication

Replication of DNA requires additional factors other than the classical enzymes present at the DNA replication fork. Such factors are foe example topoisomerases and DNA ligases.

Separation of the DNA duplex during transcription and DNA replication creates supercoiling of the DNA and consequently topological stress. Topoisomerases are enzymes that can remove this stress by transiently cleaving one or both DNA strands followed by ligation. So far, three topoisomerases have been found inside mitochondria, TOP1MT (Zhang et al., 2001), mtTOP2B (Low et al., 2003), and mtTOP3A (Wang et al., 2002) but their exact roles in mtDNA replication are still not known (Sobek and Boege, 2014).

A DNA ligases seal nicks in the phosphodiester backbone of DNA, and they are essential for both DNA replication and repair. Only one ligase, Lig3, has so far been identified in mitochondria. Mitochondrial Lig3 is formed using an alternative translation start site compared to the nuclear version (Chen et al., 1995; Husain et al., 1995; Wei et al., 1995). Inactivation of Lig3 in mice is embryonic lethal (Puebla-Osorio et al., 2006). However, the mitochondrial version of Lig3, but not the nuclear form, was shown to complement the loss of Lig3 and rescue cell viability (Simsek et al., 2011). Moreover, deletion of Lig3 in mouse nervous system leads to loss of mtDNA, indicating that Lig3 plays a crucial role in mtDNA maintenance (Gao et al., 2011).

In 2003 it was found that depletion of RNase H1 in mice caused embryonic lethality, probably as a consequence of mtDNA loss (Cerritelli et al., 2003). RNase H degrades RNA in RNA-DNA hybrids and has been suggested to take away the RNA primers during DNA replication, especially during lagging strand synthesis (Kogoma T, 1998). RNase H1 is found in the nucleus (Frank et al., 1998; ten Asbroek et al., 2002), but has also been shown to localize to mitochondria in several species (Cazenave et al., 1994; Pileur et al., 2000; Wang and Lambowitz, 1993). The loss of mtDNA observed in the *RNase H1*$^{-/-}$ mice indicates that RNase H1 could be the

enzyme that removes the primers during mtDNA replication (Cerritelli et al., 2003).

### 1.6.4 The mitochondrial D-loop

The mitochondrial D-loop was first observed studying mouse and chicken mtDNA molecules under electron microscopy (Arnberg et al., 1971; Kasamatsu et al., 1971; Robberson et al., 1972). The D-loop is a triple stranded DNA structure spanning the region from $O_H$ to TAS (Figure 9). The third strand is called 7S DNA. The D-loop form of mtDNA seems to be widely spread throughout the animal kingdom including human, mouse, and *D. melanogaster* (Brown et al., 1978; Robberson et al., 1972; Rubenstein et al., 1977). The fraction of mtDNAs containing a D-loop appears to vary depending on cell type and animal, e.g 10% for human HeLa cells and 55% for placenta tissue (Brown et al., 1978). In mouse L cells and unfertilized *X. laevis* eggs more than 75% of mtDNA contain a D-loop (Hallberg, 1974; Robberson et al., 1972). Interestingly, analyzes of different types of tissues revealed that there is a consistent and direct correlation between the 7S DNA/mtDNA ratio and oxidative metabolism (Annex and Williams, 1990). Moreover, the 7S DNA/mtDNA ratio correlated directly with mtDNA copy number and mRNA abundance.

In humans the D-loop spans about 570-650 bp of the non-coding region and this variation is due to differences at the 5´ends of the 7S DNA (Brown et al., 1978; Tapper and Clayton, 1981). However, in mouse cells 7S DNA only seems to have one 5´end (Gillum and Clayton, 1978). Since the 5´ends of 7S DNA overlapped with $O_H$, together with the fact that RNA was found attached to the 5´end of 7S DNA in mice (Gillum and Clayton, 1978; Tapper and Clayton, 1981), it was suggested that the formation of D-loops was a result of terminated replication initiated at $O_H$. In human cells the 3´ end of 7S DNA was mapped to one specific place (Doda et al., 1981; Walberg and Clayton, 1981) whereas four different 3´ ends were found in mouse (Doda et al., 1981; Gillum and Clayton, 1978). Interestingly, the 3´ends of 7S DNA coincide with some conserved consensus DNA sequences situated around 50 bp upstream of the site where DNA synthesis is prematurely terminated (Doda et al., 1981; Walberg and Clayton, 1981). Human mtDNA only contains one consensus sequence whereas mouse mtDNA contains four sequences. These sequences were named termination-associated sequence,

TAS, because of their hypothetical role in termination of DNA replication. Later, alignments of D-loop DNA sequences from different organisms revealed that there is two 60 bp conserved sequences situated at the 3´end of the D-loop, called extended TAS 1 and 2 (ETAS1 and ETAS2)(Sbisa et al., 1997).

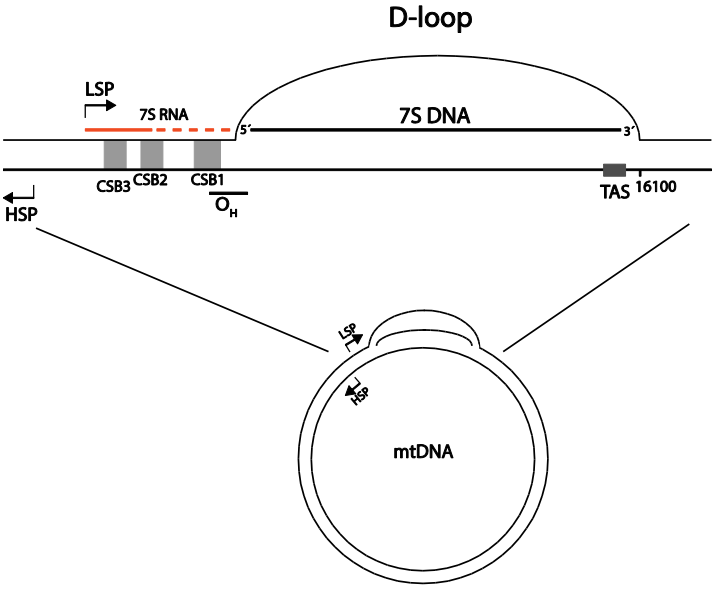


**Figure 9. The mitochondrial D-loop**
LSP transcription produces the RNA primer (red) needed for initiation of $O_H$-replication. Replication is terminated downstream of TAS, creating the triple stranded D-loop. Light strand promoter (LSP), heavy strand promoter (HSP), conserved sequence block (CSB), origin of heavy strand ($O_H$) termination associated sequence (TAS).

## Formation and hypothetical functions of the D-loop

Although premature termination of mtDNA synthesis resulting in the formation of the D-loop is a frequent event, there is no explanation for how and why it is formed. It is not known if 7S DNA acts or can act as a primer for continuation of mtDNA replication *in vivo*, even though it can do so *in vitro* (Eichler et al., 1977).

It has been proposed that the premature DNA replication termination event regulates mtDNA copy number, since depletion of mtDNA using the nucleotide analogue ddC results in an subsequent increase in DNA replication and a decrease of the premature replication termination event (Brown and Clayton, 2002). In agreement with this observation, another study, in which mtDNA replication was stimulated by activation of T lymphocyte proliferation, showed that full-length mtDNA replication was increased while initiation at $O_H$ remained unchanged (Kai et al., 1999). This result provided additional support for the idea that the control of mtDNA copy number is regulated at the 3´end of the D-loop region.

Analyses of the DNA sequence at the 3´end of the D-loop have suggested that this region is capable of forming secondary structures (Brown et al., 1986; Pereira et al., 2008). However, it is not known if the formation of this secondary structure actually occurrs *in vivo* or if the structure is involved in premature DNA replication termination. In Paper IV in this thesis we have investigated the potential of DNA secondary structure formation at the 3' end of the D-loop and its hypothetical involvement in the premature DNA replication termination event. We performed DNA replication *in vitro* using the mitochondrial replisome and a DNA template containing a sequence corresponding to the end of the D-loop (Jemt et al., manuscript). We were, however, not able to detect any premature DNA replication termination using this method, suggesting that the DNA itself is not able to terminate DNA synthesis. Instead, we found that a factor in mitochondrial extract binds specifically to the sequence corresponding to the end of the D-loop. The result proposes that a protein might be involved in the formation of the D-loop and other observations in this study suggest that the factor might possess anti-helicase activity. However, we have not been able to purify the factor to homogeneity and reveal its identity. Interestingly, several *in organello*/*in vivo* footprinting analyses of the TAS region in human, rat and bovine have observed proteins binding to these sequences, but these factors have also remained unidentified (Madsen et al., 1993; Roberti et al., 1998).

It has been pointed out that the formation of the D-loop is a persistent event even when mtDNA levels are very low and DNA replication beyond the premature termination site would be favourable (Brown and Clayton, 2002). Furthermore, calculations of DNA replication initiation events have estimated that only one out of 15-20 initiation events at $O_H$ is extended beyond the

premature termination site (Bogenhagen and Clayton, 1978; Brown and Clayton, 2002). These calculations predict that approximately 20% of all incorporated nucleotides endup in the 7S DNA strand. Evidently, a lot of energy is put into the formation of the D-loop (Clayton, 1982). These observations imply that the D-loop might be an important structural feature. In accordance with this speculation, it has been shown that mtDNA with less D-loops show increased mtDNA supercoiling (Di Re et al., 2009; Ruhanen et al., 2010), which might affect the accessibility of the mtDNA. The function of the D-loop could thus simply be to keep the mtDNA in a more open conformation and thereby more accessible for binding of proteins including the transcription and/or replication machineries.

Little is known about the turnover of 7S DNA. Free 7S DNA is not believed to exist in detectable amounts (Bogenhagen and Clayton, 1978) and the half-life of 7S DNA is estimated to be 45 min to 1 h in rat and mouse cells (Bogenhagen and Clayton, 1978; Gensler et al., 2001). The molecular mechanisms regulating 7S DNA turnover are poorly characterized, but recently a nuclease named MGME1 was shown to localize to mitochondria. A homozygous mutation in this gene was shown to cause mtDNA depletion in patients with PEO and multisystemic mitochondrial disorder (Kornblum et al., 2013; Szczesny et al., 2013). Moreover, mutations in the MGME1 gene or knock-down of MGME1 resulted in increased levels in 7S DNA (Kornblum et al., 2013; Szczesny et al., 2013), whereas overexpression of the protein lead to a decrease in 7S DNA levels (Szczesny et al., 2013). In addition, the studies also demonstrated that the protein exhibited exonuclease activity. More specifically, MGME1 degraded ssDNA efficiently, but dsDNA poorly. The increased levels of 7S DNA in patients and cells with low levels or mutated MGME1 and its pronounced preference for ssDNA suggest that MGME1 could be the nuclease that degrades 7S DNA. However, another mechanism removing 7S DNA from the mtDNA molecule probably exists since MGME1 degrades dsDNA poorly. Future studies examining the role of MGME1 in 7S DNA regulation have to be performed in order to resolve these questions.

## 1.7 Mitochondrial genetics and diseases

### 1.7.1 Genetics of mtDNA

The mitochondrial genome is inherited through the maternal line since mitochondria originating from sperm are selectively degraded by an autophagic process inside the oocyte (Al Rawi et al., 2011; Sato and Sato, 2011). The mtDNA copy number in a somatic cell is in the range of 1000-10 000 copies per cell (Falkenberg et al., 2007) and the density of mitochondria and mtDNA are believed to relate to the metabolic demand of the tissue. As a result, mitochondrial diseases usually affect tissues in which the energy demand is high such as the central nervous system, skeletal muscle or heart. However, other tissues are frequently affected such as the beta cells of the pancreas, the inner hair cells of the cochlea or the renal tubules. Therefore mitochondrial diseases often include diabetes, deafness, and kidney dysfunction (Greaves et al., 2012).

In most cases all mtDNA molecules within a cell of one individual are identical and this is referred to as homoplasmy. However, patients with mitochondrial diseases often have different populations of mtDNA, wild-type mtDNA without mutations and mtDNA containing disease causing mutations. This situation is called heteroplasmy (Ylikallio and Suomalainen, 2012). Overall, the severity of the disease correlates with the relative proportion of the mutated mtDNA. A heteroplasmic individual will only develop a disease if the mutant mtDNA load reaches a certain threshold and this threshold may be different for different mutations and different tissues. As examples, the threshold for deleted mtDNA molecules seems to be around 50-60% and for some mtDNA with tRNA point mutations >90%. When the threshold is exceeded a cellular OXPHOS defect can be observed (Greaves et al., 2012; Ylikallio and Suomalainen, 2012).

One explanation for the variation in mitochondrial disease state observed between siblings, where one sibling can be completely healthy and the other develop disease, is that mitochondria and the mtDNA are randomly segregated during early embryogenesis when primordial germ cells develop. The oocyte contains more than 100 000 mtDNA molecules, which are not replicated during the early stages of embryogenesis. Following repeated cell division the mtDNA are segregated and subsequently diluted. The cells

destined to become primordial germ cells will eventually contain about 1000 mtDNAs with different amounts of mutated mtDNA (if the mother is heteroplastic). Replication of the mtDNA is reinitiated when primordial germ cells differentiate into oocytes (Cao et al., 2007; McFarland et al., 2007; Shoubridge and Wai, 2007). This process is called the "mtDNA bottleneck" and is believed to protect the offspring from defective mtDNA. (Ylikallio and Suomalainen, 2012).

## 1.7.2 Mitochondrial diseases

Mitochondrial diseases are a heterogeneous group of diseases with a broad range of different phenotypes. Defective OXPHOS that leads to decreased ATP production is the major cause of these diseases. As mentioned above, this is also the reason for effects on tissues with high metabolic demand (Greaves et al., 2012). Defective OXPHOS can be caused by mutations in the mtDNA and includes mutations in proteins involved in the respiratory chain as well as mutations in tRNA and rRNA, which are involved in mitochondrial protein production. However, most mitochondrial diseases are caused by genetic defects found in the nuclear genome e.g mutations in genes coding for the remaining subunits of the respiratory chain and factors that assembly the respiratory chain complexes, defects in mtDNA replication factors and mitochondrial dNTP pool maintenance, mutations in genes coding for factors needed for mitochondrial translation, and mutations in genes regulating mitochondrial fission and fusion (Ylikallio and Suomalainen, 2012). The prevalence of mitochondrial disorders caused by mtDNA mutations and nuclear mutations has been estimated to be 1 in 5000 individuals, making this one of the most commonly inherited group of diseases (Schaefer et al., 2004).

### Mutations in mtDNA

MtDNA deletions were the first mitochondrial mutations found to be associated with mitochondrial disease (Holt et al., 1988). MtDNA deletions can be a single base or several kilo bases and are usually located within the major arc between the origins $O_H$ and $O_L$ (Krishnan et al., 2008; Schapira, 2006). The formation of the deletions has been suggested to result from the displacement of the ssDNA strand, found in the strand-displacment model, and subsequent misannealing of two directs repeats. The misannealing might lead to strand slippage and deletion during mtDNA replication (Samuels et

al., 2004). However, another model suggested that formation of mtDNA deletions involves repair of dsDNA breaks caused by oxidative stress or replication stalling (Krishnan et al., 2008).

Single mtDNA deletion is a common cause of sporadic mitochondrial disease and all cells within the affected tissue have identical mtDNA deletion (Schaefer et al., 2004). Another group of patients suffering from mitochondrial diseases show several mtDNA deletions in the affected tissues, and the primary genetic defect of these patients are mutations in genes coding for proteins involved in mtDNA replication or mitochondrial nucleotide metabolism. Usually over 60% of mtDNA molecules have to contain a deletion before biochemical defects can be observed (Krishnan et al., 2008). The three main clinical phenotypes associated with mutations causing deletions are the severe infantile-onset Pearson´s syndrome, KSS (Kearns-Sayre syndrome), and the more adult-onset PEO (progressive external ophthalmoplegia) (Greaves et al., 2012; Ylikallio and Suomalainen, 2012).

MtDNA point mutations in genes coding for proteins, rRNAs, or tRNAs are often maternally inherited. Mutations in the rRNA or tRNA genes lead to a general disturbance in protein synthesis, whereas mutations in a protein-coding gene affect a specific respiratory chain complex. Most of the point mutations occur in the tRNA genes (Greaves et al., 2012; Ylikallio and Suomalainen, 2012). The phenotypes associated with these diseases are broad and include mitochondrial diseases such as MELAS (myopathy encephalopathy lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepst and ragged red fibres), and Leigh syndrome (Schapira, 2006; Ylikallio and Suomalainen, 2012).

### Diseases caused by mutations in the mtDNA replisome

As mentioned above, a large number of mitochondrial diseases are caused by mutations in nuclear encoding proteins that replicate the mtDNA (the two different subunits of POLγ, and TWINKLE). The first mutation found in POLγA was associated with PEO (Van Goethem et al., 2001) and since then around 150 mutations of POLγA have been documented (Chan and Copeland, 2009). Overall, mutations in the polymerase domain of POLγA tend to cause dominant diseases such as autosomal dominant PEO since these mutations do not disturb the DNA binding activity, and mutant proteins can

compete with wild-type proteins. Recessive mutations in POLγA are in general located in the linker or exonuclease regions resulting in a reduction in DNA binding and POLγB interaction (Chan and Copeland, 2009).

Mutations in the accessory subunit POLγB have also been identified (Longley et al., 2006; Walter et al., 2010). One of these mutations causes autosomal dominant PEO and biochemical studies of this factor revealed incomplete stimulation of POLγA as a result of weak subunit interactions (Longley et al., 2006).

As discussed earlier, TWINKLE was identified because mutations within this gene were associated with families with autosomal dominant PEO (Spelbrink et al., 2001). Since TWINKLE forms a hexamer/heptamer, mutations that impair subunit interactions are often dominant and lead to PEO (Wanrooij and Falkenberg, 2010). Recessive TWINKLE mutations seem to cause mtDNA depletion but not deletions (Ylikallio and Suomalainen, 2012).

Biochemical studies of mutant autosomal dominant PEO versions of TWINKLE show that all mutant forms examined were defective in at least one biochemical aspect such as helicase activity, DNA binding activity or formation of hexamers (Holmlund et al., 2009; Korhonen et al., 2008). Moreover, expression of PEO-associated mutant versions or catalytic mutants of TWINKLE and POLγ in different *in vivo* model systems showed that these mutations cause DNA replication stalling, which might be the explanation for the deletions detected in patients with PEO (Goffart et al., 2009; Wanrooij et al., 2004).

### Mitochondrial dNTP pool maintenance and diseases

The presence and proper balance of the four dNTPs is a necessity for accurate DNA replication. MtDNA replicates independently of the cell cycle and continues to replicate in post-mitotic cells, such as neurons and skeletal muscle cells. The mitochondrial dNTP pool is produced by two different pathways. One is the *de-novo* pathway located in the cytosol where the enzyme ribonuclotide reductase (RNR) converts ribonucleotides into the corresponding deoxyribonucleotides. The other pathway is the salvage pathway where deoxyribonucleosides are phosphorylated to become dNTPs by the mitochondrial enzymes thymidine kinase 2 (TK2) and deoxyguanosine

kinase (dGK). Mutations in the mitochondrial salvage pathway enzymes have been shown to cause recessively inherited mtDNA depletion syndrome (MDS), demonstrating the importance of this pathway in mtDNA maintenance. Moreover, mutations in a subunit of RNR also lead to recessively inherited MDS.

Imbalance in the dNTP pool can be deleterious, which is shown by the mitochondrial disorder MNGIE (mitochondrial neurogastrointestinal encephalomyophathy). This disease is caused by defective thymidine phosphorylase (TP) that normally degrades thymidine. Inactive TP leads to high levels of thymidine and possibly an increase in the dTTP levels in mitochondria. MNGIE patients show mtDNA deletions, depletion, and point mutations as a result of the imbalance in the mitochondrial dNTP pool (Ylikallio and Suomalainen, 2012).

# 2 SPECIFIC AIMS

Many key factors involved in mitochondrial DNA replication have been idientified but the exact mechanisms of the different steps in mtDNA replication are not completely understood. Some factors needed for these processes remain to be identified and other factors linked to mtDNA maintenance have yet to be ascribed a clear molecular function. The aim of this thesis was to understand the molecular mechanisms of mtDNA replication, with the main focus on the initiation phase. The specific aims of each paper were the following:

**Paper I:** To examine if POLRMT specifically primes mitochondrial lagging strand DNA synthesis at the origin of light strand ($O_L$).

**Paper II:** To study if the mitochondrial replicative helicase TWINKLE requires specialized helicase loading factors for loading and initiation of DNA synthesis.

**Paper III:** To investigate the role of MTERF1 in mitochondrial transcription and mtDNA replication by characterizing an Mterf1 knock-out mouse.

**Paper IV:** To study the mechanisms responsible for premature DNA replication termination at the end of the mtDNA non-coding region.

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# 3 RESULTS AND DISCUSSION

## Paper I

### Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication

POLRMT was earlier shown to exhibit primase activity (Wanrooij et al., 2008). This promted us to investigate if the protein could be the long sought after primase required for initiation of DNA replication at $O_L$. We could indeed demonstrate that this was the case, using a combination of cell biology and *in vitro* biochemical experiments. We knocked-down POLRMT by RNA interference in human cells and analyzed the DNA replication intermediates with 2D native gel electrophoresis. A significant delay in lagging strand DNA replication initiation was found in POLRMT depleted cells, which suggested a decrease in $O_L$ initiation. We also reconstituted $O_L$-dependent initiation of lagging strand replication *in vitro* using dsDNA template with a preformed replication fork. Addition of the mitochondrial replication factors POLγ, TWINKLE, and mtSSB, together with POLRMT resulted in $O_L$ dependent lagging strand products, which was confirmed with Southern blotting and strand specific probes. Mutations that destabilized the $O_L$ stem loop structure resulted in decreased initiation of DNA replication, demonstrating the importance of this structure.

Primer initiation sites and RNA to DNA transition sites at $O_L$ in the *in vitro* assays were mapped using S1 nuclease protection assay and primer extension assay in the presence and absence of RNase H. Primer synthesis was found to be initiated at a poly-dT stretch in the loop region of $O_L$ and was in perfect correspondence with earlier mapping of initiation sites *in vivo* (Wong and Clayton, 1985a). The RNA to DNA transitions mapped to a region immediately downstream of the stem, which also was in nice agreement with previous observations (Kang et al., 1997).

In summery, our results led us to conclude that POLRMT is the primase at $O_L$. POLRMT initiates primer synthesis from a poly-dT stretch in the ssDNA loop region of $O_L$ and mutations that weaken the stem-loop structure disturb primer synthesis and origin function *in vitro*.

## Paper II

### The mitochondrial DNA helicase TWINKLE can assemble on a closed circular template and support initiation of DNA synthesis

Mitochondrial DNA replication initiated at $O_H$ depends on RNA primers that are synthesized by the mitochondrial transcription machinery. After initiation at $O_H$, H-strand replication proceeds unidirectionally, displacing the parental H-strand. The replication process requires the mitochondrial replisome consisting of POLγ, TWINKLE and mtSSB. TWINKLE forms a stable ring-shaped hexamer/heptamer in solution (Spelbrink et al., 2001; Ziebarth et al., 2010) and in this paper we investigated how TWINKLE is loaded onto DNA during initiation of mtDNA synthesis. It is known that many replicative helicases need an additional loading factor to accomplish this task.

We showed with different *in vitro* experiments that TWINKLE could load onto closed circular ssDNA without the addition of any specialized loading factor. TWINKLE therefore behaved similar to the bacteriophage T7 gene 4 protein (gp4) replicative helicase, a protein that displays high sequence similarity to TWINKLE (Ahnert et al., 2000; Spelbrink et al., 2001). We also demonstrate that the mitochondrial replication machinery, consisting of POLγ, TWINKLE, and mtSSB can assemble on a closed dsDNA template and extend a primer, a situation resembling initiation of mtDNA replication at $O_H$.

To address the characteristics of TWINKLE loading, we incubated recombinant protein with circular ssDNA, followed by cross-linking of the hexameric/heptameric structure. After denaturation, we separated the products in an electrophoresis mobility shift assay. Based on our results, we could conclude that TWINKLE can load on a closed, circular ssDNA template. TWINKLE did not bind the ssDNA outside the ring structure since it remained associated with the template when the protein was denatured. In support of this notion, TWINKLE could load onto circular ssDNA and unwind duplex DNA.

Next we designed a dsDNA template containing a ssDNA region. We annealed a short DNA primer to the ssDNA region, thus creating a triple-

stranded structure resembling the situation of mtDNA replication initiation *in vivo*. Addition of POLγ and TWINKLE resulted in unwinding of the dsDNA template and extension of the DNA primer. This finding showed that POLγ and TWINKLE could initiate DNA replication on a DNA template that closely mimics the *in vivo* substrate. Addition of only POLγ lead to extension of the DNA primer until the polymerase reached the duplex DNA.

Using the helicase assay and our "bubble template" we also showed that mtSSB does not prevent TWINKLE from loading onto ssDNA. Based on our findings, we suggest that no additional factor is needed *in vivo* for removal of mtSSB or to assist TWINKLE during the loading process of initiation of $O_H$-replication.

## Paper III

### MTERF1 binds mtDNA to prevent transcriptional interference at the light-strand promoter but is dispensable for rRNA gene transcription regulation

MTERF1 is a sequence-specific DNA binding protein, which binds to mtDNA just downsteam of the mitochondrial rRNA genes. MTERF1 has been implicated in regulation of rRNA transcription. Initally, MTERF1 was believed to terminate HSP transcription downstream of the rRNA genes (Kruse et al., 1989), which would explain the higher steady state levels of rRNA relative to downstream mRNA transcripts. Later, MTERF1 was reported to also bind in the HSP promoter region, facilitating re-initiation and stimulation of rRNA gene transcription via looping (Martin et al., 2005). However, *in vitro* biochemistry showed that MTERF1 does not have a stimulatory effect and that MTERF1 terminates LSP transcription more efficiently than transcription initiated at HSP, i.e. transcription coming from the other direction (Asin-Cayuela et al., 2005; Shang and Clayton, 1994).

In order to elucidate the function of MTERF1, we created an Mterf1 knock-out mouse model and found that mice depleted of the protein were viable. The knock-out mice showed normal OXPHOS capacity, including normal respiratory chain functions, normal ATP production and oxygen consumption. Analysis and quantification of transcript levels, including the rRNAs, showed no differences between MTERF1 knock-out and wild-type

mice. Chromatin immunoprecipitation of MTERF1 followed by next-generation sequencing revealed that MTERF1 only binds within the $tRNA^{Leu}$ gene, just downstream of the rRNA genes. No binding of MTERF1 was detected at HSP. Together these data invalidated a stimulatory role of MTERF1 via looping and demonstrated that MTERF1 is not involved in regulation of rRNA transcription.

Interestingly, depletion of MTERF1 resulted in an increase in antisense transcripts within the rRNA region (transcripts produced by the LSP promoter), as detected by Northern blotting, suggesting that MTERF1 is involved in termination of LSP transcription. This finding was consistent with the observation that MTERF1 terminates LSP transcription *in vitro* (Asin-Cayuela et al., 2005; Shang and Clayton, 1994). Furthermore, the 7S RNA transcript, which is initiated at LSP, was decreased in Mterf1 knock-out mice, as demonstrated by Northern blotting and S1 protection assays. Based on these observations, we postulated that MTERF1 terminates transcription initiated at LSP and prevents the transcription machinery from reaching the downstream LSP and to interfere with further rounds of transcription initation, a process required for expression of coding genes but also for the formation of primers needed for mtDNA replication initiated at $O_H$.

## Paper IV

## A conserved sequence element is involved in termination of mitochondrial DNA replication and transcription

MtDNA replication is initiated in a unidirectional manner at $O_H$, but most of the DNA replication events are prematurely terminated around 650 bp downstream of the origin, creating a triple stranded structure called the D-loop (Arnberg et al., 1971; Kasamatsu et al., 1971; Robberson et al., 1972). Whether the free 3´end of the annealed strand, the 7S DNA, can be used for re-initiate DNA replication of mtDNA *in vivo* is not known. In this study we investigated the mechanisms responsible for the premature DNA replication termination event and found a highly conserved DNA sequence with palindromic character located downstream of the 3´end of 7S DNA. The identified sequence appeared to function bidirectionally, since transcription initiated at HSP was also terminated at this sequence element.

Chromatin immunoprecipitation of POLγ and TWINKLE followed by next-generation sequencing or quantitative PCR showed that the replication proteins were enriched within the highly replicated D-loop region. The levels of POLγ and TWINKLE were high around $O_H$, suggesting that the proteins may be loaded at this region. Furthermore, the level of POLγ was relatively high at the 3'end of the D-loop compared to TWINKLE. POLγ may therefore remain associated with the 3´end of the 7S DNA, whereas TWINKLE is removed from this site. We speculated that the removal of TWINKLE could be an active process, possibly involving an anti-helicase activity.

To better understand the mechanisms responsible for this premature termination we first mapped the precise positions where 7S DNA terminates in human cells. Next, we mapped the termination sites of HSP transcription (transcribing the mtDNA in the opposite direction compared to $O_H$ replication) using Northern blotting and 3´RACE (Rapid Amplification of cDNA Ends). We found that HSP transcription terminates close to the 3'end of the 7S DNA. When we analyzed the short mtDNA sequence separating the 3´ends of the two termination events, we identified a 15 bp DNA sequence with palindromic character. The sequence was evolutionaly conserved within vertebrates and we decided to call it coreTAS.

The size and the palindromic character of coreTAS made us speculate that it could function as a recognition site for a sequence-specific DNA binding protein, which regulates transcription and DNA replication termination in *trans*. In agreement with this hypothesis, no termination activity at coreTAS could be found *in vitro* using our reconstituted mtDNA replication and transcription systems. In addition, using an electrophoresis mobility shift assay we observed a specific interaction between a protein factor in mitochondrial extract and a dsDNA template containing the coreTAS sequence. So far, we have not been able to purify the factor to homogeneity.

Termination of both DNA replication and HSP transcription appears to be regulated simultaneously at coreTAS. Transient depletion of mtDNA leads to a subsequent increase in DNA replication and a decrease of the premature DNA replication termination event (also observed in (Brown and Clayton, 2002)). Interestingly, under these conditions we also observed a decrease in transcription termination at coreTAS, with HSP transcription continuing

inside the D-loop region. We concluded that termination of both DNA replication and transcription appears to be functionally linked at coreTAS.

Furthermore, coreTAS is similar to a region of CSB1, located at the 5´end of the D-loop. We showed that the 3´ends of the 7S RNA transcript map to this region and that cells recovering from mtDNA depletion lack the 7S RNA transcript, indicating that the factor that terminates HSP transcription at coreTAS may also terminate transcription at CSB1.

# 4 CONCLUDING REMARKS

MtDNA was discovered about 50 years ago and the first mechanism describing how mtDNA is replicated was presented soon thereafter. Today many key factors involved in mtDNA synthesis are identified and the process has been partially reconstituted in the test tube. The identity of the primase required for initiating mitochondrial L-strand (lagging strand) DNA replication was for a long time an open question within the mtDNA replication field. In this thesis we have identified POLRMT as the missing primase and for the first time been able to reconstitute origin-specific initiation of mtDNA L-strand (lagging strand) replication in a test tube with purified proteins. Furthermore, work presented in this thesis has implications for our understanding of how mitochondrial leading strand DNA synthesis is initiated. Our data have revealed how the mitochondrial replicative helicase loads onto DNA, allowing us to reconstitute leading strand replication initiation *in vitro* on DNA that closely mimics the DNA substrate *in vivo*. We have also demonstrated that the DNA binding protein MTERF1 functions as a transcription termination factor and prevents transcription interference at LSP, an incidence that possibly could disturb the expression of coding genes, but also the formation of primers required for mtDNA replication initiated at $O_H$.

Even if we today know the basic mechanisms of mtDNA synthesis, many important aspects of this process are still not understood. One example is the mechanism of D-loop formation, which we have begun to study in this thesis. Based on our work, we have reached the conclusion that a *trans*-acting factor might be responsible for the premature DNA replication termination at the 3' end of the non-coding region. The same factor may also prevent HSP transcription from entering the non-coding region. In future work, we intend to isolate this factor and reveal its identity.

A detailed understanding of mtDNA replication is not only important for basic science, but may also have future implications for patients suffering from mitochondrial diseases. Hopefully, our work will help to define the molecular basis for mitochondrial diseases and to identify possible targets for future drug development.

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