From the DEPARTMENT OF LABORATORY MEDICINE
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# THE INTERFACE OF MITOCHONDRIAL DNA TRANSCRIPTION AND REPLICATION

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# ABSTRACT

Mitochondria are a dynamic network of subcellular organelles that produce the majority of cellular ATP through the process of oxidative phosphorylation (OXPHOS). The components of the respiratory chain are encoded by two separate genomes, nuclear DNA and mitochondrial DNA (mtDNA), and the proper maintenance of both of these genomic entities is therefore crucial for cellular ATP levels and the survival of the cell. Dysfunction of the respiratory chain leads to cellular energy deficiency and mitochondrial disease, which can manifest in a variety of ways but primarily affects tissues of higher energy demand. Although mtDNA replication and transcription are of vital importance for the cell, the molecular mechanisms behind these processes are not fully understood.

In mammalian cells, mtDNA replication initiates from two major sites, the origins of heavy and light strand replication (OriH and OriL, respectively). Activation of both origins requires a short RNA primer that is generated by the mitochondrial transcription machinery. In this way, mtDNA replication and transcription are intricately linked. At OriH, primer 3′ end formation has been suggested to rely on nucleolytic processing of full-length transcripts, but only trace amounts of the nuclease implied in this process are found in mitochondria, making this an unlikely model. In this thesis, we demonstrate that the formation of the primer 3′ end is a sequence-dependent event that is directed by the Conserved Sequence Block II (CSBII) sequence element in mtDNA. During transcription of CSBII, the nascent RNA adopts a G-quadruplex structure that causes premature termination of transcription *in vitro*. After transcription termination, the primer RNA remains stably associated with the DNA in a persistent RNA-DNA hybrid called an R-loop. We find that this interaction is mediated by hybrid G-quadruplex structures that form between the RNA primer and the DNA non-template strand. When G-quadruplex formation in either the RNA transcript or in the DNA is prevented, the stable association of the primer RNA is lost.

The mitochondrial RNA polymerase (POLRMT) is also involved in generating the primer at the origin of light strand replication (OriL). In order to define the essential sequence requirements of mammalian mitochondrial OriL, we employ an *in vivo* saturation mutagenesis approach combined with biochemical analysis. Our results support an essential role of OriL in the mouse, consistent with the strand-displacement model of mtDNA replication. Furthermore, bioinformatic analysis demonstrates conservation of the OriL structure in vertebrates.

POLRMT requires two accessory factors for transcription initiation at mitochondrial promoters. However, the necessity of the mitochondrial transcription factor A (TFAM) in this process has been questioned. We use our defined mitochondrial *in vitro* transcription system to confirm the important role TFAM in transcription initiation. The requirement for TFAM can be circumvented by conditions that promote DNA breathing, such as low salt concentrations or the use of negatively supercoiled template. We demonstrate that TFAM has the capacity to generate negative supercoils, which we speculate may contribute to melting of the promoter.

# LIST OF PUBLICATIONS

I. G-quadruplex structures in RNA stimulate mitochondrial transcription termination and primer formation.
**Wanrooij PH**, Uhler JP, Simonsson T, Falkenberg M and Gustafsson CM.
*Proc Natl Acad Sci U S A*. 2010 Sep 14; 107 (37): 16072-7.

II. A hybrid G-quadruplex structure formed between RNA and DNA explains the extraordinary stability of the mitochondrial R-loop.
**Wanrooij PH**, Uhler JP, Shi Y, Westerlund F, Falkenberg M and Gustafsson CM.
*Nucleic Acids Res.* 2012 Nov 1; 40 (20): 10334-44.

III. In vivo mutagenesis reveals that OriL is essential for mitochondrial DNA replication.
Wanrooij S, Miralles Fusté J, Stewart JB, **Wanrooij PH**, Samuelsson T, Larsson NG, Gustafsson CM and Falkenberg M.
*EMBO Rep*. 2012 Oct 23. Doi:10.1038/embor.2012.161

IV. Mammalian transcription factor A is a core component of the mitochondrial transcription machinery.
Shi Y, Dierckx A, **Wanrooij PH**, Wanrooij S, Larsson NG, Wilhelmsson LM, Falkenberg M and Gustafsson CM.
*Proc Natl Acad Sci U S A*. 2012 Oct 9; 109 (41): 16510-5.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine diphosphate |
| ANT | adenine nucleotide translocator |
| ATP | adenosine triphosphate |
| bp | base pair |
| CD | circular dichroism |
| COX | cytochrome c oxidase (complex IV) |
| CSB | Conserved Sequence Block |
| C-terminal | carboxy terminal |
| DNA | deoxyribonucleic acid |
| ER | endoplasmic reticulum |
| FRET | fluorescence resonance energy transfer |
| G4 | G-quadruplex or G-tetrad |
| HSP | heavy strand promoter |
| H-strand | heavy strand |
| IOSCA | Infantile onset spinocerebellar ataxia |
| Kb | kilobase |
| kDa | kilodalton |
| LSP | light-strand promoter |
| L-strand | light strand |
| MELAS | myopathy, encephalopathy, lactic acidosis and stroke-like episodes |
| MERFF | myoclonic epilepsy with ragged red fibers |
| mRNA | messenger RNA |
| MRP | mitochondrial RNA processing |
| mt | mitochondrial |
| mtDNA | mitochondrial DNA |
| MTERF | mitochondrial transcription termination factor |
| mtSSB | mitochondrial single-stranded DNA-binding protein |
| nt | nucleotide |
| N-terminal | amino-terminal |
| OriH | heavy-strand origin of replication |
| OriL | light-strand origin of replication |
| ORF | open reading frame |
| OXPHOS | oxidative phosphorylation |
| PEO | progressive external ophthalmoplegia |
| $P_i$ | inorganic phosphate |
| Pol γ | polymerase gamma |
| POLRMT | mitochondrial RNA polymerase |
| Q | coenzyme Q = ubiquinone |
| RITOLS | ribonucleotide incorporation throughout the lagging strand |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| SDH | succinate dehydrogenase (complex II) |
| SDM | strand-displacement model |
| ssDNA | single-stranded DNA |
| SSB | single-stranded DNA-binding protein |

| | |
|---|---|
| TCA | tricarboxylic acid cycle, citric acid cycle |
| TFAM | mitochondrial transcription factor A |
| TFB1M | mitochondrial transcription factor B1 |
| TFB2M | mitochondrial transcription factor B2 |
| tRNA | transfer RNA |
| UTR | untranslated region |

# 1 INTRODUCTION

## 1.1 MITOCHONDRIA

Mitochondria are subcellular organelles present in most eukaryotic cells. Each cell contains in the range of a few hundred to a few thousand mitochondria and they are the primary location of the cell's energy metabolism. Mitochondria contain the components of the respiratory chain, citric acid cycle and β-oxidation. They also harbor their own DNA genome of only 16 kb that encodes for 13 subunits of the respiratory chain complexes as well as the tRNAs and rRNAs required for their synthesis. The proper maintenance of the mitochondrial genome (mtDNA) is essential, as mutations in mtDNA lead to energy defects and human disease. Despite its importance for human health, the precise mechanisms behind mtDNA replication and gene expression remain unresolved at the molecular level.

### 1.1.1 Origin and structure of mitochondria

Since 1890, numerous scientists have put forward the idea that mitochondria are related to bacteria, resulting in the endosymbiotic theory that was strongly brought forward by Lynn Margulis, among others (Margulis 1981). Indeed, it is now clear that mitochondria are derived from an ancient α-proteobacterium that was engulfed by an anaerobic protoeukaryotic cell about 1.5-2 billion years ago, an event that granted the host cell the advantage of aerobic respiration (Gray *et al.* 1999; Lang *et al.* 1999). The strongest piece of support for the endosymbiotic origin of mitochondria was the groundbreaking discovery in the 1960's of a separate genome in mitochondria (Nass and Nass 1963). However, during the course of evolution, most of the gene content of the early endosymbiont has been transferred to the nuclear genome.

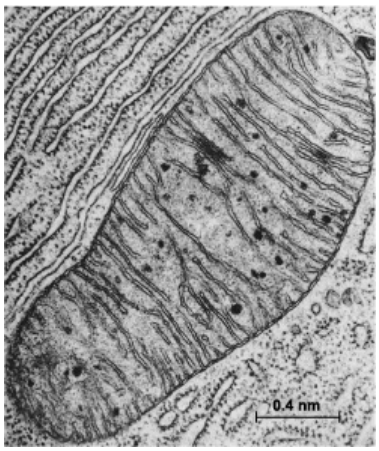


**Figure 1.** A transmission electromicrograph of a mitochondrion. The cristae are formed by extensive invagination of the inner mitochondrial membrane. Courtesy of R. K. Porter.

The word *mitochondria* is derived from the greek words for filament (*mitos*) and granules (*chondria*), thus describing the shape of these subcellular organelles when viewed under the light microscope. Early electron microscopic studies revealed that the internal space of the mitochondria, called the mitochondrial matrix, is surrounded by two membranes: the outer and inner mitochondrial membranes (Palade 1952, 1953; Sjostrand 1953). The outer membrane contains a transmembrane protein called porin or VDAC (voltage-dependent anion channel) which renders the membrane permeable to ions and smaller molecules while molecules larger than 5000 Da in molecular weight need to be actively transported across it (De Pinto and Palmieri 1992; Mannella *et al.* 1992). The mitochondrial inner membrane, on the other hand, is impermeable to hydrophilic molecules and specific transport proteins control import of metabolites across the membrane. The surface area of the inner membrane is far greater than that of the outer membrane and therefore the inner membrane is required to fold into cristae, which protrude into the inner space of the mitochondria, the matrix (Figure 1). Even though originally described as lamellar invaginations of the inner membrane, cristae have in more detailed studies been shown to have tubular rather than lamellar contacts with the inner membrane. Tubular cristae often fuse to form the lamellar compartments described in earlier studies of mitochondria (Daems and Wisse 1966; Perkins *et al.* 1997). The tubular contacts between the inner membrane and cristae are called cristae junctions and they render the intermembrane space discontinuous with the intra-cristal space, resulting in further compartmentalization of the mitochondrion. Mitochondrial membrane morphology is further organized through contacts between the outer and inner mitochondrial membranes. These contacts are transient in nature and result from the interaction of integral membrane proteins that are components of the mitochondrial protein import machinery (Hackenbrock 1966; Schatz and Dobberstein 1996).

The inner membrane has a protein:lipid ratio of up to 75:25, while in most other membranes this ratio is close to 50:50. The high protein content is partly due to the presence of the respiratory chain complexes that reside in the inner membrane, as well as the large number of proteins that carry peptides, metabolites or ions across this barrier. Furthermore, a membrane potential ($\Delta\psi$) is built up across the inner membrane through the function of the electron transport chain. $\Delta\psi$ is required for the synthesis of adenosine triphosphate (ATP), but also for the transport of various molecules across the membrane, *e.g.* for the import of peptides. The inner membrane surrounds the mitochondrial matrix, which contains the enzymes required for most pathways of fuel oxidation in the cell: the citric acid cycle, β-oxidation, and the pathways of amino acid oxidation. The matrix also contains mitochondrial DNA (mtDNA), as well as the factors required for the essential processes of mitochondrial replication, transcription and translation.

### 1.1.2 Mitochondrial dynamics

Mitochondria exist as a dynamic network of individual organelles that continuously undergo fusion and fission events (Shaw and Nunnari 2002; Karbowski and Youle 2003; Chen and Chan 2004). Therefore, the traditional view of mitochondria as static rod-shaped organelles is misleading, at least in post-mitotic cells. Mitochondrial fusion may allow the distribution of metabolites, proteins and mtDNA throughout the mitochondrial network, resulting in a homogeneous population of mitochondria within

a cell. Fission, on the other hand, allows mitochondrial "proliferation", which *e.g.* is required during cell division and that ensures distribution of mitochondria to parts of the cell with a high energy demand. The dynamic nature of mitochondria can also provide a quality control mechanism that ensures mitochondrial function in the cell (Twig, Hyde*, et al.* 2008). Fusion and fission of mitochondria are paired so that fusion triggers fission; it has been estimated that a mitochondrion undergoes approximately 5 fusion-fission cycles per hour (Twig, Elorza*, et al.* 2008). Remarkably, fission can produce two functionally different daughter mitochondria where one may be depolarized (with a reduced membrane potential $\Delta\psi$) while the other remains polarized and thus functional. Furthermore, subsequent fusion events have been shown to be dependent on the membrane potential so that depolarized mitochondria are less likely to be involved in fusion events, and are instead cleared by mitophagy (autophagy of mitochondria). Thus, paired fusion and fission events sequester damaged mitochondrial components into daughter mitochondria that are eliminated from the mitochondrial network (Twig, Hyde*, et al.* 2008).

The outer mitochondrial membrane forms contacts to the cytoskeleton, allowing the transport of mitochondria along microtubules or actin filaments (reviewed in (Bereiter-Hahn and Jendrach 2010). This phenomenon is most obvious in neurons, where transport along microtubules is required in order to localize a sufficient number of mitochondria to the axonal synapses, sites of high energy demand. About 20% of the mitochondrial surface may be in direct contact with the ER, and together these two organelles have key roles in regulating cytosolic calcium concentrations (discussed later) (Rizzuto *et al.* 1998). Although both membrane systems are dynamic, the interactions of mitochondria and the mitochondria-associated part of the ER (MAM, for mitochondria-associated membrane) seem to be somewhat stable (Lebiedzinska *et al.* 2009). Likewise, mitochondria are rarely observed in regions of the cell devoid of ER (Bereiter-Hahn and Vöth 1983), which indicates the importance of these contacts.

### 1.1.3 Mitochondrial metabolism

Mitochondria have been coined the powerhouses of the cell due to their essential function in energy production. As mentioned earlier, the mitochondrial matrix contains the enzymes required for most pathways of fuel oxidation in the cell: the citric acid cycle, β-oxidation, and the pathways of amino acid oxidation. The electrons thus derived from the oxidation of carbohydrates, fatty acids and amino acids pass through the four complexes of the respiratory chain embedded in the inner mitochondrial membrane and enable the transmembrane transport of protons into the intermembrane space to create an electrochemical gradient. Finally, this electrochemical gradient energizes the synthesis of ATP, the cell's primary energy currency, from ADP and inorganic phosphate ($P_i$) through the function of the mitochondrial ATP synthase (complex V) (Figure 2). Most of the ATP produced is exported into the cytosol in exchange for ADP by the adenine nucleotide translocator (ANT).

#### *1.1.3.1 Oxidative phosphorylation*

In the process of oxidative phosphorylation electrons move from NADH, succinate, and some other primary electron donors through flavoproteins, ubiquinone, iron-sulfur proteins and cytochromes in the respiratory chain to molecular oxygen ($O_2$). This is

coupled to the phosphorylation of ADP to form ATP. The mitochondrial inner membrane contains the multisubunit complexes that together constitute the respiratory chain. Both Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) transfer electrons from NADH and succinate, respectively, to ubiquinone (coenzyme Q). Complex II is part of the citric acid cycle, and is the only respiratory chain complex that does not contain mitochondrially-encoded subunits. Electrons from β-oxidation and from the oxidation of glycerol 3-phosphate also enter the respiratory chain at the level of ubiquinone, contributing to the pool of reduced ubiquinone. Complex III (Ubiquinone:cytochrome c oxidoreductase) then reduces ubiquinone, donating electrons to cytochrome c, a soluble heme protein of the intermembrane space. Finally, Complex IV (cytochrome c oxidase) oxidizes cytochrome c and transfers the electrons to the end recipient, molecular oxygen ($O_2$). (Nelson 2000)

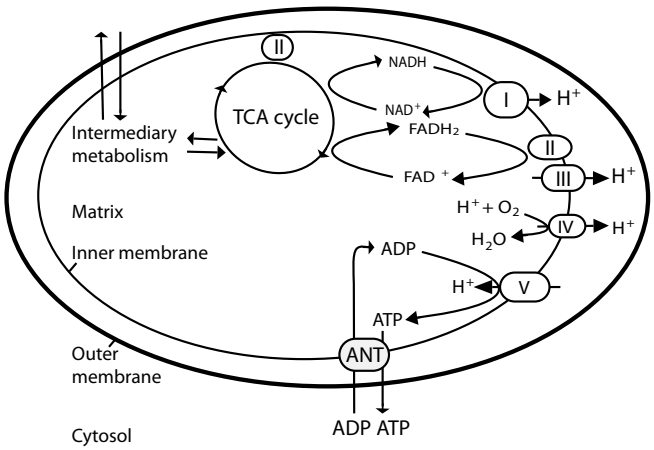


**Figure 2.** A schematic presentation of major mitochondrial metabolic pathways. The respiratory chain complexes are denoted by roman numerals. Adapted from (Toivonen 2003).

Electron movement through complexes I, III and IV is associated with the pumping of protons ($H^+$) from the matrix into the intermembrane space. The flow of these protons down their electrochemical gradient back into the matrix is coupled to the phosphorylation of ADP into ATP through the function of Complex V, the ATP synthase (Mitchell 1966). Electron transfer through the respiratory chain and ATP synthesis are obligately coupled, meaning that inhibition of ATP synthesis will block electron transfer. An exception to this strict dependence exists in brown adipose tissue, which contains an uncoupling protein (termed UCP1 or thermogenin). The uncoupling protein allows the return of protons into the matrix without passage through the ATP synthase. In this way, the energy of fuel oxidation is not conserved in ATP synthesis, but is instead dissipated as heat and contributes to maintaining body temperature (Nelson 2000).

Some organisms, including numerous plants, microorganisms and some metazoans like the sea squirt *Ciona intestinalis*, contain a protein called the alternative oxidase (AOX)

that shuttles electrons directly from the reduced quinone pool to molecular oxygen, thus bypassing complexes III-V (McDonald and Vanlerberghe 2004). In nature, it confers resistance to cyanogenic agents, inhibitors of complex IV often used by plants and microorganisms as a weapon against animal predators (Tattersall *et al.* 2001). The activity of AOX impairs aerobic ATP production, but could be a beneficial tool for alleviating the symptoms of *e.g.* Complex IV deficiency, which otherwise leads to a wide range of clinical presentations including encephalomyopathy and cardiomyopathy (McFarland *et al.* 2010). Indeed, AOX from *Ciona intestinalis* has been successfully targeted to mitochondria and found to complement Complex IV deficiency in human cells (Hakkaart *et al.* 2006; Dassa *et al.* 2009).

*1.1.3.2 Other metabolic and biochemical pathways*

Mitochondria play a central role in carbohydrate, fatty acid and amino acid catabolism. Fatty acids enter the mitochondria as fatty acyl-CoA via the acyl-carnitine/carnitine transporter in the inner mitochondrial membrane, and are broken down into acetyl-CoA in a process called β-oxidation. Each passage of β-oxidation shortens the fatty acid chain by two carbons and yields one molecule of acetyl-CoA and reducing equivalents (one copy of each $FADH_2$ and NADH). Amino acid breakdown occurs in two parts: the carbon skeleton is broken down and enters the citric acid cycle at various stages of the cycle, while the amino group is either recycled or, if present in excess, enters the urea cycle and is excreted as urea. The initial steps of the urea cycle occur in the mitochondrial matrix and it is completed in the cytosol.

The catabolic pathways of glycolysis, β-oxidation and amino acid oxidation all produce acetyl-CoA, which enters the citric acid cycle (TCA cycle) in the mitochondrial matrix. Under aerobic conditions, the TCA cycle converts acetyl-CoA into $CO_2$, yielding a molecule of ATP and reducing equivalents in the form of NADH and $FADH_2$. Both the TCA cycle and β-oxidation contain one membrane-associated enzyme complex that transfers electrons into the respiratory chain (succinate dehydrogenase and acyl-CoA dehydrogenase, respectively).

Mitochondria are involved in many other biochemical pathways of the cell, not all of which are directly linked to energy production. Intermediates of the TCA cycle provide precursors for amino acid, nucleotide and heme biosynthesis. Heme groups are an essential part of hemoglobin used to transport oxygen in the blood, but are also found in the cytochrome groups of the electron transport chain and in dehydrogenases. Mitochondria are the major site of cellular iron utilization and production of the iron prosthetic groups found in heme and Fe/S clusters. In fact, mitochondria seem to play a role in the regulation of the iron homeostasis in the entire cell ((Sheftel and Lill 2009) and references therein).

Mitochondria function as temporary calcium storages and in buffering local fluctuations in $Ca^{2+}$ concentration. Therefore, the uptake of calcium into mitochondria modulates the spread and timing of cytosolic calcium signals (David *et al.* 1998; Park *et al.* 2001; Drago *et al.* 2011). The increase of intramitochondrial $Ca^{2+}$ levels, in turn, can regulate mitochondrial metabolism via activation of three TCA cycle dehydrogenases, leading to increased ATP production (Jouaville *et al.* 1999). In apoptosis, high cytosolic $Ca^{2+}$ levels lead to increased uptake of $Ca^{2+}$ into the

mitochondria and the release of cytochrome c and other proapototic factors. In this way, mitochondria are key players in the cascade leading to activation of programmed cell death (for review, see (Giacomello *et al.* 2007)).

### 1.1.4 The mitochondrial genome

Human mitochondria contain over about 1500 proteins (Lopez *et al.* 2000), the vast majority of which are encoded in nuclear DNA, synthesized on cytosolic ribosomes and delivered post-translationally into the mitochondria. Generally, nuclear-encoded proteins destined for mitochondria contain an amino-terminal targeting signal which folds into an amphipathic α-helix and is removed upon import. The group of nuclear-encoded mitochondrial proteins includes all proteins involved in mitochondrial replication and transcription, as well as the majority of the subunits of the respiratory chain complexes. However, the human mitochondrial genome encodes 13 subunits of the respiratory chain, as well as 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) required for mitochondrial translation. All respiratory chain complexes aside from CII contain mitochondrially-encoded subunits - a fact that is exploited in diagnosis of mtDNA defects by staining for SDH (CII) and COX (CIV); COX-negative but SDH-positive fibers contain mitochondria but defective mtDNA molecules.

Metazoan mtDNA is generally a closed circular molecule of approximately 17 000 bp (16 568 bp in human, see Figure 3) that encodes for the same set of 13 peptides with very little variation between species (Anderson *et al.* 1981; Bibb *et al.* 1981; Lewis *et al.* 1995; Scheffler 1999). Strikingly, metazoan mtDNAs are very compact and economical with respect to the packaging of genetic information, as they contain almost no non-coding sequences. This is in clear contrast to the mtDNA of plants and yeasts, which can contain vast intergenic regions and have a size range of between 40-60 kb in yeasts and as much as 200 to 2400 kb in plants. It appears that some of the content of yeast and plant mtDNA is dispensable, so-called “junk” DNA. The discussion here will focus on human mtDNA, which is generally representative for metazoan mtDNAs. The compactness of genetic information in human mtDNA is further exemplified by the lack of untranslated regions (UTRs); the open reading frames (ORFs) for peptides and rRNAs are separated by one or more tRNAs, with few if any extra nucleotides in between. Furthermore, transcription of the mitochondrial genome is bidirectional and consequently ORFs can be on either strand, although they are unevenly distributed between the two strands. (Ojala *et al.* 1980; Montoya *et al.* 1981; Ojala *et al.* 1981).

The two strands of mtDNA differ significantly in base composition, which leads to different densities on alkaline CsCl gradients; hence, they are denoted as the heavy and the light strand, respectively. The light strand (L-strand) contains most of the genetic information, encoding for 12 mRNAs, 14 tRNAs and the two rRNAs, while the heavy strand (H-strand) only codes for the ND6 mRNA and 8 tRNAs. The H-strand is rich in Gs, a fact that may predispose it to G-quadruplex formation, as will be discussed in chapter 1.5.

Human mtDNA has only one major non-coding region, often referred to as the control region because it contains essential regulatory sequences including the origin of heavy strand replication (OriH), as well as the promoter sequences for transcription in both

directions (the heavy and light strand promoters; HSP and LSP). The origin of light strand replication (OriL) is located two thirds of the way around the genome (Figure 3). The control region also contains the displacement loop (D-loop) structure, which is a triple-stranded region of the mitochondrial genome that arises when a nascent H-strand displaces the parental H-strand during replication (Arnberg *et al.* 1971; ter Schegget *et al.* 1971). The D-loop stretches from the OriH region to the termination associated sequence (TAS) (Doda *et al.* 1981), as depicted in Figure 3. The sequence of the non-coding region is variable between species, but contains short stretches of conserved sequences that are functionally important (Sbisa *et al.* 1997). These include the promoters, TAS and three Conserved Sequence Blocks (CSBs I-III) (Walberg and Clayton 1981).

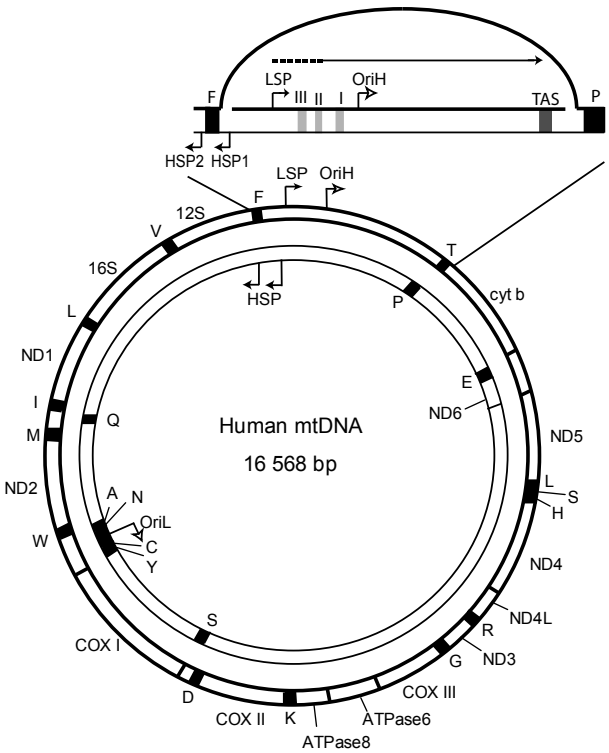


**Figure 3.** A map of the human mitochondrial genome with the control region magnified. The outer and inner rings depict the heavy and light strands, respectively. Origins of replication are illustrated as open-headed arrows and promoters as solid arrows. Conserved Sequence blocks (CSBs) I-III are shown as grey bars labeled with roman numerals. At OriH, primer RNA (dashed line) initiates at LSP and RNA-to-DNA transition points are observed in the region surrounding the CSBs.

MtDNA is present in multiple copies per cell, with a variation in copy number ranging from the 1000-6000 in most cell types to over 100 000 in the oocyte (Bogenhagen and Clayton 1974; Shmookler Reis and Goldstein 1983; Shoubridge 2000). Generally, mtDNA copy number reflects the energy demand of the cell type, with high copy number in cells that require a lot of energy. MtDNA was long considered a naked molecule, especially with regard to susceptibility to DNA damage. However, it is now

clear that mtDNA is present in discrete nucleo-protein complexes called nucleoids (Satoh and Kuroiwa 1991; Bereiter-Hahn and Vöth 1997; Spelbrink *et al.* 2001; Garrido *et al.* 2003), with hundreds of nucleoids per animal cell [(Spelbrink 2010) and references therein]. There is some dispute in the field regarding the number of mtDNA molecules per nucleoid, a factor of importance for the understanding of mitochondrial disease genetics, since nucleoids are likely to be the units of segregation (Jacobs *et al.* 2000; Gilkerson *et al.* 2008). Nonetheless, this figure is within the range of 1-10, with most recent analyses reporting an average of 1.4 copies per nucleoid (Satoh and Kuroiwa 1991; Iborra *et al.* 2004; Legros *et al.* 2004; Gilkerson *et al.* 2008; Kukat *et al.* 2011).

The most abundant and undisputed nucleoid proteins are the mitochondrial transcription factor A (TFAM) and the mitochondrial single-stranded DNA-binding protein (mtSSB) (Alam *et al.* 2003; Garrido *et al.* 2003; Bogenhagen *et al.* 2008). TFAM is the principal contributor to mtDNA organization: it binds mtDNA as a homodimer about every 30-40 bp (Ghivizzani *et al.* 1994; Takamatsu *et al.* 2002; Alam *et al.* 2003; Kaufman *et al.* 2007; Kukat *et al.* 2011; Farge *et al.* 2012) and is capable of compacting DNA and coordinating the packaging of several DNAs into single nucleoid-like structures *in vitro* (Kaufman *et al.* 2007). Which other proteins are considered nucleoid proteins depends on definition; *e.g.* should they be an integral part of the nucleoid or does transient association with mtDNA suffice to qualify? One definition of *bona fide* nucleoid proteins relies on the method of purification: in a study by Bogenhagen *et al*., proteins that copurified with mtDNA in formaldehyde cross-linked specimens under denaturing conditions were considered core nucleoid proteins. On the other hand, nucleoid proteins found only in native nucleoid preparations could include factors that were only peripherally associated with nucleoids. Using this definition, 31 core nucleoid proteins were found, including TFAM and mtSSB as well as proteins involved in mt transcription and replication. In contrast, the peripheral nucleoid fraction contained proteins responsible for translation and OXPHOS complex assembly (Bogenhagen *et al.* 2008). This reflects the view of nucleoids as the center of mitochondrial biogenesis (Capaldi *et al.* 2002; Bogenhagen *et al.* 2008). It has been suggested, although more evidence is required, that mtDNA maintenance and nucleoids may be linked to transcription, translation (both cytoplasmic and mitochondrial), protein import and OXPHOS complex assembly [see *e.g.* (Iborra *et al.* 2004; Bogenhagen *et al.* 2008; Spelbrink 2010) and references therein], possibly through a membrane-attached scaffold analogous to the ERMES complex of yeast. The membrane tethering of mtDNA was discovered already in 1969, and Attardi *et al*. reported discovery of an unknown anchoring protein in 1977 (Nass 1969; Albring *et al.* 1977). The identity of the membrane-anchoring protein and many other questions regarding nucleoid arrangement and dynamics remain to be answered by future work.

### 1.1.5 Mitochondrial translation

Mitochondria contain their own apparatus for translation of the genetic information encoded by mtDNA. In human, the RNA components of this machinery (rRNAs and tRNAs) are mtDNA-encoded, whereas all protein components required for mt translation (including ribosomal proteins, translation factors and aminoacyl-tRNA synthetases) must be imported from the cytosol (reviewed in (Jacobs and Turnbull

2005)). As a consequence of the evolutionary origin of mitochondria, mt ribosomes are related to bacterial ribosomes, at least when judged by sequence alignments and antibiotic susceptibility (Scheffler 1999; Zhang *et al.* 2005).

Mitochondria use a genetic code that differs from the "universal" genetic code used by both prokaryotes and the eukaryotic cytoplasmic translation machinery. As an example, the arginine triplets AGA and AGG have been re-assigned as termination codons in mammalian mitochondria, even though they are not "traditional" in the sense that they are not recognized by a mitochondrial release factor as stop codons. Rather, the mechanism of termination in these cases involves a strong secondary structure in the 3′ UTR that forces the mitoribosome to frameshift by -1, placing the standard UAG termination codon in the A-site (Lightowlers and Chrzanowska-Lightowlers 2010; Temperley *et al.* 2010). In addition, a simplified codon-anticodon pairing system in mitochondria allows translation with only 22 tRNAs (Attardi and Schatz 1988).

## 1.2 MITOCHONDRIAL TRANSCRIPTION

As mentioned earlier, both strands of mtDNA contain genetic information even though it is unevenly distributed over the two strands. Transcription of mtDNA is therefore bidirectional, with initiation sites in the control region. Transcription of the H-strand gives rise to 12 mRNAs, 2 rRNAs and 14 tRNAs, while the L-strand encodes only one mRNA and 8 tRNAs. Transcription initiation occurs at three different sites: two initiation sites ($H_1$ and $H_2$) for the H-strand and one initiation site for the L-strand transcription units (Montoya *et al.* 1982) (see Figure 3). The $H_1$ and $H_2$ initiation sites are closely spaced, but differ in activity and produce overlapping transcripts of different length. Transcription of the $H_1$ transcription unit initiates 19 nt upstream of the $tRNA^{Phe}$ gene and gives rise to 12S and 16S rRNAs, $tRNA^{Phe}$ and $tRNA^{Val}$, whereas the second transcription unit, initiating at $H_2$, reaches from the 5′ end of the 12S gene almost the entire way around the genome (Montoya *et al.* 1982; Montoya *et al.* 1983). In exponentially dividing HeLa cells, initiation at $H_1$ is 50-100 times more frequent than at $H_2$, reflecting the greater requirement for rRNAs over other transcripts (Gelfand and Attardi 1981). Like the $H_2$-initiated event, also transcription from the light-strand promoter (LSP) produces a near genome-length polycistronic transcript. Transcription from LSP is also coupled to replication, since it generates the primers for H-strand replication (Chang and Clayton 1985; Chang *et al.* 1985). This will be discussed in more detail in chapter 1.3.3. Monocistronic or dicistronic mRNAs are liberated from the polycistronic transcripts upon endonucleolytic excision of the tRNAs that punctuate the mRNAs and rRNAs (Ojala *et al.* 1980; Ojala *et al.* 1981). This is instantly followed by polyadenylation of mRNAs (and rRNAs) to yield the mature products. In fact, polyadenylation completes the termination codon of a number of mt-mRNAs (Ojala *et al.* 1981).

MtDNA transcription initiation has been observed from all three initiation points *in vitro*, although HSP2 activity seems to be marginal (Walberg and Clayton 1983; Bogenhagen *et al.* 1984; Falkenberg *et al.* 2002; Lodeiro *et al.* 2012). Analysis of these regions has allowed the determination of the elements required for transcription from the two major mammalian mitochondrial promoters LSP and $HSP_1$ that correspond to the L and $H_1$ initiation points. The promoters have a bipartite structure, consisting of a

promoter element and an upstream regulatory element. The promoter element is a consensus sequence motif of 15 bp that surrounds the initiation point and is essential for transcription, while the upstream regulatory element is more of an enhancer that allows optimal transcription. It contains the binding site for the mitochondrial transcription factor A (TFAM), which is located between positions -35 and -17 relative to the transcription start site (Fisher *et al.* 1987). A distance of 10 bp is required between the promoter element and the TFAM binding site (Dairaghi *et al.* 1995b).

Transcription from the $H_1$ unit has been suggested to be terminated by the mitochondrial termination factor mTERF, although direct in vivo evidence for this is lacking. The mechanism and precise site of termination of the $H_2$ and L transcription units remains unknown (Fernandez-Silva *et al.* 2003), which is partly due to the difficulty of isolating and analyzing these polycistronic transcripts that are subject to RNA processing events virtually upon transcription.

### 1.2.1 The core mitochondrial transcription machinery

The basal machinery required for transcription of the mitochondrial genome includes the mitochondrial RNA polymerase POLRMT, the mitochondrial transcription factor A and one of the two mitochondrial transcription factor Bs, TFB1M or TFB2M. The mitochondrial transcription termination factor mTERF and TERFs 2-4 are discussed briefly, as well as some other factors that play a role in mt transcription.

#### *1.2.1.1 The mitochondrial RNA polymerase*

The mitochondrial RNA polymerase activity was partially purified from yeast (Levens *et al.* 1981) and human cells (Walberg and Clayton 1983; Shuey and Attardi 1985) in the beginning of the 1980s and shown to have biochemical qualities (size, KCl optimum and antibiotic resistance) that differentiate it from the nuclear RNA polymerases. It was found to be a single subunit enzyme that requires the assistance of additional factors in order to initiate specific transcription. The C-terminal core polymerase domains of both the human mt RNA polymerase (POLRMT or mtRPOL) and the yeast mt RNA polymerase Rpo41 are homologous to the C-terminal region of the RNA polymerases of the T-odd lineage of bacteriophages (Masters *et al.* 1987; Tiranti *et al.* 1997), but they also contain an N-terminal extension unique to mitochondrial RNA polymerases. In yeast, the N-terminal extension does not affect transcription initiation *in vivo*, but deletion of the N-terminal 185 aa of Rpo41 results in decreased stability and eventual loss of the mt genome. Remarkably, this N-terminal deletion of Rpo41 can be complemented in *trans* by expression of the N-terminal region (aa 1-585) (Wang and Shadel 1999). Therefore, it seems that the N-terminal extension of the enzyme contains an independent functional domain involved in mtDNA maintenance (Asin-Cayuela and Gustafsson 2007). The N-terminal domain of Rpo41 interacts specifically with Nam1p, a matrix protein involved in RNA processing and translation (Rodeheffer *et al.* 2001), and with the inner membrane protein Sls1p (Bryan *et al.* 2002). Together, the N-terminal domain, Nam1p and Sls1p are part of a pathway that ensures efficient mt expression by localizing active transcription complexes to the inner membrane in order to coordinate transcription and translation (Rodeheffer and Shadel 2003). Whether this role of the N-terminal extension is conserved in POLRMT is so far unknown. A difference in the requirement of the N-

terminal extension for transcription initiation may exist, since human POLRMT needs the N-terminal extension to initiate promoter-specific transcription, at least *in vitro* (Ringel *et al.* 2011).

The human POLRMT is a ~140 kDa protein, the structure of which has been solved at 2,5 Å resolution (Ringel *et al.* 2011). It has a domain structure that is comprised of the N-terminal mt targeting signal that is removed upon mt import (aa 1-41), the N-terminal extension (aa 42-218), two putative pentatricopeptide repeat motifs, and a C-terminal domain that is homologous to T7 RNA pol. The 35-aa pentatricopeptide repeat (PPR) motif is present in proteins implicated in RNA editing and processing events in mitochondria and chloroplasts (Lightowlers and Chrzanowska-Lightowlers 2008). The functional importance of the PPR motifs in POLRMT is unclear, but deletion of the PPR domain abolishes transcriptional activity, presumably because the domain is required to sequester the AT-rich recognition loop that is used for promoter recognition in T7. It is however evident from the structure of POLRMT that the AT-rich loop is not used for promoter recognition in the human protein, rendering POLRMT dependent on its accessory factors (Ringel *et al.* 2011).

Recently, POLRMT has been implicated as the mitochondrial primase (Wanrooij *et al.* 2008; Fuste *et al.* 2010). This aspect of the mitochondrial RNA polymerase will be discussed further in chapter 1.3.2.4.

#### *1.2.1.2 The mitochondrial transcription factor A (TFAM)*

The mitochondrial transcription factor A (TFAM or mt-TFA) is an essential 25-kDa protein that was initially discovered as a factor that stimulated transcription from HSP and LSP by POLRMT (Fisher and Clayton 1985; Larsson *et al.* 1998). It contains two tandem High Mobility Group (HMG) box domains separated by a basic 27 aa linker region and followed by a 25-residue C-terminal tail (Parisi and Clayton 1991). The basic C-terminal tail of TFAM is important for specific DNA recognition and essential for transcriptional activation (Dairaghi *et al.* 1995a). Furthermore, it is the site of interactions with the TFBM factors (McCulloch and Shadel 2003).

TFAM exhibits a prominent non-sequence-specific dsDNA-binding activity, but the protein also binds to specific sequences in the upstream regions of mt promoters (Fisher and Clayton 1988; Fisher *et al.* 1989; Ghivizzani *et al.* 1994). In addition to transcription initiation, TFAM is required for mtDNA maintenance and there is a strict correlation between TFAM levels and mtDNA copy number *in vivo* (Poulton *et al.* 1994; Larsson *et al.* 1998; Garrido *et al.* 2003; Ekstrand *et al.* 2004; Kanki *et al.* 2004). The functions of TFAM in transcription activation and copy number regulation can be separated *in vivo*, as shown by two independent reports in 2004. Ekstrand and coworkers expressed human TFAM in mouse and witnessed an increase in mtDNA copy number while overall expression levels remained normal because human TFAM is a poor stimulator of mouse transcription (Ekstrand *et al.* 2004). Conversely, another study found that RNAi knockdown of TFAM resulted in lowered mtDNA levels in cells. Furthermore, overexpression of a C-terminal deletion mutant still increased copy number similarly to the wt TFAM, even though the C-terminus is required for transcriptional activation (Kanki *et al.* 2004). It could be concluded that the correlation between copy number and TFAM levels was not due to altered transcription and thus

altered priming of replication, but rather that TFAM directly regulates mtDNA copy number (Ekstrand *et al.* 2004; Kanki *et al.* 2004).

In general, HMG domains have the ability to interact with the minor groove of DNA and to dramatically distort DNA structure upon binding (see (Grosschedl *et al.* 1994) for review). Indeed, both TFAM and its yeast ortholog Abf2 are able to bind, wrap and bend DNA without any sequence specificity (Fisher *et al.* 1992). However, even though Abf2 contains two HMG boxes, it lacks a C-terminal tail, and is therefore dispensable for transcription, while being required for yeast mtDNA maintenance (Diffley and Stillman 1991). Recently, the crystal structure of human TFAM bound to promoter DNA has confirmed the ability of TFAM to bend DNA and has shown that it imposes a sharp turn of ~ 180° on the DNA (Ngo *et al.* 2011; Rubio-Cosials *et al.* 2011). *In vitro* experiments have demonstrated the ability of TFAM to bind DNA in a non-sequence-specific manner and organize it into nucleoid-like structures (Kaufman *et al.* 2007). Arguing for an important role of TFAM in nucleoid structure is the fact that it has been localized to nucleoids by immunocytochemistry (Garrido *et al.* 2003; Legros *et al.* 2004), and that biochemical analysis of nucleoids yields TFAM as the major component (Alam *et al.* 2003; Bogenhagen *et al.* 2008). TFAM is present in roughly a 1000-fold molar excess of mtDNA, which makes it abundant enough to coat the entire mt genome (Takamatsu *et al.* 2002; Kukat *et al.* 2011). Since it may bind as a homodimer (Kaufman *et al.* 2007), this translates to one homodimer every 30-40 bp.

As already mentioned, the function of TFAM in transcriptional activation is mediated by the C-terminal tail, which is responsible for sequence-specific binding and is the site of interaction with TFB2M (Dairaghi *et al.* 1995a; McCulloch and Shadel 2003). TFAM has high-affinity binding sites upstream of LSP and HSP, and it protects positions -35 to -14 relative to the transcription start site (Dairaghi *et al.* 1995b). The exact spacing between the TFAM binding site and the transcription start site is critical (Dairaghi *et al.* 1995b). One explanation for this was given by the structural data that shows how binding of TFAM upstream of LSP brings the C-terminal tail into close proximity of the start site, where it could be expected to interact with the TFB2M/POLRMT heterodimer (Ngo *et al.* 2011; Rubio-Cosials *et al.* 2011). The mechanism of transcription initiation is discussed in more detail in a following section. A lone report has questioned the requirement of TFAM in mt transcription *in vitro* by demonstrating a low level of LSP and HSP1 transcripts in the absence of TFAM (Shutt *et al.* 2010).

#### *1.2.1.3 The mitochondrial transcription factors B1 and B2*

The yeast mt RNA pol Rpo41 requires the transcription factor B (mt-TFB or Mtf1) in order to specifically recognize the promoter and initiate transcription (Xu and Clayton 1992). In 2002, a significant advance was made in understanding mammalian mt transcription when two human mt-TFB homologs, TFB1M (Falkenberg *et al.* 2002; McCulloch *et al.* 2002) and TFB2M (Falkenberg *et al.* 2002), were discovered. They are ubiquitously expressed, with the highest expression levels in heart, skeletal muscle and liver similarly to the expression patterns of other nucleus-encoded components of the mt transcription machinery (Asin-Cayuela and Gustafsson 2007). TFB1M and TFB2M are closely related to a family of rRNA methyltransferases, members of which dimethylate two adjacent adenosine bases near the 3′ end of the small subunit rRNA

during ribosome biogenesis. Indeed, phylogenetic analysis indicates that TFB1M and TFB2M are derived from the rRNA dimethyltransferase of the mitochondrial endosymbiont (Shutt and Gray 2006a).

Both TFB1M and TFB2M can form a heterodimeric complex with POLRMT and are essential for transcription initiation *in vitro* but probably not for elongation since POLRMT alone can carry out transcription of a duplex template with a 3′-tail at similar efficiency (Falkenberg *et al.* 2002). TFB2M is the more potent transcription activator *in vitro* and results in 10-100 times higher activation of transcription than TFB1M. Both factors still retain methyltransferase activity *in vivo* (Seidel-Rogol *et al.* 2003; Cotney and Shadel 2006; Cotney *et al.* 2007). However, the methyltransferase activity is not required for activation of transcription, as single amino acid mutations that abolish methyltransferase activity of TFB1M do not affect its ability to activate transcription *in vitro* (McCulloch and Shadel 2003).

The functional significance of having two mt-TFB homologs has been an open question, which is now starting to unravel. It seems that the two human proteins have evolved to specialize in different functions, with TFB2M having a primary role in transcriptional activation and copy number control, while TFB1M functions to methylate 12S rRNA. The first clues in this direction came from is work in *Drosophila melanogaster*, where RNAi knockdown of dm-TFB2M resulted in a reduction of specific mitochondrial transcripts and copy number (Matsushima *et al.* 2004), while knockdown of dm-TFB1M reduced mt protein synthesis, consistent with a role of TFB1M in modulation of translation (Matsushima *et al.* 2005). In agreement with this data, a more recent study found that overexpression of TFB2M in HeLa cells increases the steady-state levels of specific transcripts as well as mtDNA copy number and that, importantly, this increase is not dependent on the methyltransferase activity of TFB2M (Cotney *et al.* 2007; Cotney *et al.* 2009). In contrast, TFB1M levels did not appear to affect mt transcription, translation or copy number, but did increase mt biogenesis as evidenced by quantification of mitochondrial membranes (Cotney *et al.* 2007). Furthermore, the effect of TFB1M overexpression on mt biogenesis was through hypermethylation of 12S rRNA and, as expected, required the methyltransferase activity of the protein. Work in mice has further emphasized the importance of TFB1M for mitochondrial translation. The mouse TFB1M is essential for embryonic development and tissue-specific knockout of the gene results in severely impaired mt translation that is due to the loss of methylated 12S rRNA and the consequent instability of the small ribosomal subunit (Metodiev *et al.* 2009). These independent cellular functions of TFB1M and TFB2M are interconnected, as increased TFB2M levels trigger a coordinate upregulation of TFB1M expression (Cotney *et al.* 2009).

### 1.2.2 Recognition of promoter sequences and transcription initiation

The T7 RNA polymerase is a single subunit enzyme that interacts directly with the promoter region and can initiate transcription alone. Interestingly, the yeast mt RNA polymerase Rpo41, which is distantly related to the T7 RNA pol, is able to initiate transcription from mt promoters on negatively supercoiled or open templates without the involvement of its specificity factor Mtf1, indicating that Rpo41 itself plays a role in promoter recognition (Buzan and Low 1988; Matsunaga and Jaehning 2004; Savkina

*et al.* 2010). In contrast, initiation on linear templates requires Mtf1 involvement. In an elegant study, Savkina and coworkers clarify the role of Mtf1 in initiation, demonstrating that Mtf1 makes direct contacts with the promoter DNA, contributes to the specificity of initiation, and melts a 3-4 bp region around the start site (Savkina *et al.* 2010). A recent study confirmed a dual function for Mtf1 in stabilizing the pre-initiation complex; in addition to melting of the promoter, it also stabilizes the open state (Kim *et al.* 2012).

Human POLRMT requires the two accessory factors TFAM and TFB2M (or TFB1M) for promoter-specific transcription initiation *in vitro* (Falkenberg *et al.* 2002). The most likely model for promoter recognition involves the sequence-specific binding of TFAM at its binding site upstream of the promoter and bending of the DNA, as evidenced in the crystal structures of TFAM with an LSP-containing fragment (Ngo *et al.* 2011; Rubio-Cosials *et al.* 2011). Each HMG box of TFAM binds the promoter DNA, together inducing a turn of up to 180°. This sharp U-turn brings the C-terminal tail of TFAM into close proximity of the transcription start site. Direct protein-protein contacts between the C-terminal tail of TFAM and TFB2M have been reported (McCulloch and Shadel 2003), and these interactions may contribute to the recruitment of TFB2M-POLRMT heterodimers to the start site. Indeed, TFAM is essential for the recruitment of the POLRMT/TFB2M heterodimer to the promoter (Gaspari *et al.* 2004). The footprints of the components of the transcription machinery suggest that TFB2M may act as a bridge between TFAM, which protects positions -35 to -15 (Gaspari *et al.* 2004), and the core polymerase that is bound around the transcription start site (Sologub *et al.* 2009). TFB2M has a role in promoter melting, as well as contributing to the active site and making contacts to the priming nucleotide and the +1 position of the template (Sologub *et al.* 2009; Ringel *et al.* 2011).

However, the relative abundance of TFAM argues against the role of TFAM as the sole factor in promoter recognition. In agreement with the yeast system, it seems that POLRMT plays a part in the recognition of the promoter. This was concluded from a study assessing species-specific differences in mt transcription initiation. Specifically, it was shown that the purified recombinant human or mouse transcription machinery cannot initiate transcription from the heterologous promoter, and that this species-specificity was due to POLRMT, which makes contacts with positions -4 to -1 in the promoter (Gaspari *et al.* 2004).

### 1.2.3 Regulation of mitochondrial transcription

How mitochondrial transcription is regulated, *eg.* in response to changing metabolic conditions, cellular growth or in different tissues, is largely unknown. In yeast, there is evidence that indicates that *in vivo* mitochondrial transcript levels correlate with the *in vitro* sensitivity of mitochondrial promoters to ATP concentration. It has been suggested that Rpo41 senses levels of ATP (the starting nucleotide at yeast promoters) and that shifting ATP pools might thus influence mitochondrial transcription (Amiott and Jaehning 2006). Whether such a mechanism is present in mammalian cells remains unclear.

In human, the genes for TFAM, TFB2M and TFB1M are targets of PGC1-α and related coactivators in the nucleus. This links their expression to the induction of mitochondrial biogenesis in response to environmental and proliferative signals (Scarpulla 2008). Another master regulator of mt biogenesis is the nuclear respiratory factor 2 (NRF-2) that induces the expression of POLRMT, the transcription termination factor mTERF, and several members of the mt replisome (Bruni *et al.* 2010). PGC1-α, NRF-2 and related coactivators influence mt transcription by regulating the expression levels of the transcription machinery components. Other transcription factors, such as p53, the retinoid-X receptor (RXR) and the thyroid hormone receptor (TR) have been identified in mitochondria (reviewed in (Psarra *et al.* 2006)) and may constitute a different level of control. TR binds regulatory regions in mtDNA, and thyroid hormone activates mt transcription in an *in organello* transcription system (Enriquez *et al.* 1999). However, the in vivo relevance of these observations and the mechanisms by which these factors could stimulate transcription remain obscure.

Finally, as discussed above, TFB2M is 10-100x more active in stimulating transcription than TFB1M, yet both factors form heterodimers with POLRMT (Falkenberg *et al.* 2002). This difference in stimulatory activity would allow for the regulation of mt transcription through the balance between TFB2M and TFB1M levels. Furthermore, LSP and HSP1 show a different response to varying TFAM levels, with LSP being activated at lower levels of TFAM than HSP1. Taken together, it seems plausible that the promoter preference and activity of mt transcription can be modulated by varying the ratios between the components of the mt transcription machinery and mtDNA.

### 1.2.4 The mTERF protein family of transcriptional regulators

The mitochondrial transcription termination factor (mTERF) is a 34-kDa protein that binds to a 28-bp region at the 3′ end of the $tRNA^{Leu(UUR)}$ gene where it is believed to terminate the transcription of the HSP1-initiated transcription unit *in vitro* (Kruse *et al.* 1989; Daga *et al.* 1993; Shang and Clayton 1994). MTERF-dependent termination of downstream of the 16S rRNA, together with the higher activity of HSP1 compared to HSP2, have been considered to be the mechanisms behind the 50-100 times higher steady-state levels of the rRNA transcripts compared to HSP2 transcripts. However, whether mTERF directs transcription termination *in vivo* remains to be established, as exemplified by the effects of a mutation in the mTERF binding site that is associated with a human disease called MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes). This A3243G mutation in the mTERF binding site reduces mTERF binding *in vitro*, but does not affect the ratio of HSP1/HSP2 transcripts *in vivo* (Chomyn *et al.* 1992). Furthermore, the mTERF binding site funtions in a bidirectional, but orientation-dependent, manner, with higher termination efficiency reached when the termination site is in the opposite orientation relative to HSP (Shang and Clayton 1994; Asin-Cayuela *et al.* 2005). Therefore, the primary role of mTERF may not be termination of HSP transcription. Finally, the canonical mTERF binding site has been identified as a replication pause site and mTERF overexpression in cells causes replication stalling at the canonical binding site as well as at several other, lower-affinity, binding sites. Taken together, these data could justify a role for mTERF in coordinating the passage of transcription and replication complexes to avoid collisions (Hyvarinen *et al.* 2007).

Three mTERF-related proteins have been identified in human (Linder *et al.* 2005), all of which are targeted to mitochondria (Chen *et al.* 2005; Park *et al.* 2007; Pellegrini *et al.* 2009; Camara *et al.* 2011; Hyvarinen *et al.* 2011). The mTERF family of proteins thus includes mTERF2 (also known as MTERFD3), mTERF3 (MTERFD1) and mTERF4 (MTERFD2). MTERF2 seems to function as a positive modulator of mt transcription in mouse, as the knockout exhibits decreased mt mRNAs when challenged with a ketogenic diet (Wenz *et al.* 2009). In contrast, mTERF3 is an essential gene in mouse, and the mTERF3 protein is a negative regulator of mt transcription initiation. It interacts with the mt promoter region and tissue-specific knockout leads to increased transcription initiation on both strands (Park *et al.* 2007). The fourth member of the mTERF family, mTERF4, has been reported to regulate translation, while its effect on transcription (if any) is so far unexplored. MTERF4 forms a complex with the methyltransferase NSUN4 and targets it to the large ribosomal subunit via its ability to bind 16S rRNA. Heart-specific loss of mTERF4 causes a drastic reduction in translation that is due to defective ribosomal assembly, demonstrating that mTERF4 is essential for mt translation (Camara *et al.* 2011).

### 1.2.5 Other proteins involved in mt transcription

As is evident from the sections above, mitochondrial transcription is not entirely understood at the molecular level and new players in this process continue to be discovered. One example of a protein that has recently been suggested to be involved in transcription is the mitochondrial ribosomal protein L12 (MRPL12) that forms a complex with POLRMT both *in vitro* and *in vivo*. MRPL12 is reported to be able to stimulate transcription from LSP and HSP in an *in vitro* transcription system (Wang *et al.* 2007; Surovtseva *et al.* 2011). However, another report found no effect of MRPL12 on transcription *in vitro*, even in the presence of mt extracts (Litonin *et al.* 2010).

LRPPRC (or LRP130) is a pentatricopeptide repeat –containing protein that is localized to the mitochondrial matrix (Sterky *et al.* 2010), even though other localizations have also been reported (Tsuchiya *et al.* 2004). Mutations in LRPPRC are responsible for the French Canadian variant of Leigh syndrome, a neurodegenerative disorder characterized by cytochrome c oxidase (COX) deficiency (Merante *et al.* 1993). LRPPRC has been reported to activate mt transcription both *in vitro* and *in vivo*, and to interact directly with POLRMT (Liu *et al.* 2011). However, no change in *de novo* transcription levels could be observed in a heart-specific LRPPRC knockout mouse (Ruzzenente *et al.* 2012). Rather, knockout animals displayed decreased mRNA stability and decreased polyadenylation, as well as a pattern of misregulated translation. The role of LRPPRC in mRNA stability and polyadenylation in mouse is supported by observations from patient cell lines (Sasarman *et al.* 2010).

A recent study by Holt and coworkers has identified a proposed mitochondrial transcription elongation factor, TEFM (c17orf42). Also this protein interacts directly with POLRMT and is required for normal levels of promoter distal transcripts in cells (Minczuk *et al.* 2011). Future work will need to elaborate on the role of these and additional factors that influence expression of mtDNA at various levels.

## 1.3 MITOCHONDRIAL DNA REPLICATION

### 1.3.1 MtDNA replication models

The replication of animal mtDNA was first described in 1973, when electron microscopic studies demonstrated the presence replication intermediates that contained extensive single-stranded stretches of the heavy strand (Robberson *et al.* 1972). These observations gave rise to the strand-displacement model (SDM) of mtDNA replication, where replication is postulated to proceed in a strand-asynchronous manner that is continuous on both strands (Clayton 1982). According to this model, replication of the leading strand (H-strand) initiates at OriH and proceeds approximately two thirds of the way around the circular genome until the origin of light strand replication OriL is exposed on the displaced H-strand (Figure 4a, left-most side). The replication of the lagging strand can now initiate and proceed in the opposite direction.

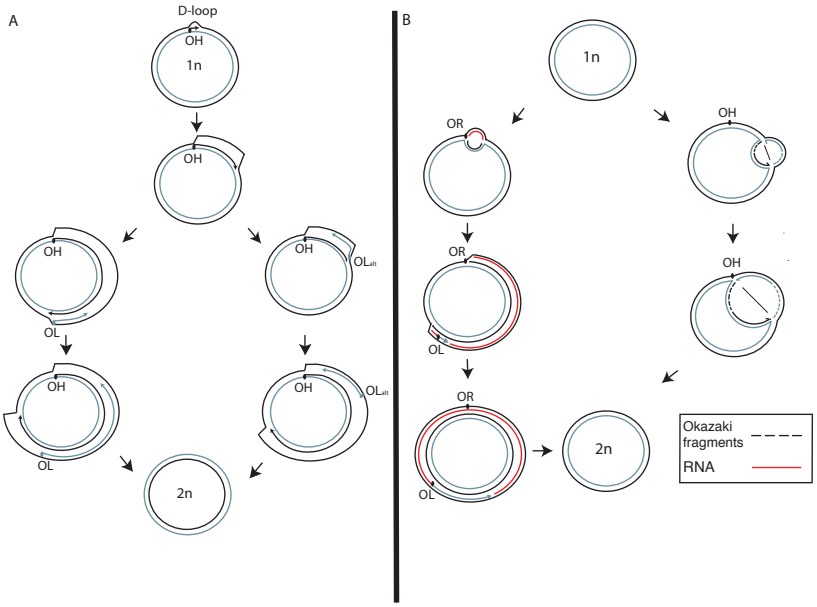


**Figure 4.** Representation of the different replication models. **a**. The strand-displacement model. Replication of the leading (H-strand) initiates at OriH and proceeds until OriL (left side) or, in the modified model, any alternative L-strand origin, becomes activated. Both strands are then synthesized until completion. **b**. *Left pathway*: In the RITOLS model, replication initiates at an origin (OR) and proceeds unidirectionally. The lagging strand is initially laid down as RNA (in red), before maturing into DNA. *Right pathway*: In the strand-coupled replication mode, replication initiates from a broad zone downstream of OriH and proceeds bidirectionally until both forks reach OriH. Synthesis of the strands is coupled, so the lagging strand is synthesized as Okazaki fragments (dashed line). Figure adapted from (Wanrooij and Falkenberg 2010).

The strand-displacement model was later challenged by Holt and colleagues based on observations from two-dimensional DNA electrophoresis analysis of replication

intermediates in cultured cells and animal tissues (Holt *et al.* 2000; Yang *et al.* 2002). These experiments revealed replication intermediates consistent with conventional strand-coupled replication, in which the lagging strand is synthesized discontinuously in the form of Okazaki fragments, as occurs during nuclear DNA replication (Figure 4b, right side). In this strand-coupled mode, replication is bidirectional and initiates from a broad zone downstream of OriH, although replication fork movement in the other direction is blocked when it reaches OriH and, effectively, replication is therefore unidirectional (Bowmaker *et al.* 2003). The different models of mtDNA replication need not be mutually exclusive, and the parallel existence of two different replication modes that predominate under different growth conditions seems by now firmly documented (Holt *et al.* 2000; Yasukawa *et al.* 2006; Pohjoismaki *et al.* 2010). Replication by the strand-coupled mode is reported to be increased during recovery from drug-induced mtDNA depletion (Yasukawa *et al.* 2005), as well as after overexpression of TFAM and expression of dominant-negative variants of the mitochondrial helicase Twinkle (Pohjoismaki *et al.* 2006; Wanrooij *et al.* 2007).

In 2002, Yang *et al*. found evidence of extensive and biased RNA incorporation in the L-strand of mt replication intermediates (Yang *et al.* 2002). This led to the introduction of the concept of RITOLS (RNA incorporation throughout the lagging strand) replication, in which the lagging strand is initially laid down as RNA before maturation into DNA (Yasukawa *et al.* 2006; Pohjoismaki *et al.* 2010). This mode of replication initiates within the noncoding region and is effectively unidirectional. As depicted in Figure 4, the strand-displacement model and the RITOLS model both involve a considerable delay between leading and lagging strand synthesis. The single-stranded replication intermediates observed by electron microscopy (Robberson *et al.* 1972) and atomic force microsopy (Brown *et al.* 2005) could thus be explained by loss of the RNA stretches of RITOLS intermediates during mtDNA purification. The mechanism that lays down the temporary lagging strand as RNA has not been elucidated and the role of transcription in this regard has not been ruled out. Therefore, it is possible that RITOLS intermediates are formed by transcripts that remain stably hybridized to the DNA template and are subsequently processed to create primers for the synthesis of the definitive lagging strand (Pohjoismaki *et al.* 2010). Arguing against the importance of RITOLS intermediates is a recent report by Reijns et al (Reijns *et al.* 2012) demonstrating that while mouse mtDNA is degraded by RNaseH2 that digests RNA-DNA hybrids at mono- and diribonucleotides, it is essentially resistant to RNaseH1 that digests RNA-DNA hybrids at stretches of four of more ribonucleotides (Nowotny *et al.* 2007).

As the SDM has recently been modified to accommodate alternative origins of lagging strand synthesis (Brown *et al.* 2005) (Figure 4a, right side), the SDM and RITOLS models mainly differ in the material that coats the displaced H-strand (mtSSB in SDM *versus* RNA in RITOLS) and the molecular mechanism behind lagging strand priming (synthesis of a short primer at OriL by a primase activity *versus* processing of the RNA lagging strand in RITOLS).

### 1.3.2 The mitochondrial replication machinery

The minimal mtDNA replisome has been reconstituted *in vitro* using purified recombinant polymerase γ (Pol γ) holoenzyme, the mitochondrial DNA helicase Twinkle and the mitochondrial single-stranded DNA-binding protein (mtSSB), which together can support the synthesis of products up to 16 kb in length (Korhonen *et al.* 2004). This minimal replisome can load on and support replication of a circular dsDNA bubble template that mimics the mtDNA D-loop region (Jemt *et al.* 2011). POLRMT can be considered a component of the replication machinery since it produces the primers required for initiation at OriH (Chang and Clayton 1985) and OriL (Wanrooij *et al.* 2008; Fuste *et al.* 2010). Priming will be discussed in more detail in the following chapter. Naturally, numerous proteins in addition to the minimal replisome are involved in the replication of mtDNA *in vivo*, some of which are briefly discussed below.

#### *1.3.2.1 The mitochondrial DNA polymerase γ*

In vertebrates, Polymerase γ (Pol γ) is the only DNA polymerase devoted to mtDNA synthesis (Clayton 1982). The mammalian Pol γ holoenzyme consists of a catalytic subunit (Pol γA) and a homodimer of the accessory subunit (Pol γB) (Carrodeguas *et al.* 2001; Fan *et al.* 2006; Yakubovskaya *et al.* 2006). Pol γA is a 140 kDa protein that harbors both DNA polymerase, 3′-5′ exonuclease and 5′-deoxyribose phosphate lyase activities and shares significant amino acid similarity with the bacteriophage T7 DNA polymerase (Kaguni 2004), while Pol γB is structurally similar to *E. coli* thioredoxin, the accessory subunit in T7 DNA polymerase (Fan *et al.* 1999). Pol γB acts as a processivity factor, accelerating the polymerization rate and enhancing the affinity of the catalytic subunit for DNA (Lim *et al.* 1999; Johnson *et al.* 2000; Kaguni 2004). The accessory subunit contains dsDNA binding activity that is required for Twinkle-dependent stimulation of Pol γ activity on a dsDNA template (Farge *et al.* 2007) as well as for the function of Pol γ in base excision repair (Pinz and Bogenhagen 2006).

#### *1.3.2.2 The mitochondrial helicase Twinkle*

The mitochondrial DNA helicase was discovered in 2001, when the genetic locus linked to some cases of autosomal dominant progressive external ophthalmoplegia (adPEO) was shown to encode a mitochondrially-localized protein with similarity to the bacteriophage T7 primase/helicase gene 4 protein (T7 gp4). Due to its punctate localization to mitochondrial nucleoids, the protein was named Twinkle (T7 gp4-like protein with intramitochondrial nucleoid localization) (Spelbrink *et al.* 2001). The sequence homology between Twinkle and T7 gp4 is restricted to the C-terminal helicase domain and, consequently, Twinkle lacks primase activity associated with the N-terminal domain of T7 gp4. Twinkle forms hexamers and heptamers in solution (Spelbrink *et al.* 2001; Farge *et al.* 2008; Ziebarth *et al.* 2010), and possesses 5′-3′ DNA helicase activity (Korhonen *et al.* 2003).

#### *1.3.2.3 The mitochondrial single-stranded DNA-binding protein*

Single-stranded DNA-binding proteins are key players in DNA metabolism across all kingdoms of life. They coat single-stranded regions of DNA, protecting it from degradation and preventing the formation of unwanted secondary structures. The mitochondrial single-stranded DNA-binding protein (mtSSB) is an essential part of the mtDNA replication machinery and is required for the maintenance of normal mtDNA

levels in human (Ruhanen *et al.* 2010), *Drosophila* (Maier *et al.* 2001) and yeast (Van Dyck *et al.* 1992). *In vitro* experiments have shown that mtSSB specifically stimulates the catalytic activities of Pol γ (Mignotte *et al.* 1988; Genuario and Wong 1993; Farr *et al.* 1999) and Twinkle (Korhonen *et al.* 2003), whereby direct interactions between the components of the minimal replisome are assumed. The mature form of human mtSSB is a polypeptide of 132 aa and contains three regions that are homologous to the *E. coli* SSB (Tiranti *et al.* 1993). The native protein is a tetramer consisting of two dimers that interact head-to-head (Yang *et al.* 1997). Human mtSSB has also been reported to be required for the synthesis of 7S DNA, the third DNA strand that is part of the D-loop form of mtDNA (Ruhanen *et al.* 2010).

#### *1.3.2.4 The role of POLRMT in priming*

Transcription generates the primer required for initiation at OriH (discussed in the next chapter), but until recently the mechanism of priming at OriL has remained elusive. Although a primase activity was discovered in human mitochondria as early as 1985 (Wong and Clayton 1985a, b), the identity of the protein responsible for the activity was never established. Twinkle was an obvious candidate due to its homology with the T7 gp4 primase/helicase, but bioinformatics analysis shows that metazoan Twinkle lacks the primase motifs that are present in homologs outside the Metazoa (Shutt and Gray 2006b). Hence, human Twinkle is not an active primase *in vitro* (Farge *et al.* 2008). More than 20 years after the initial reports of an OriL–specific mitochondrial primase, the role of POLRMT as the lagging strand primase was brought forward in a series of *in vitro* experiments (Wanrooij *et al.* 2008; Fuste *et al.* 2010).

While POLRMT is highly processive on dsDNA, on a ssDNA template it synthesizes products in the range 25-75 nts, which can be used by Polγ as primers for DNA synthesis (Wanrooij *et al.* 2008). In the presence of mtSSB, POLRMT-dependent lagging strand synthesis is specific to OriL and requires a stem-loop structure as well as the poly-dT stretch in the loop region, at position 5757-5751 of mtDNA (Fuste *et al.* 2010). This is in agreement with the *in vivo* determined starting sites of lagging strand synthesis (Kang *et al.* 1997). Knockdown of POLRMT levels in HOS cells lead to an increased delay between leading and lagging strand DNA synthesis as judged by two-dimensional neutral/neutral agarose gel (2DNAGE) analysis, providing further support for the role of POLRMT as a lagging strand primase also *in vivo* (Fuste *et al.* 2010).

#### *1.3.2.5 Other proteins involved in mtDNA replication*

Other proteins predicted to be required in the replication process include a topoisomerase, as well as a ligase and RNaseH-activity for removal of the primer. Top1mt is a mitochondrial topoisomerase that relaxes negative supercoils (Zhang *et al.* 2001), and since it is a type IB topoisomerase, it may also catalyze the removal of positive supercoils that result from DNA helicase action at the replication fork (Wang 2002). The human ligase III gene encodes two proteins, one of which is mitochondrially-targeted (Lakshmipathy and Campbell 1999). Ligase III is essential for the proper maintenance of mtDNA as RNAi-mediated knockdown of the protein resulted in mtDNA depletion (Lakshmipathy and Campbell 2001).

A mitochondrial role for the mouse RNaseH1 enzyme was demonstrated in 2003 when RNAseH1 knockout mice were found to exhibit mtDNA depletion and die during

embryonal development (Cerritelli *et al.* 2003). Even though this was taken as proof of the strand-coupled model, an RNaseH-activity is required for removal of the replication primer regardless of the replication mechanism.

### 1.3.3 Priming of mtDNA replication

While both the RITOLS and the strand-displacement model require priming at OriH, the different replication mechanisms differ in the requirement of priming for lagging strand synthesis. The strand-displacement model requires a single priming event at OriL, although the model has been modified to permit the concept of (minor) alternative origins (Brown *et al.* 2005). On the other hand, strand-coupled replication would require numerous primers for the Okazaki fragments that constitute the lagging strand, while in RITOLS replication the lagging strand is entirely comprised of RNA and OriL is more of a start site of RNA-to-DNA maturation (see chapter 1.3.1 and references therein). The ability of POLRMT to synthesize primers at OriL has been discussed above, so the focus here will be on priming of leading strand replication at OriH.


**Figure 5.** H-strand sequence in the region directly downstream of LSP. Sites of premature transcription termination are in blue font. RNA-to-DNA transition points are underlined, and free DNA 5′ ends are marked with blue dots below the sequence. See text for details. The DNA ends at 110 and 57 have been omitted for clarity.

The first report of RNA primers in mammalian mtDNA dates back to 1979 (Gillum and Clayton 1979), and it is now generally accepted that transcription from LSP provides the primers required for initiation of leading strand replication at OriH. The LSP transcript forms a stable RNA-DNA hybrid (R-loop) with the DNA at a GC-rich region called Conserved Sequence Block II (CSBII) in yeast (Xu and Clayton 1995), mouse (Lee and Clayton 1996) and human (Xu and Clayton 1996). Consistent with this, RNA-to-DNA transition points have been mapped only to the vicinity of CSBII (Kang *et al.* 1997; Pham *et al.* 2006). In contrast, free 5′ ends of H-strand DNAs have been mapped to numerous locations, the most prominent being 110, 146/147, 151, 167/168 and 190/191 (Tapper and Clayton 1981, 1982; Chang and Clayton 1985; Kang *et al.* 1997) (Figure 5). An origin at position 57 has been suggested to predominate when a steady level of mtDNA is to be maintained, but should perhaps be considered with caution since it is only based on a single report (Fish *et al.* 2004). The apparent gap between the

RNA-to-DNA transition sites and the DNA 5′ ends could be reconciled in a model where an RNA primer stretching from LSP at position 407 until the CSBII region is extended by Pol γ, and a later processing step removes the RNA primer along with a few hundred nucleotides of DNA.

Indeed, several studies have been published regarding potential nucleases that could be responsible for removal of the primer RNA at OriH. The most prominent candidate has been RNase MRP (RNase mitochondrial RNA processing), which digests model R-loops at sites that correspond to *in vivo* -mapped free 5′ ends (Lee and Clayton 1998). However, a functional role for RNase MRP in R-loop processing in the mitochondria is unlikely, since RNase MRP is barely detectable in HeLa cell mitochondria (Kiss and Filipowicz 1992). Another candidate protein for primer removal is the endonuclease EndoG (Low *et al.* 1988; Cote and Ruiz-Carrillo 1993), which has since been shown to be a non-specific apoptotic nuclease (Li *et al.* 2001) that is localized to the intermembrane space (Ohsato *et al.* 2002), thus ruling out a role for EndoG in mtDNA metabolism.

In a reconstituted *in vitro* transcription system consisting of TFAM, TFB2M and POLRMT, a significant proportion of LSP-initiated transcription events terminate at CSBII (Pham *et al.* 2006). The authors mapped the termination site to nucleotide positions 282-300 of mtDNA and demonstrated an overlap with *in vivo* RNA-to-DNA transition points in human cell lines. This premature termination of transcription was shown to be dependent on the CSBII sequence and was not specific for the mitochondrial RNA polymerase, implying the existence of a novel mechanism of primer formation for mtDNA replication. The effect of naturally occurring length polymorphisms in the CSBII sequence on transcription termination has been studied (Asari *et al.* 2007).

The use of a stably hybridized transcript in priming leading strand replication is by no means unique to mitochondria. The initiation of replication of the ColE1 plasmid has been extensively studied and involves an RNA transcript (RNAII) that initiates 555 bp upstream of the replication origin *oriV* (Itoh and Tomizawa 1980). RNAII folds into a secondary structure that allows the stable hybridization of a region of the RNA with the DNA at the origin (Tomizawa and Itoh 1982). Cleavage by RNAseH creates a 3′ hydroxyl group that can be extended by DNA Polymerase I. Primer formation is regulated by an inhibitory RNA (RNAI) that interacts with RNAII to prevent hybridization with the origin, as well as by the secondary replication repressor protein Rom that stabilizes the interaction of RNAI with the primer RNAII (Davison 1984).

## 1.4 MITOCHONDRIA AND DISEASE

### 1.4.1 MtDNA genetics

Mitochondrial DNA is strictly maternally inherited, although there is a single reported case of muscle-specific paternal mtDNA leakage (Schwartz and Vissing 2002). MtDNA has a much faster mutation frequency than the nuclear genome, perhaps because of its physical association with the inner membrane where damaging reactive oxygen species are generated, and appears to have less efficient repair mechanisms

(Brown *et al.* 1979; Richter *et al.* 1988; Pinz and Bogenhagen 1998). This high mutation rate and the maternal inheritance pattern have made mtDNA sequence analysis an interesting tool in human population genetics, evolutionary studies and forensics (Stoneking 1994). Because cells are always polyploid with regard to mtDNA, a single cell or tissue may contain mutant and wildtype copies of mtDNA, a situation referred to as heteroplasmy. However, rapid segregation of the heteroplasmic mtDNA pool is observed in the transmission from mother to offspring, a fact that is explained by a genetic bottleneck in the female germline (Chinnery *et al.* 2000; Wai *et al.* 2008). As a result, most individuals are homoplasmic for a single species of mtDNA. In contrast, most disease-causing mtDNA mutations are present in the heteroplasmic state. However, the level of heteroplasmy that leads to dysfunction varies between individuals and tissues, a phenomenon referred to as threshold effect (Rossignol *et al.* 2003). Therefore, no simple correlation between heteroplasmy level and clinical phenotype or severity of disease exists (Morgan-Hughes *et al.* 1995).

### 1.4.2 Mitochondrial disease

Mitochondrial diseases are a heterogeneous group of disorders that involve abnormalities in mitochondrial function, and owing to the key role of the mitochondria in cellular energy production, they are often, but not always, due to a defect in the function of the respiratory chain. Because the respiratory chain consists of subunits encoded by both cellular genomes, genetic defects in nuclear or mitochondrial DNA can underlie mitochondrial disease, even though most of the known mutations have been identified in mtDNA. MtDNA mutations were linked to human pathologies more than 20 years ago (Holt *et al.* 1988; Wallace *et al.* 1988; Zeviani *et al.* 1988) and to date over 200 mtDNA mutations have been described in human disease. The accumulation of mtDNA mutations is also associated with the aging process (Trifunovic *et al.* 2004). Mitochondrial abnormalities have been indicated in common neurodegenerative diseases such as Alzheimer's and Parkinson's (reviewed in (Coskun *et al.* 2012; Schapira 2012)) and are likely to play a central role in metabolic disorders such as diabetes, obesity, cardiovascular disease and cancer (Wallace 2010).

Mitochondrial disorders can be confined to single tissues (myopathy, blindness, deafness) or be complex multisystem syndromes, such as MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes) or MERFF (myoclonic epilepsy with ragged red fibers). In general, mt disorders often affect tissues of high energy demand such as the brain, muscle, heart, kidney and the endocrine system. MtDNA mutations are the primary cause of mt disease and are associated with a wide variety of clinical symptoms, including cardiomyopathy, myopathy, movement disorders, blindness, deafness *etc*. (Wallace 2010). MtDNA-linked diseases may be due to primary mutations in mtDNA, such as deletions, rearrangements or point mutations, in which case the inheritance is maternal. Mutations in the nuclear genes encoding products involved in mtDNA maintenance cause secondary mtDNA mutations and are autosomally inherited. MtDNA-associated disease can also be sporadic, caused by the accumulation of mutations due to *e.g.* oxygen radicals or faulty repair.

Disease mutations in the genes encoding mtDNA replication proteins have been found for Polγ A, Polγ B and the Twinkle helicase. Over 150 disease mutations in *POLG1*

have been identified to date. Pol γA defects are associated with disorders such as progressive external ophthalmoplegia (PEO), Alpers syndrome, ataxia-neuropathy, Charcot-Marie-Tooth disease, parkinsonism, *etc* (reviewed by (Chan and Copeland 2009)). These diseases are characterized by mtDNA deletions or depletion in the tissues affected. Pol γB mutants that are unable to stimulate the processivity of the catalytic subunit are also known to cause PEO (Longley *et al.* 2006). Mutations in *PEO1* that encodes the Twinkle helicase can be divided into two groups: the dominant ones cause autosomal dominant (ad)PEO with mtDNA deletions, while the autosomal recessive ones have been reported to cause tissue-specific mtDNA depletion (reviewed in (Suomalainen and Isohanni 2010), such as infantile onset spinocerebellar ataxia (IOSCA) (Hakonen *et al.* 2008).

## 1.5 G-QUADRUPLEXES

### 1.5.1 General introduction into G-quadruplexes

The DNA in our genomes is not always B-form DNA with the familiar structure of about 10 bases per turn of the right-handed double helix. Rather, there are several types of secondary structures that can result in the DNA adopting a non-B conformation. In recent years, non-B DNA has been suggested to play numerous roles in our genomes, including those in gene regulation, replication and recombination (Gomez-Marquez 2010). One of the secondary structures in non-B DNA is G-quadruplex (G4) DNA, also referred to in the literature as G-tetraplex DNA. G4-DNA consists of G-quartets (or G-tetrads), planar self-assemblies of four guanines hydrogen-bonded to each other by Hoogsteen bonding. Stacking of G-quartets is stabilized by monovalent cations (particularly $K^+$ or $Na^+$), which are located between two planes of G-quartets, each interacting with eight guanines. The result is intramolecular or intermolecular association of four DNA strands in a parallel or antiparallel orientation (Burge *et al.* 2006), as depicted in Figure 6.

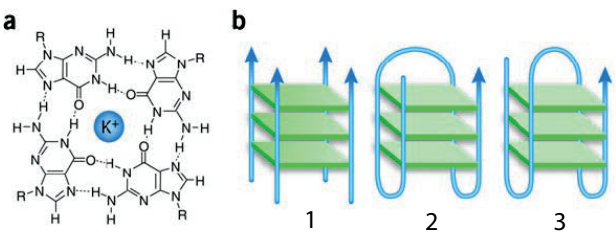


**Figure 6.** G-quartets and G-quadruplex DNA. **a.** A G-quartet. Four guanines Hoogsteen basepair, forming a planar structure that is stabilized by a monovalent cation in the central cavity. **b.** G-quadruplexes can form intermolecularly (structure 1) or intramolecularly (2 and 3), and strand orientation may be parallel (1) or antiparallel (2 and 3). From (Maizels 2006).

The first indication of the unusual ability of guanine to self-assemble into secondary structures came as early as 1910, when it was noted that guanylic acid (GMP) could spontaneously form a gel (Bang 1910). The structure behind this phenomenon was discovered over 50 years later (Gellert *et al.* 1962). The possible biological implications

of G-quadruplexes became apparent when polyguanylic acid (Arnott *et al.* 1974; Zimmerman *et al.* 1975), and, as a next step, G-rich telomeric DNA sequences were found to be able to form these secondary structures (Sen and Gilbert 1988; Sundquist and Klug 1989). G4-DNA or G4-RNA can be tetramolecular (with four separate strands contributing to the structure), bimolecular, or unimolecular and can be classified further based on the orientation of the strands. In parallel-type G-quadruplexes, all the strands have the same polarity, whereas antiparallel G4s contain at least one strand in the opposite orientation. The majority of bimolecular G4s and many unimolecular ones characterized to date are found in the anti-parallel topology (Burge *et al.* 2006). In uni- and bimolecular G-quadruplexes, the sequences connecting the guanine runs will form loops that can have various conformations (propeller-type, lateral or diagonal). The structure and sequence of the loop regions is important in controlling the stability of the resulting G-quadruplex (Burge *et al.* 2006; Bugaut and Balasubramanian 2008).

Another factor affecting the stability of G-quadruplex structures is the nature of the nucleic acid backbone, as RNA G-quadruplexes are exceedingly more stable than their DNA counterparts partly due to an extended network of hydrogen bonds that the C2′ OH groups of the ribose participate in (Bugaut and Balasubramanian 2012). Another emerging difference between RNA and DNA G4 is that while intramolecular DNA G4 are usually highly polymorphic in structure, intramolecular RNA G-quadruplexes have a tendency to adopt a parallel-stranded conformation regardless of the sequence or experimental conditions (Tang and Shafer 2006; Arora and Maiti 2009; Joachimi *et al.* 2009; Zhang and Zhi 2010).

The formation and stability of G-quadruplexes is dependent on monovalent cations. This has been ascribed to the strong negative electrostatic potential created by the guanine O6 oxygen atoms, which form a central channel that the cations fit into (Burge *et al.* 2006). $K^+$ and $Na^+$ are preferred, while $Li^+$ does not usually support G4 formation (Sen and Gilbert 1992). Depending on the particular G4-forming sequence, the identity of the monovalent cation may affect the structure that the sequence adopts, although other G4 structures are stable regardless of the type of cation present. In general, bimolecular quadruplex topology appears not to be markedly dependent on the nature of the cation (Burge *et al.* 2006).

Circular dichroism (CD) can be used to discriminate between parallel and anti-parallel G-quadruplexes, arising from different arrangements *of anti/syn* glycosidic angles (in parallel G4s, all guanine glycosidic angles are in an *anti* conformation). Parallel-type G-quadruplexes produce traces with a maximum around 260 nm and a minimum at 240 nm, while for anti-parallel G4s the maximum and minimum are located at 290 and 260 nm, respectively (Burge *et al.* 2006; Paramasivan *et al.* 2007). X-ray crystallography or NMR spectroscopy analysis allows more detailed study of G4 structures, but often requires the use of mutated sequences that crystallize (X-ray crystallography) or form only a single species of G4 (NMR) in solution because multiple structures sometimes exist in equilibrium.

Bioinformatics studies have searched the human genome for potential (unimolecular) G-quadruplex forming sequences (PGQS) and agree on a figure of about 5 700 000

PGQSs (Huppert and Balasubramanian 2005; Todd *et al.* 2005). However, this corresponds to a maximum of 375 000 non-overlapping G4 sequences, since a specific PGQS often has the potential to form several overlapping G4s. An interesting study reported that gene function correlates with its potential for G4 formation, with PGQS over-represented in proto-oncogenes and underrepresented in tumor suppressor genes (Eddy and Maizels 2006). This raises the possibility of G-quadruplexes as global regulators of gene expression, or alternatively, as factors that affect selection due to the higher genome instability that they impart.

The flagships of G4-forming sequences are the G-rich telomeric repeats that are present at the end of eukaryotic chromosomes. The 3′ ends of these telomeric repeats contain single-stranded overhangs that are able to form G-quadruplexes. These single-stranded ends are normally protected by bound protein (POT1 in humans), but if deprotected, will form G4 and trigger DNA damage-response mediated cell death (Neidle 2010). Furthermore, a stable telomeric G4 structure prevents extension of the telomere by telomerase (Zahler *et al.* 1991), leading to cell senescence and apoptosis. Therefore, although the precise role of G4 in telomere function remains to be elucidated, it no doubt has functional consequences for the cell. The G-quadruplex at the vertebrate telomeric repeat d(TTAGGG) has been extensively studied with most studies focusing on the precise structure(s) formed by this repeat depending on the surrounding conditions. Other human genome sequences that have been shown to form G-quadruplexes include rDNA repeats, immunoglobulin switch regions and many promoters. Quadruplex sequences are also found at chromosomal breakpoints, *e.g.* in the lymphoma-associated translocation of the bcl-2 gene from chromosome 14 to chromosome 18 (Raghavan *et al.* 2004). The role of G-quadruplexes in regulating transcription and replication will be discussed below.

Although most studies on G-quadruplexes have been conducted *in vitro*, *in vivo* evidence is starting to accumulate. The first description of G4 structures in the cell dates to 2001, when Schaffitzel *et al.* used an anti-G-quadruplex antibody to pinpoint these structures at the telomeres of the ciliate *Stylonychia lemnae* (Schaffitzel *et al.* 2001). G-quadruplexes may also be implicated in human disease. For example, the chromosomal instability disorders Werner's syndrome, Bloom's syndrome and Fanconi anemia complementation group J are caused by autosomal recessive mutations in genes encoding helicases that can unwind G-quadruplexes *in vitro*: WRN, BLM and FANCJ helicases, respectively (Wu and Brosh 2010). Perhaps the most compelling evidence exists for the FANCJ helicase, considering that cell lines from patients lacking FANCJ accumulate large genomic DNA deletions that map to predicted G-quadruplex forming sites (London *et al.* 2008). Taken together with the preference of FANCJ for G4 substrates *in vitro* (Wu *et al.* 2008), this speaks for a role of G4-induced genomic instability in Fanconi anemia. The enrichment of sequences with G4-forming potential to functional regions of genomes (like promoter regions) and their conservation between species indicate a strong selective pressure to maintain G4 forming sequences in the genome, even though their maintenance is problematic for the cell (Lipps and Rhodes 2009).

### 1.5.2 G4 in regulation of transcription or replication

Being such a stable structure, G-quadruplex DNA, if formed in the genome, will impede the progression of the transcription (Broxson *et al.* 2011) and replication (Lopes *et al.* 2011) machineries. Therefore, G4 formation must be tightly regulated in the cell, and under the right circumstances it provides an elaborate means of *cis*-acting control of molecular events like transcription as briefly discussed below.

Due to the G-rich nature of promoter regions, potential G4 sequences are enriched at the 5′ end of human genes (Eddy and Maizels 2009). Indeed, based on a study of *E. coli* and several other prokaryotic species, quadruplex formation has been suggested to function as a regulatory signal for transcription (Rawal *et al.* 2006)). In agreement with this notion, transcription of ORFs with G4 forming potential was specifically reduced in *S. cerevisiae sgs1* mutants lacking a functional Sgs1 helicase that unwinds G-quadruplexes (Hershman *et al.* 2008). The promoter regions of several proto-oncogenes have been found to contain G4s, most often directly upstream of the transcription start site. The most well studied example is the NHE $III_1$ region in the promoter region of the *c-myc* gene: transcriptional activation of *c-myc* is repressed when the NHE $III_1$ quadruplex structure is stabilized by the small molecule ligand TMPyP4 (Siddiqui-Jain *et al.* 2002). The effect on transcription is due to the displacement of transcription factors when NHE III is in its G-quadruplex conformation (Brooks and Hurley 2010). This and numerous other studies have brought forward the possibility of regulating cancer gene expression through small molecule therapy using compounds that target and stabilize G-quadruplexes.

# 2 SPECIFIC AIMS

The key members of the mitochondrial replication and transcription machineries have been identified and purified over the last 30 years. However, when it comes to the molecular mechanism of these essential cellular processes, many unanswered or controversial questions remain. The work presented in this thesis was designed in order to shed light on some of these questions, with the main focus lying on how components of the transcription machinery contribute to priming of mtDNA replication. The specific aims of each of the constituent papers were:

**Paper I:** To identify the mechanism behind the premature termination of LSP transcripts observed at CSBII.

**Paper II:** To assess whether G-quadruplex structures could be responsible for the stable association of primer RNA to DNA template in the CSBII region.

**Paper III:** To characterize the sequence requirements of the light strand origin of DNA replication (OriL).

**Paper IV:** To confirm the role of the mitochondrial transcription factor A (TFAM) as an essential component of the mitochondrial transcription machinery.

# 3 RESULTS AND DISCUSSION

### Paper I. G-quadruplex structures in RNA stimulate mitochondrial transcription termination and primer formation

Transcription is known to generate the primers required for initiation of replication from both OriH (Chang and Clayton 1985; Chang *et al.* 1985) and OriL (Wanrooij *et al.* 2008; Fuste *et al.* 2010). At OriH, the first evidence of this came from experiments mapping the 5′ and 3′ ends of mt nucleic acids from human KB cells. In that study, 3′ RNA ends that overlapped with 5′ DNA ends were mapped to CSBI and CSBII. Because all 5′ RNA ends were located at position 407, the starting point of LSP transcripts, it was concluded that the primer RNAs were products of transcriptional events (Chang and Clayton 1985; Chang *et al.* 1985). Different models for the switch from RNA to DNA have been presented, one of which involved processing of mt transcripts by RNase mitochondrial RNA processing (RNaseMRP) (Lee and Clayton 1997, 1998). However, only trace amounts of RNaseMRP have been identified in human mitochondria (Kiss and Filipowicz 1992), casting doubt on its role in mt primer processing. In 2006, Pham and colleagues reported termination of transcription just downstream of CSBII in an *in vitro* transcription system based on purified recombinant POLRMT, TFAM and TFB2M. The termination was absolutely dependent on the CSBII sequence and was not polymerase-specific, since it was also observed using the T7 RPOL. Most importantly, *in vivo* RNA-to-DNA transition points were mapped to the same region in thrombocytes and HeLa cells (Pham *et al.* 2006). This strongly suggests that a sequence determinant at CSBII could direct transcription termination in this region in order to produce an RNA 3′ end for priming.

In this work, we set out to determine the cause of the transcription termination observed in the CSBII region. Bioinformatic analysis using the QGRS mapper (Kikin *et al.* 2006), a tool for locating G-quadruplexes, predicted formation of a G-quadruplex structure at the G-rich CSBII. In order to find out whether such a secondary structure could form at CSBII, we made use of native gel analysis and the mitochondrial *in vitro* transcription system based on purified recombinant POLRMT, TFAM and TFB2M (Falkenberg *et al.* 2002). Indeed, while transcription on a wildtype DNA template encompassing LSP and the CSBs resulted in 30 – 40% of transcription events terminating prematurely, the levels of preterminated transcripts were clearly lower on a mutant CSBII template without the ability to form G-quadruplexes. Furthermore, the levels of pretermination were highest in the presence of $K^+$ ions, and dropped significantly in the presence of 7-deaza-GTP, which, once incorporated in the RNA, prevents Hoogsteen basepairing and thus reduces G-quadruplex stability. The ability of CSBII RNA to form G-quadruplexes was confirmed by native PAGE analysis of oligonucleotides encompassing CSBII. Finally, mutation analysis confirmed that all twelve guanines in the two G-stretches of CSBII (nt positions 315-303) were required for G-quadruplex stability, and the downstream poly-U stretch (nt positions 286-291) may play a role in fine-tuning the levels of pretermination. Taken together, the data suggest a model where a G-quadruplex forms in the nascent RNA strand upon transcription of CSBII. The formation of this stable secondary structure, perhaps in combination with the poly-U stretch downstream of CSBII, causes POLRMT to

terminate transcription at CSBII, giving rise to a product of only 110 nt instead of the near genome-length polycistronic transcript. The combination of a stable secondary structure in the transcript and a downstream poly-U stretch is reminiscent of intrinsic (rho-independent) transcription termination in prokaryotes, where a G-C rich stem-loop structure causes transcription to stall, while the position of a downstream poly-rU stretch in the RNA-DNA hybrid binding site of the RNA polymerase allows dissociation of the transcript from the template DNA due to the weak rU-dA hybrid (Farnham and Platt 1981; Wilson and von Hippel 1995).

### Paper II. A hybrid G-quadruplex structure formed between RNA and DNA explains the extraordinary stability of the mitochondrial R-loop

The RNA primer required for initiation of replication from OriH is formed by transcription from LSP. Unlike normal transcripts that do not remain bound to their DNA template, however, the mitochondrial primer RNA has been reported to be stably associated with mtDNA in an R-loop structure. R-loops are structures where one strand of the DNA double helix is locally displaced by an RNA molecule that is hybridized to the other DNA strand. The mitochondrial R-loop is observed in yeast, mouse and human, and requires the CSBII sequence (Xu and Clayton 1995; Lee and Clayton 1996; Xu and Clayton 1996). Such stable association of RNA to the template is required for its use as primer, but the molecular background of the phenomenon has never been completely clarified. The emphasis of previous work on the mt R-loop has been on determining the identity of a nuclease that could process the RNA in order to provide a free 3′-OH to be used as primer. However, the finding that a G-quadruplex structure forms in the RNA at CSBII raised the question of whether the unusual stability of the RNA-DNA hybrid could be attributed to G-quadruplexes. In support of this view, the Clayton laboratory had earlier reported that the unusual stability of the mitochondrial R-loop could not be explained by standard Watson-Crick interactions (Lee and Clayton 1996).

One could imagine that G-quadruplex formation could contribute in two ways to primer stability: the first option is that G-quadruplexes formation in the DNA non-template strand would stabilize primer binding to the template strand due to decreased competition for binding to the template strand. Alternatively, bimolecular G-quadruplex structures consisting of the nascent RNA and the DNA non-template strand could anchor the primer to the required site for priming. In order to investigate these possibilities, we first used native gel analysis of RNA and DNA oligonucleotides of the CSBII sequence. The experiments showed that RNA and DNA oligos together could form a bimolecular species. Formation of the hybrid species was abolished when using 7-deaza-dGMP-containing oligos or when oligo sequence was mutated to prevent G4 formation. For these reasons and because Watson-Crick basepairing between the two strands was not possible, we concluded that the hybrid species was dependent on G-quadruplex formation between the RNA and DNA. We further used circular dichroism to study the RNA, DNA and hybrid G-quadruplexes formed by CSBII oligonucleotides. Circular dichroism analysis allows the study of G4 conformation based on the location of the relative maxima and minima observed. The analysis confirmed G-quadruplex formation in the RNA, DNA and hybrid RNA-DNA samples

and showed that all consisted of parallel-type G-quadruplexes, with relative maxima and minima at 260 nm and 240 nm, respectively.

Next, we returned to the *in vitro* transcription assay and asked whether such a hybrid quadruplex could form during transcription. RNase treatment of transcription product revealed a species of approximately 50 nt that was resistant to RNaseA and hRNaseH1 digestion, as would be expected for a G-quadruplex. Furthermore, this product was dependent on the CSBII sequence and, by using 7-deaza-GTP and 7-deaza-dGTP, we could confirm that G-quadruplex formation in both the DNA template and the RNA transcript was required in order for this RNase resistant species to form. This led us to conclude that a G-quadruplex forms between the RNA and DNA also in the *in vitro* transcription system.

Interestingly, the hybrid we observed in our transcription system based on purified recombinant proteins differs in size from the RNase-resistant hybrid reported by the Clayton laboratory. Xu *et al*. reported a hybrid of approximately 110 nt that extends from CSBII to downstream of CSBI (Xu and Clayton 1996). The reason for this discrepancy is unclear, but may be due to differences in protein preparations, since Xu *et al*. used a partially purified fraction of KB cell mitochondrial extract, while our system is based on only three purified recombinant proteins. In fact, we could observe this longer hybrid species using the T7 RNA polymerase. Also in this case the RNase-resistant species was dependent on CSBII and G-quadruplex formation in the transcript. It is therefore likely that G-quadruplex formation at CSBII can have two consequences: either it causes POLRMT to terminate prematurely, or, if transcription can continue, this may occur in so-called R-loop extension mode, which may be energetically more favorable when the nascent transcript is anchored to the template DNA behind the elongating polymerase (Belotserkovskii *et al.* 2010).

As a last step, we wanted to explore the consequence of G-quadruplex formation for priming by Pol γ. In a primer extension experiment, it could be shown that G-quadruplex formation at the 3′ end of a DNA primer prevented extension by Pol γ. Therefore, it seems likely that if G-quadruplex formation occurs *in vivo* as a means of defining the 3′ end of the mitochondrial replication primer, the G4 structure may need to be resolved or removed before replication can take over. Further work in our laboratory will address this question and the identity of candidate G-quadruplex resolving proteins in mitochondria.

### Paper III. *In vivo* mutagenesis reveals that OriL is essential for mitochondrial DNA replication

The mechanisms of mtDNA replication have been subject to intense debate in the recent past, and to this day no consensus exists in the field. Historically, mtDNA replication has been considered to proceed according to the strand-displacement model, where both strands are synthesized continuously, but with a significant delay in the synthesis of the lagging strand. According to this model, DNA replication initiates at OriH and proceeds two thirds of the way around the genome until it reaches OriL. Here, as the parental H-strand becomes single-stranded due to the passage of the replication machinery, OriL is free to adopt a stem-loop structure that serves as an

initiation site of L-strand synthesis in the opposite direction (Wong and Clayton 1985b, 1986; Fuste *et al.* 2010). POLRMT binds OriL and initiates within the loop region of the hairpin, producing a primer of approximately 25 bases that can be elongated by Pol γ (Wanrooij *et al.* 2008; Fuste *et al.* 2010). Synthesis of both strands now proceeds until completion.

The strand-displacement model has been questioned by two-dimensional neutral-neutral agarose-gel electrophoresis analysis of replication intermediates (Holt *et al.* 2000; Yang *et al.* 2002). In short, replication intermediates with a double-stranded nature consistent with coupled leading and lagging strand synthesis (as in nuclear DNA replication) could be observed (Holt *et al.* 2000). These observations gave rise to the strand-coupled replication model in which replication initiates in a broad zone (OriZ) downstream of OriH with continuous synthesis of the leading strand coupled to the discontinuous synthesis of the lagging strand as Okazaki fragments (Bowmaker *et al.* 2003). In the strand-coupled model, OriL is dispensable. Finally, a third model, termed RITOLS replication (RNA incorporation throughout the lagging strand) has been introduced and in some respects exhibits several similarities with the strand-displacement model (Yasukawa *et al.* 2006; Pohjoismaki *et al.* 2010). It too displays a significant delay between leading and lagging strand DNA synthesis, but in the RITOLS model the lagging strand is initially laid down as RNA before being matured into DNA. In the RITOLS mode of replication, OriL has been suggested to serve as the initiation point of lagging strand maturation, but no experimental evidence exists for this.

In order to elucidate the *in vivo* functional role and the sequence requirements at OriL, we used an *in vivo* saturation mutagenesis approach combined with *in vitro* and *in silico* analyses. We used transgenic mice homozygous for a proofreading-deficient Pol γ (POL $\gamma A^{D257A}$/ POL $\gamma A^{D257A}$) that accumulate high levels of somatic mtDNA point mutations (Trifunovic *et al.* 2004). We sequenced a 974-bp region covering OriL and adjacent sequences from the somatic tissues of these mutator mice and found that the mutation frequency at tRNA genes and non-coding genes was approximately 1.3 - 1.4 mutations/1000 bp. However, the mutation frequency in the OriL region was only 0.3 mutations/1000bp, which suggests that OriL mutations are not tolerated in mice. Interestingly, the mutations that are tolerated at OriL mainly map to the purine-rich strand, which does not act as a template for primer synthesis. Furthermore, most mutations were found in the stem region, and only 4 out of 13 mutated sites were located in the loop region. A considerable variation was found in the length of the T-rich loop region, with only 42 % of the sequences being WT (11 Ts).

*In vitro* analysis of variants of human OriL was very consistent with the *in vivo* findings in mice. We found that changes in the sequence of the base of the stem, where most mutations were located in vivo, did not have a major effect on priming efficiency. Furthermore, increasing the number of Ts in the T-rich loop region did not affect priming negatively, while decreasing the loop size by 2 or 3 Ts led to a decrease or abolition of priming, respectively. Therefore, our analysis showed that all naturally occurring variants in the T-stretch could efficiently initiate lagging strand synthesis *in vitro*. We continued our analysis in search of determinants that were essential for efficient priming from OriL, and found that the most important factors were a loop

region of at least 10 nucleotides and a stable stem region, preferably with a pyrimidine-rich template strand. A stretch of Ts was also required, with the Ts at positions 5751 and 5752 being essential for initiation of lagging strand synthesis.

Finally, bioinformatics analysis showed OriL to be conserved throughout vertebrates, while it is not found in invertebrates. Conserved elements included a stable stem-loop structure, with a highly conserved T proximal to the pyrimidine-rich stretch in the stem. The pyrimidine-rich side of the stem contained an almost universally conserved CCCGCC motif. The findings in this paper show that all the determinants required for OriL activity in priming lagging strand synthesis were conserved in vertebrates. This provides strong support for a replication mechanism utilizing OriL.

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

In *S. cerevisiae*, the basic machinery for mitochondrial transcription is composed of two factors: the mitochondrial RNA polymerase (Rpo41) and its accessory factor mtTFB (Mtf1) (Xu and Clayton 1992). In contrast, the human mt transcription machinery is considered a three-component system because it also contains TFAM, the mitochondrial transcription factor A (Fisher and Clayton 1985). Together, POLRMT, TFAM and TFB2M are required and sufficient for mt transcription *in vitro* (Falkenberg *et al.* 2002). TFAM can bind, bend and compact DNA without any sequence specificity, but also exhibits sequence-specific binding to high-affinity binding sites that are located upstream of both major promoters, LSP and HSP1 (Fisher and Clayton 1988; Fisher *et al.* 1989; Ghivizzani *et al.* 1994). Mutation of the high-affinity site abolishes promoter-specific transcription (Dairaghi *et al.* 1995b; Gaspari *et al.* 2004), as does deletion of the C-terminal tail of TFAM that is thought to be the site of interaction with TFB2M/POLRMT (Dairaghi *et al.* 1995a; McCulloch and Shadel 2003; Kanki *et al.* 2004). Structural studies have provided a model for TFAM function at the promoter: upon binding to the high-affinity sites, TFAM causes the DNA to bend almost 180° and bringing the C-terminal domain of TFAM into the vicinity of the transcription start site, where POLRMT and TFB2M are expected to bind (Ngo *et al.* 2011; Rubio-Cosials *et al.* 2011). Taken together, this and other evidence suggests a model where TFAM interacts with the other components of the transcription machinery and recruits them to the right position at the promoter. However, a recent report has questioned the absolute requirement of TFAM in mtDNA transcription by demonstrating low levels of *in vitro* transcription products in the absence of TFAM (Shutt *et al.* 2010). Our aim was therefore to study the requirement of TFAM for transcription in a defined *in vitro* transcription system in order to reconcile the contradictory findings in the field.

We could show that depletion of TFAM from mitochondrial lysates abolished promoter-specific transcription from templates containing LSP or HSP1 without affecting the levels of POLRMT or TFB2M. Importantly, adding recombinant TFAM to the TFAM-depleted extracts restored transcription. The same requirement for TFAM was observed in the *in vitro* transcription assay, but could be circumvented by lowering the salt concentration to below 12 mM NaCl. HSP1 exhibited higher levels of TFAM-independent transcription than did LSP, which is in agreement with the findings of

Shutt *et al.* (2010). At the low salt concentrations required for observation of TFAM-independent transcription, we could also observe promoter-unspecific transcription events due to the exposure of single-stranded regions during DNA breathing. Likewise, the use of negatively supercoiled template relieved the absolute requirement for TFAM in transcription initiation at HSP1. Treatment of the supercoiled template with TOP1mt to relieve the negative supercoiling resulted in restoration of TFAM-dependence in transcription. Together, these results indicate that while TFAM is required for transcription initiation both in extracts and in the *in vitro* system, it is not strictly required under conditions that promote DNA breathing (low salt and negatively supercoiled template). The role of TFAM may therefore be to open up the promoter region. In agreement with this notion, TFAM could introduce negative supercoils into a circular DNA molecule that had been relaxed by TOP1mt-treatment. A similar activity has been shown for the yeast homolog of TFAM, Abf2p (Diffley and Stillman 1991). The ability of TFAM to induce structural changes upon the DNA was verified by FRET experiments using the fluorescent cytosine analog $tC_{nitro}$ and the acceptor $tC^{O}$. Surprisingly, the effect of TFAM was similar on WT DNA fragments and on ones where the high-affinity TFAM binding site had been mutated. Therefore, the observed effect of TFAM on promoter structure seems to be independent of its sequence-specific binding activity. In support of this idea, we could not observe any difference in TFAM binding to a DNA fragment containing the WT HSP1 or one with a mutated high-affinity site in an electrophoretic mobility shift assay (EMSA), even though the same mutation completely abolished transcription initiation. Therefore, it seems plausible that the most critical function of the TFAM high-affinity binding site is to direct TFAM to the right position relative to the transcription start site rather than altering the binding itself. This is consistent with earlier data showing that altering the spacing between the TFAM binding site and the transcription start site greatly impairs transcription initiation efficiency without affecting TFAM binding affinity (Dairaghi *et al.* 1995b).

Although POLRMT alone can recognize specific sequence elements in the absolute vicinity of the transcription start site, it cannot stably bind to the promoter in the absence of TFAM (Gaspari *et al.* 2004). This sequence-specificity of POLRMT was likely the reason for the observed increase in promoter-specific transcription under low-salt conditions even in the absence of TFAM as reported by Shutt *et al*. (2010). Taken together, the results of Paper IV confirm the essential role of TFAM in mitochondrial transcription initiation at physiological salt conditions, and serve to consolidate findings in the field. They also provide further insight into the mechanism of function of TFAM by illustrating the ability of the protein to introduce negative supercoils, which may facilitate promoter melting.

# 4 CONCLUDING REMARKS

The main factors involved in the processes of mitochondrial DNA replication and transcription are known and purified; yet the precise molecular mechanisms underlying these processes are still unclear. For example, the fact that transcription primes replication at OriH has been reported almost 30 years ago, but still we do not know exactly how the priming event is regulated. Our studies with the preterminated LSP transcript that forms G-quadruplexes have hopefully brought us a bit closer to unraveling the mechanism behind this essential process. The perfect overlap of the premature termination site with RNA-to-DNA transition points *in vivo* provides strong support for the importance of G-quadruplexes in this process. Nonetheless, we cannot rule out additional roles for G-quadruplex structures in the mitochondria, especially since there are many more predicted G4 sites in the genome. What, if any, is the significance of all these sites that have G4-forming potential? What is the reason underlying the extreme base bias observed in mtDNA?

Due to the stability of G-quadruplexes and our finding that the G4 oligos could not be used as primers by Pol γ, it seems likely that the G-quadruplex structure needs to be resolved or removed before the primer can be extended. The identification of such a protein in mitochondria would be a major step in the right direction. There are several mitochondrial proteins that could play a role in this process, like hRNAseH1, Dna2, and hopefully the ongoing attempts in our laboratory will be able to provide an answer to this and many more questions. Perhaps the addition of this factor to the *in vitro* system would allow reconstitution of leading strand replication in the test tube, something that has been attempted for quite some time? Is there an alternative mechanism of replication priming at the heavy strand origin? A polymorphism leading to deletion of seven Gs in the G-stretch of CSBII has been reported in the literature (MITOMAP 2012). If G4 formation at CSBII is required for defining the 3′ end of the primer, another mechanism must exist, otherwise deletion of the G-stretch would not be tolerated. A related question that concerns both origins is how the hand-over occurs from POLRMT to Pol γ. Furthermore, the field would greatly benefit from a consensus regarding the replication mechanisms. If the other suggested models are true, the biochemical machinery behind them and perhaps the regulation between different modes of replication are questions that require attention and many work hours. All of these questions are not only of scientific interest; answers may on the long run benefit patients suffering from mitochondrial disease.

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