Review Article

Replicating animal mitochondrial DNA

Emily A. McKinney and Marcos T. Oliveira

Institute of Biomedical Technology, University of Tampere, Tampere, Finland.

Abstract

The field of mitochondrial DNA (mtDNA) replication has been experiencing incredible progress in recent years, and yet little is certain about the mechanism(s) used by animal cells to replicate this plasmid-like genome. The long-standing strand-displacement model of mammalian mtDNA replication (for which single-stranded DNA intermediates are a hallmark) has been intensively challenged by a new set of data, which suggests that replication proceeds via coupled leading- and lagging-strand synthesis (resembling bacterial genome replication) and/or via long stretches of RNA intermediates laid on the mtDNA lagging-strand (the so called RITOLS). The set of proteins required for mtDNA replication is small and includes the catalytic and accessory subunits of DNA polymerase γ, the mtDNA helicase Twinkle, the mitochondrial single-stranded DNA-binding protein, and the mitochondrial RNA polymerase (which most likely functions as the mtDNA primase). Mutations in the genes coding for the first three proteins are associated with human diseases and premature aging, justifying the research interest in the genetic, biochemical and structural properties of the mtDNA replication machinery. Here we summarize these properties and discuss the current models of mtDNA replication in animal cells.

Keywords: DNA replication, mitochondria, mtSSB, pol γ, Twinkle.

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Introduction

For decades, the mitochondrial genome was thought to be a less important plasmid-like DNA, which contained residual genetic information from the ancestral α-proteobacterium that eventually became the mitochondrion, determining one of the most important endosymbiotic events in the evolutionary history of eukaryotes. We now know that an intact and functional mitochondrial genome (Figure 1) is required for normal assembly and operation of the complexes involved in mitochondrial oxidative phosphorylation (OXPHOS), and therefore, for the bulk of ATP production (Scheffler, 2008). Mitochondria also perform a myriad of functions in eukaryotic cells that are interconnected to ATP synthesis, such as autophagy, calcium signaling, apoptosis, and production of intermediates in a variety of biosynthetic pathways (reviewed in Nunnari and Suomalainen, 2012). For the correct performance of this interconnected system, and thus, for proper physiological homeostasis/adaptability, cells depend on the qualitative and quantitative status of the mitochondrial genome. It is, therefore, easy to understand how point mutations, deletions or rearrangements in the mitochondrial DNA (mtDNA) molecule, and up- or down-regulation of mtDNA copy number in a particular tissue/organ can influence the organism's

phenotype by promoting disease states or selectively (dis)advantageous conditions.

In this review, we focus on the recent discoveries in mtDNA replication in animal cells. Researchers in the last two decades have studied the subject on two fronts: 1) investigating the composition of mtDNA replication intermediates *in vivo* and in cells in culture; and 2) investigating the biochemical and physical properties of the proteins involved in mtDNA maintenance. These studies have shown that the mtDNA transactions are more diverse and complex than previously thought (for more details on these transactions, see Oliveira *et al.*, 2010), revealing new mechanisms of genome replication with implications for the understanding of human mitochondrial disorders and the evolution of animal mitochondria.

Modes of mtDNA Replication in Animal Cells: One, Two or Three Ways of Duplicating a Genome

Historically, replication of mammalian mitochondrial genomes was studied using cesium chloride-purified mtDNA, visualized by electron microscopy (Robberson *et al.*, 1972). In combination with S1 protection, radiolabeling studies and ligation-mediated PCR (Bogenhagen *et al.*, 1979; Nass, 1980a,b; Tapper and Clayton, 1981; Kang *et al.*, 1997), this extensive body of work has generated a model of mtDNA replication that has been standing for
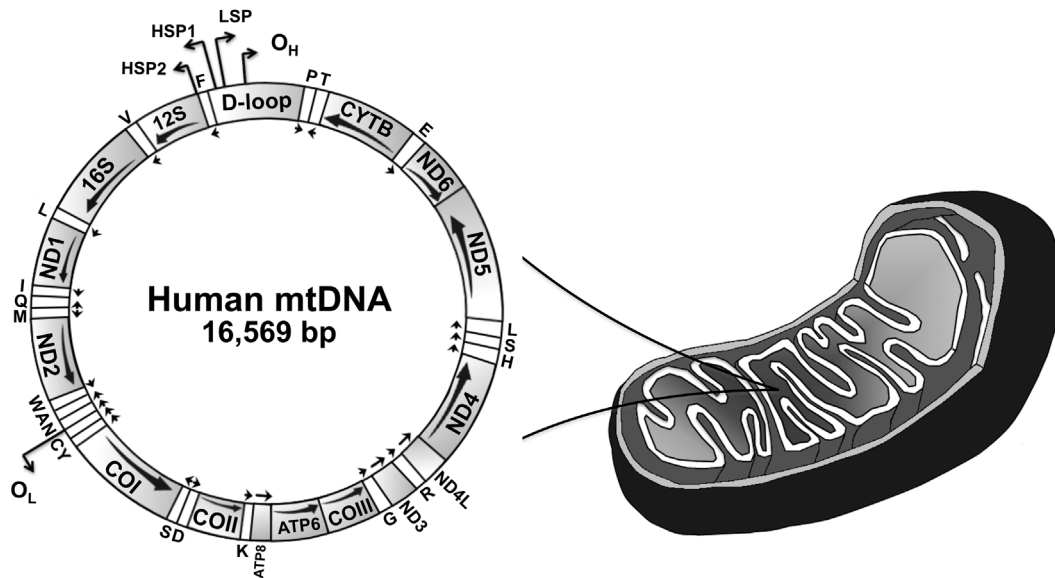
Figure 1 - Schematic representation of the human mitochondrial genome. This genome represents a typical gene content found in animal mtDNAs. The major non-coding region of mtDNA is denoted as "D-loop". The arrows below each gene indicate the direction of transcription. Transfer RNA genes are indicated by one-letter symbols, and the 12S and 16S ribosomal RNA genes appear as 12S and 16S, respectively. CYTB, gene coding for cytochrome b; COI-III, subunits I-III of cytochrome c oxidase; ND1-6, subunits 1-6 of NADH dehydrogenase; ATP6 and 8, subunits 6 and 8 of ATP synthase; O_H, origin of heavy (leading) strand synthesis; O_L, origin of light (lagging) strand synthesis, according to the strand-displacement model of mtDNA replication (see text and Figure 2 for details); LSP, light strand promoter; HSP1 and 2, heavy strand promoters 1 and 2 (reviewed in Oliveira *et al.*, 2010).

over 35 years, referred to as the strand-displacement model (Bogenhagen and Clayton, 2003a). In this model (Figure 2A), the synthesis of the leading strand (known as the heavy [H] strand in vertebrate mtDNA because of its guanine-rich composition) initiates within the non-coding D-loop region at a site designated as O_H, and proceeds continuously. As leading strand synthesis continues, replication intermediates accumulate a progressively larger displaced parental H strand, maintained in a single-stranded form. At about two thirds of the distance around the genome, the origin of the lagging strand (or light [L] strand) synthesis O_L is exposed, allowing replication of the lagging strand to be initiated and synthesis to proceed continuously. The overall process does not involve the formation of Okazaki fragments; in summary, replication is unidirectional, continuous, asymmetric and asynchronous. Recently, this model has been updated by data from atomic force microscopy, which revealed alternative L strand origins (Brown *et al.*, 2005). Moreover, biochemical data have shown that the mitochondrial RNA polymerase can in fact make an RNA primer specifically at O_L, which can be used by DNA polymerase γ (see next section for proteins of the mtDNA replisome) to initiate synthesis of the L strand (Fuste *et al.*, 2010). *In vitro*, the mitochondrial RNA polymerase is also able to prime the displaced leading strand at multiple points during synthesis of the new leading strand (see more details in the next section); the gaps between primers can then be filled by DNA polymerase γ (Wanrooij *et al.*, 2008). Although the authors concluded that this data supports the revised strand-displacement model, it also suggests the presence of Okazaki-like fragments during synthesis of the mtDNA lagging strand, a class of replication intermediates that has to date not been observed in the mitochondrion.

Since the year 2000, two other models of mtDNA replication have been reported in the literature, challenging the validity of the long-standing strand-displacement model. Studies using two-dimensional agarose gel electrophoresis (2DAGE) and a variety of nucleic acid-modifying enzymes have revealed evidence for a new class of replication intermediates: extensive segments of RNA incorporated into the newly synthesized lagging strand, hybridized to the template leading strand (Yang *et al.*, 2002; Yasukawa *et al.*, 2006). This model implies strand-coupled replication proceeding unidirectionally from the D-loop region, with the RNA replication intermediates subsequently being matured into DNA. The RITOLS (RNA Incorporated ThroughOut the Lagging Strand) model of mtDNA replication was then born (Figure 2B). More recently, this model has gained tremendous strength due to the observation via transmission electron microscopy and immunopurification using antibodies specific to RNA/DNA hybrids that replicating mammalian mtDNA molecules are essentially duplex with extensive RNA tracts present in one strand (Pohjoismaki *et al.*, 2010). Furthermore, interstrand crosslinking experiments and *in organello* DNA synthesis indicate that the RNA/DNA hybrids are present *in vivo* (Reyes *et al.*, 2013), and are not an artifact of the mitochondrial nucleic acid preparations, as previously suggested by Bogenhagen and Clayton (2003a,b).
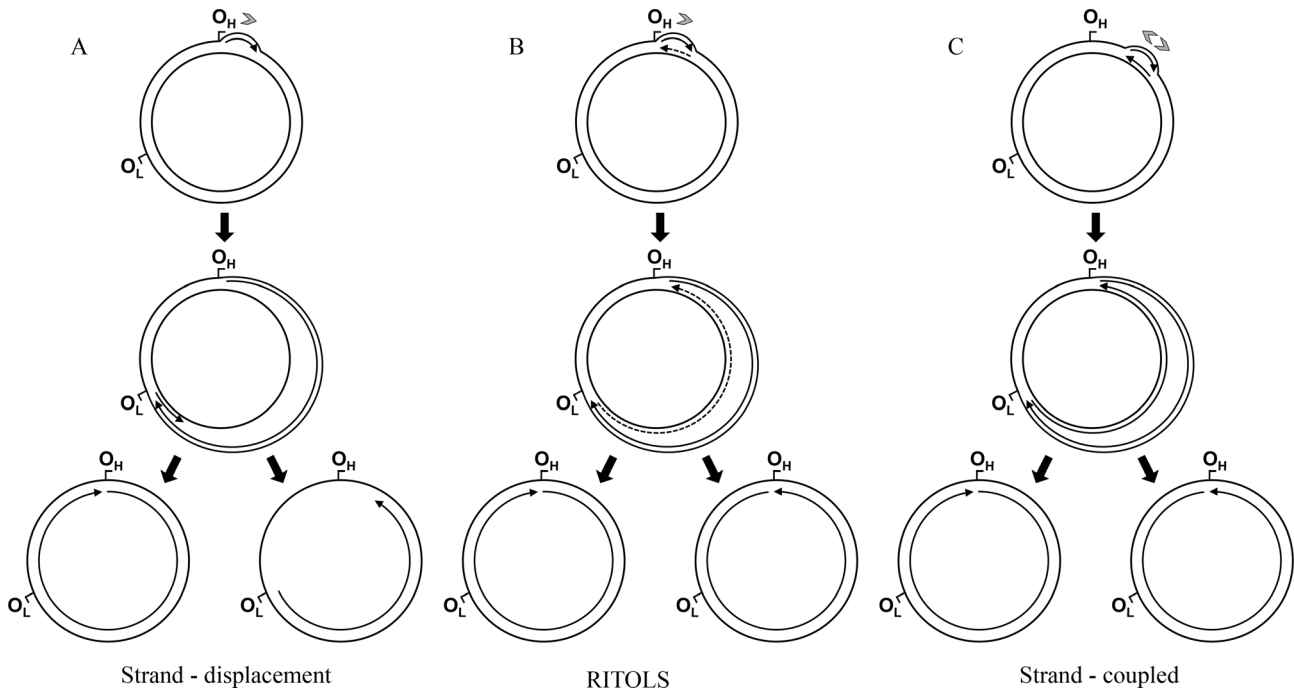
Figure 2 - Current models of mammalian mtDNA replication: the strand-displacement (A), the RNA incorporated throughout the lagging strand - RITOLS (B), and the leading and lagging strand-coupled (C) models (see text for detailed description of the models). In all models, the sites O_H and O_L are represented as reference points to the genome map (Figure 1), although these sites are primarily important for the strand-displacement model. Arrows associated with replicating mtDNA indicate the 5' to 3' direction of nucleic acid synthesis; continuous and dashed lines represent DNA and RNA, respectively (only the long stretches of RNA described in the RITOLS model are represented; the possible short RNA primers of the other models are not shown). Gray arrowheads indicate the number and directionality of replication forks generated at the origin, according to each model.

The second model, referred to as the strand-coupled model, was proposed by the same groups of researchers using 2DAGE (Holt *et al.*, 2000); this replication mode, however, appears to occur only when cells are recovering from ethidium bromide-induced mtDNA depletion. The hallmark of the model (Figure 2C) is the presence of fully double-stranded DNA theta-like replication intermediates indicative of coupled leading and lagging DNA strand synthesis, as it is found in bacterial DNA replication. Interestingly, in this case, replication starts at a broad initiation zone, in a region containing the genes *cytb*, *nad5* and *nad6* of the mammalian mitochondrial genome, and proceeds bidirectionally until finally arresting at the D-loop region (Bowmaker *et al.*, 2003). Although the 2DAGE data is substantial, this model invokes the existence of Okazaki fragments as replication intermediates and two polymerases working in a single replisome, which have yet to be shown for complete validation of the model.

In our opinion, the strand-coupled and the RITOLS models most likely describe interconnected events, as parts of the same process: the RNA hybridized to the old leading strand, during synthesis of the new leading strand, may be processed and serve as multiple priming sites for the DNA synthesis of the new lagging strand. Speculatively, the efficient action of the mitochondrial forms of DNA ligase III, Flap endonuclease 1 and Dna2 would then promote RNA removal and nick sealing, completing the maturation of the

newly replicated mtDNA (reviewed in Holt, 2009). Evidence for this interplay between strand-coupled and RITOLS has been reported by Wanrooij *et al.* (2007), who expressed catalytically mutant forms of the DNA polymerase γ and the mtDNA helicase Twinkle in human cells in culture (see next section for proteins of the mtDNA replisome). Cells expressing Twinkle mutants accumulated double-stranded mtDNA replication intermediates (the main feature of the strand-coupled model) with loss of RNA associated with the lagging strand (the hallmark of the RITOLS model), compared to control cells. This indicates an increased rate of initiation of lagging strand synthesis and/or RNA-DNA maturation relative to the rate of replication fork movement (or synthesis of the leading strand), consistent with the role of Twinkle in unwinding double-stranded DNA. On the other hand, expression of pol γ mutants induced replication stalling but maintained the RNA replication intermediates, primarily because of delayed lagging-strand DNA synthesis or its maturation.

Finding a relation between the strand-displacement model and the strand-coupled/RITOLS models has been more difficult, primarily because it appears that the regions of the mammalian mtDNA that are essentially single-stranded according to the former model, are the same regions which contain the RNA replication intermediates in accordance to the latter models. Remarkably, Pohjoismäki *et al.* (2010) showed loss of these RNA molecules when

they followed the protocol for preparation of mitochondrial nucleic acids used to describe the strand-displacement model. The single-strandness of the long-standing strand-displacement model of mtDNA replication might, in the end, just be a technical artifact. In addition, in the race to find the "right" mode of mtDNA replication, research on the strand-coupled/RITOLS models has accumulated a significant amount of supportive data, "passing" most (if not all) of the tests imposed by critics (Bogenhagen and Clayton, 2003a,b). The research has also gone beyond mammalian organisms, showing the possible conservation of mechanisms in birds (Reyes *et al.*, 2005) and in *Drosophila melanogaster* (Joers and Jacobs, 2013). Unfortunately for the field, researchers of the strand-displacement model have not maintained the same pace of investigations using *in vivo* data, which might compromise the credibility of the oldest model.

The mtDNA Replisome: The Tools to Use When Duplicating a Genome

All proteins involved in animal mtDNA maintenance are encoded by nuclear genes, and to date, only three of these proteins have been identified working at the mtDNA replication fork (Figure 3). Ahead of the fork, the replicative helicase Twinkle translocates on one DNA strand (5' to 3' direction), unwinding double-stranded DNA (dsDNA) into single-stranded DNA (ssDNA). DNA polymerase γ (pol γ) can then perform DNA synthesis *per se*, using as a template the ssDNA released by Twinkle. This ssDNA is also bound to the mitochondrial single-stranded DNA-binding protein (mtSSB), which protects it from nucleolysis, without any associated catalytic activity. Furthermore, replication does not appear to be primed by RNA derived from a dedicated primase (see discussion below), but instead by extension of processed RNA transcripts laid down by the mitochondrial RNA polymerase (mtRNApol) (Reyes *et al.*, 2013). [Technically, there is no experimental evidence showing that mtRNApol is part of the mtDNA replisome and moves along with the replication fork, but we have included this enzyme here due to its increasingly important roles in mtDNA replication]. Interestingly, sequence and structural analyses indicate that the mtDNA replisome is a "chimeric machinery", consisting of proteins of different evolutionary origins. Twinkle, the catalytic subunit of pol γ (pol γ-α) and mtRNApol share ancestry with the gp4 primase-helicase, the gp5 DNA polymerase and the gp1 RNA polymerase from the bacteriophage T7, respectively, whereas mtSSB is a homologue of the homotetrameric SSBs from eubacteria (Shutt and Gray, 2006a). The accessory subunit of pol γ (pol γ-β) appears exclusively in the metazoan lineage and has clearly evolved from the class II aminoacyl-tRNA synthetases of eubacteria (Fan *et al.*, 1999, 2006). How these proteins of originally distinct functions have evolved to participate in mtDNA replication
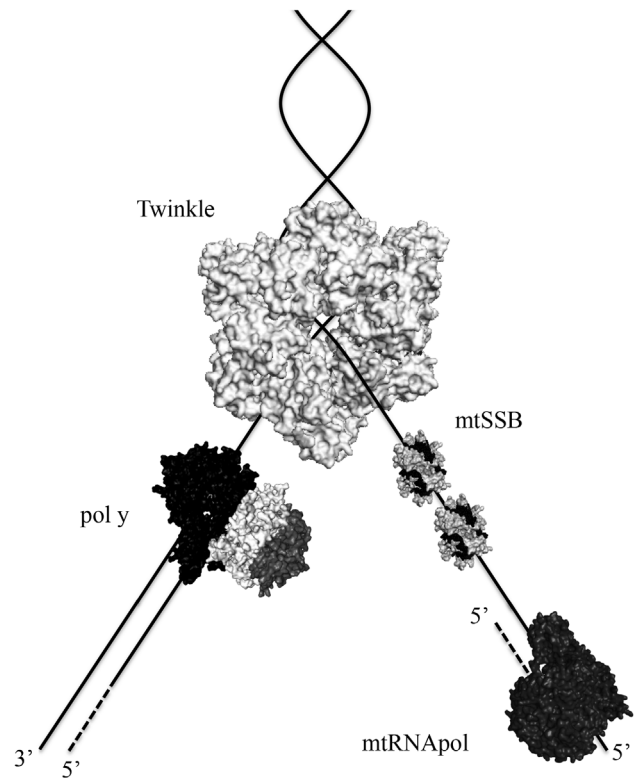

Figure 3 - Proteins at the human mtDNA replication fork. The schematic representations of the heterotrimeric pol γ, the homotetrameric mtSSB and the monomeric mtRNApol were created based on the crystal structure files (accession numbers 3IKM, 3ULL and 3SPA, respectively) deposited in the RCSB Protein Data Bank. For Twinkle, the crystal structure of the homohexameric *Bacillus stearothermophilus* DnaB (4ESV) was used, although the oligomeric state of Twinkle appears to shift from a homohexameric to a homoheptameric conformation upon interactions with cofactors (Ziebarth *et al.*, 2010). For mtSSB, two tetramers are represented wrapped around ssDNA. Technically, there is no experimental evidence showing that mtRNApol is part of the mtDNA replisome or that it moves along with the replication fork. Our intention here is to illustrate the possible roles of mtRNApol as the mtDNA primase. The diagram is not to scale, nor is it meant to depict protein or DNA structure, or specific protein-protein interactions. Solid lines represent DNA, and dashed lines represent RNA.

is still unclear, although the functional rewiring of ancestral proteins appears to have happened frequently in mitochondrial history (Shutt and Gray, 2006a; Camara *et al.*, 2011).

Twinkle, pol γ-α and pol γ-β have gained a lot of interest recently because of the discovery that mutations in these genes are associated with the outcome of human diseases (Table 1), such as progressive external ophthalmoplegia (PEO), Alpers' syndrome, ataxia-neuropathy disorders, among others (reviewed in Euro *et al.*, 2011; Martin-Negrier *et al.*, 2011; Stumpf *et al.*, 2013). The Human DNA Polymerase Gamma Mutation Database shows approximately 200 mutations in the gene coding for pol γ-α and 9 in the gene coding for pol γ-β (also known as *POLG1* and *POLG2* genes, respectively); these were found in patients with diagnosed mitochondrial disease or who presented symptoms suggestive of mitochondrial disease. Pol

Table 1 - Mutated mtDNA replication genes and associated human mitochondrial disorders.

Disorders*	Mutated gene	Frequent clinical symptoms
Alpers' syndrome	*POLG1* (pol γ-α)	Progressive spastic quadriparesis, cerebral degeneration, seizures, blindness, deafness, death before 42 months of age
Ataxia-neuropathy syndrome	*POLG1* (pol γ-α)	Peripheral neuropathy, cognitive impairment, involuntary movement, psychiatric symptoms, seizures
Autosomal dominant/ recessive PEO	*POLG1* (pol γ-α) *POLG2* (pol γ-β) *C10orf2* (Twinkle)	Bilateral ptosis, weakening of eye muscle, wasting, exercise intolerance

*Mutations in the *POLG1* gene have been associated with many diseases which are not listed here. For a more detailed list of these diseases, please refer to Stumpf and Copeland (2011) and Cohen and Naviaux (2010).

γ-α, which contains the enzyme's 5'-3' polymerase, 3'-5' exonuclease and 5' deoxyribose-5-phosphate lyase activities, is the most studied polypeptide of the mtDNA replisome at the clinical, biochemical and structural levels. The mutations found in *POLG1*, are nearly uniformly distributed along the length of the gene (Stumpf and Copeland, 2011), but they do tend to cluster within distinct functional modules in the tridimensional structure of the enzyme, at least for the mutations associated with the severe Alpers' syndrome (Euro *et al.*, 2011). Interestingly, Alpers patients typically present compound heterozygosity, but there has not yet been a case in which a combination of two mutations from the same functional module was found. The understanding of the spatial orientation of mutated residues in pol γ-α structure allows, for the first time, that computational predictions can be made to support the pathogenic role of newly discovered pol γ variants (Euro *et al.*, 2011).

In addition to its role in human diseases, pol γ has also recently been implicated in aging. A mouse model expressing a proofreading-deficient pol γ-α (the "mtDNA mutator" mouse), which has a D257A substitution in its exonuclease domain, showed increased mtDNA mutation rates and clear mitochondrial defects, as predicted (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Vermulst *et al.*, 2007, 2008; Edgar and Trifunovic, 2009). Surprisingly, the most pronounced phenotypes of these mice were reduced longevity and premature aging, characterized by a loss of weight (reduction in subcutaneous fat content and in whole-body bone mineral density and content), kyphosis, alopecia, anemia, infertility, hearing loss and reduced hair density, detected at a young age (24-25 weeks). Although it appears that the mtDNA mutator mouse does not have increased levels of reactive oxygen species (ROS) due to its mitochondrial dysfunctions, this animal model clearly establishes the role of mtDNA mutations as a cause of aging and age-related diseases. Mitochondrial ROS are generated when the transport of electrons in the respiratory chain is interrupted (by chemical inhibition or mutations in subunits of the OXPHOS complexes), causing a leak of these electrons, which will consequently react directly with molecular oxygen, creating highly reactive molecules that have DNA, lipid and protein damaging properties. This chain of events

is part of the foundation of the mitochondrial theory of aging; data from the mutator mouse suggests that this theory needs some reevaluation to accommodate the idea that mtDNA mutations might be involved with other aging mechanisms, and not with elevated ROS production (reviewed in Bratic and Larsson, 2013).

Mutations in the human Twinkle gene (*C10orf2*) were for the first time reported in 2001 as a cause of autosomal dominant PEO (Table 1), associated with multiple mtDNA deletions (Spelbrink *et al.*, 2001). It was not until 2003-2004 that biochemical studies showed the NTPase, 5'-3' ssDNA translocase and dsDNA unwinding activities of the encoded enzyme, establishing it as the replicative mtDNA helicase (Korhonen *et al.*, 2003, 2004). Combined with mutagenesis studies that demonstrated the roles of conserved residues in the Walker A and Walker B motifs and identified the arginine finger of Twinkle (Matsushima and Kaguni, 2007; Matsushima *et al.*, 2008), the discovery of the abovementioned enzymatic activities related to helicase function is not as surprising as the fact that Twinkle does not contain primase activity. The sequence similarities between Twinkle and the bifunctional gp4 primase-helicase of bacteriophage T7, which suggest that these enzymes share ancestry, are most pronounced in the C-terminal helicase domain (Spelbrink *et al.*, 2001). The N-terminal "primase"-like domain of the human Twinkle (and all other vertebrates) has diverged dramatically, losing nearly all conserved residues important for synthesis of RNA primers (Shutt and Gray, 2006b) and, most importantly, showing no detectable primase activity *in vitro* (Farge *et al.*, 2008). Even the *Drosophila melanogaster* Twinkle, which unlike its vertebrate homologues, retained most of the conserved cysteines found in the primase domain of T7 gp4 (Shutt and Gray, 2006b), does not appear to need these residues during mtDNA replication in cultured S2 cells (Matsushima and Kaguni, 2009).

These observations give us the opportunity to speculate on the roles of the N-terminal "primase"-like domain of Twinkle and on the nature of the putative mtDNA primase. Because the N-terminus of Twinkle has the ability to bind ssDNA (Farge *et al.*, 2008) and this property is required for the recently and unexpectedly identified strand annealing

activity of the enzyme (Sen *et al.*, 2012), Twinkle may play a role in recombination-mediated replication initiation, as found in the brain and heart of mammalian mitochondria (Pohjoismaki *et al.*, 2009), or in replication fork regression during repair of damaged DNA replication forks (Cheok *et al.*, 2005). Additionally, the N-terminus might be important for some of the putative roles of Twinkle as the initiator of mtDNA replication (Jemt *et al.*, 2011; Milenkovic *et al.*, 2013). Therefore, the enzyme responsible for making the RNA primers which will be used by pol γ during DNA synthesis appears to be the only DNA-dependent RNA polymerase found in the mitochondrion, mtRNApol. *In vitro* studies showed that mtRNApol can synthesize short RNA primers for DNA synthesis of mtDNA lagging strand (Wanrooij *et al.*, 2008), but *in vivo*, evidence indicate that the RNA primers come from the processing of mature transcripts, including messenger and transfer RNAs, hybridized to the lagging-strand template (Reyes *et al.*, 2013). Regardless by which mechanism mtDNA is replicated, the need for mtRNApol in this process is crucial (for a more detailed review of the interface between transcription and replication in animal mitochondria, we recommend Kasiviswanathan *et al.*, 2012).

mtSSB finalizes our list of proteins to be addressed in this review, possibly with one of the most important roles at the mtDNA replication fork: coordinating interactions of ssDNA, pol γ and Twinkle. It is believed that ssDNA wraps around mtSSB, like the stitches of a baseball, with multiple points of interactions (Oliveira and Kaguni, 2011). In fact, disturbing some of the most conserved residues of the ssDNA-binding domain by alanine substitutions disrupted the ssDNA-binding properties of recombinant mtSSB, as expected (Farr *et al.*, 2004). Remarkably, these mutants were also defective in stimulating the DNA polymerase and exonuclease activities of pol γ. The ability of mtSSB to stimulate *in vitro* the intrinsic activities of pol γ and Twinkle has been shown in individual enzymatic assays (Farr *et al.*, 1999, 2004; Korhonen *et al.*, 2003; Oliveira and Kaguni, 2010, 2011) and in the replisome reconstitution assay (Korhonen *et al.*, 2004). Interestingly, the residues of mtSSB that appear to be important for pol γ and Twinkle stimulation are distinct, indicating that the protein interacts with its partners at the mtDNA replication fork via different mechanisms (Oliveira and Kaguni, 2011). That is somewhat understandable because in the mtSSB-Twinkle interactions, the unwinding of dsDNA by Twinkle releases ssDNA, allowing mtSSB binding; whereas in the mtSSB-pol γ interactions, mtSSB is already bound to ssDNA and needs to be displaced from it to allow pol γ access to the template. We can speculate that mtSSB coordinates the functions of these two enzymes, ensuring that the replication fork progresses smoothly during mtDNA duplication. Obviously, further biochemical and physiological data are warranted to test this hypothesis, and to show the possible

roles of mtSSB-pol γ interactions in human diseases, as we have proposed previously (Oliveira and Kaguni, 2011).

Concluding Remarks

The goal of the research efforts on mtDNA replication is to understand the basic cellular mechanism(s) that promote(s) duplication of this genome, ultimately providing insight into treatment options for human patients with mtDNA replication-related diseases. However, as research in this area advances, a complex and diverse picture of these mechanisms emerges, giving the impression that treatment is far from being achieved. Nevertheless, it is plausible to speculate that the diversity and complexity of current models for mtDNA replication may reflect the different modes that operate in different tissues/cell types, and represent adaptive processes to ensure appropriate mtDNA copy number and mitochondrial gene expression, and hence ATP production via OXPHOS. Therefore, these findings need to be taken into careful consideration when developing treatments. One may never have thought that describing the replication of a ~16 kb circular genome would apparently be so complicated, involve proteins of strange evolutionary origins, and yet be so important for the health of humans and other animals.

Acknowledgments

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Internet Resources

Human DNA Polymerase Gamma Mutation Database, http://tools.niehs.nih.gov/polg (April, 2013).

RCSB Protein Data Bank, http://www.rcsb.org/pdb/home/home.do (April, 2013).

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