Critical Reviews in Biochemistry and Molecular Biology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ibmg20

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To cite this article: Maria Falkenberg & Claes M. Gustafsson (2020): Mammalian mitochondrial DNA replication and mechanisms of deletion formation, Critical Reviews in Biochemistry and Molecular Biology, DOI: 10.1080/10409238.2020.1818684

To link to this article: https://doi.org/10.1080/10409238.2020.1818684

CRITICAL REVIEWS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY
https://doi.org/10.1080/10409238.2020.1818684

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Mammalian mitochondrial DNA replication and mechanisms of deletion formation

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ABSTRACT

Mammalian mitochondria contain multiple copies of a circular, double-stranded DNA genome (mtDNA) that codes for subunits of the oxidative phosphorylation machinery. Mutations in mtDNA cause a number of rare, human disorders and are also associated with more common conditions, such as neurodegeneration and biological aging. In this review, we discuss our current understanding of mtDNA replication in mammalian cells and how this process is regulated. We also discuss how deletions can be formed during mtDNA replication.

ARTICLE HISTORY
Received 7 February 2020
Revised 25 June 2020
Accepted 31 August 2020

KEYWORDS
Mitochondrion; DNA replication; deletion; DNA helicase; DNA polymerase

In human cells, mtDNA is a circular, double-stranded molecule of 16,569 bp (Figure 1). The genome harbors 37 genes, which give rise to 13 essential components of the oxidative phosphorylation system, as well as 2 ribosomal RNA (12S and 16S rRNA) and 22 transfer RNA molecules needed for mitochondrial translation. In total, mtDNA contains genes for ~1% of all proteins located in mitochondria (Calvo et al. 2016). All other proteins, including the enzymes involved in mtDNA maintenance, are encoded by the nuclear genome, translated in the cytoplasm, and transported into mitochondria (Gustafsson et al. 2016).

Due to different base compositions, the two strands of human mtDNA can be separated based on buoyant density. The strands are therefore referred to as the heavy (H) and light (L) strand, respectively (Figure 1; Berk and Clayton 1974). The genome includes a non-coding region (NCR) of about 1 kb. The NCR contains a number of conserved sequence elements, including two promoters, the light strand promoter (LSP) and the heavy strand promoter (HSP), which serve as starting points for polycistronic transcription of mtDNA. The origin for H-strand DNA replication (OriH) is also located in the NCR, whereas a second mitochondrial origin, devoted to L-strand DNA replication (OriL), is located within a tRNA cluster about 11,000 bp downstream of OriH (Gustafsson et al. 2016). The two origins divide mtDNA into two parts: the major and minor arcs (Figure 1).

Single mtDNA molecules are packaged into nucleoprotein complexes, denoted nucleoids (Satoh and Kuroiwa 1991; Alam et al. 2003; Bogenhagen DF et al. 2008; Farge and Falkenberg 2019). The main structural component of these nucleoids is Transcription Factor A mitochondrial (TFAM), a high mobility group-box protein also required for transcription initiation (Gustafsson et al. 2016). TFAM can bind, unwind, and bend DNA without sequence specificity, covering and compacting the entire mtDNA molecule (Kaufman et al. 2007; Kukat et al. 2011; Farge et al. 2012; Kukat et al. 2015). The degree of mtDNA compaction by TFAM regulates access to regulatory sequence elements and can thus influence levels of replication and transcription (Kaufman et al. 2007; Farge et al. 2014). In addition to TFAM, a number of other proteins also associate with the nucleoid, including metabolic enzymes, indicating a possible link between nucleoid function and metabolic activity (Wang and Bogenhagen 2006; He et al. 2012; Han et al. 2017).

Mitochondrial DNA replication factors

Mammalian mtDNA is replicated by a set of replication factors, which is distinct from those needed for DNA

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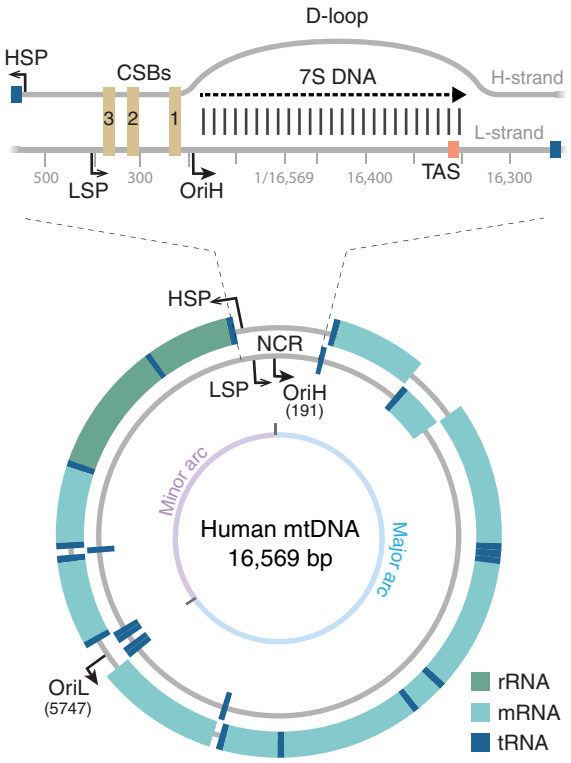

Figure 1. The human mitochondrial genome. The inner circle represents the L-strand and the outer circle the H-strand. An expanded version of the non-coding region (NCR) is shown at the top. The displacement loop (D-loop) region contains a third strand (7S DNA), which spans between OriH and the TAS regions. The minor and major arcs of mtDNA are indicated. Abbreviations. CSB: conserved sequence block; HSP: heavy-strand promoter; LSP: light-strand promoter; OriH: heavy-strand origin; OriL: light-strand origin; TAS: termination-associated sequence.

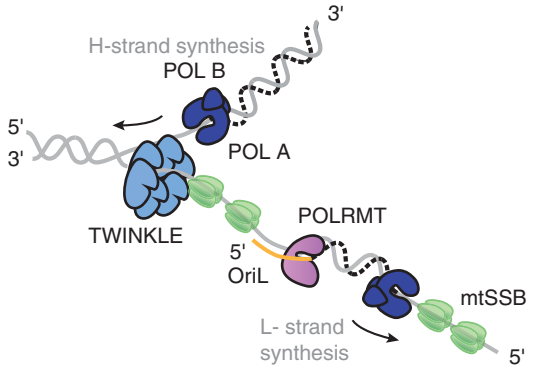

Figure 2. Schematic figure of the mtDNA replication fork. The TWINKLE DNA helicase (light blue) travels on the parental H-strand in the 5′ to 3′ direction while unwinding dsDNA. The mtSSB protein (green) binds to ssDNA and stimulates POLγ (dark blue)-dependent synthesis of the nascent H-strand. POLγ also performs L-strand DNA synthesis, using the displaced, parental H-strand as a template. POLRMT (purple) synthesizes the RNA primer (orange) needed for the initiation of L-strand synthesis at OriL. See the color version of this figure at www.tandfonline.com/ibmg

replication in the nucleus (Figure 2). DNA polymerase γ (POLγ) is essential for mtDNA maintenance and the sole polymerase responsible for both H- and L-strand DNA synthesis. The enzyme forms a heterotrimer with one catalytic subunit (POLγA) and two accessory subunits (POLγB; Gray and Wong 1992; Hance et al. 2005; Fan et al. 2006; Yakubovskaya et al. 2006; Humble et al. 2013). Interestingly, the structural composition of POLγ varies between eukaryotes. *Drosophila melanogaster* POLγ is a heterodimer of POLγA and POLγB, whereas the accessory subunit is missing in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Ravichandran et al. 2004; Fan et al. 2006).

POLγA has a molecular mass of 140 kDa and is a member of the family-A DNA polymerases, which also includes bacterial DNA polymerase I and the bacteriophage T7 DNA polymerase (Gustafsson et al. 2016). POLγA harbors a 3′ to 5′ exonuclease activity required for proofreading during DNA synthesis (Gray and Wong 1992) and the polymerase is highly accurate, with an error frequency of less than 1×10^{-6} per nucleotide (Longley et al. 2001). The accessory POLγB subunit is smaller, with a molecular mass of 55 kDa. The protein is structurally related to class IIa aminoacyl tRNA synthetases and most likely the result of a gene duplication event early in evolution. POLγB stimulates the catalytic activity and processivity of POLγA, by stabilizing interactions with template DNA (Carrodeguas et al. 2002).

In addition to POLγ, a number of other DNA polymerases have mitochondrial isoforms, including PrimPol, DNA polymerase β, DNA polymerase θ, and DNA polymerase ζ (Garcia-Gomez et al. 2013; Sykora et al. 2013; Singh et al. 2015; Wisnovsky et al. 2016). These additional polymerases are however not associated with DNA replication *per se*, but involved in different aspects of mtDNA repair. For a review on this matter, please see (Krasich and Copeland 2017).

POLγ requires the help of the replicative DNA helicase TWINKLE to unwind dsDNA at the replication fork (Figure 2; Korhonen et al. 2004). The helicase forms a hexamer with a molecular mass of 420 kDa and requires a fork structure to initiate unwinding in the 5′ to 3′ direction (Spelbrink et al. 2001; Korhonen et al. 2003; Korhonen et al. 2008). TWINKLE is similar in structure and sequence to the T7 phage gene 4 protein (Spelbrink et al. 2001), comprising a helicase and primase domain joined by a flexible linker region. In TWINKLE, the primase domain is nonfunctional and primers required to initiate mtDNA synthesis are instead synthesized by the mitochondrial RNA polymerase (POLRMT; Chang and Clayton 1985; Tsurumi and Lehman 1990; Wanrooij et al. 2008). The single subunit enzyme is structurally similar to the phage T7 RNA

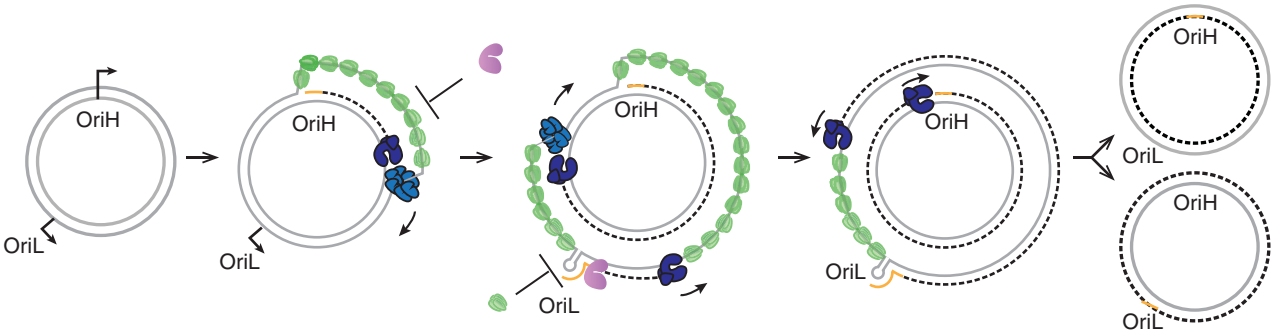

Figure 3. Strand-displacement mtDNA replication. Replication of the nascent H-strand is initiated at OriH and proceeds unidirectionally. In the process, the parental H-strand is displaced and bound by mtSSB (green), which prevents unspecific primer formation by POLRMT (purple). When the replication machinery reaches OriL, the H-strand of the origin folds into a stem-loop structure. POLRMT (purple) initiates RNA synthesis from the poly-dT stretch in the loop region, leading to the production of a short primer that is used to initiate L-strand DNA synthesis. The nascent L-strand is synthesized continuously until full-circle and two new full-length circular daughter molecules are formed. Please note that TWINKLE (light blue) is not needed for L-strand synthesis since the parental H-strand used as a template is already single-stranded. Synthesis of the daughter molecule containing the nascent H-strand is initiated and terminated at OriH, whereas synthesis of the other daughter molecule is initiated and terminated at OriL. See the color version of this figure at www.tandfonline.com/ibmg

polymerase, but the two enzymes differ in their mechanisms of transcription elongation, with the phage polymerase undergoing extensive, structural refolding during the transition to elongation (Gustafsson et al. 2016; Hillen et al. 2018). POLRMT displays low processivity on single-stranded DNA templates and only synthesizes short primers between 25–75 nt. In contrast, POLRMT displays strong processivity on dsDNA templates, allowing the formation of genomic length transcripts (Wanrooij et al. 2008).

During the progression of the replication machinery, the mitochondrial single-stranded DNA-binding protein (mtSSB) prevents the formation of secondary structures and blocks unwanted primer synthesis (see next; Mignotte et al. 1985; Tiranti et al. 1993; Miralles Fuste et al. 2014). The mtSSB protein forms a tetramer of 60 kDa, which stimulates mtDNA synthesis by increasing the helicase activity of TWINKLE and stimulating POLγ processivity (Farr et al. 1999; Korhonen et al. 2003; Korhonen et al. 2004). In contrast to TWINKLE and POLγ, mtSSB is not structurally related to its phage T7 counterpart, but instead resembles the ssDNA-binding protein (SSB) present in *Escherichia coli* (Lohman and Ferrari 1994). The precise mode of mtSSB binding is still under investigation. Conflicting reports have provided evidence for both cooperative and non-cooperative binding (Wong TS et al. 2009; Miralles Fuste et al. 2014; Qian and Johnson 2017; Kaur et al. 2018).

Mechanisms of mtDNA replication in mammalian cells

The strand displacement model for mtDNA replication was presented by Jarome Vinograd and colleagues in 1972 (Robberson et al. 1972). According to their model, both L- and H-strand DNA synthesis proceeds continuously and no Okazaki-fragment-like replication products are formed (Tapper and Clayton 1981). Replication of mtDNA is initiated from two dedicated origins of replication, OriH, and OriL (Figure 3). Replication first begins at OriH, and in the initial phase, H-strand DNA synthesis proceeds without simultaneous L-strand synthesis. During this step, TWINKLE moves on the parental H-strand in front of POLγ, with mtSSB binding and protecting the displaced, parental H-strand (Fuste et al. 2010; Miralles Fuste et al. 2014). When H-strand DNA synthesis has progressed for about 11 kb, the replication machinery passes OriL. At this point, the parental H-strand of the origin becomes single-stranded and folds into a stem-loop structure (an 11 bp stem and a 12 nt loop). The loop region contains a stretch of dT-residues, which serves as a starting point for primer synthesis by POLRMT. After initiation, primer synthesis continues for about 25 nt. POLRMT is next replaced by POLγ and L-strand DNA synthesis can commence (Martens and Clayton 1979; Fuste et al. 2010).

Once initiated, nascent H- and L-strand synthesis continue until the two replication events have reached full circle. It is worth noting that the mechanisms of H- and L-strand DNA replication are distinctly different (Figure 3). H-strand DNA synthesis uses dsDNA as a template and requires TWINKLE for duplex unwinding and fork progression. In contrast, L-strand DNA synthesis is independent of TWINKLE, since the template used is the single-stranded parental H-strand. No evidence has been presented for a physical link between the DNA polymerases working on the H- and L-strand. There is however a clear, functional connection

between replication of the two strands since H-strand DNA synthesis is required for activation of OriL. Interestingly, 2D agarose gel electrophoresis (2D-AGE) analyses have also indicated that the H-strand replication machinery pauses in the vicinity of OriL (Bailey et al. 2009). The reason for this effect is not known, but it is possible that the machinery pauses to ensure proper OriL activation and initiation of L-strand DNA synthesis, before continued progression of H-strand synthesis. The mechanisms of the observed pausing and what triggers the release of the paused replication machinery, remain to be elucidated.

Human POLRMT can initiate primer synthesis on random ssDNA using ATP as the initiating nucleotide (Wanrooij et al. 2008). During strand displacement replication, this activity is efficiently blocked by mtSSB, which covers the displaced parental H-strand and prevents unspecific primer formation (Figure 3). When OriL is activated, the double-stranded stem prevents mtSSB from binding. In addition, the single-stranded loop region is too short for mtSSB binding, but sufficiently long for transcription initiation by POLRMT, allowing primer formation and origin activation (Fuste et al. 2010; Miralles Fuste et al. 2014). The OriL structure is conserved in most vertebrates, with the possible exception of birds and reptiles. *In vivo* saturation mutagenesis has demonstrated that the origin is essential for mtDNA maintenance in the mouse (Wanrooij S et al. 2012).

By preventing nonspecific primer formation outside OriL, mtSSB efficiently restricts the initiation of L-strand mtDNA synthesis to OriL. In support of this notion, analysis of *in vivo* occupancy has demonstrated that outside the D-loop, mtSSB only binds to the H-strand. The pattern of mtSSB binding, therefore, correlates with what would be predicted by the strand displacement mode of mtDNA synthesis (Miralles Fuste et al. 2014). The mechanisms of POLRMT recruitment to OriL during activation are not known. In many other systems, a primase is constantly needed at the replication fork, where it initiates primer synthesis for each Okazaki fragment synthesis. At the *E. coli* replication fork, the primase (DnaG) interacts and travels together with the replicative helicase (DnaB; Lewis et al. 2016). If POLRMT is recruited separately to the activated OriL, or if the protein is a component of the replisome during H-strand replication, has not been addressed.

Alternative modes of mtDNA replication

Alternatives to the strand displacement mode of mtDNA replication have been reported in the literature. The RITOLS model (ribonucleotide incorporation throughout the lagging strand) is very similar to strand displacement replication but suggests that processed RNA molecules (rRNA, tRNA, and mRNA with poly A tails) cover the displaced, parental H-strand (Figure 3), forming a provisional lagging strand during mtDNA replication. The RITOLS model is primarily based on replication intermediates observed in 2D-AGE experiments (Yasukawa et al. 2006; Reyes et al. 2013). The authors of this review remain skeptical of the RITOLS model. Under normal circumstances, processed RNA molecules are folded, modified, and bound to proteins and no molecular machinery has been identified that explains how processed transcripts can be threaded onto displaced, parental H-strand during mtDNA replication. In addition, the existence of RNase H1 in mammalian mitochondria, an enzyme which actively degrades RNA molecules hybridized to ssDNA, argues against the use of RNA to stabilize ssDNA regions (Cerritelli et al. 2003; Cerritelli and Crouch 2009; Holmes et al. 2015; Al-Behadili et al. 2018; Posse et al. 2019). Finally, there are high levels of mtSSB in mammalian mitochondria and therefore no need for alternative modes of ssDNA protection, distinct from what is seen in many other systems (Miralles Fuste et al. 2014; Yao and O'Donnell 2016). In our view, RITOLS remains an unproven hypothesis until firm biochemical evidence is provided for the individual reaction steps of this model. For an alternative view, we refer to a review written by proponents of the RITOLS model (Holt and Jacobs 2014).

A third, strand-coupled model for mtDNA replication has been proposed to explain observations in certain cell-types and conditions (Holt et al. 2000). This model implies that L-strand DNA synthesis is initiated at multiple places on the parental H-strand and that the nascent L-strand synthesis is synthesized as shorter fragments that are ligated to generate a continuous strand. The model resembles conventional DNA replication seen in many other systems, with the notable exception that there are no indications of a physical link between POLγ working on the H and L-strands. The model is based on the observation of shorter, Okazaki-like replication intermediates in certain cell-types, an observation that does not necessarily contradict the strand-displacement model (Wanrooij et al. 2008; Miralles Fuste et al. 2014). POLRMT only requires a short T-stretch on ssDNA to initiate primer synthesis. Under normal conditions, mtSSB restricts primer formation to OriL (Figure 3), but if the relative levels of mtSSB decrease or the replication machinery stalls, POLRMT could potentially gain access to the parental H-strand and prime DNA synthesis from sites outside OriL. In fact, both 2D-AGE and atomic force microscopy have provided evidence for alternative origins of L-strand mtDNA synthesis

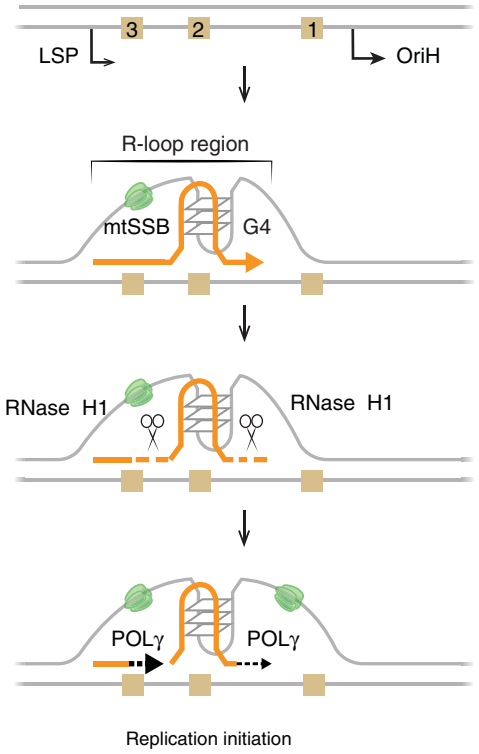

Figure 4. A schematic model for replication initiation at OriH. POLRMT initiates transcription at LSP. During transcription (yellow line) of the G-rich CSB2 region, a hybrid G-quadruplex (G4) structure is formed between RNA and the non-template H-strand. The G4 structure anchors the RNA to DNA, forming a stable R-loop. The G4 structure also causes premature transcription termination immediately downstream of CSB2 (yellow arrow). The RNA 3′ end in the nascent R-loop is not accessible to POLγ. To act as a primer, the R-loop needs to be processed by RNase H1 (scissors) This generates 3′-ends (yellow dot lines), from which POLγ can initiate DNA synthesis (black dot arrows). Both R-loop formation and DNA replication initiation are stimulated by mtSSB (green). Light brown squares; CSB 1, 2, and 3. See the color version of this figure at www.tandfonline.com/ibmg

(Brown TA et al. 2005; Pohjoismaki et al. 2011; Torregrosa-Munumer et al. 2019). If correct, one could speculate that loss of mtSSB would stimulate initiation from alternative origins. Interestingly, disease-causing mutations have recently been identified in the gene coding for mtSSB. The mutant proteins bind ssDNA less efficiently and mtDNA is depleted in affected individuals (Gustafson et al. 2019; Piro-Megy et al. 2019; Del Dotto et al. 2020) An in-depth analysis of replication intermediates in patient-derived cell lines may provide important insights about possible priming outside the OriL region.

Initiation of mtDNA replication at the origin of H-strand DNA replication

Transcription initiated from LSP does not only produce genomic-length transcripts, but also RNA molecules used as primers for initiation of H-strand DNA synthesis (Figure 4; Gillum and Clayton 1979; Chang and Clayton 1985; Chang et al. 1985). Primer formation and the mechanisms that regulate the switch to genomic length transcription are still under intense investigation (Agaronyan et al. 2015; Posse et al. 2015; Jiang et al. 2019; Posse et al. 2019).

Early studies of OriH-dependent initiation in mitochondrial extracts identified an R-loop (a triple-stranded structure with nascent RNA forming a stable hybrid with template DNA) in the region immediately downstream of LSP (Xu and Clayton 1995). The structure was linked to the formation of primers required for replication initiation and it was proposed that the R-loop was cleaved by a nuclease, to create the free 3′-OH end needed for initiation of replication at OriH. Initially, RNase MRP was identified as the responsible nuclease (Chang and Clayton 1987; Topper et al. 1992; Lee and Clayton 1997, 1998), but later studies refuted this idea, based on the lack of RNase MRP within the mitochondrial matrix (Kiss and Filipowicz 1992; Kiss et al. 1992)

R-loop formation can be reconstituted *in vitro*. On a negatively supercoiled template, the majority of all transcription events from LSP are prematurely terminated after ~120 nucleotides and the transcript produced remains stably bound to the DNA template (Posse et al. 2019). The region immediately downstream of LSP harbors three conserved sequence elements (CSB1-3) and at least one of these, CSB2, is required for R-loop formation. CSB2 is guanine-rich and during its transcription, RNA folds into a G-quadruplex structure together with non-template DNA. The hybrid G-quadruplex formed in this manner stably anchors the nascent transcript to mtDNA (Wong and Clayton 1985; Wanrooij PH et al. 2012). CSB2 also promotes premature transcription termination, since G-quadruplex formation at CSB2 coincides with the transcription of a poly-uracil sequence. In combination, the G-quadruplex structure and the weak adenine-uracil bonds in the RNA-DNA duplex lead to the dissociation of elongating POLRMT and premature termination of transcription just downstream of the CSB2 sequence (Pham et al. 2006). The structural basis for this effect has been explained in a structural study (Hillen et al. 2017).

In its native form, the mitochondrial R-loop cannot prime mtDNA synthesis, since the 3′-end of the RNA molecule is inaccessible (Posse et al. 2019). This observation revived the idea of an R-loop processing nuclease and led to the identification of RNase H1 as an essential component of the primer formation process. RNase H1 can cleave the R-loop both *in vitro* and *in vivo*, and the 3′-ends formed can be used to prime

POLγ-dependent mtDNA synthesis *in vitro* (Figure 4; Posse et al. 2019). In the reconstituted system, transitions from primer RNA to DNA synthesis cluster around CSB2 and CSB3, similar to what has been observed previously in cells (Xu and Clayton 1995; Kang et al. 1997; Pham et al. 2006). The initiation process is further stimulated by mtSSB. The molecular basis for the stimulation is not known, but single-stranded DNA-binding proteins can stabilize R-loops by binding to the single-stranded part of the structure, thereby lowering the probability of reannealing of complementary DNA-sequences (Sun et al. 2013).

Why have mitochondria evolved a complicated primer maturation process, involving an R-loop, which requires RNase H1 processing prior to initiation of mtDNA synthesis? To answer this question, we need to consider that replication initiation is a carefully regulated process that takes place from specific sequence elements, origins of replication. DNA polymerases can initiate DNA synthesis from any free 3′-OH ends located on an ssDNA template and to ensure origin-specific initiation of DNA replication it is therefore essential to have an RNase H-activity that removes nonspecific RNA molecules annealed to DNA. This point has been demonstrated in *E. coli* (Kogoma and von Meyenburg 1983; Ogawa et al. 1984). Bacterial chromosome replication is normally initiated at *oriC*. In the absence of active RNase H, this specificity is lost and initiation occurs from multiple other sites, all over the chromosome. Initiation at these alternative origins does not require the origin-binding protein, dnaA, which is needed for proper *oriC* activation. In fact, in a *rnh* (the gene encoding for RNase H) mutant background, *E. coli* can tolerate the deletion of oriC and complete inactivation of the *dnaA* gene. The role of RNase H is therefore to repress initiation from primers located outside the *oriC* region, leading to origin-specific initiation of DNA synthesis. The role of *E. coli* RNase H as a specificity factor for the initiation of DNA synthesis has also be reconstituted *in vitro* (Ogawa et al. 1984).

Similar to the situation in *E. coli*, mitochondrial RNase H1 represses the initiation of mtDNA synthesis from locations outside the OriH region (Posse et al. 2019). The enzyme efficiently removes RNA annealed to DNA throughout the mitochondrial genome. However, at OriH, the hybrid G-quadruplex structure formed renders part of the R-loop resistant to RNase H1 cleavage. The R-loop is therefore not completely removed, but partial cleavage leads to the formation of RNA 3′-ends that can be used to initiate mtDNA synthesis. In this way, RNase H1 can both repress nonspecific initiation and promote origin-specific initiation of mtDNA synthesis. The model receives support from observations in patient cells with reduced RNase H1 activity. In these cells, initiation of mtDNA replication is not restricted to OriH. Instead, RNA to DNA transitions takes place at multiple sites outside the CSB-region, not seen in normal, wild-type cells (Reyes et al. 2015; Posse et al. 2019).

R-loop formation and premature transcription termination can be strongly reduced by the mitochondrial transcription elongation factor (TEFM), which led to the suggestion that this factor could regulate primer formation (Agaronyan et al. 2015; Posse et al. 2015). According to this model, the presence of TEFM would stimulate full-length transcription, whereas the absence of TEFM would lead to transcription termination and primer formation at CSB2. Later studies have complicated this picture. As discussed above, transcripts formed by premature termination at CSB2 cannot be used directly to prime mtDNA replication, they must first be processed by RNase HI (Posse et al. 2019). Furthermore, in the mouse, loss of TEFM causes a dramatic increase of LSP proximal transcripts, terminating before the region where the switch from transcription to replication occurs. As a result, *de novo* replication is decreased, the exact opposite of what would be predicted from the proposed model (Jiang et al. 2019). Finally, when TEFM was monitored *in vitro* for effects on mtDNA replication initiation in the presence of RNase H1, the consequences were less dramatic. Equimolar levels of TEFM to POLRMT reduced initiation of mtDNA replication but did not abolish the reaction (Posse et al. 2019). Further studies are needed to determine if the levels of active TEFM actually vary in response to physiological requirements and if mild changes in TEFM concentrations, in turn, influence the relative levels of mtDNA replication *in vivo*.

Displacement loop formation

Once DNA synthesis has been initiated at OriH, only a small fraction of all replication events continue to full-length mtDNA (Figure 1, top panel). Instead, about 95% of all replication events are terminated already after about 650 nt, forming what is known as 7S DNA (not to be confused with 7S RNA, see below; Nicholls and Minczuk 2014). The 3′-end of the 7S DNA is located in a region with conserved secondary elements, known as the termination associated sequences (TAS; Bogenhagen and Clayton 1978; Doda et al. 1981). Once formed, the 7S DNA molecule can remain hybridized to the NCR, forming a triple-stranded displacement loop (D-loop).

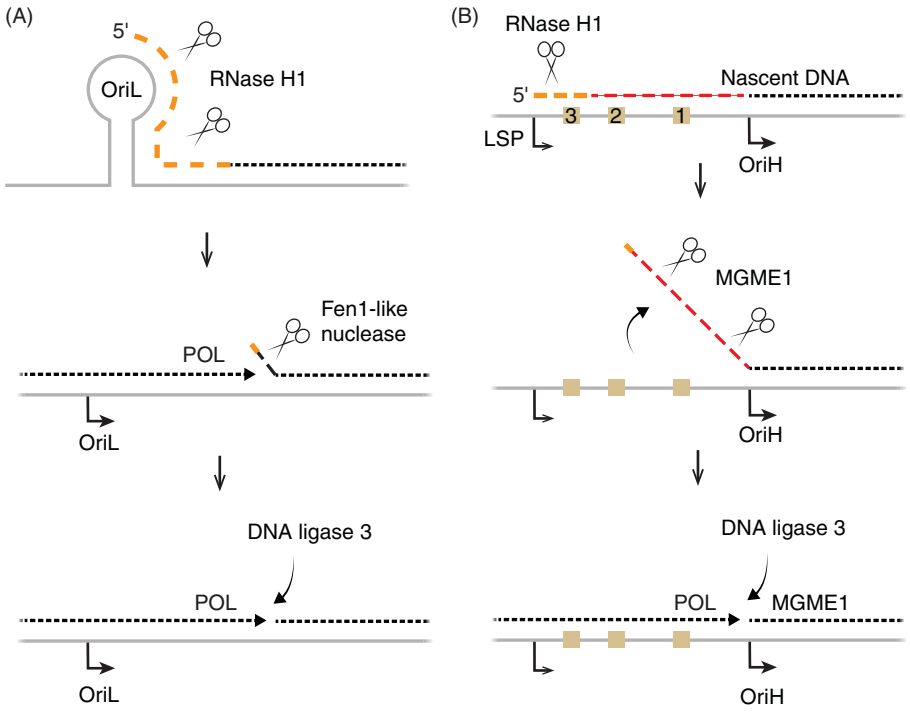

Figure 5. Primer removal at OriH and OriL. (A) At OriL, the RNA primer (~25 nt, dashed orange line) is removed by RNase H1 leaving behind 1–2 ribonucleotides attached to the 5′-end of the nascent L-strand. Replicating POLγ displaces the 5′-end during the completion of L-strand DNA synthesis, and the last ribonucleotides are removed by a FEN1-like activity. Once a ligatable nick is produced, it is sealed by Ligase 3. (B) At OriH, the 5′-end of the nascent H-strand is processed and shifted from CSB3/CSB2 to OriH at position 191. In the process, primer RNA and a stretch of nascent DNA (~100 nts) are removed. The 5′-end maturation process is independent of genome length mtDNA synthesis, since the 5′-end of the short 7S DNA is also processed. During primer removal, RNase H1 removes the primer (dashed orange line), leaving behind 1–2 ribonucleotides attached to the 5′-end of the nascent H-strand. In the next step, MGME1 removes these remaining ribonucleotides together with a stretch of nascent H-strand DNA (dashed red line). MGME1 works on a single-stranded DNA flap, but how the flap structure is formed is not known. See the color version of this figure at www.tandfonline.com/ibmg

The precise role of the D-loop in mtDNA maintenance is not understood, but there is evidence suggesting that termination at TAS may control the relative levels of abortive versus full-length mtDNA replication (Brown GG et al. 1986; Pereira et al. 2008; Jemt et al. 2015). A clue to the mechanisms governing D-loop formation came with the identification of two closely related 15 nt palindromic sequence motifs ($ATGN_9CAT$), which are found at the 5′ and 3′ borders of the triple-stranded region. The motifs are evolutionary conserved and the one coinciding with the 3′-end of 7S DNA is referred to as core-TAS, since it is located within the TAS region (Figure 1, top panel; Jemt et al. 2015). The element at the 5′-border corresponds to CSB1. The role of the $ATGN_9CAT$ motifs is not known, but it is possible that they function as binding motifs for some type of sequence-specific DNA binding activity. *In organello* footprints in the core-TAS region have been reported (Roberti et al. 1998), but in spite of considerable efforts, we have failed to isolate a protein binding to the $ATGN_9CAT$-element. Perhaps binding to these sites is a regulated event and specific signals or conditions are required. Alternatively, the two palindromic sequences may take part in the formation of some sort of secondary structure, which in turn influences the formation of 7S DNA. More work is required to elucidate the significance of the $ATGN_9CAT$ motifs.

Termination of mtDNA replication

Once mtDNA replication has been completed the two daughter molecules need to be properly ligated and separated. Before ligation, RNA primers used during the initiation of mtDNA synthesis are removed and any gaps in the nascent DNA chain are filled by POLγ. Primer removal has been studied at both OriH and OriL. Interestingly, the mechanisms and factors required for this process differ between the two origins (Figure 5; Bailey et al. 2009; Holmes et al. 2015; Uhler et al. 2016; Al-Behadili et al. 2018).

Primers used to initiate DNA synthesis at OriL are processed by RNase H1. The enzyme cleaves the RNA primer, removing nearly the entire stretch of RNA from the polydT stretch in the loop region to the transition point between RNA and DNA at the base of the stem (Figure 5(A)). Consequently, primer maturation at OriL is impaired in conditional *Rnaseh1* knockout mouse embryonic fibroblasts (Holmes et al. 2015). On its own, RNase H1 is not sufficient for complete primer removal, since the enzyme leaves 1–3 ribonucleotides at the RNA to DNA junction (Lima et al. 2007). These last remaining ribonucleotides are problematic since they block ligation by mitochondrial Ligase 3 and an additional nuclease activity is therefore required (Al-Behadili et al. 2018). The missing nuclease could potentially be a flap-endonuclease, since POLγ normally synthesizes a few nucleotides into duplex regions, causing the formation of a flap substrate. In support of this notion, the nuclear flap endonuclease 1 (FEN1) can assist RNase H1 to produce ligatable ends at OriL in a reconstituted *in vitro* mtDNA replication system. It is however unlikely that FEN1 is responsible for this activity *in vivo*, since published studies argue against the existence of active FEN1 in mitochondria (Uhler and Falkenberg 2015) and have also failed to observe effects of FEN1 depletion on OriL-ligation in cells (Al-Behadili et al. 2018). Instead, an alternative nuclease was recently suggested to play a role in primer maturation, EXOG. This protein has a clear mitochondrial localization and can efficiently remove the last ribonucleotides of the OriL primer *in vitro* (Wu et al. 2019). However, *in vivo* evidence for the role of EXOG at OriL is still missing and in unpublished experiments, we have failed to observe the formation of ligatable products when RNase H1 and EXOG are combined *in vitro*. More work is therefore needed and primer processing at OriL is not yet a resolved issue.

Primer removal at OriH is an even more complicated process (Figure 5(B)). As mentioned, the transition from RNA to DNA at OriH takes place in the CSB2/CSB3 region (Figure 4). The free 5′-end of nascent H-strand DNA is however located more than 100 nt further downstream (Clayton 1991). One major free 5′-end is located at position 191. There are also a number of additional free 5′-ends further downstream (including positions 168, 151 and 110). This discrepancy suggests that primer processing not only removes the RNA part, but also a considerable stretch of nascent H-strand DNA (Kang et al. 1997; Fish et al. 2004; Yasukawa et al. 2005; Pham et al. 2006).

Two nucleases have been identified as necessary for primer removal and nascent H-strand processing at OriH–RNase H1 and MGME1. It has been proposed that RNase H1 processes the initial part of the primer, spanning LSP to the CSB2-region, whereas the MGME1 nuclease removes the remaining part, including a stretch of nascent H-strand DNA (Kornblum et al. 2013; Uhler et al. 2016). MGME1 is a member of the RecB family and the enzyme displays a strong preference for ssDNA, cleaving both 5′ and 3′ flaps. MGME1 can also process chimeric RNA-DNA flaps, but only if the flap is sufficiently long for MGME1 to initiate DNA degradation at a position 2–5 nt downstream from the junction between RNA and DNA (Kornblum et al. 2013; Uhler et al. 2016). This substrate requirement helps to explain why MGME1 is unsuited to assist RNase H1 in processing at OriL. Loss-of-function mutations in the *MGME1* gene cause mitochondrial disease. In support of the proposed function of MGME1, the 5′-ends of 7S DNA are moved further upstream, toward CSB2, in cell lines derived from these patients (Kornblum et al. 2013; Szczesny et al. 2013; Nicholls et al. 2014).

To function, MGME1 needs a single-stranded substrate and the nascent H-strand must therefore be displaced from the template strand in order to be cleaved (Figure 5(B)). How the strand displacement takes place is not known, but there are a number of helicases in the mitochondrial matrix which are possible candidates for this reaction (Calvo et al. 2016). TWINKLE is not one of these candidates, since it requires a distinct substrate with a stretch of 10 nt of ssDNA on the 5′-side of a duplex to initiate unwinding (Korhonen et al. 2003). The mitochondrial transcription machinery could potentially also displace part of the nascent H-strand. Normally, the upstream region of the transcription bubble is reannealed once POLRMT has passed, but when a triple-stranded region is transcribed, this is not necessarily the case. Therefore, as POLRMT moves from LSP toward OriH during active transcription, the 5′-region of the nascent H-strand may be displaced and the two parental strands reanneal behind the transcription machinery. In support of this idea, an abundant, non-coding transcript denoted 7S RNA is produced by transcription from LSP to CSB1 and the synthesis of this transcript could potentially cause H-strand displacement and the formation of a suitable substrate for MGME1 processing (Amalric et al. 1978; Jemt et al. 2015).

Once a longer stretch of nascent H-strand has been removed, the DNA backbone needs to be ligated. MGME1 generates a pool of imprecisely cut products, often leaving short flaps or gaps in the H-strand, which cannot be directly sealed by mitochondrial Ligase 3. To create a perfectly aligned nick that can be efficiently ligated, POLγ must use its DNA polymerase and 3′–5′ exonuclease activities to extend or shorten the 3′-end of H-strand. During this process, POLγ works in concert with MGME1, with the polymerase displacing short

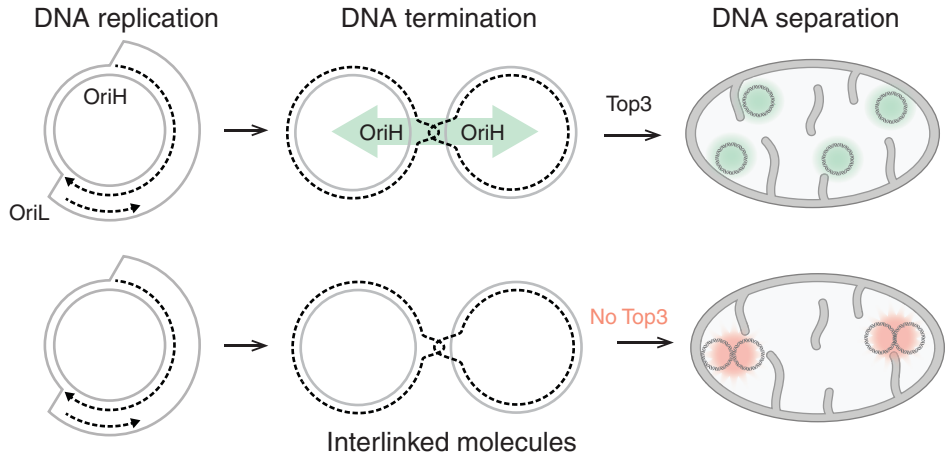

Figure 6. The role of TOP3A in the resolution of mtDNA molecules. Replication of circular mtDNA generates daughter molecules linked together by a hemicatenane structure in the OriH region. Interlinked daughter molecules are resolved by TOP3A (top panel). Lack of TOP3A activity causes the formation of catenated molecules that are unable to segregate correctly.

flaps into longer flaps that can be used for new rounds of MGME1 cleavage (Uhler et al. 2016).

Toposiomerases are required for mtDNA separation and replication fork progression

After the completion of mtDNA replication, the two daughter molecules must be separated and distributed within the mitochondrial network (Figure 6). Freshly replicated mtDNA molecules are resolved via the formation of a hemicatenane intermediate in the OriH region (Hudson and Vinograd 1967; Nicholls et al. 2018). This structure consists of two double-stranded DNA molecules associated with a single-stranded linkage, which in some systems can form from converging replication forks. How hemicatenane structures are produced during mtDNA replication remains to be established.

The newly replicated mtDNA molecules are decatenated by topoisomerase 3A (TOP3A), a type IA family topoisomerase (Nicholls et al. 2018). Disease-causing mutations in the *TOP3A* gene cause the formation of massive, catenated mtDNA assemblies, multiple mtDNA deletions, and symptoms of chronic progressive external ophthalmoplegia, a mitochondrial disorder also associated with mutations in other genes coding for mitochondrial replication factors. The nuclear isoform of TOP3A is a component of the BTR complex, which acts to resolve double Holliday junctions (Sarbajna and West 2014). The other subunits of the BTR complex (BLM, RMI1, and RMI2) are not present in mitochondria and do not affect mtDNA decatenation (Nicholls et al. 2018).

In addition to TOP3A, mitochondria also contain topoisomerase 1 mitochondrial (TOP1mt; Zhang et al. 2001), a type IB topoisomerase that acts to counteract positive supercoils produced during transcription or replication (Dalla Rosa et al. 2017). Mice lacking the *TOP1mt* gene are still viable (Douarre et al. 2012), but have changed levels of mtDNA supercoiling and symptoms of mitochondrial dysfunction (Kao et al. 1983; Zhang et al. 2014). The depletion of TOP1mt and TOP3A in combination causes a severe phenotype with a pronounced decrease in mtDNA levels (Nicholls et al. 2018).

Deletion formation in mitochondrial DNA

Deleted forms of mtDNA are associated with a number of different human diseases and are also formed in post-mitotic tissues during normal human aging (Rahman and Copeland 2019). In some diseases, for example, Kearns-Sayre Syndrome and Chronic Progressive External Ophthalmoplegia, deletions are formed sporadically. In other situations, deletions can be secondary to mutations in genes coding for proteins needed for mtDNA replication, for example., *POLG, POLG2, TWNK, DNA2, TOP3A,* and *MGME1*. The clinical symptoms associated with mtDNA deletions may vary from severe manifestations in childhood to mild symptoms in individual tissues late in life (Viscomi and Zeviani 2017). Cells contain multiple copies of mtDNA, with each cell containing hundreds to thousands of individual molecules and all mtDNA deletions are heteroplasmic, that is., they co-exist with wild type copies. A surprisingly high level of deletions can often be tolerated, but once pathogenic mtDNA molecules exceed a certain threshold, respiratory chain deficiency, and disease phenotypes develop (Hayashi et al. 1991).

The most frequent circular deletion in human cells causes the loss of an ~5.0 kb region between position

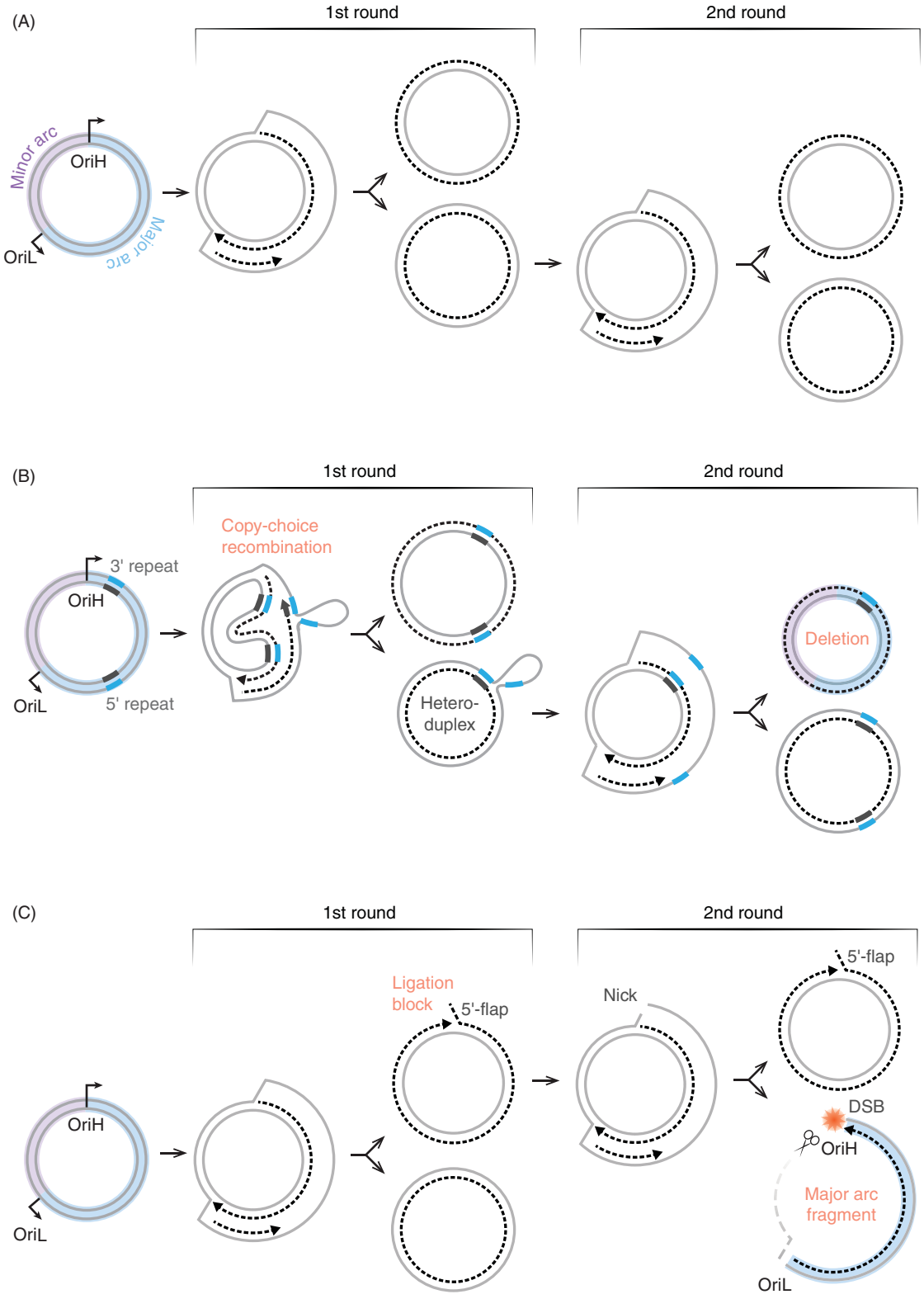

Figure 7. A model for how copy-choice recombination can lead to the formation of deleted mtDNA molecules. (A) Strand displacement of DNA replication. (B) Formation of circular deletions. During strand displacement DNA replication, repeat sequences in the parental H-strand are exposed. If POLγ dissociates from the template during replication of the first repeat, the 3′-end of the newly synthesized DNA may unpair from the template H-strand and mispair with a second repeat, located further downstream on the same strand. When POLγ resumes DNA synthesis from the mispaired 3′-end, the repeat closes to OriH is lost together with the intervening sequence. In this way, a heteroduplex molecule is formed, which when it is used as a template for the second round of mtDNA synthesis, generates one full length and one deletion-containing mtDNA molecules. Copy-choice recombination only requires the mitochondrial replication machinery and it is independent of repair mechanisms. The process may be stimulated by secondary structure elements in the H-strand or disease-causing mutations leading to reduced replication

8470 and position 13,447 in mtDNA. This "common deletion" is located between two direct repeats of 13 bp (ACCTCCCTCACCA), one of which is retained, whereas the other is lost during deletion formation (Schon et al. 1989). The common deletion leads to a depletion of essential mitochondrial genes, coding for both mRNAs and tRNAs. The deletion is seen in patients with mitochondrial myopathy, but it also accumulates in postmitotic tissues during normal aging (Cortopassi and Arnheim 1990).

How mtDNA deletions are formed has been debated, but the process requires active mtDNA replication (Phillips et al. 2017). Early work suggested a slipped-strand model, according to which the deletions are formed during H-strand DNA synthesis across the major arc (Shoffner et al. 1989). A second model proposed that double-stranded breaks followed by DNA repair could explain the formation of deletions. The second model receives support from the observation that transient expression of a mitochondria-targeted restriction endonuclease can cause double-strand breaks, which eventually results in deleted species resembling naturally-occurring mtDNA deletions (Srivastava and Moraes 2005). For the model to be correct, it requires some sort of double-strand break repair system, which remains to be identified. For a detailed discussion of double-stranded breaks and their role in mtDNA deletion formation, we refer to a recent review (Nissanka et al. 2019).

More recently, we have suggested that mtDNA deletions are formed via copy choice recombination during L-strand DNA synthesis (Figure 7(A,B)), a model similar to what has been observed in other systems, for example, *E. coli* (Persson et al. 2019). During lagging-strand DNA synthesis in bacteria, the template strand may form secondary structures that slow down or block DNA synthesis, which in turn can cause dissociation of the DNA polymerase. When this happens, the free 3′-end of the nascent, lagging strand may dissociate from the template strand and reanneal to a downstream region, of similar or identical sequence on the same template strand. When DNA synthesis resumes from the reannealed 3′-end, the intervening region is lost and deletion is formed. Copy-choice recombination can be used to explain the formation of the common deletion and other circular deletions observed in humans. In support of the model, bioinformatic analysis of deletions formed between imperfect repeats in patients has demonstrated the preferential retainment of the repeat closest to OriL, which is what would be expected if this is the 5′-repeat of the copy choice recombination event (Samuels et al. 2004; Persson et al. 2019). In addition, copy-choice recombination can be reconstituted *in vitro* using only purified mitochondrial proteins and a DNA template.

Copy-choice recombination explains why circular deletions are preferentially formed in the major arc. During strand-displacement mtDNA replication, a long stretch of the parental H-strand is present in a single-stranded conformation (Figure 7(A)). This constitutes a risk, since if POLγ stalls and dissociates during L-strand DNA synthesis, there are many opportunities for copy-choice recombination with downstream regions on the exposed H-strand (Figure 7(B)). Copy-choice recombination is stimulated by longer repeat sequences, but can also function between short repeats or even non-repeated sequences (Persson et al. 2019).

Since copy-choice recombination is a consequence of the temporary release of POLγ from the template, it is easy to understand why the process is stimulated by anything that impairs DNA replication processivity, for instance by mutations in proteins required for mtDNA replication or for maintaining correct mitochondrial nucleotide pools. Lower nucleotide pools will lead to lower processivity and a higher risk of dissociation. The model explains why pathogenic mutations in various replication factors, such as POLγ, TWINKLE, TOP3A, and the nucleotide transporter, ANT1, can all enhance mtDNA deletion formation in affected patients (Kaukonen et al. 2000; Spelbrink et al. 2001; Van Goethem et al. 2001; Nicholls et al. 2018).

Linear fragment formation in mitochondrial DNA

In addition to circular deletions, linear mtDNA deletions have also been observed. Mutations in the gene coding for the MGME1 nuclease leads to the formation of a

processivity. (C) Formation of linear fragments during mtDNA synthesis. Disease-causing mutations in MGME1 or loss of the POLγ 3′ to 5′ exonuclease activity can impair the formation of ligatable ends at OriH, leaving a nick in the H-strand (1st round). During the next round of mtDNA replication (2nd round), the initial phase of H-strand synthesis can proceed undisturbed, since the template L-strand is intact. However, DNA synthesis initiated at OriL will use the nicked H-strand as template and replication will therefore be prematurely terminated near OriH. As a result, a linear, deleted fragment will be formed. The ssDNA region of the fragment will be degraded, leaving a linear double-stranded product covering the entire major arc (right lower panel). Failure to ligate at OriH will therefore lead to the formation of two different replication products. One linear, double-stranded fragment and one circular mtDNA molecule with a nick at OriH. If the nicked molecule is used for a new round of mtDNA replication, the same two products may form again. Abbreviations: DSB, double-strand break.

linear mtDNA fragment, which corresponds to the region between OriH and OriL (Figure 7(C), major arc fragment; Nicholls et al. 2014; Matic et al. 2018). Since the fragment is linear and lacks origins, it cannot replicate and must be constantly produced *de novo*. A similar type of linear fragment is also produced in a mouse expressing a mutant form of POLγ, lacking the 3′ to 5′ exonuclease activity (Trifunovic et al. 2004; Macao et al. 2015).

The formation of the linear, deleted form of mtDNA can also be explained by the strand displacement mode of mtDNA replication. As discussed above, the MGME1 nuclease activity and the exonuclease activity of POLγ work together to produce ligatable nicks during the termination of H-strand DNA replication (Uhler et al. 2016). Loss of either activity, therefore, risks impairing H-strand ligation near OriH. A nick in the H-strand does not present a problem for new rounds of H-strand synthesis, since the template L-strand is unaffected. The nick will however be a problem for replication initiated at OriL. Once L-strand DNA synthesis initiated at OriL reaches the nick in H-strand DNA, a double-strand break will be formed. The break will cause the formation of a linear mtDNA fragment, which spans from OriH and OriL. This model, based on the strand displacement mode of mtDNA replication, may explain why mutations affecting either MGME1 or the POLγ 3′ to 5′ exonuclease activity generates a similar, linear mtDNA fragment *in vivo*.

Conclusions

In this review, we have tried to convey our current understanding of the mtDNA replication machinery, both its normal function and its role during the formation of deletions associated with human disease. As indicated, there are still a number of open questions in the field and many of the models discussed here require additional support from both *in vivo* and *in vitro* experiments to be firmly established. A fascinating aspect of mtDNA replication is its close relationship to replication processes previously studied in bacteria and bacteriophages. In many aspects, the field is guided by work performed by early pioneers of DNA replication and recombination. Another important source of information and inspiration has been the identification of a large number of disease-causing mutations in genes encoding proteins required for mtDNA maintenance. Many replication factors were first identified in this manner and the mitochondrial phenotypes observed in the affected patients have in many cases guided later biochemical work, aiming to understand the mechanistic basis for, for example, deletion formation. We are convinced that a detailed understanding of these processes will lay the foundation for the development of new therapies that can ameliorate disease phenotypes associated with mutations in the mtDNA replication machinery.

Acknowledgments

We are grateful to Dr. Jay P. Uhler who prepared the illustrations and Dr. Bradley Peter for commenting on the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The work described here was supported by the Swedish Research Council through grants [2018-02439] and [2017-01257] to M.F. and C.M.G., respectively, the Swedish Cancer Foundation through grants [2019-816] and [2017-631] to M.F. and C.M.G., respectively, the Knut and Alice Wallenberg Foundation through grants [KAW 2017.0080 and Scholar position] to M.F., the European Research Council through grant [683191] to M.F., and grants from the Swedish state under the agreement between the Swedish Government and the County Councils, the ALF agreement through grants [ALFGBG-727491] and [ALFGBG-728151] to M.F. and C.M.G., respectively.

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