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The interface of transcription and DNA replication in the mitochondria

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Abstract

DNA replication of the mitochondrial genome is unique in that replication is not primed by RNA derived from dedicated primases, but instead by extension of processed RNA transcripts laid down by the mitochondrial RNA polymerase. Thus, the RNA polymerase serves not only to generate the transcripts but also the primers needed for mitochondrial DNA replication. The interface between this transcription and DNA replication is not well understood but must be highly regulated and coordinated to carry out both mitochondrial DNA replication and transcription. This review focuses on the extension of RNA primers for DNA replication by the replication machinery and summarizes the current models of DNA replication in mitochondria as well as the proteins involved in mitochondrial DNA replication, namely, the DNA polymerase γ and its accessory subunit, the mitochondrial DNA helicase, the single-stranded DNA binding protein, toposiomerase I and IIIα and RNaseH1.

1. Introduction

The mitochondrial (mt) genome is a multicopy closed circular genome of 16,569 bp that codes for 13 proteins involved in the electron transport chain, 22 transfer RNA genes, and 2 ribosomal RNAs required for mitochondrial protein synthesis of the 13 polypeptides. Cells contain several thousand copies of mtDNA spread out over hundreds of mitochondria. The mtDNA is located in discrete nucleoids in the inner mitochondrial matrix of the mitochondrion that contain between 1–2 copies of mtDNA [1]. MtDNA is replicated by an assembly of proteins in a replisome consisting of DNA polymerase γ (pol γ), the mitochondrial single-stranded DNA binding protein (mtSSB), mitochondrial DNA helicase, topoisomerases and RNaseH activities (Table I and Fig. 1).

1.1 Current models of mitochondrial DNA replication

Two modes of DNA replication have been proposed to copy the mitochondrial genome, an asynchronous strand displacement model and a strand-coupled bidirectional replication model [2–5]. In the asynchronous strand displacement model, mtDNA is replicated in an asymmetric fashion where DNA synthesis is primed by transcription through the H-strand origin within the D-loop [6]. After two-thirds of the nascent H-strand is replicated, the L-

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strand origin is exposed, allowing initiation of nascent L-strand synthesis. In the strand-coupled model, bidirectional replication is initiated from a zone near OriH followed by progression of the two forks around the mtDNA circle [7]. Because subsequent research has shown that the strand-coupled replication intermediates contain RNaseH sensitive sites, an alternative mechanism of the strand-coupled model includes the idea that the lagging strand is initially laid down by RNA before being converted to DNA (Fig. 2), and has been termed RITOLS (RNA Incorporated Throughout Lagging Strand). [8] In all of these models, initiation is primed by an RNA primer and the DNA polymerization reaction is performed by the pol γ holoenzyme.

In the asynchronous strand displacement model, transcription initiates replication of mtDNA at OriH within the D-loop at the light-strand promoter (LSP) [9]. The primer for initiation of mtDNA replication at OriH is generated by processing the transcript starting at LSP [6, 10]. Pol γ initiates H-strand synthesis by extending the RNA primer [6, 11, 12]. When nascent H-strand synthesis is ~70% complete, the replication fork exposes the major origin for L-strand synthesis (OriL), allowing initiation of L-strand synthesis on the displaced H-strand to proceed in the opposite direction [13–15]. L-strand replication is initiated near the WANCY tRNA coding region that in a single-strand form is postulated to assume a stable stem-loop structure, and DNA synthesis proceeds along the entire length of the mtDNA strand and terminates after H-strand replication is completed [16]. In support of the strand-displacement model, L-strand replication has also been shown to initiated *in vitro* by the mitochondrial RNA polymerase [17].

The strand-coupled model of mtDNA replication is based on the ribonucleotide substitution pattern in mtDNA and analysis of replication intermediates by 2D-gel electrophoresis [7, 8, 18]. 2D-gel electrophoresis revealed two types of replication intermediates [18], one of which is resistant to nucleases that digest single-stranded DNA consistent with conventional duplex replication intermediates from symmetric, semi-discontinuous DNA replication with coupled leading and lagging strand DNA synthesis. A second class of replication intermediates, presumably derived from the strand-asynchronous mechanism of mtDNA replication, is sensitive to single-strand nuclease and is most abundant in cultured cells not treated with ethidium bromide. Although this initial report suggested coexistence of both the asynchronous and strand-coupled modes of mtDNA replication [18], later findings by the same authors indicate that mammalian mtDNA replication proceeds mainly, if not exclusively, by a strand-coupled mechanism [7, 8]. Subsequently, replication intermediates from highly purified mitochondria were demonstrated to be essentially duplex throughout their length, although they contain RNA/DNA hybrid regions, which result from the infrequent incorporation of ribonucleotides [8]. The authors suggested that *in vitro* RNaseH treatment or the process of extracting mtDNA from crude mitochondria leads to degradation of these ribonucleotide-rich regions and produces the partially single-stranded molecules previously assumed to arise by the asynchronous mechanism [18]. Analysis of mitochondrial DNA from rats, mice and humans revealed that mtDNA replication initiates at multiple origins that are distributed across a four kilobase fragment downstream from the 3'-end of the displacement loop, and that DNA replication is restricted to one direction after fork arrest near OriH [7]. Further mapping of prominent free 5' ends identified two regions of start sites, one corresponding to OriH for the strand-asynchronous model, and the other several hundred nucleotides toward the non-coding D-loop region corresponding to a possible bidirectional replication origin [19].

Analysis of two-dimensional agarose gel electrophoresis also shows that mtDNA contains ribonucleotide incorporation throughout the lagging strand, RITOLS, which is depicted in Figure 2 alongside the other models of mtDNA replication. Yasukawa *et al.* has demonstrated that these RITOLS map to the major non-coding region of birds and mammals

and occur unidirectionally. One of the more prominent RITOLS that is utilized for DNA replication starts in the region of OriL [20]. Transmission EM and antibodies specific for RNA/DNA hybrid molecules shows that duplex DNA intermediates can be found throughout mtDNA and contain extensive RNA tracts on one strand, indicative of a strand-coupled model [21]. However, Brown *et al.* observe that many mitochondrial transcripts also form stable R-loops, similar to the well-documented R-loop at the leading strand replication origin (OriH) [22]. These stable but nonreplicative, partially hybridized RNA/DNA duplexes raise questions about the function of RITOLS. There are certain elements in both models that are well supported by experimentation, but it is clear that further studies are needed to illuminate whether both models predominate in nature or are products of experimental artifacts.

Regardless of the mode of replication, both models require extension of an RNA primer and it is accepted that this is mediated by the transcription machinery to generate a nascent RNA that is cleaved, processed, and extended by DNA pol γ. DNA replication is carried out by pol γ, the mtDNA helicase Twinkle, mtSSB, topoisomerases, RNaseH1 and a host of transcription initiation and termination factors. RNA polymerase is recruited to the LSP by TFAM and TFB2 to initiate transcription and generate near-genomic length transcripts [23–25]. These transcripts from the LSP can be cleaved or prematurely terminated to generate primers for DNA replication [11, 12]. While the RNA polymerase is highly processive on double-stranded DNA, *in vitro*, the mitochondrial RNA polymerase will synthesize short RNA primers on single-stranded DNA of 25–75 nucleotides that can be extended by DNA pol γ [26]. Further evidence has suggested that the RNA polymerase synthesizes a 25 nt RNA primer at OriL, after it becomes single-stranded and adopts a stem-loop structure [17]. This RNA primer is then extended by pol γ to complete the initiation of lagging strand DNA replication [17]. Thus, RNA polymerase initiates RNA primers for DNA pol γ at OriH and OriL.

2. Human mitochondrial DNA polymerase, catalytic subunit and accessory subunit

Of the 16 DNA polymerases present in eukaryotes, pol γ is the only DNA polymerase found in the mammalian mitochondria and hence bears the burden of replicating and repairing the entire 16.5 kb circular mitochondrial genome [27, 28]. The human pol γ holoenzyme consists of a catalytic subunit (encoded by *POLG* at chromosomal locus 15q25) and a dimeric form of its accessory subunit (encoded by *POLG2* at chromosomal locus 17q24.1). The catalytic subunit is a 140 kDa enzyme (p140) that has DNA polymerase, 3'→5' exonuclease, and 5′ - deoxyribose phosphate (dRP) lyase activities [29]. The accessory subunit is a 55 kDa protein (p55) required for tight DNA binding and processive DNA synthesis [30].

Mutations in the *POLG* gene were first discovered in 2001 as the cause of progressive external ophthalmoplegia (PEO) [31] and have been found to be a frequent cause of mitochondrial disease resulting from either mtDNA depletion disorders, including Alpers and myocerebrohepatopathy spectrum disorder, or mtDNA deletion diseases such as the aforementioned PEO, ataxia-neuropathy syndromes, myoclonus epilepsy myopathy sensory ataxia, and related disorders [32–34]. Although not as frequent as *POLG* disorders, mutation in *POLG2* can cause PEO and PEO-like symptoms [35, 36].

2.1 Biochemical properties

DNA pol γ displays robust activity *in vitro* with diverse primer-template substrates including natural DNA substrates and homopolymeric templates like poly(dA)·oligo(dT)

and poly(dC)·oligo(dG). The activity of pol γ is a magnitude of order higher for homopolymeric primer-template substrates that contain high primer density compared to natural DNA substrates [37]. Pol γ can also perform efficient catalysis on ribohomopolymeric primer-templates like poly(rA)·oligo($dT_{12–18}$) in the presence of Mn^{2+} as the metal co-activator, which accelerates the catalysis of DNA polymerases at the expense of fidelity [38]. However, *in vivo*, DNA polymerases use Mg^{2+} as the co-activator, which promotes high fidelity polymerization. DNA pol γ also performs reverse transcription with a higher catalytic efficiency than HIV-1 reverse transcriptase, but the reverse transcription activity of pol γ is far less efficient than its replication of natural DNA sequences [39]. While intriguing, the physiological significance of DNA pol γ reverse transcription still needs to be elucidated. Related to this novel pol γ function, recent studies using steady state kinetic analysis have shown that the polymerase does not discriminate between deoxyribonucleotide incorporation opposite to a single deoxyribonucleotide or ribonucleotide in the template, suggesting that the single nucleotide reverse transcription activity of pol γ is very efficient [40]. Since pol γ is the sole DNA polymerase in human mitochondria, its ability to utilize a wide variety of substrates allows its involvement in all DNA transactions of the mitochondrial genome unlike the 15 nuclear DNA polymerases, which have specialized roles in replication and repair processes.

Recombinant human DNA pol γ overproduced in the baculovirus system and purified from insect (*Sf*9) cells is active over a broad pH range from 7.5 to 9.5, and requires divalent cations like Mg^{2+} or Mn^{2+}, depending on the substrates used in polymerase reactions. In addition, the enzyme has a very high affinity for dNTP binding as expected from a replicative polymerase, but is strongly inhibited by dideoxynucleotides. The protein is resistant to inhibition by aphidicolin, and the sulfhydryl blocking agent N-ethylmaleimide (NEM) inhibits the polymerase activity of the catalytic subunit > 90% at 0.5 mM NEM [37]. However, the holoenzyme displayed nearly complete resistance to NEM up to 1 mM [30], suggesting that the p55 accessory subunit protects the catalytic subunit more than 100-fold from inhibition by NEM. This property of the accessory subunit has been biochemically exploited to determine physical interactions between the subunits of the holoenzyme [41]. The optimal salt concentration for pol γ activity also depends on the presence of p55 and on the substrate used. On homopolymeric poly(rA)·oligo($dT_{12–18}$) substrates, pol γ is active at 75 mM NaCl while the optimal salt is 25 mM for the poly(dA)·oligo($dT_{12–18}$) substrate. However, the activity of pol γ is significantly inhibited at >100 mM NaCl on activated salmon sperm DNA substrates [37]. Reconstitution of the heterotrimeric holoenzyme abolishes the salt sensitivity and the complex is active over a broader range of salt concentration (75 mM to 175 mM). In addition, the processivity of the catalytic subunit is enhanced as much as 50-fold upon interaction with the accessory subunit [30]. The interaction of the accessory subunit with the catalytic subunit also enhances the affinity of the complex for DNA, which increases the processivity of the holoenzyme.

The N-terminal region of the pol γ catalytic subunit comprises an exonuclease domain responsible for degrading newly incorporated nucleotides in the nascent DNA strand. This robust activity depends on the conserved aspartic acid and glutamic acid residues [37]. The 3’→5’ exonuclease has a broad pH spectrum, requires a divalent metal cation for its activity, and is stimulated by moderate to high concentrations of NaCl. The exonuclease can efficiently degrade single-stranded DNA and shows mild preference for 3’-terminal mismatches in double-stranded DNA [42, 43]. Mutations of the catalytic aspartates in yeast mitochondrial DNA polymerase, Mip1, showed a 1,440-fold increase in the enzyme’s mutation frequency in addition to incorrect nucleotide incorporation and proficient mismatch extension [44]. Use of the human exonuclease-proficient pol γ [37] demonstrated that pol γ has high DNA synthesis fidelity due to high nucleotide selectivity and efficient proofreading [45]. The exonuclease proofreading function offered a >20-fold enhancement

of misincorporation fidelity [45]. Surprisingly, the exonuclease activity of the catalytic subunit is compromised upon interaction with its accessory subunit [45–47]. This could be due to the bias displayed by the accessory subunit upon interaction with the catalytic core to position the primer-terminus region towards the polymerase domain of the holoenzyme [48]. Alternatively, the tight binding to DNA offered by associating with the p55 subunit promotes highly processive DNA synthesis at the cost of slightly reduced fidelity [30].

2.2 Crystal structure

In 2009, Lee *et al.* described the three dimensional (3D) structure of the pol γ holoenzyme at 3.2 Å resolution using the exonuclease-deficient version of the catalytic subunit and the homodimeric accessory subunit lacking a four α-helix bundle at the dimer interface [48]. The p140 catalytic subunit contains an N-terminal exonuclease and a C-terminal polymerase domain connected by a long linker or spacer domain (~400 residues). The polymerase domain includes three subdomains, termed the “palm,” “finger,” and “thumb”. The crystal structure also revealed that a small region near the N-terminus of the spacer domain made up a portion of the thumb subdomain. The spacer region contains two subdomains, the intrinsic processivity (IP) subdomain and an accessory-interacting determinant (AID). These subdomains are essential for the interaction of p140 with the p55 dimer, which results in a highly processive holoenzyme. Sequence alignment of the catalytic subunit of pol γ revealed that the AID region in the spacer domain is conserved only in mammals and not in *Xenopus*, *Drosophila* and other lower eukaryotic organisms such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* [49]. Interestingly, the organisms that lack this AID region are also devoid of the accessory subunit.

The heterotrimeric 3D structure also contained a dimer of the accessory subunit. While the accessory subunit used for these structural studies lacked a helix bundle, the crystal structure revealed that this region of p55 is not involved in interaction with the catalytic subunit. The structure of the holoenzyme was also very unique in that the catalytic subunit mainly interacted with only one monomer of the dimeric accessory subunit [48]. Each monomer of the p55 dimer contributes differently to the overall processivity of the holoenzyme; the monomer proximal to p140 increases the DNA binding affinity of the complex whereas the distal monomer enhances the rate of polymerization. While the distal subunit of p55 contacts p140 mainly through only two of its residues (Glu-394 forms a salt bridge with Arg-232 of p140, and Arg-122 weakly contacts Gln-540 of p140 through van der Waals interactions), the proximal subunit of p55 makes extensive contact with the catalytic core of the holoenzyme predominantly via hydrophilic interactions with the N-terminal region of the p140 thumb subdomain (Glu-454 to Asp-469 and Arg-579) and positively charged residues in the palm subdomain of p140. Furthermore, the C-terminal region of the proximal p55 monomer forms hydrophobic contacts with the L-helix (Val-543 to Leu-558) of the AID of p140 [48].

2.3 Ribonucleotide incorporation

During DNA replication, DNA pol γ must preferentially incorporate deoxyribonucleotides over ribonucleotides, and it must discriminate between these two in an environment in which ribonucleotides are more abundant [50, 51]. In 1973, Grossman et al documented that mature mouse and human mtDNA contain ribonucleotides at a frequency of ~10 ribonucleotides per genome [52]. Even though pol γ selectively incorporates deoxyribonucleotides during mtDNA synthesis, mammalian mtDNA contains at least 10–30 individual ribonucleotides spaced at ~500-bp intervals across the entire mitochondrial genome [8, 52]. Ribonucleotides in mtDNA may arise from incomplete digestion of randomly spaced RNA primers, as suggested in the RITOLS model, or incorrectly incorporated during DNA synthesis. Given that pol γ is highly processive and is therefore

unlikely to fall off of DNA during mtDNA replication, pol γ is the likely source of ribonucleotide incorporation into mtDNA. Preliminary analysis revealed that pol γ can incorporate a ribonucleotide into DNA [39], and a recent study identified that human pol γ was able to differentially discriminate against each of the four ribonucleoside triphosphates [40]. The identity of the base played a role in discrimination, as rGTP was discriminated against dGTP only 1,100-fold; the discrimination for rCTP versus dCTP was 6,600-fold, and for rATP versus dATP, discrimination was 9,300-fold. Interestingly, pol γ exhibited a 77,000-fold discrimination against rUTP compared to dTTP. Prior work suggests that the stringent discrimination against rUTP is not due to the absence of the C5-methyl group compared to dTTP, because porcine liver DNA pol γ binds dUTP with only a 3-fold lower efficiency compared to dTTP [53]. Hence, the majority of discrimination against rUTP is primarily due to the 2'-OH group.

A recent study by Wheeler *et al.* revealed that in rat tissue mitochondria, the rNTP/dNTP levels varied depending on the identity of the nucleotide and tissue, but in general ATP, UTP, CTP, and GTP levels were ~1000-fold, 9 to 73-fold, 6 to 12-fold and 2 to 26-fold higher compared to their respective deoxyribonucleotide counterparts [54]. In the study of the discrimination by the human pol γ against ribonucleotides, the nucleotide binding affinity (K_m) appeared to be the greater determinant in the kinetic analysis [40]. Thus, the higher ribonucleotide concentration in mitochondria likely contributes to the high degree of ribonucleotide incorporation observed in mammalian mtDNA. However, since one source of mitochondrial dNTP pools appears to involve the reduction of ribonucleotides by ribonucleotide reductases [32, 55], the up-regulation of the reductase enzyme may alter this balance by reducing rNTP pools and increasing dNTP pools. Nevertheless, neither the biological origin nor the consequences of the presence of ribonucleotides in mtDNA is well understood.

Several studies suggest that the mitochondrial transcription machinery primes the initiation of mtDNA replication [6, 11, 12, 17, 24, 26]. Recent studies using single-nucleotide incorporation and steady-state kinetic analysis revealed that the mtDNA polymerase could extend primers with a terminal ribonucleotide, albeit with a 3–14-fold lower efficiency compared to a deoxyribonucleotide-terminated primer [40]. This difference appears to be mainly due to reduced nucleotide binding affinity and catalysis seen with the pol γ/DNA complex containing a terminal ribonucleotide since the enzyme displayed similar affinity for DNA/DNA and DNA/RNA substrates [39].

Both models of mtDNA replication, the asynchronous strand displacement model and the coupled replication model, agree that the 16.5 kb human mitochondrial genome is replicated by the pol γ holoenzyme [2]. As discussed earlier in section 1.1, in the asynchronous model, pol γ extends the H-strand DNA from an RNA primer, suggesting that it can initiate DNA synthesis from a ribonucleotide [6]. However, in the RITOLS model of mtDNA replication, the replication intermediates contain regions of RNA/DNA heteroduplexes and extensive RNA-rich sequences in the lagging strand, indicating a role for pol γ in incorporating these ribonucleotides during nascent strand synthesis [7, 8, 19, 20]. RNase H enzymes can remove the RNA incorporated into DNA, where RNase H1 processively cleaves long stretches of RNA/DNA hybrids and RNase H2 removes singly incorporated ribonucleoside residues in DNA. While both H1 and H2 enzymes are found in the nucleus, only RNase H1 has been implicated in the mitochondria [56]. Thus, the lack of a known RNase H2 activity in mitochondria helps explain why the single ribonucleotide residues persist in the mitochondrial genome, regardless of their source. Since pol γ possesses reverse transcriptase activity, these single ribonucleotides present in the template DNA strand could be bypassed during replication.

Studies investigating the physiological consequence of reverse transcriptase activity by human pol γ opposite a single ribonucleotide found that pol γ stalls after incorporating a single deoxyribonucleotide [57]. In addition, recent results reveal that the efficiency of bypassing a single ribonucleotide in the template DNA was 51% compared to a control template containing no ribonucleotides [40]. Templates with longer stretches of RNA reduced the bypass efficiency because pol γ stalled while bypassing long stretches of ribonucleotides in DNA. Pol γ was able to bypass 4 and 8 consecutive ribonucleotides in the template with only 29% and 14% efficiency, respectively, compared to the control reactions. The cause of this decline in catalytic activity is possibly due to the A-form conformation of a RNA-DNA heteroduplex, which is not well tolerated by most replicative DNA polymerases [40, 58].

2.4 Base excision repair in mitochondria

Pol γ is also implicated in base excision repair (BER) of mtDNA. BER in mitochondria can occur either by single-nucleotide BER (SN-BER) or long-patch BER (LP-BER) pathways [59]. In both cases, a specific DNA glycosylase removes the damaged DNA base and an AP endonuclease cleaves the DNA strand 5′ to the abasic site leaving a single nucleotide gap containing a 5′ -dRP group. In SN-BER, this 5′ -dRP moiety is then removed and the resulting single nucleotide gap is filled by pol γ making the substrate suitable for ligation by ligase III [60]. The rate of the dRP lyase reaction is considerably slow for pol γ compared to pol β, which is involved in nuclear BER. Since the removal of the dRP moiety by pol γ proceeds via a β-elimination reaction mechanism involving the formation of a Schiff base intermediate between the dRP-containing DNA substrate and the active site lysine residue (the Schiff base nucleophile) of the enzyme, the enzyme-dRP intermediate can be trapped as a covalent complex using sodium borohydride. Recently, LP-BER activity has been reported in mitochondrial extracts, which involves the concerted efforts of several proteins like the flap endonuclease FEN-1, which removes the 5'- flap DNA displaced by pol γ, and DNA2, a DNA helicase with endonuclease activity that processes the expanding 5'-flap structure [61–64]. In addition, ligase III is involved in sealing the gaps in DNA. However, the location of the dRP lyase active site in pol γ has remained elusive. In an attempt to delineate the dRP lyase region of pol γ, partial proteolysis of a trapped enzyme/DNA complex identified a 58 kDa fragment covalently crosslinked with the DNA substrate containing the dRP moiety. Amino-terminal sequence analysis of this 58 kDa protease-resistant fragment revealed that the polypeptide was a C-terminal fragment of the full-length enzyme that begins with the Gly723 residue. This fragment comprises the polymerase domain of pol γ and a small region of the C-terminal portion of the spacer domain of unknown function (Kasiviswanathan and Copeland, unpublished data). Mapping the dRP lyase active site in a family A DNA polymerase will be significant in elucidating the mitochondrial BER pathway.

3. The mitochondrial DNA helicase, *C10orf2* gene product or Twinkle helicase

The *C10orf2* gene encodes a 5' → 3' helicase [65] with significant sequence homology to the C-terminal end of T7 gene product 4 (gp4) helicase-primase [65, 66]. The *C10orf2* gene product contains five helicase sequence motifs in the C-terminal half of the protein, but it lacks the primase-associated sequences found in T7 gp4. However, similar to T7 gp4 [67], the mitochondrial helicase has been observed as both a hexamer and a heptamer depending on salt and cofactors [68, 69]. This helicase co-localizes with mtDNA in mitochondrial nucleoids and forms punctate mitochondrial fluorescence reminiscent of twinkling stars from which it derives its name, Twinkle [65]. Transgenic mouse lines overexpressing wild-type Twinkle demonstrated up to a 3-fold increase in mtDNA copy number in muscle and

heart, and reduction of Twinkle expression in cultured human cells by RNAi dramatically decreased mtDNA copy number in cultured human cells [70]. These observations suggest that in addition to playing a role in mtDNA maintenance, the mtDNA helicase Twinkle is essential for regulation of mtDNA copy number in mammals [70].

To help determine the specific function of Twinkle in mtDNA transactions, several lines of work have focused on the biochemical characterization of the enzyme. The helicase activity of Twinkle is specifically stimulated by human mtSSB [71], and the enzyme alone was found to only unwind short oligonucleotide substrates. With the help of mtSSB, Twinkle can promote rolling-circle DNA synthesis by the pol γ holoenzyme *in vitro* by unwinding dsDNA at the replication fork [72]. To further characterize the activity of the mitochondrial helicase, deletions and amino acid substitutions in the C-terminus of the *Drosophila* enzyme demonstrate its essential role in helicase function [73]. In addition, alanine substitution mutagenesis of conserved residues in a putative N-terminal primase domain of *Drosophila* mtDNA helicase suggests a lack of primase activity [74], consistent with the overall lack of homology to T7 gp4 in this area of the protein [65]. Despite the lack of evidence for primase activity in mitochondrial helicases, these enzymes may still play a role in elongating the RNA-primers required to initiate mitochondrial DNA replication. Recent evidence suggests that the mitochondrial DNA helicase Twinkle can load onto closed circular DNA without a specialized helicase loader [75]. Furthermore, in concert with pol γ, Twinkle could support replication of a closed circular double-stranded DNA template containing a D-loop reminiscent of that found *in vivo*. This loading onto a preformed substrate and supporting efficient extension of a primer by pol γ has direct implications for understanding initiation of mtDNA replication.

Disease mutations in the *C10orf2* gene have been evaluated *in vivo* and by study of the recombinant helicase harboring these mutations as a homogeneous population. Overexpression of mutations in *C10orf2* associated with adPEO in cultured human or Schneider cells results in stalled mtDNA replication or depletion of mtDNA [76–78]. Analysis of the recombinant helicase purified from baculoviral infected insect cells has demonstrated defects in the helicase due to disease mutations [79, 80]. Disease mutations in the linker region were shown to disrupt protein hexamerization and abolish DNA helicase activity [79]. Four mutations in the N-terminal domain demonstrated a dramatic decrease in ATPase activity [80]. A comprehensive study of recombinant disease variants overproduced and purified from *E. coli* has revealed that all of the disease variants display some level of activity, but many have partial accompanying defects. In over 20 different variants tested, mild to moderate defects were seen in helicase activity, ATP hydrolysis, and stability [81]. All of the variants displayed efficient DNA binding. This study emphasizes the need to optimize *in vitro* conditions for biochemical analysis of disease variants [81]. The moderate defects demonstrated *in vitro* is consistent with the delayed onset of the autosomal dominant PEO associated with mutation of *C10orf2*.

4. The mitochondrial single-stranded DNA binding protein

Mitochondrial single-stranded DNA binding protein was discovered in an analysis of protein-mtDNA complexes derived from rat liver mitochondria that had been lysed with SDS, which revealed nucleoprotein fibrils within the single-stranded portions of both stable and expanding D-loops in replicative intermediates of rat liver mtDNA [82]. The asynchronous model of mtDNA replication predicts the existence of large regions of single-stranded DNA, and the abundant presence of mtSSB in these nucleoprotein fibrils strongly suggests that the mtSSB protein is an essential component of the mtDNA replication machinery. Mitochondrial SSBs have been isolated and cloned from yeast and various animal sources and are between 13 and 16 kDa [83–90]. The native form of the mammalian

mtSSB is a tetramer, with a molecular weight of 56 kDa [90, 91]. The gene for the human mtSSB has been cloned [88], and the crystal structure has been determined [92]. The DNA is proposed to wrap around the tetrameric mtSSB through electropositive patches guided by flexible loops [92]. The mtSSB tetramer has high affinity for DNA, and its DNA binding site encompasses 8 to 17 nucleotides [84, 85, 93].

Although no direct interaction of mtSSB with the transcription machinery has been demonstrated, numerous interactions with the replication proteins and other factors have been documented. *In vitro* experiments with mtSSB added to purified pol γ demonstrated significant stimulation of the polymerase activity on various primer-template substrates [94, 95]. In *Drosophila*, mtSSB increases mitochondrial DNA synthesis almost 40-fold, and fruit flies with a mutated mtSSB gene display significant mtDNA depletion and dysfunction of the respiratory chain [96, 97]. In humans, an 8-fold stimulation of human pol γ was noted with human mtSSB [95]. This functional interaction with the human enzyme is modest but is negatively modulated by the terminal regions of the mtSSB, as C- and N- terminal deletion variants of human mtSSB stimulate more DNA synthesis than full length human mtSSB [98]. In a minimal *in vitro* replication system, human mtSSB stimulates DNA synthesis by pol γ and Twinkle to form products of 16 kb in length compared to 2 kb generated by pol γ and Twinkle alone [72]. Stimulation of human pol γ by human mtSSB is specific as no stimulation of bacterial and nuclear DNA polymerase is observed by mtSSB [95]. In addition to pol γ, mtSSB also specifically stimulates the mitochondrial DNA helicase Twinkle. Whereas *E. coli* SSB could not enhance the activity of the enzyme, mtSSB could promote unwinding of DNA by Twinkle [71, 98]. Recent work with human mtSSB variants demonstrated strikingly different effects on pol γ and Twinkle [99]. Human mtSSB variants that displayed defects in stimulating pol γ had unaltered effect on Twinkle. Conversely, mtSSB variant that showed reduced stimulation of helicase activity stimulated pol γ like wild type mtSSB. These experiments demonstrate distinct structural elements in the mtSSB for the functional interaction with pol γ and helicase [99].

5. Topoisomerases

DNA topoisomerases (topos) are ubiquitous enzymes that alter the topology of DNA by reversibly breaking the phosphodiester DNA backbone and allowing a second DNA strand to pass through this break (for review, [100]). While all topos induce DNA breaks via an active site tyrosine residue, they are classified as either type I (A or B) or type II (A or B) based on their mechanism of action. Type I topos break one strand of DNA at a time, while type II topos induce a concerted double-stranded break in the DNA. Topoisomerase-induced reversible strand breaks enable DNA transactions to occur as if there were no constraints. As a result, topos play essential roles in DNA transactions such as relieving supercoiling stress during replication and transcription. To date, six topoisomerases have been identified in humans. Two of the six are type II enzymes: topoisomerases IIα and IIβ. The remainder are type I enzymes: topoisomerases IIIα and IIIβ, topoisomerase I (top1), and mitochondrial topoisomerase I (top1mt). Of these six, two have been convincingly demonstrated to function in the mitochondria, and they are described below. While it is likely that other topos play a role in mitochondrial DNA transactions, minimal data is available to conclusively describe and define their identity and role(s).

5.1 Mitochondrial topoisomerase I

Until about a decade ago, it was thought that humans only had a single gene encoding for topoisomerase I. Work by Zhang *et al.* altered this perception when the group discovered a gene duplication of nuclear topo I that encoded for a polypeptide with an N-terminal mitochondrial targeting sequence, top1mt [101]. Top1mt is a type IB topoisomerase that requires a divalent metal and alkaline pH for optimal activity, and studies have shown that

this enzyme has been adapted to optimally function specifically in the mitochondria [102]. Top1mt localizes to the mitochondria, and it is highly expressed in tissues rich in mitochondria such as the heart and brain [101]. Furthermore, top1mt can be trapped to form covalent complexes with mtDNA when treated with camptothecin, a known topoisomerase poison. Top1mt cleavage sites were mapped to an asymmetric cluster near the regulatory D-loop region of mtDNA. Treatment of mitochondria with camptothecin caused a reduction in the level of 7S DNA formation, suggesting that blocking top1mt activity reduces the level of D-loop formation in the mitochondria [103]. These results imply that top1mt may play a novel role in regulating mitochondrial replication and/or transcription.

To further address the role of top1mt in mitochondrial transcription versus replication, work by Dalla Rosa *et al.* suggests that top1mt primarily affects mitochondrial transcription [102]. In an elegantly designed experiment, nuclear top1 was targeted to the mitochondria and overexpressed, but the nuclear enzyme could not function in the mitochondria. Overexpression of nuclear top1 in the mitochondria instantaneously inhibited mitochondrial transcription, and replication was inhibited after a delay. Because the kinetics of transcription inhibition versus replication inhibition are distinct, the authors hypothesized that these effects were due to inhibition of RNA-primer synthesis that is required to initiate mtDNA replication. If this were the case, one would expect that overexpression of nuclear top1 in the mitochondria would preferentially inhibit replication initiation, but replication that has already begun could still proceed. Indeed, 2D agarose gel electrophoresis of mtDNA replication intermediates showed that intermediates indicative of replication initiation were significantly reduced whereas intermediates of ongoing replication were only marginally reduced [102]. This result supports a role for top1mt in mtDNA transcription initiation, and also suggests that an alternate topoisomerase can relieve supercoiling stress during mtDNA replication. Furthermore, unpublished work shows that TOP1mt knockout mice are viable, suggesting that yet another topoisomerase may complement top1mt activity in transcription [104]. Whether TOP1mt knockout mice exhibit a pathological phenotype indicative of a mitochondrial disease remains to be determined, but work by Wang *et al.* identified human TOP1mt as a mitochondrial disease candidate, which points to an important role for top1mt in mitochondrial function [105].

5.2 Topoisomerase IIIα

While top1mt arose from a gene duplication that resulted in a dedicated mitochondrial enzyme, topo IIIα serves dual roles in the mitochondria and nucleus. Topo IIIα is a type IA topoisomerase that was previously thought to only function in the nucleus until 2002, when work by Wang *et al.* demonstrated the existence of two start codons (M1 and M26) for the topo IIIα polypeptide [100]. In HeLa cells, translation from the first codon produces an enzyme with a mitochondrial targeting sequence that directs the enzyme to the mitochondria. Translation from the second codon results in an enzyme that is primarily localized to the nucleus. This study was the first to conclusively demonstrate the existence of a type IA topoisomerase in the human mitochondria [100].

While progress has been made on the function of topo IIIα in the nucleus, little is known about its function in the mitochondria. In the nucleus, topo IIIα helps resolve recombination intermediates, but recombination of human mitochondrial DNA is rare. Clues from other organisms point to an essential role for topo IIIα in mitochondrial DNA maintenance. Wu *et al.* showed that topo IIIα is required to maintain the mtDNA genome in *Drosophila* [106]. Flies without the mitochondrial form of topo IIIα can survive to adulthood, but with accompanying fertility defects. The most dramatic effect occurs in females, which are completely sterile because the eggs they lay fail to hatch in part because of dramatically reduced mtDNA content [106]. Complementing the studies in *Drosophila*, additional clues about topo IIIα's function come from trypanosomes. Trypanosomes are protozoans that

possess a topologically intricate mtDNA (kinetoplast DNA) network in the form of thousands of interlocked circles. This network contains several dozen maxicircles interlocked with thousands of minicircles. In trypanosomes, replication of these mtDNA minicircles was found to require a type IA topoisomerase, named topo IA_{mt}. Scocca and Shapiro demonstrated that without this essential type IA topoisomerase, θ-type intermediates accumulate because the final mtDNA minicircle intertwines were not resolved at the end of replication [107]. Additional studies in bacteria show that topo III, a type IA topo, cooperates with RecQ and SSB to resolve converging replication forks at the end stage of replication [108]. Taken together, these results suggest that topo IIIα may serve an essential role in mtDNA maintenance by removing the last few intertwines of the parental mtDNA strands at the end of replication. More studies are needed to define the specific function of topo IIIα in the mitochondria, and to establish whether it is a candidate disease locus for patients suffering from mitochondrial disease.

5.3 Other mitochondrial topoisomerases

To our knowledge, top1mt and topo IIIα are the only two topoisomerases that have been convincingly shown to localize to the mitochondria in humans. Furthermore, additional evidence suggests that these two enzymes are functionally important for mitochondrial transcription and/or replication. While these two type I topoisomerases play important roles in the mitochondria, additional studies suggest that type II topoisomerases may function in the mitochondria as well. Type II topoisomerases are ubiquitous throughout nature and it would come as no surprise if they were found to be involved in mitochondrial DNA transactions, especially given the circular nature of mtDNA. A truncated form of topo IIβ, a type II topoisomerase, was putatively identified in bovine mitochondria based on MALDI-TOF analysis of an isolated polypeptide found to exhibit topoisomerase activity [109]. Other studies suggest the presence of a bacterial-like type II topoisomerase in mammalian mitochondria based on the fact that the type II topo-targeting antibacterial drug ciprofloxacin induces the formation of protein-linked mtDNA breaks in cells (for review, [110]). These studies provide evidence that other topoisomerases are present in mammalian mitochondria, but a considerable amount of work is needed to confirm these findings and define the function of these enzymes in mitochondrial transcription and replication.

6. RNase H activity in mitochondria

As mentioned above in sections 1.1 and 2.3, RNase H enzymes are necessary for the removal of RNA that is found in DNA. Two forms of RNase H exist, H1 and H2, where RNase H1 functions to processively cleave long stretches of RNA/DNA hybrids and RNase H2 removes singly incorporated ribonucleoside 5’-monophosphate (rNMP) residues in DNA. Only RNase H1 has been implicated in the mitochondria as determined by deletion of the mouse *RNaseHI* gene [56]. Mouse embryos carrying the *RNaseH1* gene knockout are embryonic lethal due to a significant decrease in mtDNA content [56]. While most RNase H1 predominantly localizes to the nucleus, a fraction of the protein is found in mitochondria. This dual localization is reminiscent of topo IIIα as discussed in section 5.2, and it results from differential translation of two potential in-frame start codons. Translation from the first codon produces an RNase H1 enzyme with a mitochondrial targeting sequence whose expression is tightly controlled since too little or too much RNase H1 in the mitochondria can lead to cell death [111]. Possible roles for RNase H1 may be removal of RNA primers at origins of replication on the leading (O_H) and lagging (O_L) DNA strand, or during processing of Okazaki fragments proposed in the coupled replication model of mtDNA replication.

7. Conclusion

The proteins and enzymes mentioned in this review must work in a coordinated fashion to initiate and carry out mtDNA replication. Figure 1 depicts a schematic cartoon of how these proteins must function in a mtDNA replication fork. While many experiments have demonstrated functional interactions between these proteins, it is unknown whether physical interactions occur between the transcription and replication proteins. Given the promiscuity of pol γ to utilize a variety of DNA and RNA substrates, a definitive interaction between the RNA polymerase and pol γ may not be needed. Future experiments are needed to address this interaction.

Highlights

Mitochondrial DNA replication is primed by transcription

An asynchronous and coupled model of mtDNA replication have been proposed

MtDNA is replicated by pol γ, helicase, SSB, topo and RNaseH1

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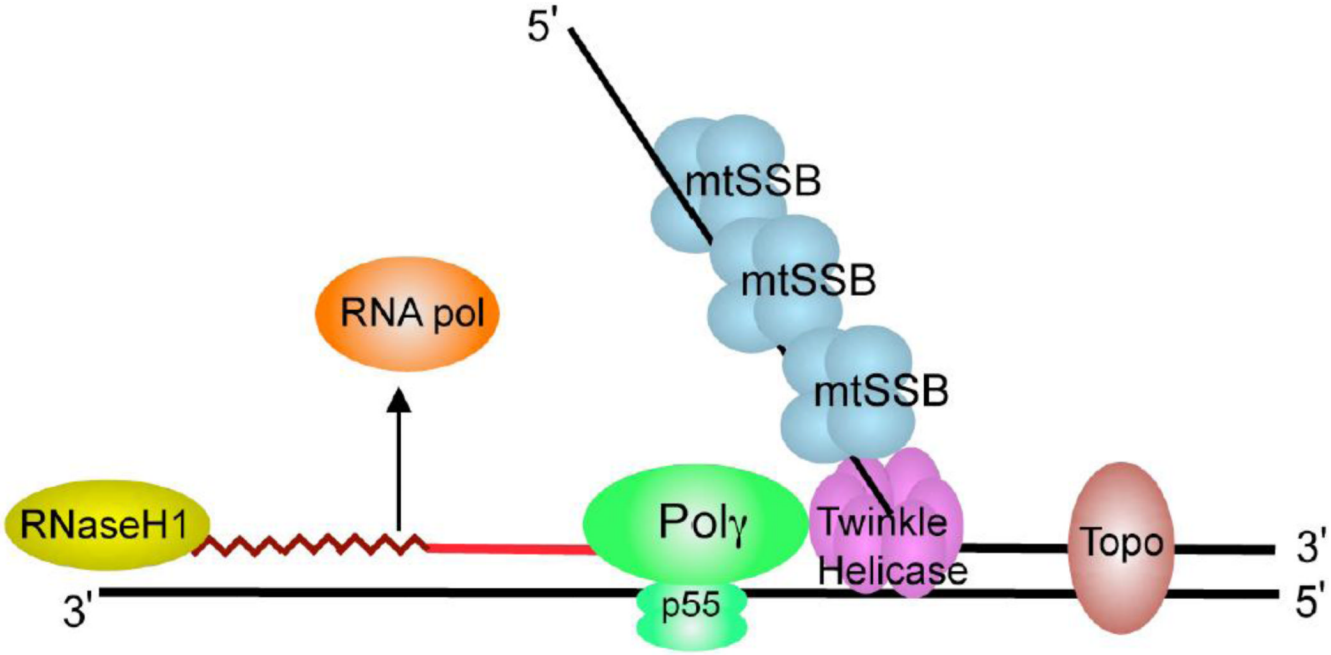

Figure 1. Schematic diagram of a mitochondrial DNA replication fork showing the critical proteins required for DNA replication

The nascent DNA synthesized by pol γ (green) is shown as a solid red line, while the RNA primer (jagged red line) created by the mitochondrial RNA polymerase (orange) is being degraded by RNase H1 (yellow). The mitochondrial DNA helicase (purple) unwinds the downstream DNA forming a single-stranded loop which is coated with mtSSB (light blue). Topoisomerases (brown) work to relieve torsional tension in the DNA created by unwinding.

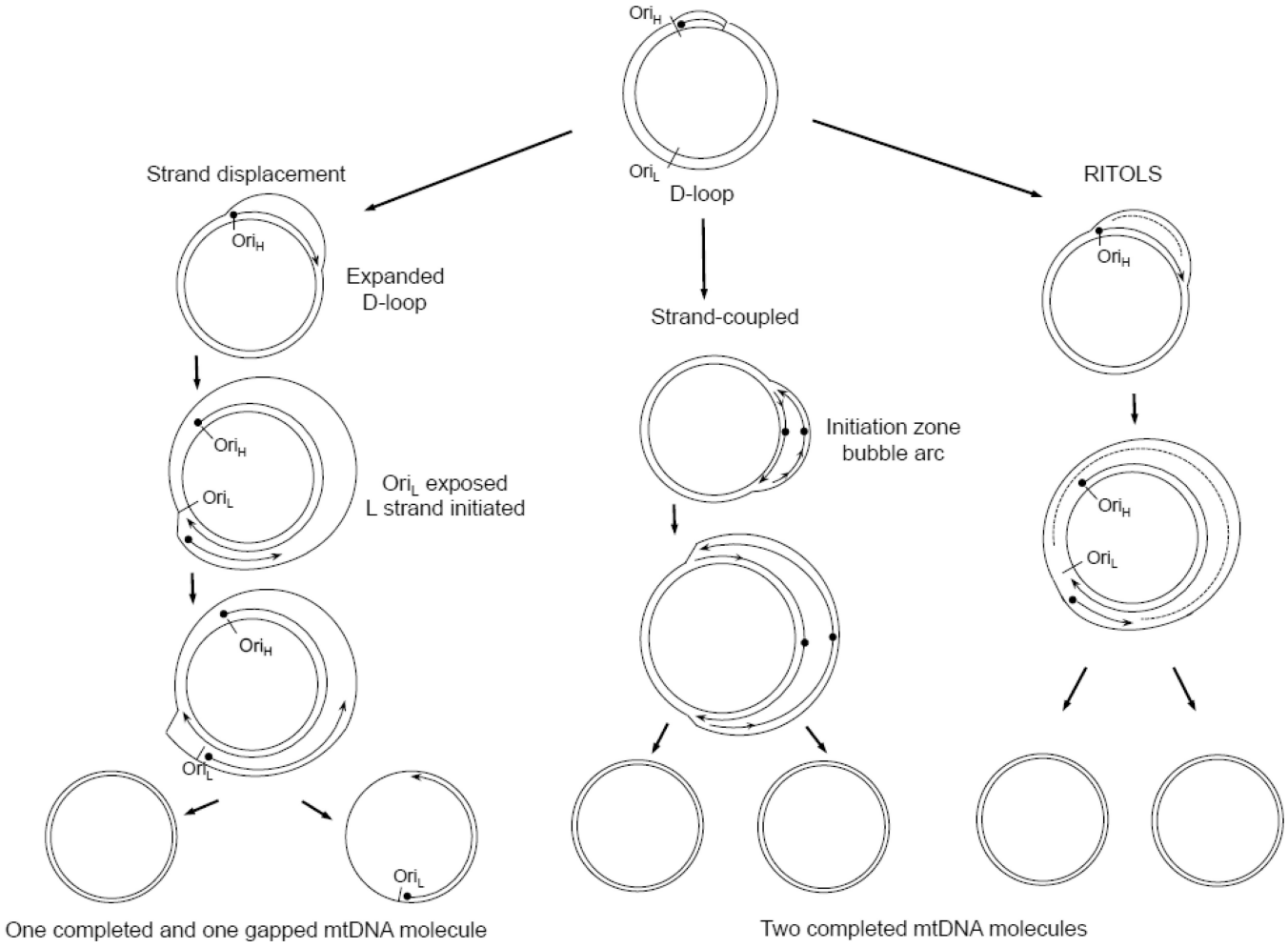

Figure 2. Models of mtDNA replication

Left panel. The asymmetric or strand displacement model. Replication of the H-strand is initiated at OriH with accompanying displacement of the H-strand thus forming a D-loop. This synthesis proceeds until OriL is exposed where synthesis of the L-stand is initiated in the opposite direction. *Middle panel*. The strand-coupled model. Bidirectional replication is initiated from a zone near OriH followed by progression of the two forks around the mtDNA circle. *Right panel*. The RITOLS model. Replication of the leading strand initiates similar to the strand-displacment model but the lagging strand is initially transcribed as RNA (dashed line) before being converted to DNA.

Table 1

Human mitochondrial DNA replication proteins

Enzyme	Size	Human Chromosome
DNA polymerase γ		
POLG	140 kDa	15q25
POLG2	55 kDa	17q23-24
Single-Stranded DNA binding protein (mtSSB)	15 kDa	7q34
Helicase		
C10orf2 (Twinkle)	77 kDa	10q24
Topoisomerases		
Topo Imt	67 kDa	8q24.3
Topo IIIα	112 kDa	17p12-11.2
RNase H1	32 kDa	19p13.2