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REVIEW

Mitochondrial Matrix Processes

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Abstract—Mitochondria possess their own genome that, despite its small size, is critically important for their functioning, as it encodes several dozens of RNAs and proteins. All biochemical processes typical for bacterial and nuclear DNA are described in mitochondrial matrix: replication, repair, recombination, and transcription. Commonly, their mechanisms are similar to those found in bacteria, but they are characterized by several unique features. In this review, we provide an overall description of mitochondrial matrix processes paying special attention to the typical features of such mechanisms.

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STRUCTURE AND DYNAMICS OF NUCLEOID

In mammals, mitochondrial DNA (mtDNA) is organized as DNA–protein complexes called nucleoids similarly to the structures representing the genetic apparatus in prokaryotes [1, 2]. The organization of the mitochondrial nucleoid has been examined in numerous studies, as it is responsible for such processes as mitotic segregation and inheritance of mtDNA. However, despite the fact that nucleoid is considered as a discrete unit of mtDNA segregation, the exact composition of its protein component and its stability remain unclear [1].

Daniel Bogenhagen and his team were able to isolate proteins being most tightly associated with mtDNA within the nucleoid. Their data suggested a model for layered organization of the mitochondrial nucleoid [3]. According to the model, the proteins responsible for replication and transcription of mtDNA are concentrated in the central core of nucleoids. Among them are mitochondrial transcription factors A (TFAM), B1 (TFB1M), and B2 (TFB2M), mitochondrial protein binding to single-stranded DNA (mtSSB), mitochondrial RNA polymerase (POLRMT), mitochondrial DNA polymerase (POLG), mitochondrial transcription termination factor (mTERF), mitochondrial DNA helicase (Twinkle), mitochondrial topoisomerase I, and at least 20 more proteins. Proteins functionally related to RNA processing and translation are located on the periphery of nucleoids. TFAM is the major protein associated with mtDNA involved in both packaging of mtDNA and its local melting by regulating its transcription and replication. Such proteins as POLG, mtSSB, and Twinkle are the minimum factors required for replication of the mitochondrial genome. Similarly, proteins POLRMT, TFB1M, TFB2M, and mTERF are required for transcription of mtDNA. All these proteins from the central core of the nucleoid physically interact with mtDNA. It is now generally accepted that nucleoids are linked to the inner mitochondrial membrane via protein–protein interactions.

At present, there is no unified opinion regarding the number of mtDNA copies contained within each nucleoid. For instance, it was earlier shown that each nucleoid contained one or two molecules of mtDNA [4]. Other studies demonstrated that the nucleoid combines six to ten molecules of mtDNA [5], two to eight molecules of mtDNA [6] or, on average, five molecules of mtDNA [7]. The last study examining this issue was published in 2011, showing that by using microscopy with

Abbreviations: BER, base excision repair; MMR, mismatch repair; mtDNA, mitochondrial DNA; mTERF, mitochondrial transcription termination factor; mtSSB, mitochondrial protein binding to single-stranded DNA; POLG, mitochondrial DNA polymerase; POLRMT, mitochondrial RNA polymerase; TFAM, TFB1M and TFB2M, mitochondrial transcription factors A, B1 and B2; Twinkle, mitochondrial DNA helicase.

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suppression of spontaneous emission (STED) each nucleoid contained at least two molecules of mtDNA [8]. Also, it was suggested that nucleoids combining five to seven molecules of mtDNA are typical for somatic cells, whereas gametes contained one or two molecules of mtDNA [9]. Bogenhagen et al. proposed that such organization of the mitochondrial genome (one or two molecules per nucleoid) allows molecular and biological mitochondrial systems to more effectively detect pathogenic mutations compared to nucleoids consisting of several molecules of mtDNA [10]. In turn, defective mitochondria must be eliminated by mitophages [11]. In due time, this suggested that nucleoids containing more than one molecule of DNA facilitated preserving normal structure of mtDNA, whereas close position of several molecules to each other may trigger a repair process via gene conversion [12].

Another topical issue logically following from the problem regarding a number of mtDNA copies within the nucleoid was exchange of molecules of mtDNA among nucleoids. Two models of functional behavior for mtDNA within nucleoids were proposed: "stable nucleoid" [13] and "dynamic nucleoid" [14]. The former model states that nucleoids do not exchange their mtDNA, so that after division the daughter nucleoids coincide with parental mtDNA both qualitatively and quantitatively. According to the second model, mtDNA can be transferred between nucleoids followed by subsequent recombination. The latter was concluded by Manfredi et al. based on data on fusion of cells containing mtDNA of two different haplotypes. In particular, one lineage possessed a point mutation in the mtDNA, whereas another lineage contained a deletion. As a result, the respiratory activity of hybrid cells was close to normal range, and their mitochondria bore mtDNA resulting from recombination of the initial molecules [14].

In 2008, Schon et al. examined segregation of two different types of mtDNA to check for a true model of the behavior of mtDNA within nucleoids. For this, cells lines bearing genetically distinct nucleoids labeled by mutations in two different loci were fused. Populations of these types of mtDNAs were detected using color hybridization probes (FISH): probe with green fluorescence was complimentary to mtDNA with deletion No. 1, and red – deletion No. 2. Results of the study unequivocally showed that mitochondria from hybrid cells contained both types of mtDNA, but within different nucleoids. The fact that the two fluorescent signals did not overlap between two populations of different nucleoids supported the "stable nucleoid" model [7]. Moreover, using STED-microscopy, Larsson et al. also obtained data supporting a "stable nucleoid" model [8]. In particular, they quantified the amount of mtDNA per nucleoid as being on average 1.4. It is hard to imagine that nucleoids might exchange their mtDNA if they possessed only one molecule of mtDNA.

An assumption about lack of mtDNA recombination was stated based on broad phylogeographic data using mtDNA as a genetic marker [15]. Using electron microscopy, as early as in the 1960s, it became possible to visualize linked two or three circular mtDNAs in patients with leukemia [16, 17]. Later, similar data were also obtained in cultured cells [18, 19]. In 1990, it was demonstrated that homologous recombination at repeats flanking a deleted region is the major cause of large-scale deletions in humans [20]. In 2009, using electron microscopy it was demonstrated that mtDNA in cultured human heart cells had complex organization and might exist both as oligomers and multimeric complexes as well as branched structures such as Holliday and three-way (Y-) junction structures [21]. Similar molecular structures were found only in cardiac muscle cells and brain neurons, but not in other examined tissues. These data suggested that homologous recombination occurs extensively in mitochondria enriched in reactive oxygen species. Likewise, appearance of recombinant mtDNA was documented in murine models [22].

Important proofs that the recombination process exists in human cells were obtained while investigating human tissues from a person who was confirmed to inherit mitochondria from both parents [23]. Sequence analysis of single-molecule PCR products revealed the existence of two types of recombinant molecules containing regions from the paternal and maternal mtDNA. The regions from mtDNA were mainly exchanged in three areas of the genome – the initiation site for alternative replication of the light strand as well as two sites near the 5′-region of 7S DNA, which is formed during premature termination of mtDNA replication. Exchange of DNA regions occurring right at these sites can be explained by formation of recombinogenic protruding 3′-ends upon stalling of recombination. Such free ends can invade a neighboring homologous mtDNA, thereby inducing recombination. Similar results were obtained while investigating the presence of recombination products in mitochondria from muscle cells of patients with high degree of heteroplasmy [24].

Another proof for a mechanism of homologous recombination in mammalian mitochondria was obtained in murine cells [25]. In particular, a guided system of ScaI endonuclease expressing mitochondrial targeting sequence was developed that enabled double-strand breaks in mtDNA. After transient expression of the endonuclease and a short "recovery" period, it was demonstrated that both intramolecular and (much less frequently) intermolecular homologous recombination close to ScaI recognition sites occurred in the mtDNA of modified cells. Hence, the rate of recombination depended on relative position of the recognition site for restrictase and the D-loop.

Identification of factors participating at each stage of this process is an important issue in studying homologous

recombination in mitochondria. The classical prokaryotic recombination pathway includes several key proteins such as: endonucleases creating 3′-free ends after double-strand breaks; SSB (Single Strand Binding) protein required to stabilize single-strand 3′-ends; Rad52 recombination mediator attracting recombinase; Rad51/RecA recombinase performing homologous pairing and strand transfer; and specific endonucleases – resolvases, which finally resolve Holliday junctions formed after recombination [26]. Only a few of the abovementioned components have been securely identified in mitochondria. First, the conservative mitochondrial protein SSB having prokaryotic origin and existing as a homotrimer in both yeast and human mitochondria was described [27]. CCE1 resolvase from yeast mitochondria was also well characterized [28, 29]. In human mitochondria, it was indirectly shown that transcription factor A (TFAM) and DNA helicase Twinkle were also involved in a recombination process, and their impaired expression can result in an upregulated number of Holliday junctions in mtDNA [30].

Multiple copies of mtDNA existing in cells can often result in heteroplasmy, i.e. a condition characterized by several variants of mtDNA simultaneously occurring in a cell, tissue, or organ, compared to homoplasmy when all mtDNAs are identical. It is believed that neutral polymorphisms characterizing that mtDNA (with few exceptions) belong to a certain haplogroup existing as homoplasmy, whereas pathogenic mutations are usually exhibited in heteroplasmy [31]. However, an increasing amount of evidence has been recently appearing indicating that mtDNA in the human body can be present as a mixture of related variants of mtDNA haplotypes, in other words being heteroplasmic at multiple regions in the mtDNA [32, 33].

During cell division, mitochondria are randomly distributed between daughter cells due to mitotic segregation, so that the latter can differ in the degree of their heteroplasmy [34]. According to the "stable nucleoid" hypothesis and data that a nucleoid can contain several molecules of mtDNA, Schon et al. assumed that the rate of skewing towards either mutant or WT mtDNA is determined by the composition of the nucleoid from parental cells. Both mutant and WT mtDNAs can be present within the same nucleoid (heteroplasmic nucleoid) or within separate nucleoids (homoplasmic nucleoid). In case a maternal cell contains heteroplasmic nucleoids, then overall fluctuations in heteroplasmy degree in daughter cells would be insignificant; however, if there were homoplasmic nucleoids it might account for pronounced differences in heteroplasmy degree in daughter cells depending on selective pressure and random genetic drift [35, 36]. If a nucleoid contains a single variant of an mtDNA molecule, then all nucleoids would become homoplasmic, and their segregation between daughter cells could be primarily determined by selection among various variants of mtDNA. Aside from that, mtDNA with deletion would be replicated faster due to advantages in replication of its shorter molecule. Postmitotic cells represent the main part of such tissues and, perhaps, accumulate mutant mtDNA, a process known as "clonal amplification" of mutant mtDNA [37], which is considered as an attempt by cells to restore the proper level of energy production by increasing the number of copies of mitochondria. Some point mutations may also provide replicative advantage to mtDNAs. For instance, the m.3243A>G mutation is located at the recognition site for mTERT, and it shortens replicative pausing, thereby providing mtDNA with advantage in replication rate [38].

Interestingly, the level of heteroplasmy in mitochondria observed within the first three zygotic divisions is the same in all blastomeres [39]. This is probably because up to the eight-cell stage, embryonic cells are totipotent and undergo their first differentiation starting only from the 16-cell stage (differentiate into trophoblast and germ cell mass). It can be supposed that this is the exact time when mitochondrial mitotic segregation is "switched on", i.e. a random distribution of mitochondria between daughter cells occurs. It seems that this process is "switched off" at the stage of oocyte maturation, as both oocyte and polar body (the first and the second ones) were characterized by similar level of heteroplasmy [39]. It was also suggested that mitotic segregation occurs at post-gastrulation [40].

PROTEIN COMPOSITION OF NUCLEOID

POLRMT. Despite the fact that the endosymbiotic hypothesis explaining the origin of mitochondria is commonly accepted, mitochondrial RNA polymerase is evolutionarily closer to RNA polymerase from T3/T7 bacteriophages. RNA polymerase from human mitochondria is a 1230 a.a. protein that consists of three domains: *C*-terminal polymerase domain (648-1230 a.a.), *N*-terminal polymerase domain (369-647 a.a.), and *N*-terminal tail (1-368 a.a.). Only the sequence from the *C*-terminal domain was shown to have similarity between mitochondrial RNA polymerase and bacteriophage RNA polymerase: conserved regions were required for catalytic activity of the two enzymes [41]. Interestingly, no similarity was found within the *N*-terminal polymerase domain of POLRMT and bacteriophage RNA polymerase; however, they had structural similarity [42]. The *N*-terminal tail in POLRMT has a unique structure: it consists of a short proline-rich linker region (355-367 a.a.) that connects the *N*-terminal tail to the *N*-terminal polymerase domain; PPR-domain (218-355 a.a.); a large region with unknown function (42-217 a.a.); and signal sequence targeting protein precursor into mitochondria after its synthesis in the cytosol (1-41 a.a.). The *C*-terminal domain consists of subdomains known as "finger" (939-

1124 a.a.), "palm" (791-831, 912-938, 1125-1176 a.a.), and "thumb" (705-790 a.a.). It seems that the thumb subdomain functions by keeping RNA polymerase on the DNA template during transcription elongation [43]. The finger subdomains are also involved in binding to DNA template and keeping the entire complex from dissociating during transcription elongation.

The *N*-terminal tail of POLRMT is characterized by several activities including regulation of transcription initiation, regulation of mtDNA stability, and its replication, as well as providing transfer and RNA processing [44]. The PPR-subdomain has superhelical structure that specifically binds to a single-strand RNA, thereby modulating its expression [45]. On the other hand, by protein–protein interactions, POLRMT can bind LRPPRC via the PPR-subdomain, thus linking transcription with posttranscriptional events such as RNA processing, its transfer, and translation [46].

The major function of POLRMT is to synthesize RNA from ribonucleoside triphosphates on DNA template during a polymerization process known as the "two-metal-ion mechanism" [47]. In brief, divalent magnesium ions, on one hand, coordinate β- and γ-phosphate groups of a nucleotide to be incorporated into the strand and, on the other hand, motif A of the *C*-terminal domain of POLRMT. Another magnesium cation coordinates the 3′-OH group of an already incorporated nucleotide, α-phosphate group of the nucleotide to be incorporated, as well as motif A and C from the *C*-terminal domain of POLRMT. Such position of the second magnesium cation stimulates nucleophilic attack on the α-phosphate atom. This reaction results in formation of a covalent bond between the 3′-oxygen of the preceding ribonucleotide and the α-phosphate of the next ribonucleotide. This mechanism is typical for all polymerases and, therefore, for polymerization of both ribonucleotides and deoxyribonucleotides. In general, a process of incorporating a new nucleotide into a strand occurs as a four-stage process: nucleotide binding, conformational changes in enzymes relative to nucleotide, formation of chemical bond between incorporated and preceding nucleotides, and release of the pyrophosphate group.

TFAM. TFAM is an mtDNA-binding protein that participates in its local cleavage and bending during the compaction process upon formation of nucleoids. At low TFAM concentration, *in vitro* experiments showed TFAM activates transcription from LSP promoter, whereas at higher TFAM concentration it switches transcription towards HSP1 promoter [48].

TFAM is highly conservative ~25 kDa protein that consists of several well-characterized domains, such as the *N*-terminal mitochondrial leader sequence of ~45 a.a. length that is subsequently removed during maturation process the protein enters the mitochondrial matrix [49]. TFAM contains two DNA-binding domains known as HMG-boxes (high-mobility group box) made of three α-helices bound with two peptide loops. A structure that binds the minor groove of DNA is formed, thereby resulting in its bending. A linker region between two HMG-boxes is supposed to additionally bind to the sugar-phosphate backbone of DNA [50, 51]. According to available data, binding of the HMG-box to DNA is accompanied by interaction between polar amino acids from the HMG-box and the phosphate backbone of DNA, whereas hydrophobic sites of the strands intercalate between nitrogenous bases. Such interaction moves the bases apart, thereby increasing the length of the minor groove of DNA and shortening length of the major groove of DNA. In turn, this results in DNA bending. Thus, TFAM contributes to negative supercoiling of double-stranded DNA [52]. It is assumed that binding of one TFAM protein alters local structure of the DNA, thereby stimulating interaction of other TFAMs with DNA. Moreover, TFAM binds DNA as a dimer, and the length of the binding site on DNA is 37.2 bp [53]. In *in vitro* and *in vivo* experiments, it was shown that a high amount of TFAM bound to DNA can inhibit both transcription and replication of mtDNA [54, 55]. It was found that 900-1600 TFAM molecules fit one mtDNA [8, 56], i.e. ~500 TFAM dimers [57]. Considering that each cell contains numerous copies of mtDNAs, it is likely that some of them are present in a "switched off" state due to TFAM-dependent inactivation of the mitochondrial genome. As a certain amount of mtDNA is preserved in cells even being in a transiently inactive state, it does not disturb cellular energy metabolism that occurs upon lowered number of mtDNA copies [53].

It is assumed that frequent binding of TFAM to LSP promoter increases the rate of generating primer RNA for subsequent replication of mtDNA. On the other hand, binding of TFAM to multiple sites on mtDNA results in arrested replication, thereby stabilizing the number of mitochondrial genomes [53, 58]. Also, TFAM stimulates formation of D-loop *in vitro* [59].

TFB1M and TFB2M. Initially, TFB1M and TFB2M proteins were characterized as participants of the transcription process in yeast [59]. It was shown that these proteins stimulate transcription in the presence of POLRMT and TFAM in an *in vitro* system, although TFB2M does it 10 times better than TFB1M [60]. It was found that during transcription, TFB1M and TFB2M bind to TFAM by directly interacting with its *C*-terminal activation domain.

Nevertheless, it was found that mice with TFB1M knockout died during embryogenesis [61]. By analyzing nucleotide sequence of the genes encoding these proteins, it was revealed that they were homologous to bacterial rRNA-dimethyl transferase, which was later proved by the ability of TFB1M to methylate two adenosine bases within 12S rRNA [62]. It was found that participation of these proteins in methylation and transcription are two independent processes, as a mutation inserted into the

methyl transferase motif did not suppress the capacity of the protein to activate transcription [63]. TFB1M gene knockout and knockdown are characterized by downregulated mitochondrial translation, perhaps due to impaired assembly of mitochondrial ribosome subunits [64]. Overexpression of TFB1M results in upregulated methylation of 12S rRNA, thereby inhibiting mitochondrial biogenesis, and it does not contribute to stimulating transcription of mtDNA as opposed to overexpression of *TFB2M* gene, which does [65]. It is likely that in mammals TFB2M evolved by skewing to be a specialized transcription factor [66]. Interestingly, TFB2M knockdown in *Drosophila* lowers the level of transcripts, whereas TFB1M knockdown was accompanied by downregulated protein synthesis [67].

The protein composition of nucleoids is still not fully determined. Occasionally, studies appear describing novel components identified in mitochondrial nucleoids. However, most commonly their functions remain unclear. To some degree, it is generally viewed that apart from the abovementioned components, mitochondrial nucleoids contain the following proteins [2, 68]: 1) mtSSB, a mitochondrial ortholog of bacterial SSB protein able to bind to single-stranded regions on DNA created during replication; 2) DNA-polymerase γ (POLG), *per se* performing replication of mtDNA; 3) DNA-helicase Twinkle, also required for replication; 4) mTERF, a mitochondrial transcription termination factor.

Functions of these proteins will be discussed later in the corresponding paragraphs.

TRANSCRIPTION OF mtDNA

In mammals, the sequences of HSP1 and HSP2 promoters were characterized on the heavy strand of mtDNA [69] located ~100 bp from each other; they initiate unidirectional transcription, although a role for HSP2 has still not been elucidated in *in vitro* experiments [70]. Initiation of transcription from HSP1 promoter gives rise to a short mRNA including genes of two rRNAs (12S and 16S) and two tRNA (valine- and phenylalanine-specific tRNAs). On the other hand, synthesis of a long polycistronic unit including both short transcript and remaining part of the heavy strand (12 mRNAs and 11 tRNAs) starts from the HSP2 promoter. Each gene of mRNA and rRNA is flanked by at least one gene of tRNA. After transcription, tRNAs are excised from transcripts by specialized processing RNases generally known as RNase P, which outlines a tRNA punctuation model [71]. Transcription of the light strand of mtDNA is initiated from the LSP promoter and results in synthesis of a polycistronic unit containing one mRNA (ND6) and eight tRNAs [72].

TFAM presumably acting as a dimer binds to a motif located slightly upstream from the HSP and LSP promoters [73]. It is assumed that the next TFAM performs local cleavage of mtDNA, thereby enhancing interaction of promoter with other proteins from the transcriptional apparatus. In particular, it was demonstrated that the *C*-terminal domain of TRAM directly interacts with transcription factors TFB1M and TFB2M [63]. Moreover, TFAM bends DNA within the promoter region, which is a critical event upon transcription initiation [74]. There is a direct relationship between the amount of TFAM and mtDNA in cells: in particular, decrease in TFAM expression by 50% results in a similar decrease in mtDNA [75]. It should be noted, however, that the total level of mitochondrial gene expression is kept within a normal range, thereby determining the impact of TFAM only on the amount of mtDNA, but not on the number of transcripts. On the other hand, complete absence of the *TFAM* gene results not only in decreased number of mtDNA copies, but also in decreased number of mitochondrial transcripts [56]. Decreased number of mtDNA copies under reduced amount of TFAM is due to interaction between transcription and replication, as RNA synthesis must be initiated to start replication of mtDNA [76]. It was demonstrated that a heterodimer made of POLRMT and TFB2M covers nucleotides at positions from +10 to –4 in the promoter of the light strand. It was found that to initiate transcription not only a certain nucleotide sequence within promoter of the light strand (LSP) is required, but an upstream nucleotide motif as well. In particular, by replacing the nucleotide motif upstream from the human LSP promoter with the murine LSP motif, this region started to recognize components of transcriptional apparatus from mice [77]. The exact mechanism for initiating transcription of mammalian mtDNA is unclear; however, there are indirect data obtained with cultured cells showing that β-hairpin within the *C*-terminal domain of POLRMT was required for local melting of duplex DNA upon initiating transcription [42]. In contrast to T7 phage RNA polymerase, POLRMT *per se* is unable to recognize a certain nucleotide sequence, uncoil it, and start RNA synthesis. To do this, additional factors are required. Due to the fact that POLRMT recognizes three different promoters and a start site of replication, it seems that the surface of POLRMT is highly labile, although additional factors play an important role in these processes. Because POLRMT cannot continuously transcribe an entire mitochondrial genome [44], long-term RNA synthesis on DNA template requires additional factors similar to those that contribute to transcription elongation in both prokaryotes and eukaryotes [78]. It was found that mitochondrial transcription elongation factor (TEFM) upregulates enzyme processivity by interacting with the *C*-terminal domain of POLRMT and newly synthesized mRNA [79]. It is known that the finger subdomain contains the O-loop (968-1000 a.a.), presumed to mainly bind nucleotides followed by release of pyrophosphate anions [80].

Another factor, such as mitochondrial ribosomal protein L12 (MRPL12), was identified as a partner for

POLRMT [81]: it also regulates RNA metabolism [82], but details of its action remain unclear.

Transcription termination was examined only in case of HSP1 promoter and found to occur with participation of MTERF1 protein, which binds to a 28-bp region at the 3′-end of leucyl-tRNA. This region is called a site of transcription termination [83]. However, MTERF1 was also shown to take part in transcription initiation by binding to a nucleotide motif located close to HSP1. It is assumed that interaction of the protein with both regions of mtDNA facilitates formation of the loop used for looping transcription, which, in turn, upregulates the number of short transcripts in mitochondria [70]. Also, additional recognition sites for MTERF1 were found in mtDNA, which are located on D-loop, the start site of replication for the light strand, *ND1* gene, and a cluster consisting of isoleucine-, glutamine-, and methionine-specific tRNAs. Binding of MTERF1 to these sites perhaps contributes to replicative pausing, thereby regulating the rate of replication [84]. Termination of transcription on the HSP2 initiation site seems to occur in an area of control region, but the proteins involved have not been identified yet [85].

REPLICATION of mtDNA

Three models of mtDNA replication are now being discussed. Two of them operate via asynchronous mechanisms, whereas the other model implies simultaneous copying of DNA strands. A thorough analysis of models describing mtDNA replication is reviewed elsewhere [38, 86]. In brief, a transcript of the light strand serves as a primer during mtDNA replication [76]. It is assumed that replacement of POLRMT for POLG with subsequent DNA synthesis occurs slightly downstream from the CSBII site, presumably between 282-300 bp [87]. It was found that *in vitro* this region plays a role as a terminator of transcription that was started from LSP [88]. TFAM differentially regulates the rate of transcription and replication events and apparently depends on TFAM/mtDNA molar ratio [55]. Low level of TFAM seems trigger replication. The nascent heavy strand of mtDNA is often terminated 700 bp away from the start site of replication of the heavy strand, which results in formation of D-loop. Earlier it was mentioned that the function of the D-loop seems to be in binding mitochondrial nucleoid to the inner mitochondrial membrane via ATAD3 protein [12]. In the absence of mtSSB helicase, Twinkle is unable to uncoil double-stranded mtDNA by more than 55 bp, similarly to POLG, which cannot use double-stranded DNA to synthesize strands without additional enzymes. The presence of POLG, Twinkle, and mtSSB in *in vitro* reactions is sufficient for replication of the mitochondrial genome with calculated rate 270 bp/min [89].

An asynchronous replication model was proposed half a century ago [90] and continues to be updated without being disproved [91-93]. According to this model, replication starts at OriH by triggering synthesis of the heavy strand. This site is located within a control region of mtDNA. While synthesis of heavy strand is executed, the light strand adopts a single-strand form and interacts with mtSSB. Such single-strand state facilitates altering conformation of mtDNA bearing an ~80 bp OriL region. The latter adopts a hairpin shape to be recognized by POLRMT, which acts in this case as a primase. The head of the hairpin consists of ~12 bp and contains six thymidines, which are critical for starting replication of the light strand, the very site for starting synthesis of primer. After synthesizing ~25 bp, POLRMT is replaced by POLG, which continues DNA synthesis on the light strand [94]. Helicase Twinkle uncoils double-stranded DNA for further action of replication enzymes. RNase H1 removes RNA primers followed by ligating DNA fragments via ligase III, whereas topoisomerases relax torsional stress upon movement of the replication fork. A question about the existence of a factor helping POLRMT to recognize DNA hairpin within OriL for synthesis of primer and further replication of the light strand according to asynchronous model remains open.

In 2002, Holt et al. applied 2D agarose gel electrophoresis and a set of specific nucleases to show the existence of RNA molecules that were complimentarily bound to DNA (the firmer had to be as single stranded molecules according to the previous replication model) [95]. Henceforth, such model was called RITOLS (RNA Incorporated ThroughOut the Lagging Strand) [96]. At present, several studies confirmed that the RNA molecule binds to an extended region earlier considered to be single-stranded DNA [97, 98]. Discovery of an RNA intermediate involved in the process of mtDNA replication was originally interpreted as an artefact, and it provoked debates between two scientific schools in high-impact journals [99-101]. Recently, according to the asynchronous replication model, an RNA/DNA duplex was confirmed to exist in *Drosophila melanogaster* [102]. Continuing development of this model, Holt et al. noted that binding of DNA to RNA instead of mtSSB has several advantages. In particular, RNA contains the same genetic information as DNA, thus it can serve as a template for repairing lesions upon replication of the lagging strand [38]. The asynchronous model is probably related to RITOLS, but a relationship of RNA and mtSSB relative to a single-stranded DNA during replication of mitochondrial genome remains unclear.

Also, a model of synchronous replication for mtDNA was proposed by Holt et al. [103]. This model corresponds to the replication model for nuclear DNA and implies leading and lagging strands. By now, it has been shown that the mitochondrial proteome includes proteins necessary for maturation of Okazaki fragments, particularly endonuclease Pif1, Fen1, and Dna2 [104]. In this case, replication starts as a bidirectional process;

however, on reaching a certain site in the D-loop, it is arrested and continues as a unidirectional process [105].

Currently, it is believed that mitochondria use one of these replication mechanisms depending on energy demands in the cells. At stationary growth phase, replication of mtDNA seems to occur according to the synchronous mechanism, which shifts to asynchronous in case it is required to rapidly increase the number of mitochondria [106].

A special regulatory mechanism known as a "bottleneck", which controls replication of mtDNA, was observed in gametes [107]. It was found that before fertilization, the oocyte contains ~200,000 mitochondria, with one or two molecules of mtDNA in each, i.e. one, on average, nucleoid per mitochondrion. After fertilization, due to a series of zygotic divisions, the number of mitochondria declines two-fold with each cell division. The blastocyst contains ~1000 mitochondria, i.e. ~100 mitochondria per blastomere. After implantation, primary gametes known as gonocytes appear during further cell differentiation. These first germ cell lineages first accumulate in endoderm of the yolk sac and then migrate via mesenchyme into gonad rudiments. Before migration, each gonocyte contains ~10 mitochondria; however, after migration their number increases up to 100, in oogonia – up to 200, and primordial follicles – up to 5000. Following sexual maturation, the number of mitochondria in maturing oocytes elevates up to 200,000. Division of mitochondria is directly linked to replication of mtDNA and number of nucleoids in mitochondria. It was shown that after fertilization replication of mtDNA becomes arrested, thereby restricting division of mitochondria.

REPAIR AND RECOMBINATION OF mtDNA

Compared to the nucleus, the milieu in mitochondria is more aggressive, which results in higher rate of mutations [108]. Hence, maintaining stability of the mitochondrial genome is very important for functioning both of the mitochondria and the cell as a whole. However, not much is known about mechanisms involved in repairing damage in mtDNA.

In particular, two repair pathways were demonstrated in mitochondria – base excision repair (BER) and mismatch repair (MMR).

BER is the most investigated repair mechanism described in mitochondria. Repair of modified nucleotides via BER involves several steps. First, it is necessary to identify a damaged nucleotide and hydrolyze the β-N-glycoside bond between the modified nucleotide and the sugar-phosphate backbone. A quite broad class of enzymes such as DNA glycosidases exhibits this activity in both nuclei and mitochondria. These enzymes are divided into mono- and bifunctional DNA glycosidases. Monofunctional DNA glycosidases remove damaged nucleotides only from double-stranded DNA and create an apurinic/apyrimidinic site (AP-site), and include uracil-DNA glycosylase UNG1 removing deaminated cytosine or erroneously inserted dUMP, as well as a homolog of *E. coli* MutY – MYH, which removes adenine or guanine erroneously inserted opposite 8-hydroxyguanine. These gene products undergo alternative splicing, which results in their targeting to both the nucleus and mitochondria [109-111]. Because monofunctional DNA glycosidases eventually create an AP-site, specific AP-lyases are also required for making free 3′-OH to repair damage. APE1 endonuclease serves as a mitochondrial lyase, which is also localized in the nucleus [112].

Among bifunctional DNA glycosidases, mitochondria contain OGG1 (8-Oxoguanine DNA Glycosylase 1), NTH1 (a homolog of *E. coli* endonuclease III), and NEIL1 and NEIL2 (homologs of *E. coli* Fpg and Nei glycosidases, respectively). Similar to UNG1 and MYH, OGG1 and NTH1 are localized both in nuclei and mitochondria owing to alternative splicing of their pre-mRNAs, whereas a pathway underlying import of NEIL-proteins into mitochondria is unknown [111, 113]. Bifunctional DNA glycosidases can recognize damaged nucleotides and remove them in various structures both in single- and double-stranded DNAs. Bifunctional glycosidases bear AP-lyase activity apart from serving as DNA glycosidase [114, 115].

Aside from DNA glycosidases and AP-lyases, mitochondria were also shown to contain other players of the BER process, such as polynucleotide kinase/phosphatase (PNKP) repairing a single-stranded break at the 3′-end [116]; Polγ1 subunit of mitochondrial DNA polymerase Polγ bearing dRP-lyase activity and making a ligatable phosphate at the 5′-end and attaching a "correct" nucleotide instead of the removed one [117], as well as DNA lyase III rejoining the repaired strand [118].

Detailed information regarding molecular mechanisms of BER repair and some players in the MMR process occurring in mitochondria can be found in a review by Kazak et al. [119].

Previously, it was mentioned that so far the scientific community still lacks a univocal view whether homologous recombination similar to that in nuclei takes place in mammalian mitochondria. Nonetheless, an evidence-rich database has accumulated in favor of it.

Thus, it can be assumed that mechanisms for conducting homologous recombination of DNA exist in mammalian mitochondria, which, however, should be further investigated in more detail.

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