DNA Replication and Transcription in Mammalian Mitochondria

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Abstract

The mitochondrion was originally a free-living prokaryotic organism, which explains the presence of a compact mammalian mitochondrial DNA (mtDNA) in contempory mammalian cells. The genome encodes for key subunits of the electron transport chain and RNA components needed for mitochondrial translation. Nuclear genes encode the enzyme systems responsible for mtDNA replication and transcription. Several of the key components of these systems are related to proteins replicating and transcribing DNA in bacteriophages. This observation has led to the proposition that some genes required for DNA replication and transcription were acquired together from a phage early in the evolution of the eukaryotic cell, already at the time of the mitochondrial endosymbiosis. Recent years have seen a rapid development in our molecular understanding of these machineries, but many aspects still remain unknown.

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INTRODUCTION

mtDNA: mitochondrial DNA

The existence of a separate mitochondrial DNA (mtDNA) genome is explained by the widely accepted endosymbiotic theory, according to which the mitochondrion developed from an α-proteobacterium (1). During the course of time, ancestral bacterial genes have been transferred from the mitochondrial to the nuclear genome, as is evident from the presence of orthologous genes in the mitochondrial genome in some species and in the nuclear genome of other species (2). Gene transfer explains why all proteins necessary for mtDNA replication, as well as transcription and translation of mtDNA-encoded genes, are encoded in the nucleus. There are open questions regarding why mitochondria have retained their genetic material and why elaborate enzymatic machineries are necessary to replicate and express a separate genome containing only a few genes (3). One explanation suggests that some hydrophobic proteins are difficult to import across the mitochondrial membranes and then send to the correct location. These proteins may therefore need to be produced within the mitochondrion (4, 5). In support of this notion, the genes, conserved in every completely sequenced mitochondrial genome, for cytochrome *c* oxidase subunit 1 and cytochrome *b* encode the most hydrophobic of all proteins present in the mitochondrion (6). An alternative explanation for the retention of mtDNA could be the differences in codon usage between the nuclear and mitochondrial genomes, which may make further gene transfer from the mitochondrion difficult. A third explanation suggests that the regulated expression of mitochondrial genes is important for metabolic control in eukaryotic cells (7). The molecular machines governing mitochondrial gene expression may be directly influenced by components of the respiratory chain and also affected by the reduction/oxidation (redox) status of the mitochondrion. In support of this notion, data from plants demonstrate that chloroplast gene transcription is dependent on the organellar redox status (8).

GENETICS OF mtDNA

The mammalian mtDNA is maternally inherited, and mitochondria in mammalian sperm are destroyed in the fertilized oocyte. Sperm mitochondria are ubiquitinated inside the oocyte cytoplasm and later subjected to proteolysis during preimplantation development (9). This block against transmission of paternal mtDNA is not absolute but can be bypassed in some situations. It has been reported

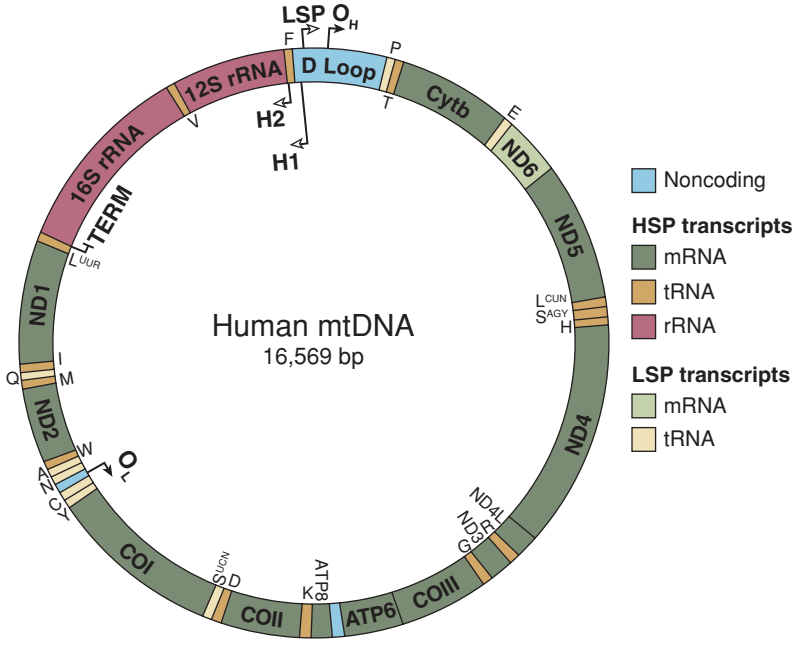

Figure 1

Map of human mtDNA. Light-strand promoter (LSP) transcription produces the ND6 mRNA molecule and primers for initiation of DNA synthesis at O_H. HSP transcription is initiated from two sites. The H1 site is located 16 bp upstream of the $tRNA^{Phe}$ gene and produces a transcript, which terminates at the 3′ end of the 16S rRNA gene (TERM). The mTERF protein binds to the TERM region and promotes termination of transcription. The H2 site is close to the 5′ end of the 12S rRNA gene and produces a polycistronic molecule, which corresponds to almost the entire H strand. The tRNA genes encoded on each of the two strands are indicated with the standard one-letter symbols for amino acids. Abbreviations: COI, cytochrome *c* oxidase subunit I; COII, cytochrome *c* oxidase subunit II; COIII, cytochrome *c* oxidase subunit III; Cytb, cytochrome *b*; HSP, heavy-strand promoter; ND1, NADH dehydrogenase subunit 1; ND2, NADH dehydrogenase subunit 2; ND4, NADH dehydrogenase subunit 4; ND6, NADH dehydrogenase subunit 6; O_H, origin of H-strand DNA replication.

that interspecific crosses of mice lead to transmission of low levels of paternal mtDNA (10). The circular double-stranded mtDNA genome, which is about 16,600 base pairs (bp) in humans and 16,300 bp in mouse cells, encodes 13 of the ~90 different proteins present in the respiratory chain of mammalian mitochondria (**Figure 1**). The remaining components of the respiratory chain are encoded by nuclear genes and imported to the mitochondrion via specialized import systems (11). The genes encoded in mtDNA are all essential for cellular ATP production by oxidative phosphorylation. In addition to mRNA molecules, the mitochondrial genome also encodes 2 ribosomal RNAs and 22 transfer RNAs. A somatic mammalian cell contains ~1000–10,000 copies of mtDNA, and a pathogenic mutation can be present in all copies (homoplasmy) or only in a fraction of all copies (heteroplasmy) (12). Heteroplasmic mtDNA mutations segregate during cell division because there is no mechanism to ensure that every mtDNA molecule is replicated once and only once during each cell cycle (13). It should be emphasized that mtDNA is continuously turned over and replicated during the entire cell cycle, displaying no strict phase specificity as is the case with nuclear DNA synthesis (14). Segregation of heteroplasmic mtDNA mutations will therefore occur as a cell divides or as mtDNA is renewed

LSP: light-strand promoter

HSP: heavy-strand promoter

within a postmitotic cell. This segregation phenomenon explains the mosaic distribution of mutated mtDNA. Patients with heteroplasmic mtDNA mutations often have widely varying levels of mutated mtDNA in different organs, and even in different cells of a single organ. A minimum threshold level of a pathogenic mtDNA mutation must be present in a cell to cause respiratory chain deficiency. It has been shown that different types of heteroplasmic mtDNA mutations have different threshold levels for induction of respiratory chain dysfunction, ranging from 50% to 60% for mtDNA deletions and up to >90% for some tRNA mutations (15). The transmission of mtDNA is, for all practical purposes, maternal, and a mother with heteroplasmy may transmit widely varying levels of mutated mtDNA to her children, provided that the mutation is present in the maternal germ line. The risk for transmission of mtDNA mutations is dependent on the mutation levels in the maternal germ line, and below a certain threshold, mutated mtDNA may not be transmitted at all (16). In addition, different types of mtDNA mutations have differing risks for transmission. Transmission of heteroplasmic mtDNA deletions from mother to child is rare, whereas transmission of heteroplasmic point mutations is common in human pedigrees. The uneven distribution of mutated mtDNA among siblings has been attributed to a bottleneck phenomenon during mammalian oogenesis. A mammalian oocyte contains ~100,000 copies of mtDNA, and all of these copies are probably derived by replication of just a few mtDNA copies in a precursor cell. The purpose of this mechanism could be to reset the mtDNA mutation rate between generations. Experimental data suggest that the segregation of heteroplasmic mtDNA mutations occurs at a very early stage in oogenesis (17). The molecular basis for the bottleneck phenomenon remains unexplained, and future studies of this central event in mammalian mitochondrial genetics are clearly needed.

MITOCHONDRIAL TRANSCRIPTION

The individual strands of the mtDNA molecules are denoted heavy (H) strand and light (L) strand because of their different buoyant densities in a cesium chloride gradient. The observed difference is due to uneven nucleotide content of the two strands; the H strand is guanine rich, whereas the L strand is guanine poor. The strongest strand-specific biases are found at fourfold degenerate sites (18), where patterns of variation most likely result from base substitution processes that are unaffected by natural selection. This observation has led to the hypothesis that mutation pressure acting on mtDNA is strand specific (18–20). The combination of small genomes and extreme nucleotide bias may also explain why some codons are rare or absent in mitochondria. Studies of 111 complete mitochondrial genome sequences revealed that no less than 76 lack one or more codons (mean, 1.6), and 101 have at least one codon (mean, 4.3) that occurs fewer than three times (21).

The compact mammalian mtDNA genome lacks introns and the only longer noncoding region contains the control elements for transcription and replication of mtDNA (22). In human cells each strand contains a single promoter region for transcriptional initiation, the light-strand promoter (LSP) and the heavy-strand promoter (HSP) (**Figure 2**). Transcription from the mitochondrial promoters produce polycistronic precursor RNAs, encompassing all of the genetic information encoded in each of the specific strands. These primary transcripts are processed to produce the individual mRNA, rRNA, and tRNA molecules (23–25). HSP transcription is initiated from two specific sites, H1 and H2 (26). The H1 site is located 16 bp upstream of the $tRNA^{Phe}$ gene and produces a transcript, which terminates at the 3′ end of the 16S rRNA gene. The H2 site is close to the 5′ end of the 12S rRNA gene and produces a polycistronic molecule, which corresponds

to almost the entire H strand. Each of the protein and rRNA genes is immediately flanked by at least one tRNA gene. Excision of tRNA molecules is required to produce mature mRNA and rRNA molecules. Folding of the tRNA structures presumably presents a substrate for mitochondrial RNase P and other unidentified RNases. This mode of RNA processing is known as the "tRNA punctuation model" (24).

Mitochondria contain enzyme systems responsible for mtDNA replication and expression, which are distinct from those found in the nucleus. In contrast to what might be expected, several of the key components of these systems are not related to those of eubacteria. Surprisingly, the mitochondrial DNA-directed RNA polymerase (POLRMT), the catalytic subunit of mtDNA polymerase (POLγA), and the replicative mitochondrial helicase (TWINKLE) are similar to proteins encoded by the T-odd lineage of bacteriophages, which include bacteriophages T7 and T3. This has led to the proposition that some genes required for mtDNA replication and expression were acquired from an ancestor of T-odd phage early in the evolution of the eukaryotic cell, perhaps already at the time when mitochondrial endosymbiosis occurred (27).

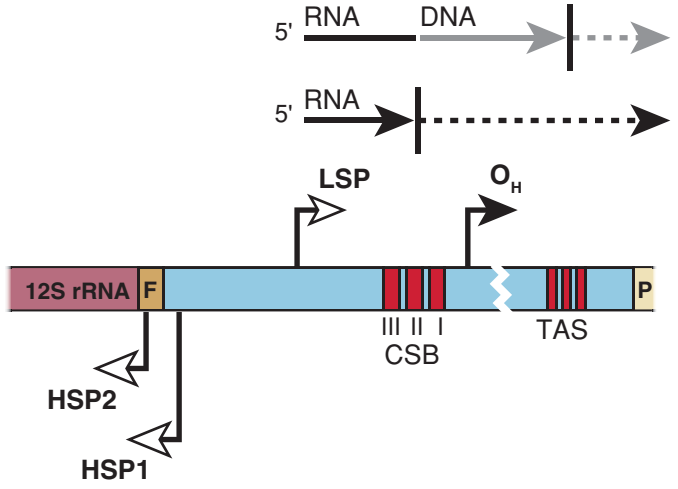

Figure 2

Schematic representation of the D-loop regulatory region. The three conserved sequence blocks (CSB I, CSB II, and CSB III) are located just downstream of light-strand promoter (LSP). Transitions from the RNA primer to the newly synthesized DNA have been mapped to sequences within or near CSB II. The conserved termination-associated sequence (TAS) elements are located at the 3′ of the nascent D-loop strands and are proposed as a major regulation point of mtDNA replication. Abbreviations: HSP, heavy-strand promoter; LSP, light-strand promoter; O_H, origin of H-strand DNA replication.

Mitochondrial RNA Polymerase

The existence of a single-subunit RNA polymerase in mitochondria was first reported in yeast (28–28c) and later in human cells (29). The human POLRMT gene encodes a protein of 1230 amino acid residues, including a 41-amino acid N-terminal targeting peptide, which is cleaved after import into mitochondria. The C-terminal part of the protein (amino acids 520 to 1230) contains a series of conserved motifs, also found in bacteriophage RNA polymerases (28). In addition, POLRMT contains a unique N-terminal extension with unknown function. In *Saccharomyces cerevisiae*, deletion of 185 N-terminal amino acids of the RNA polymerase results in decreased stability and eventual loss of the mitochondrial genome (30). The N-terminal region of mammalian POLRMT contains two putative pentatricopeptide repeat (PPR) motifs in the N-terminal extension (31). The 35-amino acid PPR motif is present in proteins implicated in RNA-processing events in mitochondria and chloroplasts (32). Whether the PPR motif itself constitutes an RNA-binding domain, however, is not known, and the functional importance of this motif in mammalian mitochondrial transcription and DNA replication remains to be established. In contrast to the phage T7 RNA polymerase, POLRMT cannot interact with promoter DNA and initiate transcription on its own. It requires the assistance of mitochondrial transcription factor A (TFAM) and one of the two mitochondrial transcription factor B paralogues (TFB1M and TFB2M, also called mtTFB1 and mtTFB2) (33, 34).

POLRMT: mitochondrial DNA-directed RNA polymerase

POLγA: mitochondrial DNA polymerase γ catalytic subunit

TWINKLE: mitochondrial TWINKLE helicase

TFAM: mitochondrial transcription factor A

TFB1M and TFB2M: mitochondrial transcription factor B1 and B2

Endosymbiont: any organism that lives within the body or cells of another organism, i.e., forming an endosymbiosis

Mitochondrial Transcription Factors B1 and B2

The transcription machinery in budding yeast is less complicated than the mammalian machinery and contains one single TFB1M/TFB2M homologue, which is denoted mt-TFB or Mtf1. The *S. cerevisiae* mitochondrial RNA polymerase (Rpo41) and mt-TFB forms a heterodimer that recognizes mitochondrial promoters and initiates transcription (35, 36). An Rpo41 mutant (E1224A), which displayed reduced interactions with mt-TFB in a two-hybrid assay, has been identified (37). The E1224A mutant is temperature sensitive for promoter-specific initiation of transcription but does not affect the elongation stage. These results suggest a role for mt-TFB in promoter utilization.

In agreement with the observations in budding yeast, both TFB1M and TFB2M can form a heterodimeric complex with POLRMT (33). TFB1M and TFB2M display primary sequence similarity to a family of rRNA methyltransferases, which dimethylates two adjacent adenosine bases near the 3′ end of the small subunit rRNA during ribosome biogenesis (33, 34). Phylogenetic analyses suggest that the TFBM factors are derived from the rRNA dimethyltransferase of the mitochondrial endosymbiont. Even if the recruitment of an RNA-modifying enzyme to the mitochondrial transcription machinery is surprising, it is not without precedent. The smaller B subunit of the mtDNA polymerase is structurally homologous to a family of tRNA synthetases (38). Human TFB1M is in fact a dual function protein, which not only supports mitochondrial transcription in vitro but also acts as an rRNA methyltransferase (39). Point mutations in the conserved methyltransferase motifs of TFB1M do not affect transcription in vitro (40). The dimethyltransferase modification is universally conserved in prokaryotes and eukaryotes, with the only known exception being *S. cerevisiae* mitochondrial 12S rRNA (41). In accordance with this observation, primary sequence similarities between yeast mt-TFB and rRNA methyltransferases are limited, but the X-ray structure of mt-TFB revealed striking homology to the *Escherichia coli* rRNA methyltransferase ErmC′ (42). It therefore appears that the original methyltransferase activity has been lost in *S. cerevisiae*. TFB2M also has rRNA methyltransferase activity but is a less efficient enzyme than TFB1M (42a). It has been suggested that TFB2M has evolved as a specialized transcription factor in mammalian mitochondria (43). In support of this idea, it has been demonstrated that TFB2M is a much more active transcription factor than TFB1M in vitro (33). Furthermore, RNAi knock down of *Drosophila* TFB2M in *Drosophila* Schneider cells reduces the abundance of specific mitochondrial RNA transcripts two- to eightfold (44). In contrast, RNAi knock down of *Drosophila* TFB1M does not change the abundance of specific mitochondrial RNA transcripts but instead reduces mitochondrial protein synthesis, suggesting a primary role in modulating translation (45). These experiments are intriguing but cannot rule out that TFB1M also has a role in transcriptional regulation under specific conditions or for specific mitochondrial transcripts. Manipulation of TFB1M and TFB2M expression in the mouse will shed further light on the individual roles of these two factors.

Mitochondrial Transcription Factor A

The levels of TFAM directly regulate the activity of both TFB1M/POLRMT- and TFB2M/POLRMT-dependent mtDNA transcription in vitro (33). The TFAM protein contains two tandem High Mobility Group (HMG) box domains separated by a 27-amino acid residue linker region and followed by a 25-residue C-terminal tail. Mutational analysis of TFAM has revealed that the tail region is important for specific DNA recognition and is essential for transcriptional activation (46). TFAM can bind, unwind, and bend DNA without sequence specificity, similar to other

proteins of the HMG domain family (47). It therefore appears likely that TFAM binding introduces specific structural alterations in mtDNA, e.g., unwinding of the promoter region, which can facilitate transcription initiation. The sequence-specific binding of TFAM upstream of HSP and LSP may allow the protein to introduce these structural alterations at a precise position in the promoter region and perhaps partially unwind the start site for transcription. This model may explain why the exact distance between the TFAM-binding site and the start site for LSP transcription is of critical importance (48). Because all human factors required for basal mitochondrial transcription now are available in pure and active form, it is possible to address this model experimentally.

Mechanisms of Promoter Recognition and Transcription Initiation

Promoter recognition by the T7 RNA polymerase is achieved by the insertion of a "specificity loop" into the DNA major groove at position −8 to −12 relative to the transcription start site (49). Primary sequence analysis suggests that POLRMT also contains a specificity loop (42), and footprinting analyses demonstrated that the POLRMT–TFB2M heterodimer protects the +10 to −4 region of LSP (50). Promoter recognition in mammals has also been investigated by taking advantage of species-specific differences (50). In lysates, the mouse mitochondrial transcription machinery cannot recognize a human promoter sequence and initiate transcription (50a). TFAM and TFB2M are not responsible for this species specificity because the mouse factors can work together with the human POLRMT and initiate transcription from human LSP. By contrast, mouse POLRMT cannot replace the human polymerase and seems to be largely responsible for species-specific promoter recognition, interacting functionally with several nucleotides immediately adjacent to the transcription start site (50).

The idea that POLRMT recognizes specific promoter elements is also supported by findings in *S. cerevisiae* (51). On linear templates, the yeast mitochondrial RNA polymerase Rpo41 is strictly dependent on the TFB2M homologue mt-TFB for initiation of promoter-specific transcription. However, if the templates are negatively supercoiled, Rpo41 has the intrinsic ability to initiate transcription from promoters without mt-TFB. The sequence element in yeast mitochondrial promoters recognized by Rpo41 is localized between positions +1 and −8 relative to the transcription start site.

The mitochondrial RNA polymerase may also play a role in the coordinated control of nuclear and mitochondrial transcription. In *S. cerevisiae*, there is a direct correlation between in vivo changes in mitochondrial transcript abundance and in vitro sensitivity of mitochondrial promoters to ATP concentration (52). It appears that the Rpo41 itself senses in vivo ATP levels and that shifting cellular pools of ATP may influence mitochondrial transcription. Whether a similar mechanism exists in mammalian mitochondria remains to be established.

The molecular roles of TFB1M and TFB2M are still unknown. The strong homology to a family of RNA-binding rRNA methyltransferases suggests that TFB1M and TFB2M have the capacity to bind RNA and/or single-stranded DNA (ssDNA) (33, 34). One possible role for TFB2M could be binding newly synthesized RNA and thus preventing the formation of an RNA/DNA hybrid at the promoter, which could inhibit further rounds of transcription initiation. Alternatively, TFB2M could bind ssDNA and stabilize a partially unwound promoter during transcription initiation. The phage N4 RNA polymerase II, which also belongs to the family of T7-like RNA polymerases, is recruited to DNA by a ssDNA-binding protein, gp2 (53). It has been suggested that a sequence-specific DNA-binding protein could create single-stranded stretches of DNA in the promoter regions. The protein would bind to

mTERF: mitochondrial transcription termination factor

phage promoter regions and induce unwinding of the double-stranded DNA (dsDNA) template (53). The model proposed for N4 RNA polymerase II-dependent initiation of transcription may serve as a paradigm for our understanding of transcription initiation in mammalian mitochondria. It is possible that binding of TFAM will bend DNA and introduce a sequence-dependent partial unwinding of the mitochondrial promoter, which will allow TFB2M to bind the ssDNA and thereby recruit POLRMT to the promoter. TFAM interacts directly with TFB1M and TFB2M, and these protein-protein interactions may also contribute to the recruitment of the transcription machinery to mitochondrial promoters (40).

The mechanisms of transcription initiation differ significantly between budding yeast and mammalian mitochondria because the yeast TFAM homologue, Abf2, lacks the C-terminal transcriptional activation domain. Abf2 is not required for transcription of yeast mtDNA (46, 54, 55) and instead has a role in mtDNA packaging and maintenance. It is possible that the evolution of multicellular organisms with specialized cell types has necessitated the development of more complex regulation of mtDNA transcription. In support of this notion is the existence of specific mechanisms for termination of mtDNA transcription in metazoan cells.

Regulation of Transcription Termination

There are three mitochondrial transcription units (those starting at H1, H2, and LSP), but only the one starting at H1 has a clearly established termination site, which is located at the end of the 16S rRNA coding gene (56). Transcription termination at this site is dependent on the mitochondrial transcription termination factor (mTERF) protein, a 39-kDa protein, which binds sequence specifically to a 28-bp region at the 3′ end of the $tRNA^{Leu(UUR)}$ gene (57, 58). A termination site for the H2 transcript has been identified just beyond the control region, immediately upstream of the $tRNA^{Phe}$ coding gene. Two proteins of 45 and 70 kDa associate with this region, but their identities have not been reported (59). Termination of the L transcription unit has not been studied in detail. The mTERF protein can terminate transcription in vitro, but the functional role of the protein in vivo remains to be established. A mutation in the mTERF-binding site is associated with a human disease known as MELAS. The MELAS mutation reduces the binding affinity for mTERF but does not change the ratio between H1/H2 transcripts (60). This finding may suggest that the main role of mTERF is not to regulate transcription termination from HSP. In vitro transcription assays also showed that mTERF-mediated termination of transcription at its binding site occurs bidirectionally (61, 62). The mTERF protein may therefore be responsible for termination of the L-strand transcription unit. This possibility is supported by the facts that heterologous RNA polymerases are stopped only in the L-strand direction of transcription and that no genes are located downstream of the mTERF-binding site on the L strand. Furthermore, recombinant human mTERF completely blocks L-strand transcription in a fully recombinant transcription system, whereas blockage of H-strand transcription is far less effective (61). The mTERF protein may also stimulate initiation of transcription (57, 63). A second binding site for mTERF was recently identified at H1 and shown essential for stimulation of transcription initiation in vitro (64). These observations led to the proposition that mTERF is controlling the expression of the H1 transcript by creating a loop between H1 and the end of the 16S rRNA gene. The loop has been visualized by electron microscopy and may facilitate reinitiation of transcription.

Recent bioinformatics analyses suggest that regulation of transcription termination in mammals may be more complex than previously anticipated. Similarity searches and phylogenetic analyses have identified three novel

genes in vertebrates coding for proteins homologous to mTERF, all of them with predicted mitochondrial localization (65). These transcription termination factors have been denoted mTERF1 to mTERF4. mTERF1 corresponds to the previously characterized human mTERF protein. The mTERF1 and mTERF2 proteins appear to be unique to vertebrates, whereas *mTERF3* and *mTERF4* are the ancestral genes in metazoans, present also in worms and insects. So far, only two reports have been published about the new members of the mTERF family of proteins. The mitochondrial localization of human mTERF2 (mTERFL) has been confirmed, and gene expression analysis has shown high expression in heart, liver, and skeletal muscle, a pattern typical for a mitochondrial protein (66). Expression of mTERF2 is inhibited by addition of serum in serum-starved cells, whereas the expression pattern of mTERF1 was the exact opposite. Although the actual function of mTERF2 remains to be elucidated, this observation may indicate that the expression of these two members of the mTERF family is tightly coordinated. In the second report (67), RNA interference was used to knock down *Drosophila* mTERF3 in D.MeL-2 cells. The depletion of D-mTERF3 decreased the synthesis of some mitochondrially encoded proteins, but the levels of mitochondrial mRNAs remained unaltered. The characterization of the mTERF family of proteins is just emerging, and we may expect interesting progress in the coming years. Advances in this direction will help define the mechanisms for the control of mtDNA transcription and might even force us to revisit some of the generally accepted models for transcriptional regulation.

mtDNA REPLICATION

Mitochondria contain a unique enzymatic machinery devoted to mtDNA replication. Many basic components of this system have been characterized, but certain enzymatic activities have still not been identified, e.g., the primase for lagging-strand DNA synthesis. There is also an intense debate about the exact mode of mtDNA replication.

Initiation of mtDNA Synthesis

Transcription from the LSP is not only necessary for gene expression, but also produces the RNA primers required for initiation of mtDNA replication at the origin of H-strand DNA replication (O_H) (23, 68, 69). RNA covalently linked to the newly synthesized H strand has been detected in both mouse and human cells (70, 71). The molecular mechanism governing the switch between genomic length transcription and primer formation is still not completely elucidated. Sequence comparisons in vertebrates have revealed three conserved sequence blocks (termed CSB I, CSB II, and CSB III) downstream of LSP (72). CSB II increases the stability of an RNA-DNA hybrid, and transitions from the RNA primer to the newly synthesized DNA have been mapped to sequences within or near CSB II (70). According to one model, the primary LSP transcript is cleaved by an endonuclease activity at certain locations in the O_H region. These processed transcripts are proposed as primers for DNA synthesis. RNase mitochondrial RNA processing (RNase MRP) may execute this role in primer formation as this enzyme cleaves LSP transcripts in vitro in regions corresponding to the major initiation sites of leading-strand mtDNA synthesis mapped in vivo (73, 74). The relevance of these findings has been questioned because RNase MRP is mainly localized to the nucleolus, where it plays an important role in rRNA processing. In addition the amount of detectable full-length MRP RNA in mitochondria has been judged too small to attribute a mitochondrial function to RNase MRP (75–77). Finally, there are no robust genetic data distinguishing affects on rRNA processing in the nucleolus from primer formation in mtDNA replication. It was recently reported that CSB II is a powerful transcription termination element in vitro (78). Mutations in CSB II abolish transcription termination,

demonstrating the requirement of specific DNA sequences. The 3′ ends of the prematurely terminated transcripts are localized just downstream of CSB II, between positions 300–282 in the mitochondrial genome. These sites coincide with the major RNA-DNA transition points in the D loop of human mitochondria. The excellent correspondence between transcription termination and initiation of DNA replication does not exclude the RNase MRP-dependent model for primer formation. However, it suggests an alternative mechanism whereby primer formation may be directed by sequence-specific DNA elements in human mitochondria, independent of RNase MRP action (78, 79).

Models for mtDNA Replication

DNA synthesis from O_H is unidirectional and proceeds to displace the parental heavy strand. The nascent H strand frequently terminates 700 bp downstream of O_H, giving rise to the 7S DNA. This termination event produces a characteristic triple-stranded structure called the D loop (23). The frequency of the D-loop structures in the overall cellular mtDNA population is variable depending on the sources and growth state of cells and tissues. The functions of the D loop and of the mechanism that decides if the replication should proceed or not are still unknown. An mTERF orthologue in *Paracentrotus lividus* (mtDBP) stops transcription bidirectionally in the control region at a site where H-strand DNA synthesis stops to generate the D loop (80). Recently, in vitro analyses have revealed that mtDBP has contrahelicase activity, and it may therefore block the mitochondrial replication fork and contribute to control of mtDNA replication (81). A similar mechanism may exist in mammalian cells. Termination-associated sequences (TAS) are short (15 bp) DNA elements conserved in vertebrates and located at the 3′ end of nascent D-loop H strands (**Figure 2**) (82). The TAS sequences are proposed as a major regulation point of mtDNA replication (83). In vivo footprints have revealed protein-binding sites in the TAS region in rat and human mitochondria (84). A 48-kDa protein of unknown identity binds the TAS sequence and promotes D-loop formation in bovine mitochondria (85). Because mTERF proteins from other species display contrahelicase activity, it is tempting to speculate that one of the uncharacterized mTERF proteins may be the long-sought-after TAS-binding protein in mammals.

Early studies of mtDNA replication in mammalian cell lines led to the definition of a strand-asymmetric replication model (23). In this model, transcription from LSP provides primers for initiation of leading heavy (H) strand DNA replication. When leading-strand synthesis has reached two thirds of the genome, it exposes another major origin, the origin of L-strand DNA replication (O_L), which is activated, and lagging-strand DNA synthesis then initiates in the opposite direction. New mtDNA molecules with completed strand synthesis are ligated to form closed circles prior to the introduction of superhelical turns. The strand-asynchronous model for mammalian mtDNA replication is based on examination of replicative intermediates by electron microscopy, biochemical isolation, and analysis of these intermediates, as well as on pulse and pulse-chase labeling experiments to identify bona fide replicative intermediates (86, 87).

In recent years, a different mode of mammalian mtDNA replication has been proposed (88–91). Neutral/neutral two-dimensional agarose gel electrophoresis may be used to distinguish DNA molecules on the basis of size and shape. With this technique, DNA fragments are separated in the first dimension by size and in the second dimension by strand configuration. Particular types of replication intermediates can therefore be resolved in specific and predictable ways. DNA fragments with classic synchronous leading- and lagging-strand replication forks are predicted to form a so-called y arc. Such y arcs are present within the major arc between the two

origins, O_H and O_L, of mtDNA. This finding may be inconsistent with the asymmetric strand-displacement model of replication (88). This and related observations led to the conclusion that mtDNA actually replicates symmetrically, with leading- and lagging-strand synthesis progressing from multiple, bidirectional replication forks within a broad zone (90). These results led to vivid discussion between proponents of the two models. There is still a lack of consensus in the field, and more work is clearly needed to clarify these issues. Recently, attempts have been made to reconcile the two models. The most active origin for lagging-strand synthesis is likely O_L, but there is evidence for other points of initiation of L-strand synthesis (92). A recent study has provided atomic force microscopy evidence for the predominance of the strand-displacement mode of replication and the existence of multiple alternative origins of lagging-strand DNA synthesis (93). The combination of multiple lagging-strand origins and branch migration of nascent strands in isolated replicative intermediates could potentially provide an explanation for the detection of various y-arc forms upon two-dimensional gel electrophoresis of mtDNA (93).

In general, it is not possible to distinguish the free 5′ end of replicating mtDNA molecules arrested at the TAS region or progressing beyond this site. This observation suggests that abortion of replication is independent of the 5′ end (79). A novel major replication origin with distinct functional properties was identified at position 57 in the D-loop region (94). This novel origin has been described as responsible for mtDNA maintenance under steady-state conditions, whereas mtDNA synthesis from the previously characterized D-loop origins may be more important for recovery after mtDNA depletion and for accelerating mtDNA replication in response to physiological demands. Another recent development concerns the identification of a new bidirectional origin of replication, which may support the strand-coupled model for mtDNA replication. This origin maps to a region distal to O_H, with initiation most frequently taking place at positions 16184 (L strand) and 16197 (H strand) in the noncoding region of mtDNA (95).

The mtDNA Polymerase γ

POLγB: mitochondrial DNA polymerase γ accessory subunit

The POLγ was first identified as an RNA-dependent DNA polymerase in human HeLa cells (96). POLγ could be distinguished from other cellular DNA polymerases by a number of chemical criteria, including high activity, using synthetic RNA templates in vitro and resistance to aphidicolin (97). The enzyme belongs to the family A group of DNA polymerases, which also include the well-characterized *E. coli* DNA polymerase I and the T7 DNA polymerase (96, 98–100). The human catalytic subunit POLγA has a molecular mass of 140 kDa (101) and harbors polymerase, 3′-5′ exonuclease, and 5′-deoxyribose phosphate (dRP) lyase activities (102–104). Characterization of POLγ purified from frog and human cells demonstrated that the catalytic subunit POLγA was associated with a smaller protein, the mtDNA polymerase γ accessory subunit (POLγB) (101, 105), which does not exist in yeast (106). POLγA and POLγB form a heterodimer in *Drosophila* but form a heterotrimer ($POL\gamma AB_2$) in mammalian cells (91, 107). Human POLγB has a molecular mass of 55 kDa, and crystal structures of both mouse and human POLγB have been determined (107, 108). Interestingly, POLγB shares a high level of structural similarity to class IIa aminoacyl tRNA synthetases and forms a dimer in the crystal. The *Drosophila* POLγB homologue lacks over 100 of the first 250 residues present in the mouse and human accessory subunits. The residues missing map to domain 1 and 2, which contain critical amino acid residues for dimerization. The structure provides a molecular explanation for the observation that *Drosophila* POLγB forms a heterodimer with POLγA (103). The human POLγB subunit binds to double-stranded DNA stretches longer than 45 bp. The function of this DNA-binding

mtSSB: mitochondrial single-stranded DNA-binding protein

activity is unclear, but it is not required for stimulation of DNA synthesis on a single-stranded template (109). The POLγB subunit substantially increases both the catalytic activity and the processivity of POLγA. This effect is partially explained by enhanced DNA binding but also by increased nucleotide binding, suggesting that the accessory subunit directly influences the function of the catalytic subunit and thereby promotes tighter substrate binding (103).

The Mitochondrial TWINKLE Helicase

The TWINKLE gene was identified by positional cloning and was found mutated in some cases of progressive external ophthalmoplegia (PEO), a human disorder associated with multiple mtDNA deletions (110, 111). Affected patients have decreased function of extraocular muscles and additional symptoms inherited in an autosomal dominant (adPEO) or autosomal recessive (arPEO) fashion. A variety of nuclear mutations causing adPEO and arPEO have been identified in the genes for POLγA (112), POLγB (113), TWINKLE (114), and the adenosine nucleotide translocator 1 (ANT1) (115). It is at present unclear how ANT1 mutations cause disease, and one possibility is that such mutations affect intramitochondrial deoxyribonucleoside pools.

The observation that the TWINKLE protein was similar in sequence to the T7 primase/helicase (gene 4 protein, gp4) immediately suggested that TWINKLE was the long-sought-after replicative helicase in mammalian mitochondria. The TWINKLE helicase domain displays the same conserved organization as other members of the RecA/DnaB superfamily, and invariant amino acids of this superfamily are strictly conserved in TWINKLE. In contrast, the primase domain motifs found in the N-terminal region of phage T7 gp4 protein were not readily identifiable in mammalian TWINKLE, and no reports of mammalian TWINKLE being a primase have been published (114). Recently, bioinformatics analyses revealed homologues of TWINKLE throughout the eukaryotic tree, and the essential residues required for gp4 primase activity were present in many eukaryotes, with the notable exception of Metazoa (27, 116). In addition, the TWINKLE homologue identified in the malaria parasite *Plasmodium falciparum* has been shown to have both primase and helicase activities (117). These observations suggest that nonmetazoan TWINKLE orthologues might still function as a mitochondrial primase.

Similar to the gp4 protein and related ring helicases, TWINKLE forms a hexamer in solution (114). Recombinant TWINKLE also displays all of the classical features of a DNA helicase (118). The protein catalyzes the ATP-dependent unwinding of a DNA duplex (20 bp) with a distinct polarity (5′ to 3′), and it requires specific substrates with a single-stranded 5′-DNA loading site and a short 3′ tail to initiate unwinding. The preferred substrate thus resembles the conformation of a DNA replication fork, a structure with which the TWINKLE protein would be expected to interact in vivo. The substrate requirement is also similar to what has been described previously for the T7 gp4 protein and other hexameric helicases, such as DnaB (118). The ability of the TWINKLE protein to utilize various nucleoside 5′-triphosphates as coeffectors for helicase activity is interesting. ATP efficiently supports TWINKLE-mediated DNA unwinding, but UTP is clearly a much more potent cofactor. The physiological relevance of this observation remains to be established.

Mitochondrial Single-Stranded DNA-Binding Protein

In order to copy or repair DNA, the double-stranded helix must be unwound to reveal the two complementary strands. The need to manipulate DNA in its single-stranded form has given rise to a specialized group of ssDNA-binding proteins (97). The mitochondrial ssDNA-binding (mtSSB) protein has a molecular weight of about 13–16 kDa

and has been purified from several species. mtSSB displays sequence similarity to *E. coli* SSB and both proteins form a tetramer in solution (103). mtSSB binds cooperatively to DNA with a binding site size of 50–70 nucleotides per tetramer. Deletion of the mtSSB gene (*RIM1*) in budding yeast causes loss of mitochondrial DNA. The crystal structure of homotetrameric human mtSSB has been solved at 2.4-Å resolution and led to the proposition that ssDNA wraps around the tetrameric mtSSB protein through electropositive channels guided by flexible loops (119). The mtSSB protein has a stimulatory effect on the rate of DNA unwinding by TWINKLE, and this effect is specific because *E. coli* SSB cannot substitute for mtSSB (118). The observed specificity may be due to a direct protein-protein interaction between mtSSB and TWINKLE. Physical interactions between replicative helicases and their endogenous ssDNA-binding proteins have been demonstrated in other systems, e.g., the herpes simplex virus type 1 helicase-primase complex is specifically stimulated by the viral SSB, denoted ICP8 (120).

The Minimal Replisome

Consistent with its central role in mtDNA replication, mtSSB has been shown to stimulate the DNA helicase activity of TWINKLE (118). In spite of this stimulatory activity, TWINKLE is unable to unwind longer stretches of dsDNA (>55 bp) even in the presence of mtSSB. Similarly, the POLγ holoenzyme cannot use dsDNA as template for DNA synthesis. However, POLγ and TWINKLE together form a processive replication machinery, which can use dsDNA as template to synthesize ssDNA molecules of about 2 kb (**Figure 3**). The addition of mtSSB stimulates this reaction further, generating DNA products of about 16 kb, which correspond to the size of the mammalian mtDNA molecule. The observed DNA synthesis rate is 180 bp/min, agreeing reasonably well with the previously calculated value of 270 bp/min for in vivo DNA replication (13, 121). The functional interactions observed between the mtDNA replication proteins appear specific because TWINKLE cannot support T4 and T7 DNA polymerase-dependent rolling-circle DNA replication, even in the presence of the T4 ssDNA-binding protein (121). Furthermore, the presence of both POLγA and TWINKLE in the minimal mtDNA replisome may explain why mutations in either of these two components can cause very similar clinical phenotypes, as discussed above.

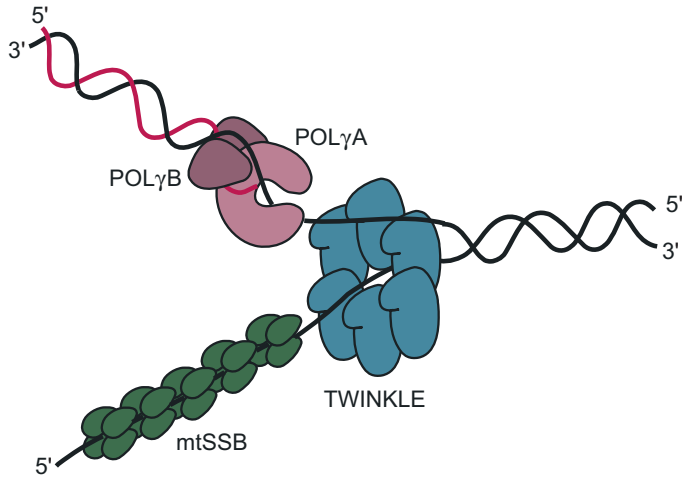

Figure 3

The mtDNA replication machinery. The TWINKLE helicase has 5′ to 3′ directionality and unwinds the duplex DNA template. The mtSSB protein stabilizes the unwound conformation and stimulates DNA synthesis by the POLγ holoenzyme.

Characterization of the components of the mitochondrial replisome should inform us about the mechanism of mtDNA replication in vivo. A comparison of the replication mechanisms in the two bacteriophages T4 and T7 is particularly informative (97). The T4 DNA replication machinery includes a dimeric DNA polymerase. The polymerase subunit on the lagging strand recycles in an ATP-dependent manner and requires a sliding clamp and a clamp loader. In contrast, recycling of the processive T7 DNA polymerase is independent of ATP, and it does not form a homodimer. It has instead been suggested

that physical interactions between the T7 SSB and the leading-strand T7 DNA polymerase coordinate leading- and lagging-strand synthesis (122). Similar to the T7 DNA polymerase, the catalytic POLγA subunit does not form a dimer, and no sliding clamp or clamp loader has been identified in mitochondria. In this respect, it is interesting to note that the monomeric T7 SSB protein, which coordinates leading- and lagging-strand synthesis in the bacteriophage, is structurally distinct from mtSSB. The mtSSB is similar to the tetrameric *E. coli* SSB protein (123, 124), which is dispensable for strand-coordinated DNA synthesis at the bacterial replication fork (122). If coordinated leading- and lagging-strand DNA synthesis takes place at the mitochondrial replication fork, as suggested by Holt and colleagues (88, 89), then the coordination must be achieved by a mechanism that is functionally distinct from the mechanisms previously described for *E. coli* or bacteriophages T4 and T7.

Other Enzymatic Activities Required for mtDNA Replication

A primase activity was reported in extracts from human mitochondria in 1985, but the enzyme has not yet been purified to homogeneity, and the corresponding gene has not been identified (125, 126). A conventional primase activity may not be required for mammalian mtDNA replication if the mammalian mtDNA replicates by the asymmetric strand-displacement mechanism involving two unidirectional origins. POLRMT provides the primer needed for initiation of mtDNA replication at O_H, and it is possible that POLRMT also can prime initiation of L-strand replication O_L (22, 78). Alternatively, the POLγ holoenzyme may use mitochondrial tRNAs as primers. In support of this idea, the POLγ holoenzyme can bind and copy RNA templates, and the accessory subunit POLγB has a tRNA-like binding fold (103). An understanding of the mechanism of primer synthesis at O_L is an important challenge for the future, and the solution of this problem will undoubtedly help us clarify the mode of mtDNA replication. Interestingly, the mode of mtDNA replication may not be evolutionary conserved because TWINKLE contains a primase activity in lower nonmetazoan eukaryotes (117). In addition, yeast and trypanosomes lack identifiable TWINKLE homologues despite the fact that mitochondrial primase activities have been purified from both organisms (27, 116).

RNase H1 may play an important role in mammalian mtDNA replication because *RNaseh1−/−* mice have been reported to have a significant decrease in mtDNA content, leading to apoptotic cell death (127). Ribonucleases' H activities have mostly been implicated in eliminating short RNA primers used for initiation of lagging-strand DNA synthesis. RNase H1 may therefore be required for primer removal at O_H, and/or at O_L, or for primer removal in Okazaki fragment synthesis. Interestingly, whereas *E. coli* RNase H is a distributive enzyme, mammalian RNase H1 is a processive enzyme and therefore able to effectively process long RNA-DNA hybrids (128). It remains to be determined if the processive action of RNase H1 is essential for digestion of specific intermediates in mtDNA replication.

The mtDNA are closed circular molecules, and topoisomerase activity is required to relieve the torsional strain that is generated by mtDNA replication. The TOP1mt gene encodes a mitochondrial topoisomerase, which appears unique to vertebrate cells. The protein has a molecular mass of about 72 kDa and the capacity to relax negative supercoils (129, 130).

Organization into Nucleoids

Mammalian cells contain thousands of mtDNA molecules that are organized in several hundred nucleoids. The distribution of nucleoids may be an important determinant of the segregation of heteroplasmic mitochondrial genomes in cells bearing a mixture of wild-type and mutant mtDNA molecules.

In vivo time-lapse imaging of mammlian nucleoids has demonstrated that they are dynamic structures able to divide and redistribute in the mitochondrial network and suggests that nucleoids are the mitochondrial units of inheritance (131). The packaging of multiple mtDNA molecules into a single nucleoid may therefore provide a molecular explanation for the observation that the segregation of mtDNA mutants is faster than would be expected given the large number of mtDNA genomes in a cell. The human nucleoid has been isolated by immunoaffinity purification, and the protein content has been characterized (132). This approach led to the identification of 21 proteins, including many proteins with previously known roles in mtDNA replication and transcription, e.g., TFAM, mtSSB, and TWINKLE. Several of the nucleoid proteins were previously unidentified mitochondrial proteins, including a specific isoform of the DEAH-box helicase DHX30. mtDNA molecules packaged in nucleoids are engaged in a variety of dynamic processes, including replication and transcription. Nucleoids also contain proteins known to reside in the mitochondrial inner membrane, such as ANT1 and prohibitin, which suggests that mtDNA is membrane-associated. A molecular understanding of the nucleoid structure may also have direct implications for mtDNA copy number control. The major nucleoid component, TFAM, may serve as a key regulator of the mtDNA copy number. The human TFAM protein is a poor activator of mouse mtDNA transcription, despite its high capacity for unspecific DNA binding. By overexpressing human TFAM in P1 artificial chromosome (PAC) transgenic mice, it was therefore possible to investigate effects of TFAM independent of its role in mitochondrial transcription (133). Interestingly, the overexpression of human TFAM in the mouse results in upregulation of mtDNA copy number without increasing respiratory chain capacity or mitochondrial mass. This experiment therefore experimentally dissociated the role of TFAM in mtDNA copy number regulation from mtDNA expression and mitochondrial biogenesis in mammals in vivo.

Somatic mtDNA Mutations and Aging

A role for mitochondrial dysfunction in mammalian aging is supported by the finding of progressive accumulation of somatic mtDNA mutations and decline of respiratory chain function with increasing age (134). Increased amount of rearrangements (deletions/small duplications) and point mutations of mtDNA have been reported during aging in several mammalian species. A possible causative role has been much debated because the abundance of individual somatic mtDNA mutations in tissue homogenates is well below the threshold levels needed to cause respiratory chain dysfunction. The highest levels of age-associated multiple mtDNA deletions range from 0.5% to 2% and are observed in postmitotic tissues, such as heart, skeletal muscle, and brain. Studies of different tissues have shown that the somatic mtDNA mutations are not always evenly distributed but can accumulate clonally in single cells and cause a severe respiratory chain deficiency in these cells. It is probable that clonal expansion of pathogenic mtDNA mutations in individual cells will lead to their loss with age, but is unclear how this phenomenon will affect organismal survival.

The recently created mtDNA-mutator mice are homozygous for a knockin allele expressing an mtDNA polymerase that is inherently error prone (135). This defective mtDNA polymerase has a normal DNA synthesis capacity, but the 5′-3′ exonucleolytic proofreading activity is severely reduced. As a consequence, these mutant mice have a three- to fivefold increase in levels of somatic mtDNA point mutations and also contain a linearly deleted mtDNA form. The defects in mtDNA cause a progressive respiratory chain deficiency, which reduces life span and generates a variety of premature aging phenotypes, including osteoporosis, anemia, cardiomyopathy, reduced fertility, weight loss,

alopecia, hair loss, and lean body composition. It is thus clear that high levels of somatic mtDNA mutations can cause aging phenotypes in mammals, and these experimental data therefore suggest that the occurrence of somatic mtDNA mutations may be causally related to mammalian aging. The role of mitochondrial dysfunction in human aging must now be revisited, and additional studies of respiratory chain capacity and mtDNA mutation load in different organs from humans of different ages must be performed to further validate this hypothesis. It may also be possible to perform additional genetic experiments in the mouse to test this hypothesis, e.g., creating mice with enhanced mtDNA polymerase proofreading capacity or enhanced mtDNA repair capacity. Such mouse mutants should have a longer than normal life span if somatic mtDNA mutations have a causative role in aging. Subsequent studies examining the consequences of a proofreading deficiency in the mtDNA polymerase of the mouse have led to the conclusion that cellular apoptosis events may be enhanced by accumulation of induced mtDNA mutations (136).

CONCLUSIONS AND FUTURE PROSPECTS

In recent years, there have been rapid developments in our understanding of the basal molecular machineries that are responsible for mammalian mtDNA replication and transcription. Even so, many questions still remain unanswered. There is no generally accepted model for mtDNA replication, and new experimental approaches must be used to conclusively resolve this issue. The mechanisms of lagging-strand DNA synthesis are not understood, and it is necessary to establish the mechanisms of primer formation. Key to this understanding is to identify and further characterize the primase, which previously only has been described as an activity in mammalian mitochondria. The mechanisms of D-loop formation and the functional role of these structures in regulation of mtDNA replication must be addressed. Experiments suggest the existence of a specific TAS-binding protein, and the identification of this factor should provide important insights into the regulation of mtDNA replication. An mTERF orthologue in *P. lividus* displays a contrahelicase and binds to a site where DNA synthesis stops to generate the D loop (81). This observation may suggest that one of the uncharacterized mTERF proteins is the long-sought-after TAS-binding protein in mammals.

The basal components of the mitochondrial transcription machinery are known, but how these are regulated in response to the metabolic requirements of the mammalian cell remains to be established. The possibility that the respiratory chain and the redox status of the cell directly influence mitochondrial gene transcription must be further explored. The existence of two homologous transcription factors, TFB1M and TFB2M, is intriguing, but the physiological importance of this increased complexity in mammalian cells is still not understood. Characterization of the newly identified mTERF proteins should provide important insights into regulation of transcriptional termination. Most certainly, there are other yet-to-be-identified factors, which may influence mitochondrial gene expression.

SUMMARY POINTS

1. Many key components of the mtDNA replication and transcription machineries in mammalian mitochondria are related to proteins found in bacteriophages.

2. Transcription from the mitochondrial promoters produces polycistronic precursor RNA molecules encompassing all of the genetic information encoded in each of the specific strands.
3. Regulation of transcriptional termination is an important principle in the control of mitochondrial gene expression. A new family of transcription termination factors may play an essential role in this process.
4. Mitochondrial transcription and DNA replication are coupled events. LSP transcription generates the primers required for initiation of leading-strand DNA synthesis at O_H.
5. The mode of mtDNA replication is currently under debate.
6. The mtDNA molecules are organized into nucleoprotein complexes, denoted nucleoids. These structures may be involved in epigenetic regulation of mtDNA replication and transcription.

FUTURE ISSUES

1. Establishing the functional role of the mTERF family for regulation of mammalian mtDNA replication and transcription is necessary.
2. Further experiments are required to arrive at a generally accepted model for mtDNA replication.
3. The mechanisms and regulation of D-loop formation must be determined.
4. The nucleoid structure and its role in epigenetic regulation of mtDNA replication and transcription need to be further addressed.

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