

REVIEW

The Genetics of Childhood Disease and Development: A Series of Review Articles

This review is the seventh in this series. Dr. Elpeleg describes the exciting, new field of mitochondrial DNA depletion syndromes. The genetic defects responsible for mitochondrial DNA depletion are discussed as well as the variations in clinical manifestations and tissue expression.

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Inherited Mitochondrial DNA Depletion

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ABSTRACT

Mitochondrial DNA (mtDNA) depletion is associated with heterogeneous clinical phenotypes. The recent identification of the mutated genes in three groups of patients with mtDNA depletion had underscored the importance of the synthetic pathway of the mitochondrial nucleotides for mtDNA replication. Future goals include understanding how the defective proteins perturb replication, why it affects only some tissues and spares others, and which other genes should be considered in other patients with mtDNA depletion. (*Pediatr Res* 54: 153–159, 2003)

Abbreviations

mtDNA, mitochondrial DNA

dNTP, deoxyribonucleoside triphosphate
dNDP, deoxynucleoside diphosphate
TP, thymidine phosphorylase
dGK, deoxyguanosine kinase
TK2, thymidine kinase 2
dThd, deoxythymidine
dCyt, deoxycytidine
dCK, deoxycytidine kinase
dNT2, mitochondrial nucleotidase
Tfam, mitochondrial transcription factor A
POLG, mitochondrial DNA polymerase γ
TMP, thymidine monophosphate

Mitochondria are believed to be direct descendants of a bacterial endosymbiont (most likely *Rickettsia prowazekii*) that became established ~1.5 billion years ago, in a nucleus-containing host cell. The low gene content of mammalian mtDNA compared with even the smallest known eubacterial genomes appears to imply a relatively rapid and extensive loss

or transfer of genetic information from the mitochondria to the nucleus and a compensatory import of nuclear encoded proteins into the mitochondria (1). It is not completely understood why mitochondrial genes moved to the nuclear genome, how they survived their voyage, and whether this process seized to occur. Transfer of genes to the nucleus may have been favored by the genetic principle, which predicts that deleterious, but sub-lethal, mutations accumulate more rapidly in an asexually propagated genome (mtDNA) than in a sexually propagated one (nuclear DNA), ultimately dooming the former to extinction (2). Gene retention in the mitochondria, on the other hand,

Received April 2, 2002; accepted February 18, 2003.
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DOI: 10.1203/01.PDR.0000072796.25097.A5

may stem from the need of the cell to protect itself from oxidative damage, having redox control on the organelle gene expression (3). The difficulty of importing extremely hydrophobic proteins from the cytosol, most notably cytochrome b and COXI with their 8 and 12 transmembrane regions, respectively, may also contribute to the mitochondrial localization of their genes. The net result is the retention of a small number of genes in the mitochondria, with proteins involved in mtDNA replication and maintenance encoded by the nucleus. Defects of mtDNA replication and maintenance, resulting in mtDNA depletion or multiple deletions, are therefore transmitted mainly in an autosomal manner.

The recent elucidation of the molecular basis of some of the disorders associated with mtDNA depletion is the subject of this review. Since the first description of inherited mtDNA depletion a decade ago, more than 50 patients have been reported (4). Most patients presented in the neonatal period with muscle weakness, hepatic failure, or renal tubulopathy accompanied by lactic acidemia and died during the 1st year of life. The remaining patients presented with isolated myopathy associated with motor regression or with a slowly progressive encephalomyopathy that began in early childhood (5–9). Of special clinical interest was the finding of mtDNA depletion in infants with Navajo neurohepatopathy, an autosomal recessive condition characterized by severe sensory motor peripheral neuropathy, cerebral white matter disease, and recurrent episodes of liver failure (10). Unusually late onset and slower progression was reported in three children with mtDNA depletion who developed glomerular disease, deafness, retinitis pigmentosa, white matter disease, muscle weakness with ptosis, and external ophthalmoplegia during childhood and survived to adolescence (11). mtDNA depletion was usually suspected in patients with normal activity of complex II (succinate:ubiquinone reductase) and decreased enzymatic activity of all other mitochondrial respiratory chain complexes (12). This is because complex II is the only one that is solely composed of nuclear encoded proteins; all other enzymatic complexes contain both mitochondrial and nuclear encoded subunits. mtDNA content was quantified by Southern blot with hybridization of mitochondrial and nuclear probes and the genomes ratio was calculated. Because it is unclear what constitutes normal mtDNA levels in different tissues at different ages and to compensate for variations in hybridization conditions, data were compared within a single blotting experiment that included tissue-specific and age-matched control samples. For the diagnosis of mtDNA depletion, most authorities regarded mtDNA/nDNA ratio of 30% as a cut-off point (5, 9). Autosomal inheritance was repeatedly suggested by the families' clinical history and was elegantly demonstrated by the restoration of mtDNA replication following fusion of the patient cells with control mtDNA-less (ρ^0) cells and failure of the control mtDNA molecules to replicate after being fused with patient fibroblasts (13).

The vast array of symptoms suggested that mtDNA depletion is the common outcome in several monogenic disorders. Elucidation of the underlying molecular background required prior assignment of the patients to complementation groups;

however, the normal mitochondrial respiratory chain function in skin fibroblasts of most patients precluded complementation studies. Clinical classification based on the combination of organs involved was hampered by the fact that mtDNA levels have not been examined in unaffected tissues to the same extent as in affected tissues and by the short life span of the patients. While decreased mtDNA content was invariably detected in affected tissues, unaffected tissues exhibited a wide range of mtDNA abundance levels. For example, in a patient who presented with neonatal encephalopathy and expired in infancy muscle mtDNA content was 23%, liver 7%, kidney 24%, and brain 37% of the control (14). It is a matter of conjecture whether organs such as kidney or muscle would become involved had this patient lived longer, given the known variability of clinical threshold among tissues (15).

Clinical classification was additionally complicated by the postnatal changes of mtDNA abundance in a given organ, exemplified by the finding of mtDNA/nDNA ratio of 27% of the control in muscle at 2 mo of age and 9% of the control at 8 mo of age in the same patient (6). Estimation of the full extent of organ involvement should therefore be repeated throughout the course of the disease.

Finally, for the purpose of classification, secondary mtDNA depletion, as may occur in viral hepatitis, iron storage disease, or chronic cirrhosis with severe asthenia, has to be sorted out (10, 16), and epigenetic factors must be considered. In the first reported family, the index case suffered from hypotonia, external ophthalmoplegia, and lactic acidemia, and expired at 3 mo of age. Mitochondrial respiratory chain defect and markedly decreased mtDNA content was evident in his muscle but not in his liver, kidney, brain, and heart (4, 17). A similarly affected sister whose tissues were not available for molecular studies had cytochrome b deficiency in liver and a second cousin died from hepatic failure with markedly decreased mtDNA content in liver. This variable organ involvement within the same family is hard to understand in simple genetic terms and cast doubt on the reliability of clinical classification in mtDNA depletion syndromes. Altogether, attempts toward elucidation of the underlying molecular background were impeded by the lack of well-defined patient groups.

In the absence of proper clinical delineation, the search for mutated genes relied on linkage analysis of large kindred and on attempts to extrapolate from the mechanism underlying acquired forms of mtDNA depletion and animal mutants. For instance, mtDNA depletion has been reported in HIV patients treated with antiviral nucleoside analogs, which were incorporated into the mtDNA and inhibited DNA synthesis (18–20). Similarly, in patients with chronic hepatitis B infection, a therapeutic trial of the nucleoside analogue fialuridine was stopped because of lactic acidosis, hepatic failure, and skeletal and cardiac myopathy due to inhibition of mtDNA replication (21, 22). Finally, deletions and depletion of mtDNA were observed in yeast mutants with defects in the nucleotide metabolizing enzymes thymidylate synthetase, thymidylate kinase, or ribonucleotide reductase (23). These examples suggested that well-balanced pools of mitochondrial nucleotides are a prerequisite for mtDNA replication. In contrast to nuclear

DNA, mtDNA is replicating throughout the cell cycle, requiring stable supply of deoxyribonucleoside triphosphate (dNTP) molecules for its synthesis. Even in replicating cells, where cytosolic dNTPs are also produced, they can only traverse the mitochondrial inner membrane *via* dedicated transporters because of their charge (24–26). To date, only one mitochondrial nucleotide transporter, the deoxynucleotide carrier (DNC) has been characterized; it preferentially transports the four deoxynucleoside diphosphate (dNDPs) and less efficiently the dNTPs (27). Nonetheless, in nonreplicating cells, mtDNA synthesis relies on the salvage pathway to maintain the mitochondrial dNTP pool (Fig. 1).

As expected, linkage analysis of large kindred and clinically homogenous patients revealed that some of the genes mutated in mtDNA depletion are involved in the metabolism of the mitochondrial dNTPs. The first gene so linked was the thymidine phosphorylase (TP), whose product catalyzes the reversible conversion of thymidine (dThd) and deoxyuridine to their bases and 2-deoxy D-ribose 1-phosphate. Mutations in the *TP* gene were found in patients with mitochondrial neurogastro-

intestinal encephalomyopathy (MNGIE) syndrome characterized by white matter disease, ptosis, progressive external ophthalmoparesis, gastrointestinal dysmotility, cachexia, and peripheral neuropathy. In all the examined patients mtDNA depletion was identified and in half of these, multiple mtDNA deletions were also detected (28, 29). Because of the increased plasma dThd levels, it was proposed that the defective mtDNA synthesis is attributed to an overload on the mitochondrial dThd salvage pathway. It was also suggested that alternative forms of nucleoside dysmetabolism might cause other mitochondrial diseases characterized by mtDNA depletion (30, 31). In agreement, linkage analysis of large kindred of Druze origin yielded mutations in the *DGUOK* gene, which encodes deoxyguanosine kinase (dGK), a mitochondrial salvage enzyme that phosphorylates deoxyguanosine and deoxyadenosine. Patients originated from this kindred presented in infancy with fatal liver failure, encephalopathy, and mtDNA depletion in liver but not in muscle. All the patients were homozygous for a frame shift mutation associated with an undetectable protein (32). Since the activity

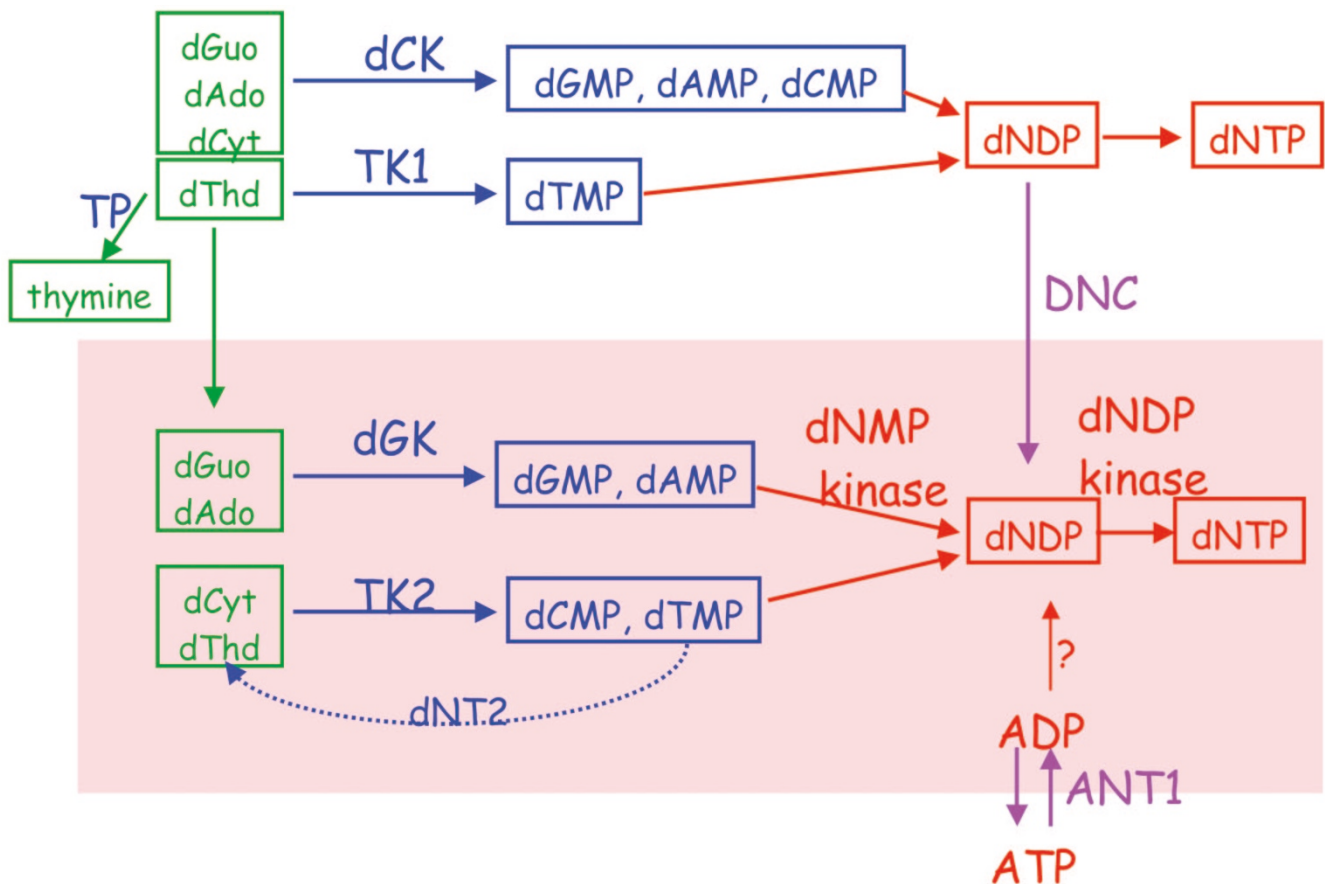


Figure 1. Schematic representation of the synthetic pathway of the deoxyribonucleotides in the mitochondria. The mitochondrial inner membrane is impermeable to charged molecules and the mitochondrial dNTP pool is maintained by either import of cytosolic deoxyribonucleotide diphosphates (dNDP) *via* a dedicated transporter (DNC) or by salvage of deoxyribonucleosides within the mitochondria. The substrate specificity and the number of the dNMP and dNDP kinases are currently unknown. ANT1, an adenine nucleotide translocator, is involved in mtDNA stability as mutations in the gene are associated with multiple mtDNA deletions in muscle (61). DGuo, deoxyguanosine; dAdo, deoxyadenosine; dCyt, deoxycytidine; dThd, thymidine; dCK, deoxycytidine kinase; TK1, cytosolic thymidine kinase; dGMP, deoxyguanosine monophosphate; dAMP, deoxyadenosine monophosphate; dCMP, deoxycytidine monophosphate; dTMP, thymidine monophosphate; dNDP, deoxynucleotide diphosphate; dNTP, deoxynucleotide triphosphate; dGK, deoxyguanosine kinase; TK2, mitochondrial thymidine kinase.

of dGK toward deoxypurines is similar to that of the mitochondrial thymidine kinase (TK2) toward deoxypyrimidines (Fig. 1), the *TK2* gene was sequenced in patients with other mtDNA depletion phenotypes. This analysis revealed missense mutations in the *TK2* gene in infants with isolated myopathy and muscle mtDNA depletion (33). Genetic heterogeneity is evident for both phenotypes, as mutations in the *DGUOK* and *TK2* genes were identified in only ~10% of the patients with similar phenotypes (34–36).

The findings are not only useful for the genetic counseling of the affected families, but also underscore the indispensability of the salvage pathway for mitochondrial dNTP metabolism. The ability of the mitochondria to use ribonucleosides as precursors of dNTPs, *i.e.* the existence of a mitochondrial *de novo* pathway of dNTP synthesis, was previously suggested by the demonstration of a mitochondrial specific ribonucleotide reductase activity, a key enzyme of the *de novo* pathway (37). The finding of mtDNA depletion in patients with defective salvage pathway negates the possibility of a significant contribution of a mitochondrial *de novo* pathway to dNTP synthesis.

The selective nature of tissue involvement in patients with TK2 and dGK deficiencies exemplifies the role of the overlapping specificities of the deoxyribonucleotide kinases in the determination of the clinical phenotype. In mammals there are four deoxyribonucleoside kinases with marked overlapping specificities; the two mitochondrial enzymes, TK2 and dGK, and two cytoplasmic enzymes, thymidine kinase 1 (TK1), which phosphorylates dThd and deoxyuridine, and deoxycytidine kinase (dCK) which phosphorylates deoxyadenosine, deoxyguanosine, and deoxycytidine (dCyt). In contrast, the fruit fly *Drosophila melanogaster* and the insect *Bombyx mori* have only one deoxyribonucleoside kinase, which phosphorylates all naturally occurring deoxyribonucleosides (38, 39). It is therefore likely that all mammalian deoxyribonucleoside kinases have originated from a common progenitor kinase, with the progenitor of TK1-like kinases and the progenitor of the TK2-, dCK/dGK-like kinases being generated by the first gene duplication. The modern TK1-like enzymes are all strictly specific for pyrimidine substrates, while the other phylogenetic group includes pyrimidine, purine, and multisubstrate deoxyribonucleoside kinases, giving rise at a later stage to TK2-like enzymes, which include the *D. melanogaster* and the *B. mori* single multisubstrate kinase and the pyrimidine deoxyribonucleoside kinases (including *X. laevis* TK2) and to a dCK/dGK-like enzymes, which are dCyt and/or purine deoxyribonucleoside-specific kinases (40, 41). The selective involvement of tissues in dGK and TK2 deficiency, notwithstanding with the ubiquitous expression of these mitochondrial genes, could be partly explained by the overlapping specificities of deoxyribonucleoside kinases. For example, the preferential involvement of liver and brain in dGK deficiency may derive from the low expression of the cytosolic dCK, which like dGK phosphorylates deoxyadenosine and deoxyguanosine. The deoxypurine monophosphate molecules so formed may be further phosphorylated in the cytosol and imported into the mitochondria by DNC, the deoxynucleotide carrier. The low activity of dCK in brain, liver, and muscle suggests that the mitochondria

will be dependent on dGK activity. The fact that the original patients who died in early infancy had normal mitochondrial respiratory chain activity and mtDNA content in muscle despite an undetectable dGK protein is therefore puzzling (32). A recently identified patient, homozygous for a milder mutation in the *DGOUK* gene, had low mitochondrial respiratory chain activity in liver and also in muscle (M. Zeviani, personal communication), suggesting that muscle mitochondria have slightly better compensation for reduced dGK activity, deteriorating later in the course of the disease.

The selective involvement of muscle in patients with TK2 deficiency could not be solely explained by overlapping specificities of the deoxyribonucleoside kinases. The two cytosolic kinases perform together the role of TK2; dCK phosphorylates dCyt and TK1 phosphorylates dThd. Because TK1 is cell-cycle regulated, the mitochondria of nonreplicating tissues is entirely dependent on TK2 activity. Deficiency of TK2 would not manifest in replicating cells because of cytosolic compensation but is bound to affect all quiescent tissues. Preliminary data suggest that sparing of some quiescent tissues, such as liver and heart, may be attributed to the low TK2 activity and relatively high mitochondrial encoded proteins content in control human muscle when compared with liver or heart tissue. This decreased ratio likely renders the muscle especially vulnerable to TK2 dysfunction (Saada-Reisch A, Shaag A, Elpeleg O, unpublished data). Because none of the reported patients survived past the 4th birthday and only missense mutations were identified, it is a matter of conjecture whether other postmitotic tissues would show clinical symptoms in the course of the disease or in patients harboring more deleterious mutations.

The exact aberrations of the mitochondrial dNTP pools are not easily predicted. The ratio of the deoxypyrimidine:deoxypurine monophosphates is likely decreased in TK2 deficiency. However, in patients' cells the phosphorylation of dCyt was more perturbed than that of dThd possibly due to a higher affinity of normal TK2 toward dThd. This would not necessarily result in a decreased ratio of dCyt monophosphate to thymidine monophosphate (TMP) because TMP is the preferred substrate of the only known mitochondrial nucleotidase, dNT2 (42). The determination of the mitochondrial dNTP pools, which is bound to resolve these uncertainties, is hampered by the need for an exceptionally large number of affected mitochondria for these experiments. The use of animal models for similar studies was heretofore unsuccessful; a condition mimicking TP deficiency in mice, with disruption of the two murine pyrimidine phosphorylase genes, resulted in only moderate elevation of plasma dThd level with preservation of mtDNA abundance suggesting an inter-species variability of the expression pattern of other dThd modulating proteins (43).

At present, the gene defect is known for only a small number of patients with mtDNA depletion. Where should we look next? Other kinases involved with the phosphorylation of the products of the reactions catalyzed by TK2 and dGK are immediate candidates. Little is known about the mitochondrial deoxynucleotide monophosphate kinases, but two dNDP kinases have been identified in the mitochondria, nm23-H4 and

nm23-H6 (44–46). The affinity of these kinases toward the various dNDPs has not been clarified. Nonetheless the relatively low expression of nm23-H4 in brain, kidney, and leukocytes may imply that mutations that further decrease the expression would primarily result in encephalopathy and tubulopathy with relative preservation of mitochondrial respiratory chain function in other tissues.

Mitochondrial nucleotide imbalance may also result from a defect in the nucleotidase dNT2, which functions in the fine-tuning of dThd triphosphate levels (42). Mutations in dNT2 might lead to surplus of its main substrate TMP with subsequent accumulation of dThd triphosphate, causing mtDNA depletion as in TP dysfunction. However, because dNT2 is hardly expressed in human liver but is abundant in brain, skeletal muscle, and heart, isolated liver failure may be the only symptom in dNT2 deficiency.

A defect in DNC, the deoxynucleotide carrier is not expected to result in mtDNA depletion, because the mitochondria are capable of producing its own dNTPs throughout cell cycle. However, a defect in the DNC was recently reported in patients with severe microcephaly (47, 48). Although mtDNA content was not determined in these patients, the associated massive α -ketoglutaric aciduria might reflect mitochondrial dysfunction.

Defects in proteins that participate in the process of mtDNA synthesis but are not part of the dNTP synthesis machinery are also expected to result in mtDNA depletion (Fig. 2). The

mtDNA is a double stranded, 16.5-kb circular molecule. It consists of a heavy (H) strand and a light (L) strand, which differ in their G+T base content. The molecule replicates in an asynchronous manner, with the L-strand lagging behind the H-strand synthesis. The replication of the H-strand is primed by a short L-strand transcript, which is synthesized by the mitochondrial RNA polymerase. To allow access to the mtDNA duplex, a conformation change is induced through the binding of mitochondrial transcription factor A (Tfam). Two additional proteins, mitochondrial transcription factor B1 and B2, were recently shown to be essential for the formation of the L-strand transcript (49). The indispensable role of the transcript for mtDNA replication was underscored by the development of mtDNA depletion in a yeast strain mutated at its mitochondrial RNA polymerase gene and by the low mtDNA abundance and embryonic lethality in *Tfam* $-/-$ mice (50, 51). A search for mutations in the *Tfam* gene was initiated by the finding of decreased abundance of the *Tfam* protein in the cells of several patients with mtDNA depletion but none were found. Thus, in the study of candidate genes, proteins that require mtDNA molecules for their own stability and are secondarily decreased, should be distinguished from those that are the primary cause of the disease (14, 52).

Most L-strand transcripts are cleaved by an RNA processing endonuclease at the origin of replication of the H-strand and remain attached to the two parental strands. This endonuclease can probably be excluded from the list of candidate genes because mutations in its gene, although not associated with complete abolition of activity, did not result in mtDNA depletion but with another disease, cartilage-hair hypoplasia (53). Subsequent to the cleavage of the short L-strand transcript, bases are added to its free 3'-terminus by the mitochondrial DNA polymerase γ (POLG). Only missense mutations in the *POLG* gene have been reported, and all were associated with multiple mtDNA deletions and adult onset myopathy (54). It is possible that the mutated *POLG* retains some catalytic activity but loses its proof reading ability, resulting in markedly increased error rate for certain mismatches (55); in homozygotes, a complete lack of *POLG* activity is probably lethal *in utero* due to severe mtDNA depletion but with no symptoms in heterozygotes.

In addition to *POLG*, multiple *trans*-acting factors participate in the elongation and maturation of the growing strands of the human mtDNA and should thus be included in linkage analyses of patients with mtDNA depletion syndrome. Topoisomerase I, which introduces reversible breaks in the DNA phosphodiester backbone so to relieve the strain that arises during the closed-circle replication (56), and the mitochondrial single stranded binding protein, which prevents large single-stranded DNA regions from renaturation (57), are two well-characterized candidate genes. A less attractive candidate is the recently described topoisomerase III, which probably participates in the resolution of mtDNA rings in their final stage of replication. Mutations in this gene would likely result in a nuclear rather than mitochondria DNA pathology, as most topoisomerase III transcripts lack mitochondrial targeting signal and are localized to the nucleus (58).

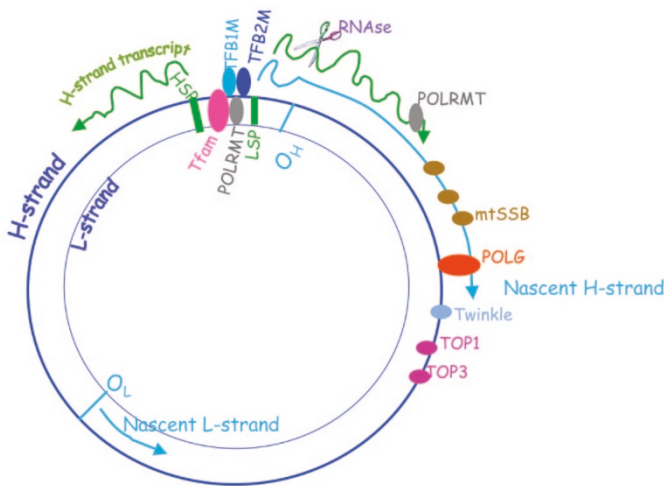


Figure 2. Schematic drawing of the major steps of mtDNA replication. The circular, double stranded molecule of mtDNA is replicated in a strand-asynchronous manner and the L-strand replication starts at its origin of replication (O_L) only after the nascent H-strand synthesis has completed about two-thirds of the circle and proceeds in the opposite direction. The H-strand replication is initiated using a short L-strand transcript that is synthesized by a mitochondrial RNA polymerase (POLRMT). POLRMT gains access to its DNA template by the binding of mitochondrial transcription factor A, B1, and B2 (Tfam, TFB1M, and TFB2M) to the mtDNA duplex. The precursor RNA primer is cleaved by mitochondrial RNA processing endonuclease (RNase) at the origin of replication of the H-strand (O_H). Bases are added to the free 3'-termini of the transcript by mitochondrial DNA polymerase gamma (POLG). The Twinkle protein and the topoisomerase 1 and 3 (TOP1 and TOP3) are required for the preparation of single stranded templates for POLG activity. HSP and LSP, H-strand and L-strand promoters; mtSSB, mitochondrial single stranded binding protein.

Mutations were identified in the human *C10orf2* (Twinkle) gene, which encodes a protein homologous to the helicase domain of the T7 bacteriophage and thus may be involved in the preparation of single stranded templates for POLG activity by unwinding the double strand. As with POLG, patients heterozygous for mutations in the *Twinkle* gene suffer from myopathy and multiple mtDNA deletions (59).

Mutations should be sought in genes yet to be identified, which are involved with the modeling of mtDNA, its segregation, and the expression level or import of replication proteins. Their number should not be underestimated as 36 complementation groups of yeast mutants exhibit temperature-induced loss of mtDNA (60).

Acknowledgment. Dr. Ann Saada-Reich is acknowledged for fruitful discussions.

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