DNA Polymerase γ in Mitochondrial DNA Replication and Repair

Maria A. Graziewicz, Matthew J. Longley, and William C. Copeland*

Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, North Carolina 27709

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* To whom correspondence should be addressed. Telephone: 919-541-4792. Fax: 919-541-7613. E-mail: copelan1@niehs.nih.gov.

1. Introduction

DNA polymerases are indispensable for maintaining the integrity of the genome, both through faithful replication of DNA and by repairing damage to DNA. Among the 16 highly specialized mammalian polymerases, 15 are involved in maintaining nuclear genetic information. Polymerases α, β, δ, and ϵ function in replication and repair of nuclear DNA, and the remaining nuclear DNA polymerases participate in either translesion synthesis to prevent the arrest of nuclear DNA replication, in DNA repair, or in somatic hypermutation, reviewed in refs 1 and 2. In contrast, replication and maintenance of the mitochondrial genome relies on a relatively modest enzyme repertoire. As the only DNA polymerase found in animal cell mitochondria, DNA polymerase γ (pol γ) bears sole responsibility for DNA synthesis in all replication, recombination, and repair transactions involving mitochondrial DNA (mtDNA). Mutations accumulate in mtDNA with age, and mutation of mtDNA has been shown to promote premature aging in mice. Also, mutation and/or depletion of mtDNA has been observed in certain human diseases, and several recent reports link some forms of these diseases to heritable defects in the pol γ gene. This review summarizes the current knowledge of pol γ roles in maintaining mitochondrial DNA, mitochondrial DNA replication and repair, diseases, and aging.

1.1. History

Mitochondria became a symbiotic element of the eukaryotic cell about 1.8 billion years ago, when a small α-proteobacterium was endocytosed to become an intracellular generator of ATP.[3] Mitochondria were first visualized as discrete organelles by light microscopy in 1840. Their isolation, however, was not possible until 1948, when zonal centrifugation methods were developed. In the early 1960s it was determined that these cytoplasmic organelles contain their own DNA. The entire DNA sequence of the human mitochondrial genome (16 569 nucleotides) was determined in 1981, well in advance of the Human Genome Project.[4] Four years later, in 1985, mitochondrial gene products were assigned. Unlike nuclear DNA, double-stranded mtDNA is very compact and does not contain introns or long noncoding segments. The genome contains 37 genes, all of which are directly or indirectly involved in the production and storage of energy in the form of ATP. Thirteen of these genes encode protein subunits involved in electron transport or oxidative

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Maria A. Graziewicz received her M.Sci. from the University of Warsaw, Poland. She obtained her Ph.D. in 1999 in Biochemistry and Molecular Biology working with Professor Celina Janion and Dr. Barbara Tudek at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. She is currently a postdoctoral fellow for Dr. William C. Copeland in the Mitochondrial DNA Replication Group, at the National Institute of Environmental Health Sciences, NIH. Her research interests are focused on DNA replication, damage, and repair processes in association with human diseases.

Matthew J. Longley received his B.A. in Biochemistry in 1986 and his Ph.D. in Chemistry in 1991 from the University of Texas at Austin, where he characterized porcine DNA polymerase γ with Dr. Dale W. Mosbaugh. He did postdoctoral work with Dr. Paul Modrich in the Department of Biochemistry at Duke University from 1991 to 1995. He is currently a Staff Scientist in the Laboratory of Molecular Genetics at the National Institute of Environmental Health Sciences, NIH, where his research focuses on the structural and biochemical characterization of the protein components that replicate and repair the human mitochondrial genome.

William C. Copeland received his B.S. in Chemistry from the University of Florida and his Ph.D. in Chemistry from the University of Texas at Austin in 1988 under the supervision of Dr. Jon Robertus. He completed his postdoctoral training at Stanford University School of Medicine, Department of Pathology, with Professor Teresa Wang studying the human DNA polymerase α·primase complex. In 1993 he joined the National Institute of Environmental Health Sciences, NIH, in the Laboratory of Molecular Genetics as head of the Mitochondrial DNA Replication Group within the Division of Intramural Research. His major research interests are in the field of DNA replication and mitochondrial disorders in human diseases. He also serves as president of the Mitochondrial Research Society.

phosphorylation. The remaining 24 genes encode the transfer RNAs (22 genes) and ribosomal RNAs (2 genes) required for mitochondrial protein synthesis.[5,6] A noncoding segment referred to as the displacement loop bears several *cis*-acting elements required for initiation of transcription and replication. Several hundred mitochondria can be present in individual cells, and each mitochondrion is estimated to contain 2−10 copies of mtDNA.[7,8] Since mitochondria have such a critical role in providing energy to eukaryotic cells, the enzymes responsible for maintaining the mitochondrial genome are essential for normal function of the living cell. The DNA polymerase involved in mtDNA replication was first reported as an RNA-dependent DNA polymerase activity in 1970.[9,10] This activity differed from viral reverse transcriptases in that it failed to utilize natural RNA as a substrate.[11,12] The first evidence indicating that this new activity was distinct from pol α and pol β came from column fractionation of HeLa cell extracts.[13] By 1975 the new polymerase was officially designated as pol γ, although the cellular function was still unclear at that time.[14] In 1977 pol γ was localized to the mitochondrial compartment,[15] and evidence supporting the functional role of pol γ in mitochondria was obtained two years later in a study of isolated brain synaptosomes.[16] Further evidence for the role of pol γ was provided by inactivation of the yeast pol γ gene (*MIP1*)[17] and by inhibition of mtDNA replication in mitochondrial extracts with antibodies directed against pol γ.[18] With the exception of trypanosomatid *Crithidia fasciculata*, pol γ is the only DNA polymerase found in mitochondria.[19] In trypanosomes, mitochondrial DNA is wrapped up in kinetoplasts (kDNA), where it is replicated and/or repaired by a number of β-like DNA polymerases.[19−21] Interestingly, a γ-like DNA polymerase has never been isolated or identified in trypanosome mitochondria. In addition to earlier localization of pol β from trypanosomatid mitochondria,[22] other DNA polymerases were detected in mitochondrial extracts from *Trypanosoma brucei*.[23] Four different mitochondrial DNA polymerases, TbPOLIA, IB, IC, and ID, have been identified in *T. brucei*, raising the total number of mtDNA polymerases in trypanosome mitochondria to five.[24] None of the four new proteins are homologous to pol γ, although all four are related to bacterial pol I. Interference with TbPOLIB and TbPOLIC activities led to shrinkage of kDNA. Silencing TbPOLIC caused depletion of kDNA minicircles and maxicircles, and the concomitant accumulation of minicircle replication intermediates clearly indicates a role for TbPOLIC in kDNA replication.[24]

Among the 16 known eukaryotic DNA polymerases, pol γ is the only DNA polymerase to have been detected in mammalian mitochondria.[1,2,25] Nevertheless, the presence of other polymerases in mitochondria, perhaps within specialized cell types or expressed transiently during development, remains a formal possibility. Pol β, however, is clearly absent from mouse embryonic fibroblasts after purification of mitochondria over Percoll gradients.[26]

2. Pol γ Proteins and Activities

2.1. Purification and Subunit Composition of Pol γ

Mitochondrial DNA accounts for approximately 1% of the cellular DNA, and pol γ activity comprises only 1−5% of the total cellular DNA polymerase activity.[11,12] The low natural abundance of pol γ and its sensitivity to proteolysis and oxidative damage have been major obstacles in the purification and biochemical characterization of pol γ from a variety of sources. High-molecular-weight forms of pol γ were first isolated from chicken,[27] and by the mid 1980s various groups had reported native molecular weights for pol γ ranging from 47 kDa to 330 kDa (reviewed in ref 11). Today it is well established that pol γ in animal cells possesses two distinct subunits, and yeast pol γ has only one polypeptide. Pol γ from *Drosophila melanogaster* has 125 kDa and 35 kDa subunits, and the native form sediments as a 7.6 S heterodimer.[28] Subsequently DNA pol γ was purified from *Xenopus laevis*,[29] pig,[30−32] and human cells.[33] The *Xenopus* enzyme consists of 140 kDa and 50 kDa subunits,[29] and the initial report on Hela cell pol γ identified a 7.8 S heterodimer containing 140 kDa and 54 kDa polypeptides in the most purified fraction.[33] The mitochondrial polymerase (MIP1) gene was first cloned from *Saccharomyces cerevisiae*.[34] Subsequently, the coding sequences for the catalytic subunit have been determined for human, mouse, chicken, *Xenopus*, *Drosophila*, *Schizosaccharomyces pombe*, and *Pychia pastoris*.[35−38] The predicted sizes for the catalytic subunit range from 115 kDa in *S. pombe* to 143 kDa in *S. cerevisiae*, and all the genes contain conserved sequence motifs for DNA polymerase and 3′ → 5′ exonuclease functions. Alignment of predicted amino acid sequences[39] revealed that pol γ is clearly homologous to the family A of DNA polymerases, which includes the Klenow fragments of *Escherichia coli* and *Bacillus stearothermophilus* polymerase I and *Thermus aquaticus* and bacteriophage T7 DNA polymerases (Figure 1). Recently this class of

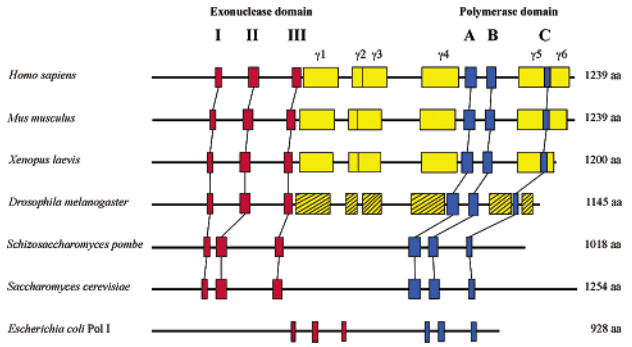

Figure 1. Schematic linear organization of the pol γ catalytic subunit with conserved domains. The conserved 3′-5′ exonuclease domains (*red*) are encoded by the three motifs I, II, and III while the DNA polymerase domains (*blue*) are encoded by the three ABC motifs. *Yellow* boxes indicate DNA polymerase γ-specific sequences highly conserved in vertebrates, weakly conserved in insects, and absent in single-cell eukaryotes. The *E. coli* DNA pol I linear organization is included for comparison. Adapted with permission from ref 57. Copyright 2004 Annual Reviews.

polymerases was joined by two new polymerases found in mice and humans, and both are related to *Drosophila Mus 308*: pol θ[40,41] and pol ν.[42] We have optimized the expression and purification of the recombinant form of the human

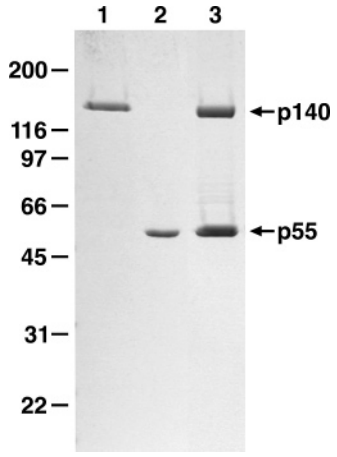

Figure 2. Subunit composition of human pol γ. The two subunits of human pol γ were resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lane 1, free catalytic subunit (500 ng p140); Lane 2, recombinant accessory subunit (300 ng p55); Lane 3, reconstituted two subunit polymerase γ complex (∼800 ng recombinant p140·p55) resolved on DNA·cellulose. Adapted with permission from ref 46. Copyright 1999 The American Society for Biochemistry.

catalytic subunit (p140) from baculoviral infected insect cells[43,44] (Figure 2). Human pol γ can utilize a wide variety of DNA substrates, including activated DNA, singly primed M13 DNA, and several homopolymers such as poly(dA)·oligo(dT) and poly(dC)·oligo(dG). Pol γ also possesses reverse transcriptase activity which permits differentiation of this activity from most other cellular DNA polymerases by assay on poly(rA)·oligo(dT).

In 1998 a detailed characterization of the isolated native and recombinant forms of the human catalytic subunit[43] demonstrated identical sizes and activities with no evidence for post-translational modification. This comparative analysis revealed its salt sensitivity and moderate processivity, which suggested that an additional subunit or other factors were required to restore the salt tolerance and highly processive DNA synthesis typical for γ polymerases. Several groups have reported additional polypeptides associated with the catalytic subunit of pol γ from a variety of organisms.[28,29,31,33,43] The gene for a smaller subunit of *Drosophila* pol γ was first isolated by Kaguni and colleagues.[45] A BLAST search of the human genome based upon the *Drosophila* polypeptide sequence identified a partial cDNA sequence of the human pol γ accessory subunit.[45] Subsequently, the full-length cDNA for the human accessory subunit was cloned, and the 55 kDa human protein (p55) was overexpressed in *E. coli* and purified to homogeneity (Figure 2, lane 2).[46] The isolated catalytic subunits from *Drosophila* and humans have significant differences in enzymatic activity. The isolated *Drosophila* catalytic subunit displays only marginal catalytic activity when compared to the native heterodimeric enzyme isolated from mitochondrial extracts of *Drosophila* embryos (∼2% of the holoenzyme activity),[38,47] whereas the isolated human catalytic subunit retains significant activity at low salt concentrations.[43] Similarly, when the *Drosophila* heterodimeric enzyme is reconstituted by coexpression of both subunits in a baculovirus system, the purified complex has physical and biochemical properties identical with that of the native enzyme. Therefore, the human accessory subunit is required for highly processive DNA synthesis.[46,48,49] The accessory subunit forms a high affinity, salt-stable complex with p140,

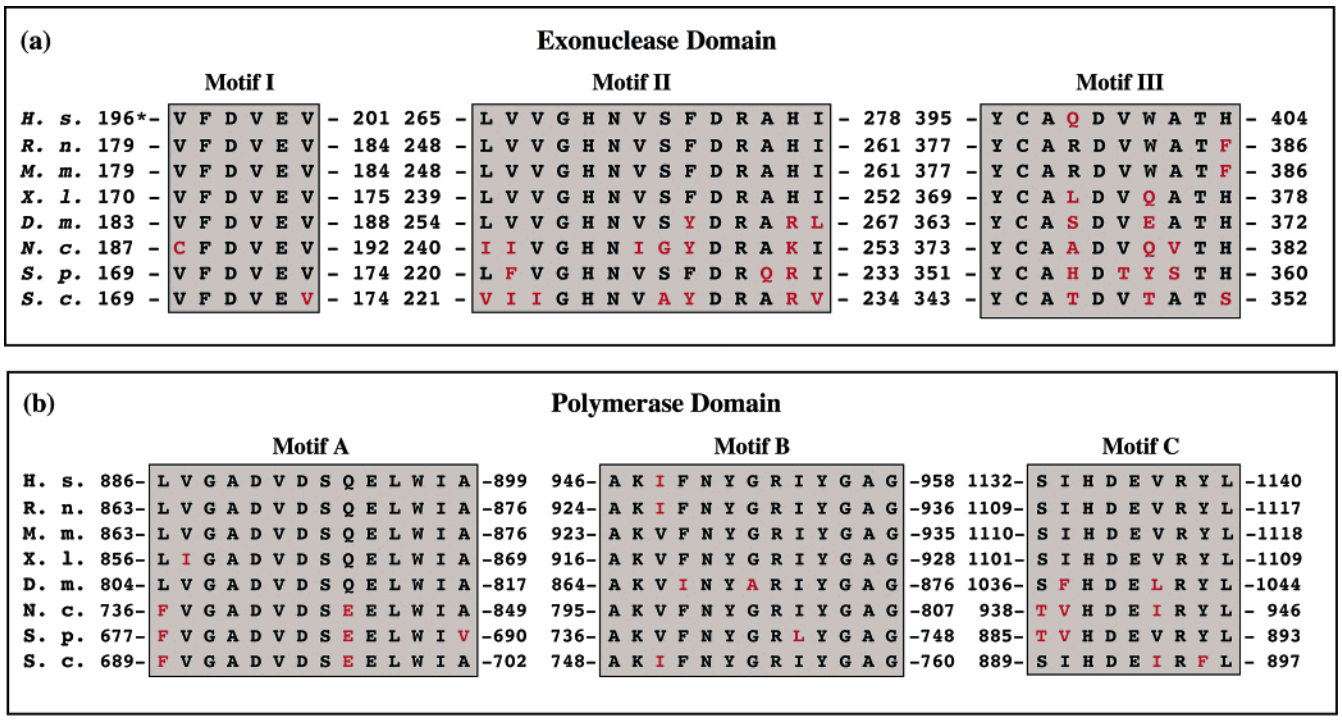

Figure 3. Sequence alignment of conserved pol γ exonuclease and polymerase motifs from the indicated organisms: (a) amino acid sequences of the three 3′ → 5′ exonuclease motifs; (b) amino acid sequences of the three polymerase motifs. Gray boxes frame sequences that are highly conserved in the family A group. Amino acid residues indicated in red are nonconserved residues. *H. s.* = *Homo sapiens*; *R. n.* = *Rattus norvegicus*; *M. m.* = *Mus musculus*; *X. l.* = *Xenopus laevis*; *D.* m. = *Drosophila melanogaster*; *N. c.* = *Neurospora crassa*; *S. p.* = *Schizosaccharomyces pombe*; *S. c.* = *Saccharomyces cerevisiae*.

and gel filtration and sedimentation analyses reveal a 195 kDa complex.[46] Reconstitution of the human complex with recombinant subunits restores the salt tolerance of p140, stimulates the polymerase and exonuclease activities, and increases the processivity of the enzyme by several 100-fold. Similar to p140, isolated p55 binds double-stranded primer·template DNA with moderate strength and specificity. Interestingly, no coding sequences for the accessory subunit have been found in the yeasts *S. cerevisiae* and *S. pombe*. Also, amino acid alignment of the *Drosophila*, *Xenopus*, and human accessory subunits revealed significant homology to the class II aminoacyl-tRNA synthetases, although the ATP binding site and the anticodon binding site are impaired.[50]

2.2. Molecular Structure of the Pol γ Catalytic Subunit

Biochemical analysis of γ-polymerases isolated from different animal sources revealed copurification of a 3′ → 5′ exonuclease activity.[28,30,31,33,51−54] The first genomic clone of pol γ was the *MIP1* gene from *S. cerevisiae*.[34] Sequence alignment of the MIP1 amino acid sequence with several bacterial DNA polymerases identified three conserved motifs for 3′ → 5′ exonuclease function and also classified this polymerase among the family A DNA polymerases.[55] Genetic inactivation of the MIP1 exonuclease activity produced a 500-fold increase in the frequency of spontaneous mutation in yeast mtDNA.[56] In addition to the highly conserved polymerase motifs (A, B, and C) and exonuclease motifs (I, II, and III), presented in Figures 1 and 3, analysis of cDNA clones from *Drosophila*,[38] *X. laevis*,[37] and humans[36] revealed six moderately conserved sequence elements, γ1, γ2, γ3, γ4, γ5, and γ6, in the pol γ catalytic subunit[57] (Figure 1). Two of these elements are near the C-terminal end of the catalytic subunit, and four elements are located between the exonuclease and polymerase catalytic domains. The linker region that separates the two domains in pol γ ranges from 337 amino acids in yeast to 482 amino acids in humans, which is almost twice as long as this region in other family A polymerases. Doublie and colleagues have demonstrated that the 293 amino acid spacer fragment in T7 polymerase contains a 71 amino acid loop within its thumb subdomain that enables interaction with its accessory subunit, *E. coli* thioredoxin.[58] Although no function has yet been assigned to the linker element in pol γ, this region has been speculated to be involved in subunit interaction (see discussion below), DNA binding, and/or functional coupling between the polymerase and exonuclease activities. Mutations in this region have been linked to several human mitochondrial disorders[59−65] that are discussed later in this review. A recent structure−function analysis of *Drosophila* pol γ addressed the roles of four conserved sequence elements within the linker region.[66] Conserved elements γ1, γ3, and γ4, corresponding to *Drosophila* pol γ amino acid residues 413−470, 536−581, and 666−742, respectively, were shown to play a role in polymerase activity. Interestingly, different mutations within these elements had differing effects on pol γ performance, altering to various extents the DNA binding affinity, polymerase activity, and processivity of each mutant enzyme. Deletion mutants $\Delta\gamma$1 and $\Delta\gamma$4 demonstrated weaker interaction with the pol γ accessory subunit. The L558A and F578A mutations within the conserved γ3 region of *Drosophila* pol γ exhibited reduced processivity and DNA binding on a single-stranded DNA template.[66]

Although an X-ray crystal structure of the pol γ catalytic subunit does not exist, the structures of the family A polymerases *E. coli* Klenow polymerase I,[67−71] *Thermus*

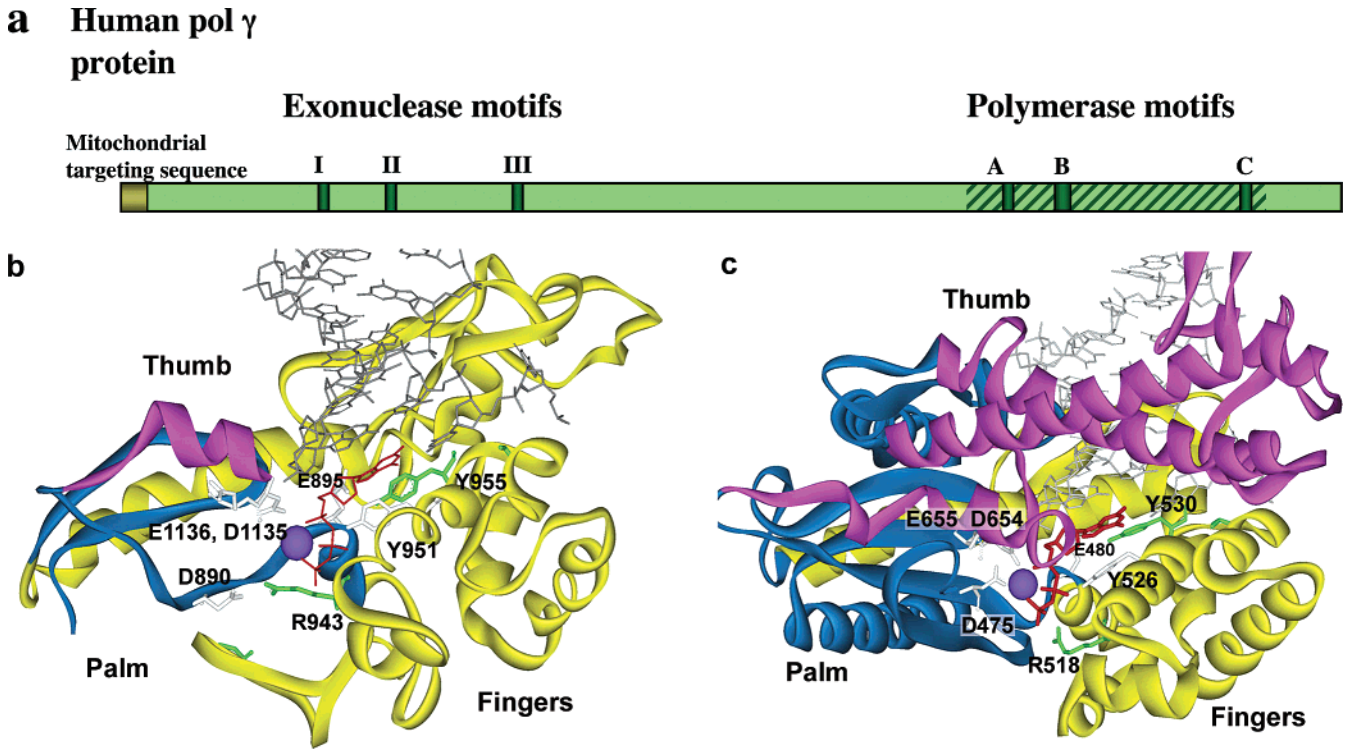

Figure 4. Molecular model of the p140 polymerase domain: (a) schematic diagram of human DNA pol γ protein (the striped section marks amino acid residues 871−1145 encompassed by the p140 polymerase domain model); (b) structural homology model of the wild-type human DNA pol γ active site developed from solved family A polymerase structures;[82] (c) T7 structure for comparison derived from PDB 1T7P. In panels b and c, the thumb, fingers, and palm subdomains are magenta, yellow, and blue, respectively. Primer and template DNA strands are gray, and the incoming ddGTP is red. The purple sphere is an Mg^{2+} ion.

aquaticus KlenTaq polymerase I,[72−78] *Bacillus stearothermophilus* DNA polymerase I large fragment,[79,80] and bacteriophage T7 polymerase[58] have been solved. Some are binary and/or ternary complexes with template·primer DNA, an incoming dNTP, and metal ions. These polymerases share substantial structural homology and conserved structural features in both the polymerase and exonuclease domains (Figure 3). Based on these similarities and the previously solved X-ray crystal structures of other family A DNA polymerases, our group developed a structural model of the polymerase domain of human pol γ.[81,82] The model encompasses amino acid residues 871−1145 of pol γ (Figure 4a) and includes all three highly conserved polymerase motifs. Coordinates for the modeled pol γ structure were taken largely from the closed conformation of the T7 DNA polymerase in complex with an incoming nucleotide in the active site.[58] Since the incoming nucleotide is solvent-accessible in the open conformation and it is completely buried in the closed form, the closed conformation provides greater insight into the structure of the active site. The predicted structure of the pol γ polymerase domain displays well-defined fingers, thumb, and palm subdomains (Figure 4b). The fingers subdomain is rotated inward toward the template·primer DNA, so that the O-helix is closer to the nucleotide-binding site, allowing amino acid residues R943, K947, and Y951 to contact the sugar and incoming nucleotide phosphate (Figure 4b). Residues R943 and K947 in pol γ are analogous to residues R518 and K522 in T7 polymerase (Figure 4b and c) and residues K65 and R72 in HIV-RT, the human immunodeficiency virus reverse transcriptase. The K ϵ-amino group and R guanidinium group of these conserved residues form salt bridges with the α- and γ-phosphates of the incoming dNTP. Binding the dNTP and metal ions induces repositioning of both the 3′ primer terminus and active site carboxylate side chains (D890 in pol γ corresponds to D110 in HIV-RT and D610 in KlenTaq). By simple analogy to the structure of HIV-RT and the solved structures of family A polymerases, the V891, D1135, and E1136 side chains in the pol γ palm subdomain likely participate with the V891 and D890 residues in the thumb to bind the dNTP·Mg^{2+} complex. In our closed pol γ model, the incoming nucleotide remains close to the active site carboxylate side chains D1135, E1136, and D890. Changing from the open to closed conformation affects the orientation of the O-helix, which affects dNTP binding and incorporation.[79] In the open conformation the tyrosine residue at the C-terminal end of the O-helix (motif B) in the family A polymerases (Y955 in pol γ) inserts and base stacks with the template DNA, whereas the I-helix forms most of the DNA contacts. From our structural model it is readily seen that Y955 is in close proximity and interacts with Y951, a residue involved in the recognition of the incoming dNTP and sugar ring. Tyrosine residues 951 and 955, together with E895, form a hydrophobic pocket near the C2′ sugar position in the active site that provides a structural basis for deoxynucleotide selectivity. This pol γ active site model has provided structural insights into the function of many of the conserved amino acids in the active site, which has been useful in predicting the potential effect of disease mutations, as well as the ability of pol γ to select nucleotide reverse transcriptase inhibitors.[81,82]

2.3. Molecular Structure of the Pol γ Accessory Subunit

The pol γ accessory subunit was first cloned from *Drosophila*.[45] Sequence alignment with accessory subunits from mouse and human reveals a moderate homology, with

a 120 amino acid fragment in the C-terminal region being especially well conserved among animal homologues. Striking sequence and structural similarities between these homologues and class IIa aminoacyl-tRNA synthetases were reported in 1999.[83,84] A three-dimensional structural model of the C-terminal region of the pol γ accessory subunit was also developed.[83] This model depicted a five-stranded β-sheet surrounded by four α-helices, which share structural homology with the anticodon-binding domain of class IIa aminoacyl-tRNA synthetases. The model also shares structural similarities with two processivity factors: the N-terminal domain of the δ' subunit of the γ complex in *E. coli* DNA polymerase III and *E. coli* thioredoxin.[83]

In 2001 the three-dimensional structure of the mouse pol γ accessory subunit was determined, which showed the subunit crystallized as a dimer[50] (Figure 5). Although the

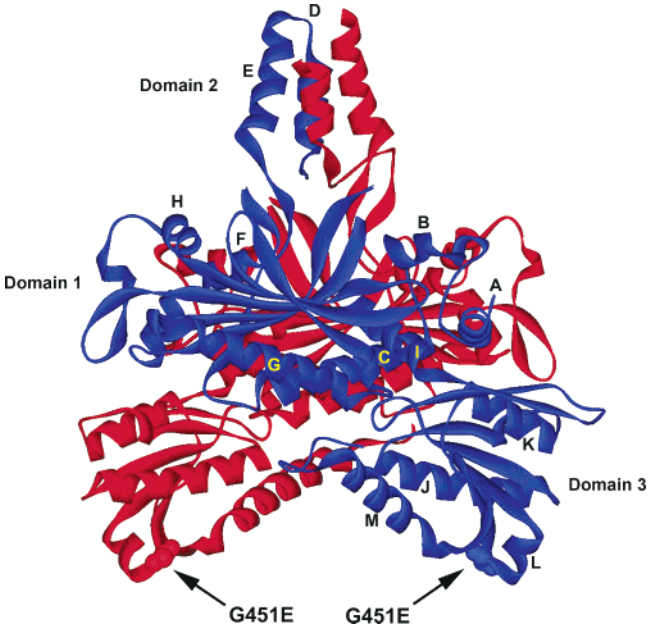

Figure 5. Crystal structure of the mouse accessory subunit dimer. Homologous mutation G451E in human PEO is highlighted. The structure was obtained from PDB file 1G5H,[50] and the image was generated with the Swiss PDB viewer.

overall crystal structure is remarkably similar to glycyl-tRNA synthetase, the functional sites of aminoacyl-tRNA synthetases are not conserved in the pol γ accessory subunit.[50] The structure indicates that each monomer has three distinct domains. Domain 1 consists of a seven-stranded β-sheet and all strands except one are antiparallel. One face of the twisted β-sheet is covered with helices G and C, whereas the other solvent accessible β-sheet forms a pocket lined with helices F and H. Domain 2 encompasses secondary structural elements that interact with their symmetric counterparts in the other monomer. Within this domain three strands from each monomer form a six-stranded antiparallel β-sheet across the dimer interface. Helices D and E of each monomer form a four-helix bundle across the dimer, and the helical axes are roughly parallel to the 2-fold axis relating both monomers. Domain 3 contains a five-stranded mixed β-sheet located between helices J and M on one side and a β-hairpin (formed by two other strands) and helix K on the other side. Biochemical data from studies of the *Drosophila* accessory subunit support the presence of three distinct domains[85] and suggest multiple contacts between the large and small pol γ subunits. In contrast, only a single contact site exists between thioredoxin and the tip of the thumb subdomain of T7 DNA polymerase.[58]

The dimer interface contains a predominantly hydrophilic region in domain 1 and a large hydrophobic region in domain 2. The majority of the subunit interactions are contacts between side chains pointing toward the inside of the four-helix bundle.[50] Two metal ions are positioned within the dimer interface and bind the amino acid residues V93, R96, and V99. Although the ions used for developing crystals were Na^+ (from sodium citrate), the authors suggest the metal ions may be Mg^{2+} or Ca^{2+} cations *in vivo*. Binding with the catalytic subunit occurs via surface residues of domain 3, which show the highest degree of sequence conservation. Further analysis demonstrated that a C-terminal deletion mutant of the accessory subunit does not bind to the catalytic subunit.[48] We have previously shown that interaction between the human catalytic and accessory subunits is stable at salt concentrations as high as 0.5 M,[46] which suggests the involvement of hydrophobic residues. Two regions with solvent-exposed hydrophobic residues in the C-terminal region are visible in the crystal structure of the mouse accessory subunit, and these residues may participate in salt-stable interactions with hydrophobic sites on the catalytic subunit. Based on the crystal structure of the accessory subunit and deletion analysis, Carrodeguas et al. concluded that functional mammalian pol γ is a heterotrimer composed of one catalytic subunit and a dimeric accessory subunit.[50] Recent physical studies using a derivative of the accessory subunit lacking the four helix bundle (I4 mutation) that fails to dimerize further demonstrate a heterotrimer complex.[86] Analytical ultracentrifugation, Biacore analysis, isothermal titration calorimetry, and electrophoretic mobility shift assay demonstrate that the accessory subunit functionally binds as a homodimer to the catalytic subunit with a Kd of 27 nM.[86] However, hydrodynamic analysis of the native human, *Xenopus*, and *Drosophila* pol γ complexes indicates a heterodimer composed of one catalytic and one accessory subunit.[28,29,46] The discrepancy between the hydrodynamic analysis of native proteins and the recent work with highly purified recombinant proteins may result from the low protein concentrations used in the original measurements, which could have yielded intermediate *S* values due to dissociation of p55 homodimers from the heterotrimeric complex during the extended time required for sedimentation.

Several lines of evidence suggest the early linker region of the catalytic polypeptide, following the exonuclease domain, is the site of interaction with the accessory subunit. A deletion mutagenesis study of the *Drosophila* pol γ catalytic subunit suggested that early linker region γ1, as well as γ4, might be responsible for subunit interaction.[85] Also, the YED mutant in γ1of *Drosophila* pol γ exhibits a 40% reduction in processivity, further suggesting that this region participates in subunit interaction.[66] The A467T disease mutation occurs in this area of the human polypeptide, and biochemical analyses suggest this mutation causes a dramatic decrease in catalysis and impaired interaction with the accessory subunit.[87,88]

2.4. Enzymatic Activities of Pol γ

DNA polymerase γ is unusual in its ability to utilize a wide variety of DNA substrates. In addition to natural DNA templates, pol γ efficiently utilizes homopolymeric DNA

templates such as poly(dA)·oligo(dT) or poly(dC)·oligo(dG). Pol γ activity is generally most efficient on substrates with high primer density. The specific activity of both native and recombinant forms of human pol γ is 5- to 10-fold higher on homopolymeric templates than on activated, natural sequence DNA.[43] The steady-state kinetic constants determined for the native human enzyme using poly(dA)·oligo(dT) as a substrate, in the presence of 100 mM salt, were K_m(dTTP) = 4.0 μM and k_{cat}(dTTP) = 0.28 s^{-1}.[46] Pol γ also efficiently uses ribohomopolymeric templates. In contrast to previous findings,[11,12,17,41] a recent single report indicates that pol γ can utilize a synthetic RNA template,[89] although the significance of this activity *in vivo* remains unclear. The catalytic rate of reverse transcription is higher for pol γ than for HIV-1 reverse transcriptase, and the authors report a proofreading activity with the RNA template. This remarkable variety of potential template·primer substrates utilized by pol γ can perhaps be explained by the fact that, as the sole mitochondrial DNA polymerase, pol γ is involved in all DNA synthesis events within mitochondria.

Due to the lack of activity or expression of the *Drosophila* and *Xenopus* pol γ catalytic subunits, a comprehensive analysis of the enzymology of the isolated catalytic subunit has only been performed on the human p140 subunit. Pol γ is active between pH values of 7.5 and 9.5, and the enzyme requires a divalent metal cation. Mg^{2+} cations are preferred on DNA templates, whereas Mn^{2+} are required for efficient utilization of ribopolymeric templates. The enzyme is resistant to inhibition by aphidicolin, but it is strongly inhibited by dideoxynucleotides. The human pol γ catalytic subunit is sensitive to the sulfhydryl blocking agent *N*-ethylmaleimide (NEM).[43] The polymerase activity is inhibited ~50% with <0.1 mM NEM and >90% at 0.5 mM NEM.[43] Similar sensitivity was also reported for *Xenopus* pol γ holoenzyme, which lost 95% of polymerase activity at 1 mM NEM.[29] The sensitivity to NEM was once thought to be one of the few inhibitory characteristics to differentiate pol γ from NEM-resistant DNA polymerase β.[12,90] However, further analysis of the two subunit native and recombinant forms of human pol γ indicated the holoenzyme displayed nearly complete resistance to NEM up to 1 mM,[46] revealing that the accessory subunit protects the catalytic subunit greater than 100-fold from inhibition by NEM. The protective effect of the accessory subunit was also observed when pol γ was subjected to reactive oxygen species.[91] The optimal salt concentration for DNA polymerase activity of isolated p140 is 50 mM on the homopolymeric template poly(rA)·oligo$(dT)_{12-18}$, whereas polymerase activity on activated salmon sperm DNA is significantly inhibited at physiological salt concentrations (>100 mM NaCl).[43] Reconstitution of the two subunit pol γ complex (p140·p55) abolishes this salt sensitivity, and p140·p55 acts over a broad range of ionic strength, with optimal activity from 75 to 175 mM NaCl.[46] This value is slightly lower than the salt optima of the *Drosophila* holoenzyme (~200 mM KCl) or porcine pol γ (150−200 mM) on natural DNA.[28,30]

The presence of the pol γ accessory subunit was demonstrated to stimulate polymerase and exonuclease activities and to increase the processivity of the enzyme.[46,47,49,84] Early analysis of *Drosophila* pol γ[92] reported a processivity of 30-nucleotides. However, at low ionic strength the two subunit *Drosophila* holoenzyme is capable of highly efficient DNA synthesis, yielding products exceeding 1000 nucleotides.[93] The isolated human catalytic subunit elongates singly primed M13 DNA most efficiently under low salt conditions, but the average length of the DNA product is only 100 nucleotides.[46] A hybrid pol γ complex of the human catalytic subunit and *Xenopus laevis* accessory subunit displays a processivity of around 250 nucleotides at 100 mM NaCl.[84] The reconstituted human holoenzyme displays markedly enhanced processivity, with the two subunit complex synthesizing products as long as 7 kilobases at 150 mM salt.[46] The association of the accessory subunit with the catalytic subunit dramatically increases the affinity of the two proteins to DNA,[46] and this enhanced binding helps to increase the processivity of the holoenzyme.

2.5. 3′ → 5′ Exonuclease Activity

An exonuclease activity associated with pol γ was first identified in the chicken enzyme[51] and subsequently in the porcine,[31] *Drosophila*,[52] and *Xenopus*[53] forms of pol γ. Using the yeast *MIP1* DNA sequence,[34] Ito et al. identified three exonuclease motifs in the N-terminal fragment of MIP1 that possessed significant homology to other family A polymerases.[55] Active site mutations were generated in MIP1 by substituting conserved aspartic acid residues with alanine or glycine in exonuclease motifs Exo1, Exo2, and/or Exo3.[56] The mutant forms of the polymerase were capable of replicating mtDNA, but the strains exhibited a several 100-fold increase in the frequency of spontaneous mutation of mtDNA relative to the wild-type enzyme with its intact, mismatch specific 3′ → 5′ exonuclease activity. Subsequently, the intrinsic 3′ → 5′ exonuclease activity of pol γ from higher eukaryotes was demonstrated in the human catalytic subunit[43,94] and reconstituted forms of the recombinant *Drosophila*[47,95] and human[46,49] holoenzymes.

Like the DNA polymerase activity, the 3′ → 5′ exonuclease activity has a broad pH optimum, requires a divalent metal cation, and is stimulated by moderate to high concentrations of NaCl. The exonuclease shows a slight preference for 3′-terminal mispairs in double-stranded DNA, and it can efficiently degrade single-stranded DNA. Native pol γ has a high mismatch specificity that ranges from 5- to 34-fold, depending on the DNA substrate and specific nucleotides forming the mispair.[43,52,53,96,97]

2.6. Fidelity of DNA Synthesis by Pol γ

Pol γ purified from chicken embryos or from pig liver mitochondria accurately replicates DNA *in vitro*, with measured error frequencies at a three nucleotide mutational target of $<3.8 \times 10^{-6}$ per nucleotide and $<2.0 \times 10^{-6}$ per nucleotide, respectively.[31,51] Pol γ contains an intrinsic 3′ to 5′ exonuclease activity that prefers mispaired 3′-termini, and several lines of evidence indicate that the exonuclease contributes to replication fidelity. Partial inhibition of the exonuclease activity with 20 mM dGMP increases the frequency of errors, suggesting the exonucleases proofread replication errors *in vitro*.[31,51,92] Proofreading-deficient forms of yeast pol γ and their resulting mutator effect have been recently reviewed.[98] All family A DNA polymerases with intrinsic exonuclease activity contain conserved aspartate and glutamate residues in their exonuclease active sites, which coordinate the two metal ions involved in catalysis.[99] As we mentioned in the previous section, in yeast, site-directed mutagenesis of these highly conserved aspartate residues in MIP1 and reintroduction of the mutant allele produce mutator

phenotypes.[56,100] Individually, the amino acid substitutions D171G in ExoI, D230A in ExoII, and D347A in ExoIII led to a several 100-fold increase in frequency of spontaneous mutation of mtDNA *in vivo*, and the D171G/D230A double mutant enhanced mutagenesis up to 1500-fold. Biochemically, a 10^4-fold decrease in exonuclease activity was observed *in vitro*. Studies of random mutations in all three exonuclease motifs in yeast pol γ produced up to a 500-fold increase in the frequency of replication errors.[101] In human pol γ, substitution of Asp198 and Glu200 with alanine in the ExoI motif eliminated detectable 3′-5′ exonuclease function *in vitro*.[43] Comparing the *in vitro* rates of base substitution errors for the exonuclease deficient and proficient forms of human pol γ indicated that the proofreading function contributes at least 20-fold to the fidelity of base selection.[102] Expression of an exonuclease-deficient pol γ fusion protein in cultured human cells resulted in the accumulation of point mutations in mitochondrial DNA.[103] Additionally, the loss of pol γ's exonuclease function in transgenic mice resulted in the rapid accumulation of point mutations and deletions in cardiac mtDNA, and the mutagenesis was accompanied by cardiomyopathy.[104] Substitution of a critical aspartate residue with alanine in the second exonuclease domain (D257A) produced homozygous knock-in mice expressing a proofreading deficient form of the catalytic subunit of pol γ.[105] These knock-in mice developed a mtDNA mutator phenotype with up to a 5-fold increase in the number of point mutations as well as a significant increase in mtDNA deletions. The increase in somatic mtDNA mutations was associated with reduced life span and premature aging, indicating that an increase in mtDNA mutations participates in the aging process.[108] A more recent study of a similar exonuclease deficient knock-in mouse showed that the increase in mtDNA mutations induces apoptosis but is not associated with increased production of reactive oxygen species.[106] Additionally, seven mutations in the exonuclease domain of human pol γ have been linked to the mitochondrial disorder progressive external ophthalmoplegia,[60,62,107] described in greater detail later in this review. Interestingly, all seven mutations map within the exonuclease domain but outside the conserved exonuclease motifs, and three mutations (T2511, L304R, and R309L) are transmitted as recessive traits.

The human catalytic subunit of pol γ has high base substitution fidelity that results from high nucleotide selectivity and exonucleolytic proofreading.[102] Pol γ is also relatively accurate for base incorporation in noniterated and short repetitive sequences where a misinsertion event occurs, on average, once per 500 000 nucleotides synthesized.[102] However, when copying homopolymeric sequences longer than four nucleotides, pol γ has a lower frameshift fidelity, suggesting that homopolymeric runs in mtDNA may be particularly prone to frameshift mutation *in vivo* due to replication errors by pol γ. Pol γ also generates base substitutions inferred to result from a primer dislocation mechanism. Inclusion of the 55 kDa accessory subunit, which confers processivity to the pol γ catalytic subunit, decreases frameshift and base substitution fidelity. Kinetic analyses indicate that p55 lowers fidelity of replication by promoting extension of mismatched termini.[102]

2.7. dRP-lyase Activity

Repair of damaged mtDNA can occur through base excision repair pathways in which the damaged base is recognized and removed by DNA glycosylase and AP endonuclease activities. In the late 1990s, a 5′-deoxyribose phosphate (dRP) lyase activity was discovered in *X. laevis* pol γ.[108,109] This dRP lyase activity, which is intrinsic to the catalytic subunit of human pol γ, catalyzes the release 5′-terminal dRP sugar moiety from incised apurinic/apyrimidinic sites.[110] The rate of the dRP lyase reaction is significantly slower for pol γ than for pol β, which is involved in nuclear base excision repair.[111,112] The ability to trap covalent enzyme·DNA complexes with $NaBH_4$ strongly suggests the presence of a Shiff base intermediate in a β-elimination reaction mechanism.[109,110] Pinz and Bogenhagen[109] demonstrated that pol γ's dRP lyase reaction proceeds by formation of a covalent enzyme·DNA complex that is converted into an enzyme·dRP intermediate following elimination of the DNA. The high stability of the pol γ·dRP complex slows the release of the dRP group from the enzyme, which is believed to be the rate-limiting step of the reaction.[109] The intrinsic dRP lyase activity allows pol γ to remove the dRP moiety and fill the resulting single-nucleotide gap, thereby generating a substrate for DNA ligase.[110] The accessory subunit can incease the efficiency of this reaction by increasing the lyase reaction and the ability of the enzyme to locate the damage site on DNA, presumably by enhancement of DNA binding.[113]

3. Regulation of Pol γ Gene Expression

Proliferation of eukaryotic cells relies on efficient mitochondrial biogenesis, and replication of mtDNA is a vital element of this process. Maintaining the content of mtDNA in dividing cells depends on the regulation of expression of the nuclear genes encoding both pol γ subunits. In yeast, deletion of the *MIP1* gene leads to loss of mtDNA and the formation of petites.[17] In contrast, overexpression of the catalytic subunit in *Drosophila* leads to depletion of mtDNA,[114] whereas mutation of the gene for the *Drosophila* accessory subunit causes loss of mtDNA and lethality.[115] Expression of the *Drosophila* pol γ accessory subunit is controlled, in part, by a DNA replication-related element (DRE) that normally regulates genes involved in nuclear DNA replication.[116] The nuclear respiratory factor 1 (NRF-1) is a transcription factor that regulates many of the nuclear-encoded mitochondrial proteins needed for oxidative phosphorylation and some components of the mitochondrial transcription machinery. Binding of NRF-1 to DNA is regulated by the ATP requirements of the cell. Promoters of the human genes for the pol γ catalytic subunit, the accessory subunit, and the mitochondrial transcription factor (mtTFA) all contain consensus binding motifs for NRF-1.

Pol γ activity has been detected in yeast rho^0 cells, which lack mtDNA.[17] Pol γ is expressed and translated in cultured human cell lines either containing or lacking mitochondrial DNA, indicating that the pol γ protein is stable in the absence of mitochondrial DNA *in vivo*.[117] In contrast, the same study demonstrated that the stability of mtTFA was highly dependent on the mtDNA content of the cell.[117] Synthesis of mitochondrial DNA, as estimated by incorporation of 5-bromo-2-deoxyuridine (BrdU), occurs preferentially in perinuclear mitochondria.[118] However, the observation of pol γ in both peripheral and perinuclear mitochondria[117] indicates pol γ is also present in nonreplicating mitochondria, suggesting a maintenance role for pol γ such as mtDNA repair.

4. Mitochondrial DNA Replication

4.1. Proteins Involved in Mitochondrial DNA Replication

Mitochondrial DNA in animal cells is a double-stranded circular molecule that ranges in size from ~16 to 20 kb. The genetic information encoded by the mitochondrial genome is limited to 37 genes, which is quite a modest number when compared to the thousands of genes in the nuclear genome. Replication of mtDNA requires the concerted action of pol γ and a growing list of accessory proteins, transcription factors, and enzymes (Table 1). The mitochondrial single-stranded DNA binding (mtSSB) protein enhances helix destabilization for DNA helicases and pol γ to support mtDNA replication, recombination, and repair processes. The sizes of the mitochondrial SSBs isolated and cloned from yeast and various animal sources are between 13 and 16 kDa.[119−126] The native form of the mammalian mtSSB is a tetramer, with a molecular weight of 56 kDa.[126] The gene for the human mtSSB has been cloned,[124] and the crystal structure has been determined.[127] Based on the crystal structure, single-stranded DNA is proposed to wrap around the tetrameric mtSSB through electropositive patches guided by flexible loops.[127] The mtSSB tetramer has high affinity for DNA, and its DNA binding site encompasses 8 to 17 nucleotides.[120,121,128] *In vitro* experiments with mtSSB added to purified pol γ demonstrated significant stimulation of the polymerase activity on various primer·template substrates.[129,130] In *Drosophila*, mtSSB increases mitochondrial DNA synthesis almost 40-fold, and fruit flies with a mutated mtSSB gene display significant mtDNA depletion and dysfunction of the respiratory chain.[131,132]

Table 1. Known Nuclear Gene Products Required for mtDNA Replication and Repair

enzyme	size	human chromosome
	Replication Genes	
DNA polymerase γ:		
POLG	140 kDa	15q25
POLG2	55 kDa	17q23-24
single-stranded DNA binding protein	15 kDa	7q34
helicase:		
TWINKLE	77 kDa	10q24
Transcription:		
core RNA polymerase	150 kDa	19q13.3
mtTFA	24 kDa	10q21
mtTFB1	39 kDa	6q25.1-q25.3
mtTFB2	45 kDa	1q44
RNase MRP RNA	275−285-nt RNA	9p13
topoisomerases:		
Topo I	67 kDa	8q24.3
Topo IIIα	112 kDa	17p12-11.2
ligase:		
DNA ligase III	96 kDa	17q11.2-12
RNase H1	32 kDa	19p13.2
	Repair Genes	
glycosylases:		
UDG	27.5 kDa	12q23-q24.1
OGG1	38 kDa	3p26.2
NTH1	34 kDa	16p13.3
MUTYH	60 kDa	1p34.3-p32.1
AP endonucleases:		
APE1	35 kDa	14q11.2-q12
APE2	57 kDa	Xp11.22
Endo G	30 kDa	9q34.1

A putative human mitochondrial DNA primase has been described,[133,134] and the enzyme is proposed to contain an essential RNA component.[135] However, no further reports exist to confirm this activity or to identify any genes. Most models of mtDNA replication rely on transcription as the priming event in mtDNA replication.

An ATP-dependent mtDNA helicase was initially described in sea urchin[136] and bovine brain.[137] However, the replicative mitochondrial helicase remained elusive until the gene for a T7 gene 4 homologue was identified as a locus for PEO. This *TWINKLE* gene encodes a 5′ to 3′ helicase[138] with significant sequence homology to the C terminal end of T7 gene 4 helicase-primase.[138,139] Similar to T7 gene 4, *TWINKLE* contains five helicase sequence motifs, but it lacks the primase-associated sequences found in T7 gene 4. *TWINKLE* colocalizes with mtDNA in mitochondrial nucleoids. A recent study reported stimulation of *TWINKLE*-dependent unwinding of short fragments in dsDNA by human mtSSB,[140] but *TWINKLE* alone was unable to unwind longer dsDNA molecules. However, the helicase stimulates rolling-circle DNA synthesis by pol γ holoenzyme *in vitro* by supporting unwinding of dsDNA at the replication fork.[141] Transgenic mouse lines overexpressing wild-type *TWINKLE* demonstrated up to a 3-fold increase of mtDNA copy number in muscle and heart, and reduction of *TWINKLE* expression by RNAi dramatically decreased mtDNA copy number in cultured human cells.[142] These observations suggest that *TWINKLE* helicase, in addition to playing a role in mtDNA maintenance, is essential for regulation of mtDNA copy number in mammals.[142]

Mouse embryos carrying the RNase H1 gene knockout suffer a significant decrease in mtDNA content that leads to apoptotic cell death.[143] RNase H1 predominantly localizes to the nucleus; however, a fraction of the protein is found in mitochondria. Possible roles for RNase H1 may be removal of RNA primers at origins of replication on the heavy and light strands (Figure 6), as well as the processing of Okazaki fragments proposed in some models of mtDNA replication.

Topoisomerases belonging to the three topoisomerase subfamilies IA, IB, and IIA have been identified in the mitochondria of various organisms.[144,145] Several studies have implicated the presence of type IIA DNA topoisomerase in the mitochondria of protozoan parasites,[19,146] the slime mold *Dictiostelium discoideum*,[147] *Plasmodium falciparum*,[148] and bovine heart tissue.[149] A gene encoding the mitochondrial-targeted topoisomerase I, a member of the IB subfamily, has been identified in humans.[144] The human enzyme mitochondrial topoisomerase I (TOPOImt) is a 72 kDa protein that relaxes negative supercoils and has been suggested to remove positive supercoils created by helicase activity.[144] In addition, the human gene TOP3α that encodes DNA topoisomerase IIIα (hTop3α), a member of the IA subfamily, has two potential start codons. One start site would produce an enzyme with an N-terminal sequence that may function as a mitochondrial-targeting signal,[145,150] and the presence of hTop3α in the mitochondria of HeLa cells has been demonstrated.[145] Analysis of the amino acid sequence of DNA topoisomerase IIIα from mouse and *Drosophila*, as well as DNA topoisomerase III from *S. pombe*, points to the possible presence of this IA subfamily member within the mitochondria of other eukaryotes.[151]

Mitochondrial DNA ligase activities were initially purified from *X. laevis* and identified as ligases III and IV, and DNA ligase III functioned with pol γ in reconstituted base excision repair *in vitro*.[108] A year later cDNA analysis identified conserved mitochondrial localization signals (MLS) in the

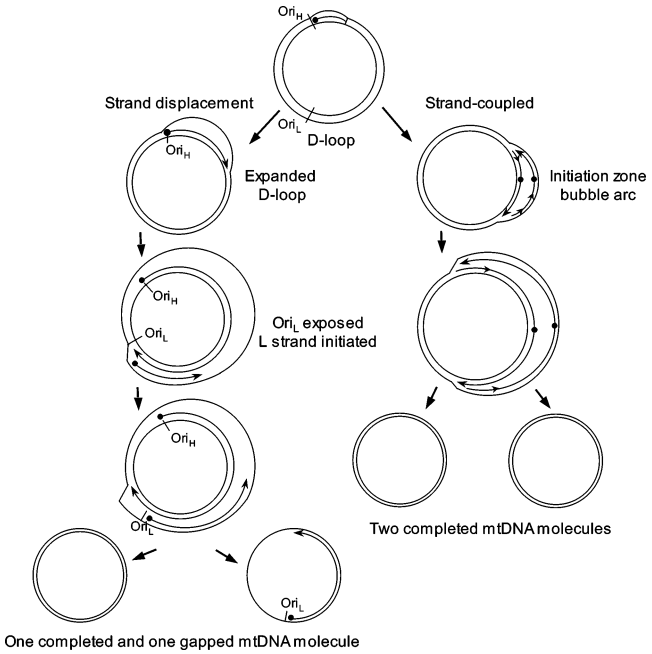

Figure 6. Models of mitochondrial DNA replication. The asymmetric or strand displacement model is shown in the left pathway while the strand-coupled model is shown in the right pathway. Replication by the strand displacement model is initiated at OriH with single-stranded replication of the H-strand with displacement of the D-loop. This synthesis proceeds until OriL is exposed where synthesis of the L-stand is initiated in the opposite direction. In the strand-coupled model, bidirectional replication is initiated from a zone near OriH followed by progression of the two forks around the mtDNA circle. Details of these models are described in ref 187.

DNA ligase III gene, and antisera raised against a DNA ligase III-specific epitope was shown to cross-react with the mtDNA ligase.[152,153] Also, reduced mtDNA content and multiple single-stranded nicks in mtDNA were reported in cultured human cells with limited DNA ligase III expression.[154] These observations demonstrate that mtDNA ligase III is needed in mitochondrial DNA replication and repair. A role for ligase IV in mtDNA maintenance may be possible, since the gene for this enzyme contains a putative MLS region.[155]

Mitochondria transcription factor (mtTFA, also called mtTF-1 and Tfam) is a high-mobility-group (HMG) box protein involved in mtDNA replication. MtTFA binds conserved regulatory sequences within the D-loop of mtDNA, and it recruits other replication factors to the D-loop.[156] Heterozygous mtTFA knockout mice have reduced levels of mtDNA while homozygous knockout embryos are defective in oxidative phosphorylation and have severely depleted mtDNA content. Homozygous mutant embryos proceed through implantation and gastrulation but die prior to embryonic day (E)10.5.[157] Because normal mtTFA cellular levels significantly exceed those needed for transcription, mtTFA has also been postulated to act in a histone-like fashion, providing protection to the mitochondrial genome.[156,158,159]

4.2. Mechanisms of DNA Replication in Mitochondria

The basic processes of mitochondrial DNA replication were initially elucidated with studies on budding yeast. As facultative anaerobes, yeast do not rely solely on oxidative phosphorylation for generation of ATP and NAD^+, which means they can survive without functional mitochondria via glycolysis and fermentation. Because yeast grown on a fermentable carbon source can survive without functional mitochondria (rho$^-$ mutants) or even mtDNA (rho^0 mutants), respiration-deficient yeast mutants provide a valuable model for characterization of nuclear-encoded enzymes that participate in mitochondrial DNA maintenance. Petite mutants (rho$^-$) are spontaneously generated under normal conditions. The retained mtDNA sequences in the rho$^-$ mutants often consist of amplified head-to-tail repeats of a portion of the genome and most often contain less than 1% of the normal sequence. In the majority of hypersupressive (HP) yeast mutants, this repeated region includes a 280 bp ori/rep sequence[160] structurally similar to the vertebrate O_H (the origin of heavy strand mtDNA replication). Yeast mtDNA is ~80 kb, almost five times the size of human mtDNA, and it contains four bidirectional origins of replication (ori/rep sequences numbered 1−8). Of these sequences, ori1, ori2, ori3, and ori5, harboring an intact transcriptional promoter, are most likely the origins of mtDNA replication. Studies of HP strains revealed that RNA transcripts initiated at these promoters serve as primers for initiation of mtDNA synthesis.[161] The essential oligoribonucleotide primers hybridize at various sites encompassing the conserved sequence block II (CSB II), which is one of three CSB elements present in O_H.[162] In at least two of the four origin of replication sequences, CSB II is the site of transcription-dependent formation of RNA/DNA hybrids *in vitro*, suggesting that a longer RNA species transcribed in this region serves as the precursor of the oligoribonucleotide primers used in replication.[163] Yeast lacking either mtRNA polymerase or the mitochondrial transcription-initiation factor sc-mtTFB generates rho$^-$ and rho^0 daughters at high rates,[164,165] which supports the notion that an ori/rep-dependent, transcription-primed replication mechanism is required for leading-strand synthesis of yeast mtDNA. This mechanism is similar to that of mtDNA replication in vertebrates. Two isoforms of human mitochondrial transcription specificity factors, TFB1M and TFB2M, have been identified.[166,167] Both have been demonstrated to interact with mtTFA and the mitochondrial RNA polymerase during transcription initiation. Expression of human TFB1M and TFB2M is regulated by two nuclear respiratory factors, NRF-1 and NRF-2, as well as PGC-1 family coactivators, all of which are essential factors for mitochondrial biogenesis.[168]

Over 20 years ago, Clayton and colleagues derived a model of mitochondrial DNA replication based on experiments with mouse L-cells in which replication occurs in a unidirectional, asymmetric fashion[169−171] (Figure 6). In this model, transcription initiates replication of mtDNA. Transcription is initiated at two major mtDNA promoters within the D-loop—the light strand promotor (LSP) and the heavy strand promotor (HSP)—and the resulting transcripts are processed into individual mitochondrial mRNAs, tRNAs, and rRNAs.[172] The primer for initiation of mtDNA replication at O_H is generated by processing the transcript starting at LSP.[171,173] The nascent transcript remains hybridized to a sequence upstream of O_H, forming a stable R-loop structure that is cut by the MRP RNase at positions adjacent to the R-loop. Pol γ initiates H-strand synthesis by extending the RNA primer.[171,174,175] When nascent H-strand synthesis is ~70% complete, the replication fork exposes the major origin for L-strand synthesis (O_L), allowing initiation of L-strand synthesis on the displaced H-strand to proceed in the opposite

direction[176−178] (Figure 6). L-strand replication is initiated near the WANCY tRNA coding region that in a single-strand form is postulated to assume a stable stem loop structure, and DNA synthesis proceeds along the entire length of the mitochondrial DNA strand and terminates after H-strand replication is completed.[133] According to this model, DNA synthesis is continuous but asynchronous on both the H-strand and the L-strand. The steps required for separation of daughter DNA molecules, removal of RNA primers, ligation, and reintroduction of supercoils are not well understood.[179]

Recently the asynchronous replication model has been challenged by an alternative mechanism of mtDNA replication that is based on the ribonucleotide substitution pattern in mtDNA and analysis of replication intermediates by 2D-gel electrophoresis (Figure 6).[180−182] The 2D-gel electrophoresis revealed two types of replication intermediates.[180] The first class was resistant to nucleases that digest single-stranded DNA, had electrophoretic mobilities typical of coupled leading and lagging strand replication intermediates, was found in mouse liver and human placenta, and was predominant in cultured cells recovering from transient mtDNA depletion. The presence of conventional duplex replication intermediates indicates symmetric, semidiscontinuous DNA replication with coupled leading and lagging strand DNA synthesis. The second class of replication intermediates, presumably derived from the strand-asynchronous mechanism of mtDNA replication, was sensitive to single-strand nuclease and was most abundant in untreated cultured cells. Although this initial report suggested coexistence of both the asynchronous and strand-coupled modes of mtDNA replication,[180] later findings by the same authors indicate that mammalian mtDNA replication proceeds mainly, if not exclusively, by a strand-coupled mechanism.[181,182] Replication intermediates from highly purified mitochondria were demonstrated to be essentially duplex throughout their length, although they contain RNA:DNA hybrid regions which result from the infrequent incorporation of ribonucleotides.[181] The authors suggested that *in vitro* RNaseH treatment or the process of extracting mtDNA from crude mitochondria leads to degradation of these ribonucleotide-rich regions and produces the partially single-stranded molecules previously assumed to arise by the asynchronous mechanism.[180] Analysis of mitochondrial DNA from rats, mice, and humans revealed that mtDNA replication initiates at multiple origins that are distributed across a 4 kb fragment downstream from the 3′-end of the displacement loop and that DNA replication is restricted to one direction after fork arrest near O_H.[182] In contrast to this, Holt and co-workers find by 2D agarose gel analysis that initiation of mtDNA replication in cultured human cells is concentrated around the displacement loop.[183] A review of both models of DNA replication with their supporting evidence has recently been published in a series of published arguments.[184−186] The controversy between the strand displacement and strand-coupled modes of replication may be resolved with a new analysis of replication intermediates by atomic force microscopy. AFM analysis of mouse liver mtDNA replication intermediates demonstrated not only single-stranded displaced loops but also alternative origins of light strand synthesis.[187] Although utilized less frequently, the alternate origins apparently produce y-arc and bubble-arc intermediates on 2D agarose gels, and these findings are consistent with branch migration of the asymmetrically replicating nascent strand or the strand displacement model. The finding of alternative origins of light strands also helps to explain second strand synthesis in chicken mitochondrial DNA, for which a canonical light strand origin is conspicuously absent.

In 2004 a new site of mtDNA replication was discovered in several different human cell lines at position-57 of the D-loop.[188] Whereas nascent DNA strands that initiated at the previously identified H-strand origins were shown to terminate prematurely at the 3′ end of the D-loop, nascent chains starting at position-57 proceeded well beyond the end of the D-loop. Attardi and co-workers propose that there are two pathways of mtDNA replication in human cells. Under steady-state conditions the position-57 origin appears to be responsible for the maintenance of mtDNA copy number, whereas the previously discovered multiple D-loop origins participate in the recovery of mtDNA following depletion.[188]

Recent *in vitro* reconstitution of a minimal mammalian mtDNA replisome by Korhonen and colleagues[141] provided an important insight into biochemical aspects of mtDNA replication. The replisome contained recombinant pol γ (both catalytic and accessory subunits), *TWINKLE*, and the mtSSB. The combination of two of the proteins, pol γ and *TWINKLE*, demonstrated efficient synthesis of single-stranded DNA of approximately 2000 nt in length using double-stranded minicircle DNA as a template. The double-stranded substrate was constructed by annealing a 90-nt oligonucleotide to a 70-nt ssDNA minicircle. Addition of mtSSB to the complex permitted synthesis of single-stranded DNA products more than 15 000 nt in length, a size similar to the mammalian mitochondrial genome. The authors estimated the rate of DNA synthesis for this minimal replisome to be approximately 180 nt/min, which agrees with a previous estimate of DNA synthesis *in vivo* of 270 nt/min.[169]

5. Pol γ in Mitochondrial DNA Repair

5.1. Background of Mitochondrial DNA Repair

Unlike nuclear DNA, mitochondrial DNA is not protected by histone proteins but instead is associated with the mitochondrial inner membrane, which is the site of reactive oxygen species (ROS) generation. These two factors make the mitochondrial genome more prone to damage than its nuclear counterpart. Indeed, point mutation frequency in mtDNA exceeds 10-fold that of nuclear DNA,[189] and in different organisms such as yeast, rodents, and humans, oxidative stress alone results in up to a 10-fold greater increase in damage to mtDNA over nuclear DNA.[190−193] Also, DNA repair after hydrogen peroxide treatment has been shown to be slow in mitochondria as compared to the nucleus.[190] In addition to damage from ROS, mtDNA is also damaged by exposure to ionizing or ultraviolet (UV) radiation, chemicals, and antiviral compounds. Because the human mitochondrial genome undergoes approximately 1000 more cycles of replication than the nuclear genome,[194] repairing the damage to mtDNA is necessary to avoid the accumulation of point mutations and/or deletions. If the fraction of mutant mitochondrial genomes exceeds the energetic threshold (40−80% mutant genome copies), tissue degeneration may result. Along these lines of thinking, an accumulation of point mutations and deletions in mitochondrial DNA is associated with aging.[195−198] These observations have fueled interest in the ability of mitochondria to repair DNA damage. In the early 1970s Clayton and Friedberg showed that UV-induced pyrimidine dimers were not repaired in mouse cell

mtDNA.[199] This observation led to the assumption that there was no DNA repair in mitochondria. However, subsequent studies have demonstrated that certain types of DNA damage can be efficiently repaired in the mitochondria. As the only DNA polymerase present in mitochondria, pol γ is inevitably implicated in all of these mtDNA repair processes.

5.2. Base Excision Repair in Mitochondria

Repair of damaged DNA bases via the base excision repair (BER) mode is the major DNA repair mechanism acting in mitochondria.[200−205] In general, BER starts with recognition and removal of a damaged or inappropriate base by a DNA glycosylase that cleaves the N-glycosylic bond between the base and the sugar, as depicted in Figure 7. This is followed

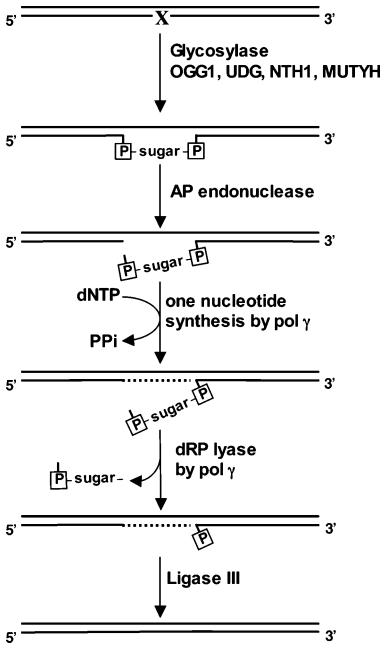

Figure 7. Mitochondrial base excision repair scheme showing the enzymatic steps and enzymes involved at each stage.

by an apurinic/apyrimidinic endonuclease (AP endonuclease) activity that catalyzes incision of the DNA phosphate backbone at the AP site. A lyase activity then removes the 5′-terminal 2-deoxyribose-5-phosphate (dRP) sugar moiety from the downstream DNA (Figure 7). Some glycosylases have the additional AP lyase activity that cleaves the DNA backbone. In this latter case the AP-endo activity removes the 3′ deoxyribose group. In both cases the resulting 3′-hydroxyl moiety can be extended by a DNA polymerase and DNA ligase activity completes the repair event by joining the free DNA ends.[206]

Mitochondrial BER has been demonstrated by numerous groups and shown to target oxidatively modified DNA bases, such as 7,8-dihydro-8-oxoguanine (8-oxo-G) and thymine glycol.[200−205,207,208] DNA repair enzymes isolated from mitochondria include several types of damage-specific DNA glycosylases,[200,202,208−210] AP endonuclease,[211] and DNA ligase III[108,152] (Table 1). Although Ape1 appears to be the predominant AP endonuclease,[211,212] a second weaker AP endonuclease, Ape2, has also been found in the mitochondria.[213,214] Certain DNA glycosylases are expressed as nuclear and mitochondrial isoforms encoded by the same gene, including uracil DNA-glycosylase (UDG)[215] and an 8-oxo-G DNA-glycosylase/AP lyase (OGG1).[216] MUTYH (homologue of the *E. coli* MutY glycosylase), NTH (thymine glycol glycosylase), and OGG1 have been demonstrated to localize to mitochondria in rat neurons[217] and human cells.[216,218,219] Efficient repair of methylated and ethylated bases in mtDNA has also been documented.[205,220−223]

Pol γ participates in uracil-provoked base excision repair reconstituted *in vitro* with purified components, where, after actions of uracil-DNA glycosylase and AP endonuclease, pol γ fills a single-nucleotide gap in the presence of a 5′ terminal deoxyribose phosphate (dRP) flap.[110] The removal of the dRP moiety can proceed via simple hydrolysis or by enzyme catalyzed β-elimination.[206] A complete system for the repair of abasic sites in DNA has been reconstituted with mitochondrial enzymes purified from *X. laevis* and identified the dRP lyase function to be in either ligase or pol γ.[108] The catalytic subunit of human pol γ was subsequently shown to catalyze the release of the dRP residue from incised apurinic/apyrimidinic sites to produce a substrate for DNA ligase.[110] The repair of base damage in mitochondria proceeds with a one nucleotide gap filling reaction.[208]

The effect of caloric restriction on mitochondrial and nuclear DNA base excision repair has been demonstrated in mice.[224] BER was studied by measuring the repair of uracil by protein extracts from brain, kidney, and liver mitochondria. Caloric restriction lowered overall BER in brain and kidney but not in liver mitochondria. Individual analysis of three stages of BER revealed a subtle increase in uracil DNA glycosylase activity, while AP endonuclease activity was decreased in all studied tissues and the efficiency of pol γ, estimated by the gap filling assay, was decreased in brain and kidney 20−30%. The influence of caloric restriction on nuclear DNA BER was the opposite, where an increase of 26% to 42% was observed in liver and kidney, respectively, which is in agreement with a previous report.[225] The observed decrease in BER activity in mitochondria in response to caloric restriction was somewhat surprising since a restricted diet has been demonstrated to promote a decrease in oxidative mtDNA damage and mtDNA mutations.[224] However, caloric restriction does limit generation of ROS in mitochondria, and the authors suggest that mitochondrial BER activity is regulated by mitochondrial ROS production.[224]

DNA repair activities such as 8-oxoguanine-DNA glycosylase (OGG1), uracil-DNA glycosylase, and AP-endonuclease (APE1) appear to increase in aging cells, which coincides with the accumulation of oxidative damage to both mtDNA and nuclear DNA. Although the total OGG1 activity is higher in mitochondria isolated from livers of older mice, a large fraction of the enzyme remains bound to the membrane in its precursor form.[226] This results in inhibition of the enzyme translocation and processing in the mitochondrial matrix.[226] Reported differences in OGG1 and APE1 import efficiencies between young and older cells could help to account for the observed oxidative DNA lesions that accumulate in both nuclear and mitochondrial DNA during aging.[226,227]

5.3. Lack of Nucleotide Excision Repair in Mitochondria

Nucleotide excision repair (NER) is a mechanism responsible for removing bulky adducts from bacterial DNA or nuclear DNA in eukaryotes by excision and resynthesis of a large gap of DNA encompassing the lesion. In general, these types of DNA lesions are not repaired in mitochondrial DNA.[199,228] However, mitochondrial DNA has been dem-

onstrated to be a major cellular target for carcinogenic compounds that induce bulky adducts, such as the dihydrodiol-epoxide derivative of benzo[*a*]pyrene (BaP), bleomycin, and aflatoxin B_1.[229−233] Also, the commonly used anticancer drug cisplatin generates intrastrand and interstrand DNA cross-links, the former being the dominant type of lesion arising from cisplatin contact with DNA.[234,235] These lesions are removed from nuclear DNA by NER, but repair of cisplatin intrastrand cross-links is not observed in mtDNA as detected by the gene specific repair assay or by a DNA relaxation assay.[200,236] The fact that the majority of known bulky adducts are not removed from mtDNA is quite striking when one considers that many chemical carcinogens do enter mitochondria and interact with mtDNA.[230,237,238] Unrepaired bulky lesions pose a replication block for DNA polymerase γ, causing polymerase stalling[239] that can lead to mtDNA deletions or rearrangements.

Interestingly, removal of interstrand cisplatin adducts[200] and 4-nitroquinoline 1-oxide (4NQO)[240] adducts from mtDNA has been demonstrated in Chinese hamster ovary cells. The significance of these discoveries is still not clear, and it is not known whether any nuclear NER proteins are involved.

5.4. Mismatch Repair in Yeast Mitochondria

The presence of mismatch repair (MMR) pathways required for the removal of base mismatches and short insertions and deletions in nuclear DNA is well established. MMR machinery present in mammalian nuclei shares significant similarities with bacterial MutHLS, reviewed in ref 241. Briefly, repair is initiated by two protein complexes, MutSα and MutSβ. MutSα consists of a MSH2/MSH6 (homologues of bacterial MutS) heterodimer that acts with MutLα, composed of MLH1 (MutL homologue) and (h)PMS2, to remove base/base mismatches. MutSβ is a MSH2/MSH3 heterodimer that can act in concert with MutLβ, composed of MLH1 and (h)PMS1, to repair small DNA loops.[242,243] No mammalian homologue for bacterial MutH has been found.[244] The presence of MMR in mitochondria was reported in *S. cerevisiae* and *S. pombe*[245−247] but not in higher eukaryotes. *S. cerevisiae* encodes *msh-1*, a homologue of *mutS*, and mutations in *msh-1* have been demonstrated to induce a high mutation rate in yeast mtDNA.[100,248] No homologue of *msh-1* has been found in mammals. A homologue of bacterial MutS (MSH) gene has been found in the mitochondrial genome of the soft coral *Sarcophyton glaucum*.[249,250] The presence of this homologue suggests a mismatch repair activity in *S. glaucum* mitochondria.[250] Phylogenic analysis of MutS family protein sequences revealed that *S. glaucum* mtMSH protein is more closely related to the *S. cerevisiae* nuclear DNA-encoded mitochondrial mismatch repair protein MSH1 than to eukaryotic or bacterial homologues.[250] Recently MMR that shows no strand bias for correcting point mutations has been reported in mammalian mitochondria.[251] This is in contrast to the strand bias MMR systems operating in bacteria and animal cell nuclei.[252,253] Rat liver mitochondrial lysates repaired GT and GG mismatches, and this activity was shown to be mismatch selective, bidirectional, and ATP-dependent. The reported activity was much less potent than that of nuclear MMR and no MSHs were detected in purified mitochondrial extract, suggesting that different enzymes/mechanisms may be involved.[251] Further studies are needed to fully understand this single observation.

5.5. Recombination and Nonhomologous End-Joining in Yeast Mitochondria

Repair of double-strand breaks in mtDNA may be conducted by recombination or nonhomologous end-joining (NHEJ) mechanisms, as occurs in the nucleus. Recombinantion of mtDNA has been readily observed in yeast, and yeast has proven an excellent model system to study recombination in mtDNA. Two proteins involved in recombination of nuclear DNA, the dsDNA binding protein Rad50 and the nuclease Mre11, have been reported to colocalize in yeast mitochondria.[254] Several other proteins involved in recombination have also been found in yeast mitochondria. The Pif1 5′-3′ DNA helicase promotes recombination between ρ− and ρ+ genomes.[255] The cruciform cutting endonuclease Cce1 is possibly involved in resolving Holiday junctions.[256] Loss of the 5′-3′ exonuclease Din7, which is expressed in response to DNA damage, increases the petite frequency and destabilizes mitochondrial poly GT tracts.[257,258] The Mhr1 protein has been reported to pair ssDNA and form heteroduplex joints *in vitro*, and mutational inactivation of this gene leads to loss of homologous mtDNA recombination.[259] Also, double mutation of *Cce1* and *Mhr1* leads to formation of ρ^0 daughter cells, suggesting that recombination may be a crucial mechanism of maintenance of mtDNA in yeast.[259]

Recombination of mitochondrial DNA in mammalian cells has been much less clear.[260−270] Recombination activity has been reported in mammalian cell culture both *in vitro* and *in vivo*.[271,272] Mammalian mitochondria can rejoin blunt-ended and cohesive linearized plasmid DNA at a low level.[273] Although rare, mtDNA structures consistent with recombination intermediates have been isolated from human heart muscle.[274] Data concerning the repair of interstrand cross-links in mtDNA are mixed. In the nucleus, these lesions are repaired by homologous recombination. Cisplatin interstrand cross-links and adriamycin DNA adducts were demonstrated to be efficiently removed from mtDNA,[200,275] whereas cross-links produced by psoralen were not removed.[276] Recent studies by one group have reported mtDNA recombination in the skeletal muscle of individuals with multiple mtDNA heteroplasmy.[277,278] Nevertheless, the biological significance of the low frequency of recombination observed in mammalian cells is unclear.

5.6. Translesion Synthesis by Pol γ

Reports of translesion synthesis past DNA adducts by pol γ are limited. Translesion DNA synthesis past platinated DNA adducts by human pol γ has been demonstrated.[279] Human pol γ displays a specificity for translesion synthesis past dien Pt-DNA adducts, with less synthesis past oxaliplatin and still less for cisplatin.[279] The ability of pol γ to synthesize past abasic sites has been studied, and the enzyme was found to stall the majority of the time.[280] When pol γ does perform translesion synthesis past an abasic site, it prefers to incorporate dAMP opposite the site.[280] Translesion synthesis by pol γ is readily accomplished opposite 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxo-dG). *Xenopus laevis* pol γ incorporates dCMP 73% of the time while misincorporating dAMP 27% of the time opposite an 8-oxo-dG adduct.[280]

Mitochondrial DNA has long been suspected as a major cellular target of other chemical carcinogens.[229,233,281−287] Benzo[*a*]pyrene and benzo[*c*]phenanthrene (BcPh) have been found to localize specifically in mitochondria.[282] Several

studies have demonstrated that mtDNA suffers more modification than nuclear DNA after exposure of cells to BaP.[229,230] In isolated, whole mitochondria from BaP-treated rats, DNA synthesis is reduced but mitochondrial DNA polymerase activity increases, most likely as a feedback response to the high degree of mtDNA modification and the decrease in ATP production.[285] Treatment of cells with BaP inhibits mitochondrial DNA synthesis and leads to formation of 4−15 times more adducts per unit length of mtDNA than nuclear DNA.[288] Recently, using a highly sensitive chemiluminescence immunoassay, levels of BaP 7,8-diol 9,10-epoxide (BaP DE) adducts at dG were found to be 10-fold higher in mtDNA than nuclear DNA per unit length of DNA.[238] Antioxidant anticancer treatment with *N*-acetylcysteine decreased the amount of mtDNA adducts in rats exposed to cigarette smoke,[237] which contains polycyclic aromatic hydrocarbon (PAH) carcinogens, possibly by trapping the reactive diol epoxide metabolites. Due to the lipophilic character of PAHs and the high ratio of lipid to DNA in mitochondria, the mtDNA may be a more significant biological target for PAHs than nuclear DNA.

Pol γ has limited ability to perform translesion synthesis opposite BaP dG, BaP dA, and BcPh dA adducts in DNA. The catalytic subunit alone has weak ability to incorporate opposite all of the DE adducts studied and showed no translesion synthesis.[239] Even in the presence of the p55 accessory subunit, only limited, one-base extension beyond the BaP dG adducts is observed, and even weaker extension or no extension beyond dA adducts. For all the adducts studied, kinetic experiments in the presence of p55 indicated that the total incorporation of both correct and incorrect nucleotides opposite these adducts was decreased by 3−4 orders of magnitude relative to the unmodified control template.[239] The nucleotides most frequently inserted opposite a dG adduct were the purine nucleotides dG and dA. Thus, these adducts present a substantial block to DNA replication *in vitro*. This blockage is consistent with the inhibition of mitochondrial DNA replication that was previously demonstrated with intact mitochondria upon treatment with BaP.[285,288]

6. Induced Mitochondrial Toxicity and Pol γ Inhibition by NRTIs

NRTI (nucleoside reverse transcriptase inhibitor) therapy in HIV-infected patients has been beneficial in extending life and slowing the progression of AIDS, but treatment with nucleoside analogues is accompanied by certain side effects. The most pronounced side effects from NRTI therapy are damage to the mitochondria and loss of mitochondrial function. First observed in 1990 by Dalakas et al. as mitochondrial myopathies in patients on AZT (3′-azido-3′-deoxythmidine) therapy,[289] NRTI-induced mitochondrial dysfunction is termed mitochondrial toxicity. These patients had induced myopathies with ragged red fibers and reduced amounts of mitochondrial DNA.[290] Further clinical evidence demonstrated that mitochondrial myopathy slowly and cumulatively develops during AZT treatment.[291] Mitochondrial toxicity from NRTI treatment mimics mitochondrial genetic diseases and induces similar clinical syndromes, including ragged-red muscle fibers, lactic acidosis, myopathies, cardiomyopathies, hepatic steatosis, lipodystrophy, and neuropathy.[292−294]

Early investigations into the observed mitochondrial toxicity implicated pol γ in the process of toxicity. Pol γ is unique among the cellular replicative DNA polymerases in that it is highly sensitive to inhibition by anti-HIV nucleotide analogues such as AZT-TP (3′-azido-3′-deoxythmidine-5′-triphosphate), dideoxynucleotides, and other antiviral nucleotide analogues.[96,295−306] The general inhibitory effect of NRTIs on polymerases is as follows: HIV-RT $\gg$ pol γ > pol β > pol α = pol ϵ.[307] Mitochondrial toxicity may be caused by (1) direct inhibition of pol γ activity without incorporation; (2) termination of the growing nascent DNA strand by incorporation of these chain-terminating analogues into mitochondrial DNA; (3) alteration of the fidelity of DNA synthesis of pol γ; (4) the persistence of these analogues in mtDNA due to inefficient excision; or (5) a combination of any of these effects. Kinetic studies indicate that the apparent *in vitro* hierarchy of mitochondrial toxicity for the approved NRTIs is as follows: ddC (dideoxycytidine, zalcitabine) ≥ ddI (dideoxyinosine, didanosine) ≥ D4T (2′,3′-didehydro-2′,3′-dideoxythymidine, stavudine) $\gg$ 3TC (2′,3′-dideoxy-3′-thiacytidine, lamivudine) > PMPA (9-(*R*)-2-(phosphonomethoxypropyl)adenine, tenofovir) > AZT (zidovudine) > CBV (guanine analogue, abacavir).[305,306] During *in vitro* chain elongation by pol γ, dideoxynucleotides and D4T-TP are utilized at least as efficiently as natural deoxynucleotides, whereas AZT-TP, 3TC-TP, PMPA, and CBV-TP are only moderate inhibitors of DNA chain elongation.[305,306] Once incorporated, the polymerase may remove the terminal NRTI with the exonuclease activity intrinsic to pol γ. We previously found that pol γ is inefficient in removing terminally incorporated dideoxynucleotides, D4T, AZT, and CBV, from DNA.[305] This finding predicts persistence of these analogues *in vivo* following successful incorporation. In contrast, removal of 3′-terminal 3TC residues is 50% as efficient as natural 3′-termini, predicting reduced persistence and lower toxicity for this analogue. In addition to the triphosphate form of these analogues, metabolic intermediates have the potential to inhibit pol γ or other cellular polymerases. The cellular conversion of AZT to AZT-TP has been shown to accumulate the monophosphate intermediate *in vivo* at millimolar concentration.[308,309] We have shown that the pol γ exonuclease activity is inhibited by AZT-monophosphate at concentrations known to occur in cells.[305] Thus, although their greatest inhibitory effects are through incorporation and chain termination, persistence of these analogues in DNA and inhibition of exonucleolytic proofreading are also likely to contribute to mitochondrial toxicity. 3TC-TP is one of the analogues least likely to be incorporated and yet is one of those most efficiently removed. This may explain the low mitochondrial toxicity induced by 3TC *in vivo*. Although AZT-TP is one of the analogues least likely to be incorporated into DNA by pol γ, once incorporated it is not efficiently removed from DNA by the pol γ exonuclease function. The inefficiency of pol γ to remove AZT from DNA may help to explain some of the AZT-induced mtDNA depletion observed *in vivo*.

The kinetics of NRTIs incorporation suggests that AZT-TP is only a moderate inhibitor of pol γ. Despite kinetic explanations, the wide range of clinical toxicities reported from AZT therapy indicates a more potent toxicity from this analogue. This has prompted many investigators to seek alternative mechanisms of toxicity such as oxidation,[310] inhibition of glycosylation,[311−313] and inhibition of the ADP/ATP translocator.[314,315] Over 10 years ago, AZT was shown to be reduced *in vitro* by thiols to produce a wide range of products.[316,317] The most common product upon reduction of AZT by DTT is D4T, where 31% of the products resulted

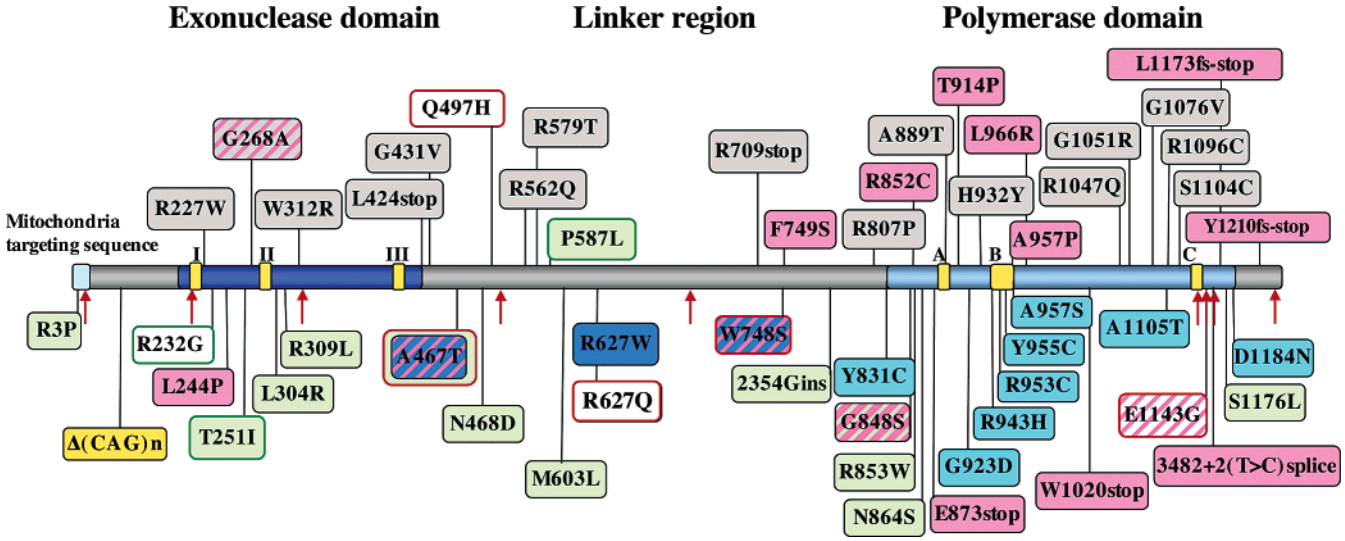

Figure 8. Schematic diagram of human pol γ protein showing the location of amino acid substitutions resulting from disease and polymorphism mutations. The disease substitutions are represented by the following boxes: *light blue boxes*, autosomal dominant PEO; *light green boxes*, autosomal recessive mutations; *gray boxes*, sporadic PEO; *pink boxes*, Alpers syndrome; *dark blue boxes*, SANDO; *yellow boxes*, male infertility. Boxes highlighted with *red lines* represent mutations found in spino-cerebellar ataxia-epilepsy, while boxes highlighted in *dark green lines* are found in infantile hepatocerebral syndrome. Striped boxes represent disease substitutions found in more than one disease. *Red arrows* depict the nonsynonymous polymorphic amino acid changes.

in D4T.[318] Recently, significant levels of D4T were found in HIV-infected patients undergoing only AZT treatment, suggesting intracellular reduction of AZT to D4T.[319] Given the moderate inhibition by AZT-TP of pol γ and the strong inhibitory affect of D4T-TP on pol γ, these new findings indicate the majority of AZT toxicity may result from *in vivo* conversion of AZT to D4T. For example, a mere 0.05% conversion of AZT to D4T would result in toxicity seen with D4T therapy alone.

Based on sequence alignment of the bacterial DNA polymerases within family A, mutagenesis studies, and available three-dimensional structures, three amino acids, Tyr951, Tyr955, and Glu895, in human DNA pol γ were studied for their role in NRTI selection.[320] The function of these three residues accounts for the majority of the selection of incoming dNTPs. The cause of dideoxynucleoside and D4T sensitivity is mainly attributed to a single tyrosine in motif B, Tyr951, of human pol γ.[43,320] This tyrosine is invariant in all of the pol γ sequences and corresponds to Y526 of T7 DNA polymerase. The sensitivity of T7 DNA polymerase to ddNTPs is due to Y526,[321] and the presence of this tyrosine in the γ polymerases is consistent with their sensitivity to ddNTPs. Substitution of this tyrosine residue with phenylalanine in the human enzyme reduces inhibition by dideoxynucleotide or D4T-TP by several 1000-fold with only minor effects on overall polymerase function.[43,320] The hydroxyl group of the Tyr951 side chain appears to form a hydrogen bond network with the β-phosphoryl oxygen and the 3′-OH of the incoming dNTP. In the absence of the 3′-OH moiety, as with ddC or D4T, the incoming nucleotide analogue is stabilized in the active site by the Tyr951 hydrogen bond to the β-phosphoryl oxygen, allowing efficient incorporation. Tyr955 and Glu895 appear to interact together to form the steric block against ribonucleotides as well as to interact with the rigid sugar rings of D4T-TP and CBV-TP. Interestingly, none of the pol γ active site mutant proteins displayed enhanced discrimination against AZT-TP, and the structural attributes in pol γ that allow AZT-TP to be incorporated must wait until a crystal structure is solved.

7. Human Mitochondrial Disorders Associated with Pol γ

7.1. Background of Mitochondrial Diseases

POLG, the gene for the catalytic subunit of Pol γ, is one of several nuclear genes that is associated with mitochondrial DNA depletion or deletion disorders. Dysfunction of the mitochondrial DNA polymerase has been associated with such disorders as progressive external ophthalmoplegia (PEO), parkinsonism, premature menopause, Alpers syndrome, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) or sensory ataxic neuropathy, dysarthria, and ophthalmoparesis (SANDO) (Figure 8). Also, alteration of the $(CAG)_{10}$ repeat in the second exon of *POLG* has been implicated in male infertility. Mutations in *POLG*, *TWINKLE*, and *ANT1* are associated with PEO while mutations in several enzymes involved in mitochondrial nucleotide metabolism can cause mitochondrial syndromes resulting in point mutations, deletions, or depletion of mtDNA (Table 2).

Table 2. Nuclear Loci That Affect the Stability of Mitochondrial DNA

chromosome locus	disorder	gene	function
15q25	PEO/Alpers	*POLG*	mitochondrial DNA polymerase
17q23-24	PEO	*POLG2*	Pol γ accessory subunit
10q24	PEO	*TWINKLE*	mitochondrial helicase
4q34-35	PEO	*ANT1*	adenine nucleotide translocator
22q13.32	MNGIE	*TPase*	thymidine salvage
2p13	MtDNA depletion	*DGUOK*	deoxyguanosine kinase
16q22	MtDNA depletion	*TK2*	thymidine kinase 2
17q25.4	MCPHA	*DNC*	deoxynucleotide carrier

7.2. Progressive External Ophthalmoplegia

PEO is a mitochondrial disorder associated with mtDNA depletion and/or accumulation of mtDNA mutations and deletions.[60,322−325] PEO is usually transmitted in an autosomal dominant trait (adPEO).[322,326] PEO is characterized by late

onset (between 18 and 40 years of age) bilateral ptosis and progressive weakening of the external eye muscle, resulting in blepharoptosis and ophthalmoparesis, proximal muscle weakness and wasting, as well as exercise intolerance. The disease is often accompanied by cataract, hypogonadism, dysphagia, and hearing loss, and it may, within several years, lead to development of neuromuscular problems.[325,327] Neurological problems may include depression or avoidant personality.[328] Skeletal muscles of PEO patients present red ragged fibers and lowered activity of respiratory chain enzymes. Multiple large-scale deletions of mtDNA isolated from muscle biopsies were first demonstrated in Italian families with the heritable autosomal dominant form of PEO (adPEO) by Zeviani and colleagues.[322] This observation led to the hypothesis that a specific autosomal gene may be responsible for mtDNA instability. Positional cloning studies have linked PEO to four different chromosomal loci (Table 2): a gene at locus 4q34-35 that encodes adenine nucleotide translocator (ANT1),[329] chromosome 10q24 encoding a mitochondrial helicase (*TWINKLE*),[138] a locus at chromosome 22q13.32-qter, corresponding to the thymidine phosphorylase gene, associated with mitochondrial neurogastrointestinal encelophalomyopathy (MNGIE), a recessive form of PEO,[330] and chromosome 15q22-26 that encodes the gene for the catalytic subunit of the mitochondrial DNA polymerase.[60] Most recently a patient with autosomal dominant PEO was found to harbor a mutation in the gene for the pol γ accessory subunit (Clark, Longley, Copeland, and Chinnery, personal communication). All five nuclear loci encode enzymes that participate in maintenance of mtDNA or nucleotide metabolism.

Over 60 pathogenic mutations have been found in *POLG* that are associated with PEO (Figure 8). To date, all dominant *POLG* mutations responsible for developing PEO have been mapped to the polymerase domain of pol γ[331] (see also the Human Polymerase Gamma Mutation Database http://dir-apps.niehs.nih.gov/polg). A structural homology model developed in our laboratory[82] allowed the investigation of four amino acid substitutions, G923D, R943H, Y955C, and A957S, associated with adPEO. Recombinant proteins with these substitutions were characterized biochemically.[82] Two of the substitutions, R943H and Y955C, change side chains that interact with the incoming dNTP. Recombinant proteins carrying these substitutions retain less than 1% of the wild-type polymerase activity and display a severe decrease in processivity. The significant stalling of DNA synthesis and extremely low catalytic activities of both enzymes are the two most likely causes of the severe clinical presentation in R943H and Y955C heterozygotes.[82] Additionally, several patients carrying the Y955C or A1105T mutation were reported to develop parkinsonism later in life.[63] The substitution of Y955 to cysteine also increases nucleotide misinsertion replication errors 10−100-fold in the absence of exonucleolytic proofreading.[332] The G923D and A957S recombinant forms of pol γ retained less than 30% of wild-type polymerase activity. This is consistent with the reduced clinical severity of PEO in individuals heterozygous for the G923D and A957S mutations.[82]

Because both copies of *POLG* are expressed, autosomal dominant mutations are thought to produce proteins that compete with the wild-type pol γ in a dominant negative fashion. Indeed, a slight increase in DNA binding efficiency was observed in three of the four autosomal dominant mutants described above: G923D, R943H, and A957S.[82] However, most of the mutations found in *POLG* are associated with autosomal recessive PEO (arPEO), and patients with PEO are often compound heterozygotes with two arPEO alleles. For example, the A467T mutation has been found *trans* to other *POLG* missense mutations in PEO, SANDO, and Alpers syndrome.[59,333] The A467T mutation was found in two pedigrees as a homozygous mutation and associated with severe ataxia in midlife.[334] Biochemical analysis indicates that the A467T mutant pol γ possesses only about 4% of the wild-type DNA polymerase activity with only a modest effect on the exonuclease.[88] Additionally, the A467T pol γ protein fails to interact with the p55 accessory subunit that is normally required for highly processive DNA synthesis.[88] Nevertheless, A467T is a common mutation present in 0.6% of the Belgian population.[333] The recessive T251I mutation is also common and frequently found *cis* to the P587L mutation in PEO. Both mutations also exist as single mutations in *POLG*, perhaps suggesting that the double mutant arose through a recombination of the two single-mutant alleles. A yeast model for PEO has been developed in which PEO mutations in amino acids that are conserved between man and yeast were constructed in *MIP1*.[335] This yeast model is useful to quickly ascertain the severity of PEO mutations. Many of the more severe PEO mutations in human also cause rapid loss of yeast mtDNA and are accompanied by elevated damage to mtDNA as well as nuclear DNA.

Recently a single mutation in the gene encoding the accessory subunit has been reported in a patient with PEO (Clark, Longley, Copeland, and Chinnery, personal communication). The mutation results in G451E substitution in a loop region not involved in p55 dimerization (Figure 5). Characterization of the recombinant G451E mutant of p55 demonstrates that the mutant accessory subunit fails to stimulate processive synthesis in the catalytic subunit (Longley, Clark, Copeland, and Chinnery, personal communication). The failure to enhance processivity in the catalytic subunit would cause the complex to stall during DNA replication and is consistent with the accumulation of mtDNA deletions detected in PEO.

7.3. Parkinsonism and Premature Menopause

Parkinson's disease is a frequent neurodegenerative disease that affects up to 2.2% of the population in North America.[336] A link between the disease and mitochondrial dysfunction has been suggested in the past.[337,338] The presence of a deleted mtDNA fraction in idiopathic brains, from patients with Parkinson's disease, was reported by two groups.[339,340] Parkinsonism has been described in several families suffering from progressive external opthalmoplegia with multiple mtDNA deletions and mutations.[341−343] In 2004 significant cosegregation of parkinsonism with mutations in the *POLG* gene was described.[63] Positron emission tomography (PET) findings demonstrated loss of dopaminergic neurons, and postmortem examination revealed in two individuals loss of pigmented neurons as well as pigment phagocythosis in substantia nigra without Levy bodies. Levy bodies, which are protein aggregates formed within neurons, consistent with defective protein degradation, are often seen in other forms of the disease, not associated with mutations in *POLG*. The authors suggest that Parkinson's disease related to mutations within *POLG* may proceed via an alternative pathogenic pathway, where accumulation of mtDNA mutations might lead to lower ATP production and/or oxidative stress, which

in turn could cause neurodegeneration. Treatment of Parkinson's disease with large doses of coenzyme Q10, a powerful antioxidant, led to slowing down the functional decline in the Parkinson's patients,[344] which may support this possibility. In all cases, parkinsonism manifested several years after initial disease symptoms.

In addition, most PEO women included in the study had menopause before the age of 35 and were suffering from high gonadotropin and low estrogen concentrations, which points to premature ovarian failure.[63] Also, in one PEO family studied by Luoma and colleagues,[63] affected men had been earlier diagnosed with testicular atrophy.[345] Since mitochondria have been indicated to have a significant role in the regulation of steroidogenesis,[346] there may be a link between these observations and alterations in the *POLG* gene.

7.4. Alpers Syndrome

Alpers syndrome is a rare but severe heritable, autosomal recessive disease that afflicts young children. Within the first few years of life, patients exhibit progressive spastic quadriparesis, progressive cerebral degeneration leading to mental deterioration and seizures, cortical blindness, deafness, and eventual death. Naviaux *et al.* reported an Alpers patient with reduced electron transport chain function, dicarboxylic aciduria, fulminant hepatic failure, refractory epilepsy, and lactic acidosis which resulted in death at 42 months.[347] Skeletal muscle biopsy indicated a reduction of mitochondrial DNA content to 30% of normal with no detectable pol γ activity.[347] Sequencing of the *POLG* gene in this Alpers pedigree as well as in unrelated Alpers pedigrees revealed a heterozygous G to T nonsense mutation in exon 17 of *POLG* that converts Glu873 (GAG) to a stop codon (TAG) just upstream of the polymerase domain of the protein.[59,348] In addition, each affected child was heterozygous in exon 7 for the A467T amino acid substitution between the exonuclease and polymerase domains of pol γ.[59,348] Pol γ mRNAs with the E873stop mutation are removed from the pool of mRNAs by nonsense mediated decay resulting in monoallelic expression of *POLG* containing only the A467T mutation.[349] Recently, reports by Zeviani and co-workers and Naviaux and co-workers have found several other mutations in *POLG* in 20 independent pedigrees.[350,351] Sequence analysis in these families has identified a total of at least 16 mutations associated with Alpers syndrome. In nearly all cases, the *POLG* mutations found in Alpers affected patients are recessive and both alleles must contain a mutation in order for the disease to develop. Many of these mutations also occur in PEO as autosomal recessive mutations. Thus, certain combinations of recessive mutations may only produce mild forms of PEO while other combinations of recessive mutations lead to the devastating Alpers disease. The A467T mutation commonly found as a compound mutation in arPEO has also been frequently found in Alpers patients either as a homozygous or as a heterozygous mutation in compound with other mutations.

7.5. Ataxia—Neuropathy

Mutations in *POLG* are also associated with an ataxia-neuropathy syndrome with onset in the early teens to late thirties. This ataxia, also termed mitochondrial associated ataxia syndrome (MIRAS)[352] or spino-cerebellar ataxia-epilepsy syndrome (SCAE), is caused by autosomal recessive mutations in *POLG* which then produce multiple mtDNA deletions in affected individuals. Symptoms of ataxia involving mutations in *POLG* include peripheral neuropathy, dysarthria, mild cognitive impairment, involuntary movements, psychiatric symptoms, myoclonus, and epileptic seizures. Ataxic patients who are homozygous for the A467T present with symptoms in their early to late teens[334,353] while one patient homozygous for the A467T mutation presented with MERRF-like syndrome at age 15, exhibiting low levels of mtDNA deletions in the muscle.[354] SANDO patients have also been found to have compound heterozygous mutations with the A467T mutation in one *POLG* allele and either R3P, L304R, or R627W in the other.[333] One patient with ataxia-myopathy syndrome was shown to have A467T in one *POLG* allele, with R627Q and Q1236H mutations in the second *POLG* allele.[87] Other patients with ataxia were found to be heterozygous with the A467T mutation on one allele and W748S *cis* to the E1143G mutation in the other allele. The latter mutation, E1143G, was originally identified as an SNP in 4% of the general population.[331] The W748S mutation in combination E1143G has been found as a frequent cause of ataxia.[352,353] Haplotype analysis of the Finnish population demonstrates a carrier frequency of 1:125 for the W748S mutation with a common ancestor origin of this diseased allele in ancient European.[352]

7.6. Male Infertility

The human *POLG* gene contains a 10 unit CAG trinucleotide tract encoding a poly-glutamine stretch near the N-terminus of the mature protein.[36] A shorter CAG trinucleotide repeat is found in African great apes, but this feature is not found in other eukaryotic *POLG* genes.[355] Although deletion of the CAG repeat has no detectable effect on mitochondrial function in tissue culture cells,[103] some studies suggest that alteration of the CAG repeat is associated with loss of sperm quality and contributes to 5−10% of the male infertility cases in the European population.[356,357] Since polyglutamine tracts can be sites for protein−protein interactions, altering the tract in pol γ may result in suboptimal or improper mtDNA replication. In contrast to these studies, two independent studies reported alterations in *POLG*'s CAG trinucleotide tract at the same frequency in both normal and infertile men and failed to confirm a relationship between the polymorphic CAG repeat in the *POLG* gene and male infertility.[358,359]

7.7. Nonsynonymous Single-Nucleotide Polymorphisms in *POLG*

In addition to disease mutations in *POLG*, several other mutations exist as single-nucleotide polymorphism (SNP) mutations. The NIEHS Environmental Genome Project evaluated SNP mutations both in the NIH Polymorphism Discovery Resource (NIHPDR) cell lines and in DNA from 450 anonymous, unrelated individuals with equal representation of females and males belonging to diverse ethnic groups including European-Americans, African-Americans, Mexican-Americans, Native Americans, and Asian-Americans. To date, the *POLG* gene contains 304 individual SNP mutations including six synonymous and nine nonsynonymous mutations in the coding region. Three nonsynonymous SNPs fall within motif C at frequencies from 1 to 4% of the study group. At the 3′ end of motif C the E1143G SNP occurs in 3−4% of the population, and it has also been found in patients with PEO,[360] ataxia,[334] and Alpers syndrome.[350,351]

The E1143G SNP mutation is predicted to be damaging to the function of the enzyme based on the degree of conservation at this position (http://www.snps3d.org/modules.php?name=SnpAnalysis&locus_ac=5428). The biochemical effects of these SNP mutations on polymerase function have not been established.

7.8. Replication Fidelity with Asymmetric dNTP Pools in Normal and Diseased Tissues

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder caused by mutations in the gene for thymidine phosphorylase (TP).[330,361] Similarly to PEO, the disease is associated with multiple deletions and depletion of mitochondrial DNA.[325,362] The onset usually takes place between the second and fifth decade of life, and typical clinical features include ptosis, progressive external ophthalmoparesis, gastrointestinal dysmotility, cachexia, peripheral neuropathy, myopathy, and leukoencephalopathy.[363,364] In a recessive family with MNGIE features, but without leukoencephalopathy, three missense mutations in *POLG* have been detected: T251I, P587L, and N846S.[365] Therefore, it has been recommended that, in patients with MNGIE features but without mutations in the gene encoding TP, *POLG* should also be sequenced.[365] TP deficiency leads to increased concentrations of circulating deoxythymidine (dThd)[366] and deoxyuridine,[367] which generates imbalanced mitochondrial deoxyribonucleoside triphosphate pools and can be responsible for increased mtDNA mutagenesis.[368]

The majority (>80%) of mtDNA mutations found in tissues from MNGIE patients are T to C transitions.[369] Also, most of the identified mtDNA point mutations were preceded by at least one adenine residue, and almost half of them were preceded by 5′-AAA sequences.[369] Based on the sequence specificity of the observed mutations, a "next-nucleotide effect", leading to high level of direct misincorporation,[370] was proposed as a main mechanism leading to detected alterations. The next-nucleotide effect may be responsible for 5′-AAT to 5′-AAC transitions. Direct misincorporation of dGMP opposite the thymine residue (T:dGMP) in a template is a common phenomenon.[102,371] After such misincorporation, elevated concentrations of dTTP, resulting from TP deficiency in the mitochondria of MNGIE cells, may lead to an increase of polymerase activity along the 5′-AA template region and compromise the exonucleolytic removal of the mismatched T:dGMP nucleotide.[369] Such a next-nucleotide effect has been observed *in vitro* with bacterial and mammalian polymerases possessing exonuclease activity, including pol γ.[370,372,373] Elevated concentrations of dTMP derived from the increased dThd were reported to inhibit pol γ.[305,373] Therefore, in cells of MNGIE patients, higher levels of dThd derived dTMP and dTTP may lead to inhibition of pol γ exonuclease activity, and in a subsequent round of mtDNA replication, T:G misincorporation would result in T-to-C mutation. Song et al. reported that, in HeLa cells grown in the media supplemented with 50 μM thymidine, mitochondrial pools of dTTP and dGTP increased significantly while at the same time dATP depletion was detected.[368] These results support a mutagenic mechanism involving competition between dGTP and dATP for incorporation opposite template T reported by Nishigaki and colleagues.[369]

Asymmetric dNTP pools in the mitochondria may not be confined to disease states such as MNGIE. Several research groups have demonstrated equal dNTP pools in isolated mitochondria from cultured cells.[368,374−376] In contrast, analysis of the mitochondrial dNTP pools in rat tissues such as liver, heart, and brain does show naturally occurring asymmetries in which dGTP pools are elevated by over 10-fold in comparison to the other three dNTPs.[377] *In vitro* fidelity measurements that mimic the observed *in vivo* dNTP concentrations demonstrate a reduced fidelity due to the formation of template T-dGTP mismatches that are inefficiently corrected by proofreading. This suggests that the naturally occurring dNTP pool asymmetries may contribute to spontaneous mutations in the mammalian mitochondrial genome.

In conclusion, mutations in the *POLG* gene are responsible for several mitochondrial disorders, including fatal childhood diseases such as Alpers syndrome, PEO with varied clinical severity, and possibly male infertility. While most *POLG* mutations likely result in an inactive or truncated protein, the dominant mutations commonly found in PEO produce biochemically deficient dominant negative pol γ proteins that compete with pol γ encoded by the wild-type allele. The resulting polymerase stalling and/or error-prone DNA synthesis promotes the pathogenic depletion, deletion, and mutagenesis of the mitochondrial genome. *In vitro* biochemical analysis of mutant pol γ proteins has become an essential tool for predicting the *in vivo* consequences of heritable mutations in the *POLG* gene.

8. Future Directions

Although a wealth of information has been collected over the past decades, the roles of pol γ in replication and repair of mtDNA are still not fully understood. The recent identification of *POLG* as a major disease locus for human mitochondrial disorders has stimulated research on pol γ and provided many new unanswered questions about the biology of mtDNA and human disease. Most of the major factors needed for mtDNA replication have likely been identified, and work to reconstitute the mtDNA replication fork has begun. However, two competing models of mtDNA replication have been described, and further biochemical investigation is needed to develop a unified model. Our hope is that pursuing the complete *in vitro* reconstitution of double-stranded mtDNA replication will both aid the search for new factors and inform the clinical community of new disease loci for heritable mitochondrial disorders. Similarly, studying dysfunctional enzymes resulting from disease mutations should yield new insights on the enzymology of mitochondrial DNA replication. Finally, much attention has been focused on understanding selection of nucleoside analogues by pol γ and the response to chemical modifiers of mtDNA. However, a full appreciation of pol γ's selectivity will have to wait until a three-dimensional crystal structure is determined.

9. Abbreviations

3TC	(−)-2′,3′-dideoxy-3′-thiacytidine, lamivudine
4NQO	4-nitroquinoline 1-oxide
8-oxo-dG	7,8-dihydro-8-oxo-2′-deoxyguanosine
ANT1	adenine nucleotide translocator
AP	apurinic/apyrimidinic
APE1	apurinic/apyrimidinic endonuclease
AZT	3′-azido-3′-deoxythmidine, zidovudine
AZT-TP	3′-azido-3′-deoxythmidine-5′-triphosphate
BaP	benzo[*a*]pyrene

BaP DE	BaP 7,8-diol 9,10-epoxide
BcPh	benzo[*c*]phenanthrene
BER	base excision repair
BrdU	5-bromo-2-deoxyuridine
CBV	2′,3′-didehydro-2′,3′-dideoxyguanosine, abacavir
d4T	2′,3′-didehydro-2′,3′-dideoxythymidine, stavudine
dA	deoxyadenosine
ddC	2′,3′-dideoxycytidine, zalcitabine
ddI	2′,3′-dideoxyinosine, didanosine
dG	deoxyguanosine
dNTP	deoxynucleotide triphosphate
DRE	DNA replication related element
dRP	5′ terminal deoxyribose phosphate
HIV-RT	human immunodeficiency virus reverse transcriptase
HP	hypersupressive
HSP	heavy strand promotor
kDNA	kinetoplast DNA
LSP	light strand promotor
MLS	mitochondrial localization signal
MMR	mismatch repair
MNGIE	mitochondrial neurogastrointestinal encelophalomyopathy
mt	mitochondrial
mtTFA, TFB1M, TFB2M	mitochondrial transcription factors
MUTYH	homologue of MutY, *E. coli* A:G glycosylase
$NaBH_4$	sodium tetrahydridoborate
NEM	*N*-ethylmaleimide
NER	nucleotide excision repair
NRF-1, NRF-2	nuclear respiratory factors 1 and 2
NRTI	nucleoside reverse transcriptase inhibitor
NTH	thymine glycol glycosylase
OGG1	8-oxo-G DNA-glycosylase/AP lyase
O_H	the origin of heavy strand mtDNA replication
O_L	the origin of light strand mtDNA replication
ori	origin of replication
PAH	polycyclic aromatic hydrocarbon
PEO	progressive external ophthalmoplegia
PET	positron emission tomography
PMPA	(+)-9-2-(phosphonylmethoxypropyl)adenine, tenofovir
rho^0	yeast mutants without mtDNA
rho^-	yeast mutants without functional mitochondria
ROS	reactive oxygen species
SANDO	sensory ataxic neuropathy, dysarthria and ophthalmoparesis
SNP	single-nucleotide polymorphism
SSB	single-stranded DNA-binding protein
TP	thymidine phosphorylase
TWINKLE	mitochondrial helicase
UDG	uracil DNA-glycosylase
UV	ultraviolet

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