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BIOCHEMICAL AND GENETIC STUDY OF THE MITOCHONDRIAL DNA REPLICASE AND DEVELOPMENT OF A *DROSOPHILA* MODEL OF MITOCHONDRIAL TOXICITY IN ANTIVIRAL THERAPY

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ABSTRACT

BIOCHEMICAL AND GENETIC STUDY OF THE MITOCHONDRIAL DNA REPLICASE AND DEVELOPMENT OF A *DROSOPHILA* MODEL OF MITOCHONDRIAL TOXICITY IN ANTIVIRAL THERAPY

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Progressive external ophthalmoplegia (PEO) is a Mendelian disorder characterized by the accumulation of multiple deletions in human mitochondrial DNA (mtDNA). The disease has been linked to several nuclear genes controlling replication and maintenance of mtDNA, including the mitochondrial DNA polymerase.

Mitochondrial DNA polymerase (pol γ) is the key enzyme in mitochondrial DNA replication. The catalytic (α) subunit of pol γ shares conserved DNA polymerase (pol) and 3'-5' exonuclease (exo) active site motifs with *E. coli* DNA polymerase I and bacteriophage T7 DNA polymerase. A major difference between the prokaryotic and mitochondrial proteins is the size and sequence of the intervening region, referred as the spacer in pol γ-α. A genetic study showed that a single missense mutation in the spacer region of *Drosophila* pol γ-α causes mitochondrial and nervous system dysfunction, and developmental lethality in the larval third instar. Several mutations in the spacer region of human pol γ-α have also been reported to be associated with PEO.

We constructed recombinant proteins to study the effect of various spacer region mutations on the mechanism of both *Drosophila* and human pol γ. Enzymatic analysis shows that a number of conserved amino acids are critical to maintain enzyme function. Enzyme activity, processivity, DNA binding affinity, and the balance between the pol and exo activities are affected differentially among the various spacer region mutants. In the crystal structures of *E. coli* DNA pol I and T7 pol, a cleft is formed between the exonuclease and DNA polymerase domains that binds the template-primer. We propose that together with other replicative proteins, the conserved sequence blocks play a role in positioning the substrate in the enzyme: template-primer complex to enhance catalytic activity and enzyme processivity.

To investigate the function of spacer region mutants *in vivo*, an approach of targeted gene replacement by homologous recombination was used for mutagenesis in *Drosophila*. Several *Drosophila* pol γ-α mutations that are associated with PEO in humans have been introduced in *Drosophila*, and their effects on development, mitochondrial function, adult life span and behavior will be addressed in future studies.

Nucleoside reverse transcriptase inhibitors (NRTIs) are a group of nucleoside analogs used in antiviral therapy in humans. mtDNA depletion and phenotypes that are typical in mitochondrial genetic diseases become key clinical observations after long-term NRTIs treatment. Biochemical studies have demonstrated that NRTIs are substrates for both HIV-RT and for human DNA pol γ. In preliminary studies, we documented significant decreases in mtDNA content after feeding both larvae and adult flies with the nucleoside analog dideoxycytidine. Our results suggest that *Drosophila* may provide a suitable animal model to study the mitochondrial toxicity of NRTIs.

To my husband Youjin Ma
And my son Daniel Linchuan Ma

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3TC	lamivudine
aaRSs	aminoacyl-tRNA synthetases
ad/arPEO	autosomal dominant/recessive PEO
AIDS	acquired immune deficiency syndromes
ANT1	adenine nucleotide translocator
ATP	adenosine triphosphate
AZT	zidovudine
bp(s)	base pair(s)
CBV	abacavir/carbovir
C-terminus	carboxyl-terminus
CTL	cytotoxic T-lymphocyte
DSB	double-strand break
DC	dentritic cells
d4T	stavudine
ddI/ddA	didanosine
ddC	zalcitabine
D-loop	displacement loop
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsRNA	double strand RNA

DTT	dithiothreitol
E. coli	*Escherichia coli*
HAART	highly active antiretroviral therapy
HIV	human immunodeficiency virus
H-strand	heavy strand
HU	hydroxyurea
IN	integrase
kbp	kilo base pair
kDa	kiloDalton
LC	Langerhans cells
LSP	light strand promoter
L-strand	light strand
LTR	long terminal repeat
MELAS	Mitochondrial Encephalomyopathy; Lactic Acidosis; Stroke
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mtSSB	mitochondrial single-stranded DNA binding protein
nDNA	nuclear DNA
N-terminus	amino terminus
Ni-NTA	Nickel chelated nitrilo-tri-acetic acid
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NRTIs	nucleoside reverse transcriptase inhibitors
nt	nucleotide

OXPHOS	oxidative phosphorylation
PCR	polymerase chain reaction
PEO	progressive external ophthalmoplegia
PI	protease inhibitors
PMPA	tenofovir
pol γ	mitochondrial DNA polymerase
pol γ-α	catalytic subunit of pol γ
pol γ-β	accessory subunit of pol γ
PR	protease
RNA	ribonucleic acid
RNAi	RNA interference
RNase MRP	mitochondrial RNA processing endonuclease
RT	reverse transcriptase
SSB	single-stranded DNA binding protein
ssDNA	single-stranded DNA
tRNA	transfer RNA
UV	ultraviolet light

CHAPTER I

INTRODUCTION

MITOCHONDRIA AND MITOCHONDRIAL DYSFUNCTIONS

Mitochondria are double-membrane organelles and exist ubiquitously throughout the eukaryotic organisms. They originate from a eubacterial endosymbiotic α-proteobacterial ancestor and are mainly involved in cellular energy production by using electron transport coupled with oxidative phosphorylation (OXPHOS). In addition, mitochondria participate in several other important functions, including ion homeostasis, intermediary metabolism and apoptosis (1).

I. Mitochondrial Structure and Mitochondrial OXPHOS

The mitochondrion is the site of OXPHOS in eukaryotes. There are four compartments in each mitochondrion: 1) the central mitochondrial matrix, which contains mitochondrial DNA (mtDNA) and proteins/ enzymes necessary for mtDNA replication and transcription, translation, and other metabolic functions including the tricarboxylic acid cycle and β-oxidation; 2) the inner membrane, which is impermeable to most small molecules and ions, including protons (H^+). Besides oxygen and carbon dioxide, the only molecules that can cross the inner membrane are some specific transporter proteins. The relative impermeability of the inner membrane is essential for maintaining a proton gradient required for the synthesis of ATP. The inner membrane contains OXPHOS complexes and forms cristae to create an enormous surface area on which OXPHOS can take place; 3) the intermembrane space, in which key proteins are stored; and 4) the outer membrane, which is permeable to small molecules up to 10 kDa and ions. Transmembrane channels composed of porin protein allow most molecules

<5,000 kDa to pass easily. Thus, the outer membrane mediates interaction between mitochondrion and cytosol (2).

The fundamental role of mitochondria is in cellular energy metabolism, which includes fatty acid β oxidation, the urea cycle, and the final common pathway for ATP production – the respiratory chain (3). The mitochondrial respiratory chain is made of a group of five enzyme complexes and each of them is composed of multiple subunits encoded by nuclear and/or mitochondrial DNA. Reduced cofactors (NADH and $FADH_2$) generated from the intermediary metabolism of carbohydrates, proteins, and fats donate electrons to complex I and II. These electrons flow between the complexes and electrochemical gradient, shuttled by complexes III and IV and by two mobile electron carriers, ubiquinone and cytochrome *c*. The liberated energy is used by complexes I, III, and IV to pump protons out of the mitochondrial matrix into the intermembrane space. This proton gradient, which generates the bulk of the mitochondrial membrane potential, is harnessed by complex V to synthesize ATP from ADP. ATP must be released from the mitochondrion in exchange for cytosolic ADP. The OXPHOS capacity is strictly regulated during embryonic development and in different physiological situations. In many mitochondrial diseases, OXPHOS capacity is impaired.

II. Mitochondrial Genetics

Mitochondria have their own self-replicating genome and associated replication, transcription, and translation systems. However, the information encoded in the mitochondrial genome is extremely specialized for the synthesis of components of the OXPHOS system, and more than 95% of all proteins located in the mitochondrial compartments are encoded by nuclear DNA. These nuclear DNA gene products include most subunits of the OXPHOS complexes and factors controlling mtDNA expression, assembly, function and turnover (4). Thus, the biogenesis of functional mitochondria depends on the coordination of both nuclear and mitochondrial genetic sources. Proteins from nuclear genes are synthesized in the cytoplasm with mitochondrial targeting sequences that direct them to pass through the outer and inner membranes. The targeting sequences are then cleaved before subunits assemble on the inner membrane (3).

Each human cell contains more than one mitochondrion and each mitochondrion contains hundreds of mtDNA. Mammalian mtDNAs are double-stranded circular molecules. They do not contain introns and both strands of a circular mtDNA molecule encode genes. These genes code for 13 proteins, 2 rRNAs, and 22 tRNAs. These 22 tRNAs can interpret the entire mtDNA genetic code and several mtDNA codons are different from the universal nuclear genetic code. For example, the Tyr tRNA reads both the normal UGG codon as well as the stop codon UGA; the Met tRNA recognizes both AUG and AUA codons as Met; the tRNA for recognizing Arg codons AGA and AGG is absent in mitochondrial code. Instead, AGA and AGG are stop codons in mitochondria (5).

In most healthy individuals, only wild-type mtDNA is present in mitochondria (homoplasmy). However, when a mutation arises in cellular mtDNA, a mix with both wild-type and mutant mtDNA is created (heteroplasmy) (6). The percentage of mutant mtDNA can vary widely from individual to individual, and also from organ to organ, and tissue to tissue. The multiplicity of mtDNA molecules within a cell indicates that mitochondrial mutations are accumulated gradually, without immediate deleterious impact. Studies on maternal pedigrees harboring heteroplasmic mtDNA mutations indicate that the percentage level of mutant mtDNA in clinically relevant tissues correlates with the severity of disease (7). Different tissues and organs have different demands on mitochondrial energy generation. In decreasing order, the bio-energetic expression thresholds for human organs are the central nervous system, heart and skeletal muscle, the renal system, the endocrine system and the liver (8).

Human mtDNA is strictly maternally inherited (9) and this maternal inheritance arises from the selective destruction of sperm mitochondria (10). During the early stage of oogenesis, there is a restriction in the number of mitochondrial genomes and this creates a functional "genetic bottleneck" (11). The same random mechanism may operate during the transmission of pathogenic mtDNA mutations. Due to variability in the transmitted mutation load to the offspring, differences in the severity of mitochondrial disorders are often seen among different family members.

mtDNA is continuously recycled even in nondividing tissues such as the brain (12) and mtDNA replication is independent of the cell cycle. It is possible that mutant and wild-type mtDNA replicate at subtly different rates. This may lead to changes in the proportion of mutant mtDNA in a heteroplasmic cell. Also, when a heteroplasmic cell divides, subtle differences in the proportion of mutant mtDNA may be passed to the daughter cells, leading to changes in the level of mutant mtDNA within a dividing tissue. Theoretically, uneven partitioning of mitochondria to daughter cells may explain the late onset and progression of some mtDNA disorders and the tissue/organ specific syndromes in some patients with mitochondrial diseases (3).

III. Human Mitochondrial Diseases

mtDNA commonly undergoes higher rates of evolutionary change than the nuclear genome (13) and mtDNA has been widely employed as an evolutionary marker to study the processes of molecular evolution and to infer phylogeographic and phylogenetic patterns. Possible reasons for the high mutation rate in mtDNA are: 1) Absence of protective histone covering mtDNA; 2) Primitive DNA repair system within mitochondria; 3) Increased damage of mtDNA by reactive oxygen species produced as by-products of OXPHOS within mitochondria (14).

Mutations can occur spontaneously in the germline, allowing maternal inheritance, or in somatic cells, causing sporadic cases of disease. Rearrangements including depletions and duplications are usually sporadic and invariably heteroplasmic. Point mutations, on the other hand, are usually transmitted through the maternal lineage and may be either heteroplasmic or homoplasmic (4). Since the first connections were made between mtDNA mutations and human diseases in 1988 (15), over 150 mutations with documented pathogenicity have been identified within the human mitochondrial genome. More than half of the disease-related mutations are located within tRNA genes, even though these sequences comprise only 10% of the genome (16). Mutations accumulated in postmitotic tissues may contribute to normal aging or to the development of common disorders such as heart failure or neurodegenerative disorders.

The segregation pattern of pathogenic mtDNA mutants somehow determines the nature and severity of mitochondrial diseases, but it varies with the specific mutation, mtDNA and nDNA background, cell type, and environment. For example, it has been reported that different mitochondrial genomes were favored in different tissues (17), and mtDNA segregation may be controlled by some specific nuclear genetic loci (18).

Besides disease-related mtDNA mutations, quite a few nuclear OXPHOS- related genes have been proven to be associated with mitochondrial syndromes. These include genes encoding structure components and/ or assembly factors of OXPHOS complexes, genes encoding factors involved in the biogenesis of mitochondria, and genes altering the stability of mtDNA (4). Autosomal progressive external ophthalmoplegia (PEO) is a Mendelian disorder with multiple deletions or depletion of mtDNA. Most PEO families carry mutations in one of three genes: *POLGA*, encoding the catalytic subunit of mtDNA polymerase; *Twinkle*, encoding mtDNA helicase; and *ANT1*, encoding adenine nucleotide translocator (19).

Many mitochondrial diseases include neurological symptoms, but the clinical features are often non-specific and diffuse. Patients with mutations in nDNA and mtDNA can have a similar clinical phenotype. On the other hand, the same genetic defect can cause very different clinical phenotypes. For example, clinically indistinguishable Leigh syndrome can be caused by mutations both in the nuclear COX assembly gene SURF1 and the mtDNA ATPase6 gene, whereas the A3243G mtDNA mutation in the tRNA Leu(UUR) gene can be present with classical MELAS, with CPEO, or with diabetes and deafness (3).

MITOCHONDRIAL DNA REPLICATION

mtDNA replication is much simpler than DNA replication in bacterial and nuclear systems. A minimal group of critical proteins are required in the mtDNA replication process. Animal mtDNA is a double-stranded circular molecule with a highly conserved gene content and organization (20). mtDNA genomes encode 2 rRNAs, 22 tRNAs, and 13 mRNAs and the size of mtDNA from different animals ranges from ~16-20 kb. Mitochondrial rRNAs and tRNAs are sufficient for protein synthesis and all of the protein products of mtDNA are essential for the OXPHOS. Nuclear DNA encodes all the proteins involved in mtDNA replication.

I. Models of Mitochondrial DNA Replication

It has been a long time since the unidirectional and strand-asymmetric DNA synthesis model was established (Figure 1.1) (21). The model postulates two sites of initiation of DNA synthesis, one for each strand. Mammalian mtDNA replication begins at O_H, which is located directly downstream of the light strand promoter (LSP) in the noncoding control region of the molecule. The mtDNA heavy (H) strand transcription initiates at the LSP and a RNA/DNA hybrid induces as transcription proceeds across the origin. The RNA/DNA hybrid forms a stable R-loop configuration, which is cleaved by the mitochondrial RNA processing endonuclease (RNase MRP) to provide the mtRNA primer for H-strand DNA replication (22). The newly synthesized region of the H-strand displaces the H-strand origin to create the displacement-loop (D-loop). Synthesis of the daughter H-strand is initiated at the D-loop by using the RNA primer and continued in a unidirectional manner until completion. When H-strand synthesis is 67% complete, the light (L) strand initiation site is exposed as a single-stranded template and folds into a stem-loop structure. Initiation of L-strand synthesis then occurs in the opposite direction (21). In *Drosophila*, H-strand initiates at an AT-rich region, and DNA replication on one

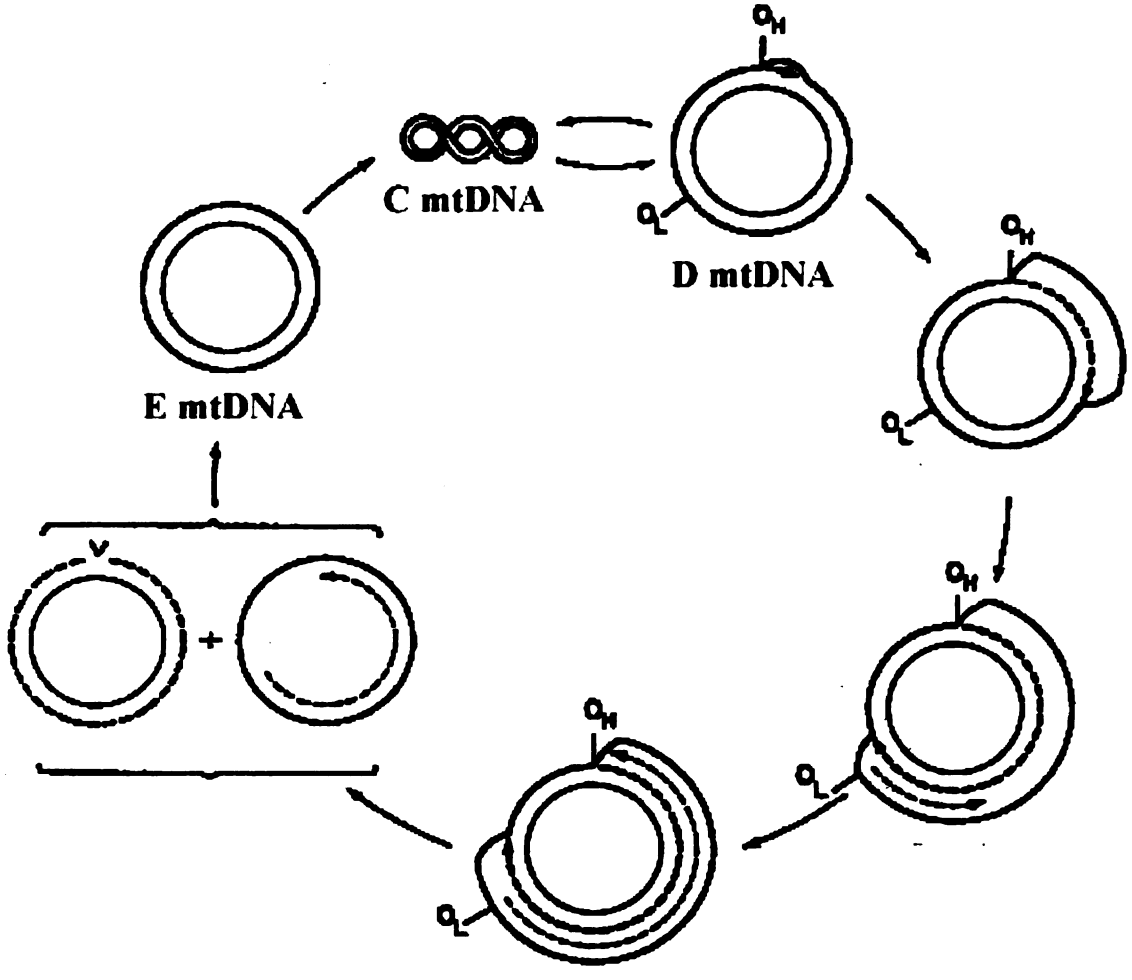


Figure 1.1. **Replication model of mammalian mtDNA replication.** Thick solid lines: parental heavy (H) strands. Thin solid lines: parental light (L) strands. Thick dashed lines: progeny H strands. Thin dashed lines: progeny L strands. The order of replication is clockwise starting at D mtDNA, the D-loop containing molecule. C mtDNA: supercoiled mtDNA molecule. OH and OL: ignition sites for H- and L- strand synthesis, respectively. The double arrows reflect the equilibrium between D mtDNA and C mtDNA. E mtDNA: complete progeny molecule.

strand is up to 99% complete before synthesis on the second strand is initiated (23). Therefore, during a single round of replication, most L-strand synthesis occurs on the displaced single strand template. Mitochondrial single-stranded DNA-binding protein (mtSSB) binds to the displaced parental single strand *in vivo* (24) and stimulates pol γ activity *in vitro* (25).

Recently, Dr. Holt's group proposed an alternative bi-directional, strand-coupled mtDNA replication model. Highly purified mtDNA replication intermediates were prepared from sucrose gradient and these intermediates were almost entirely duplex. This result is not consistent with the previous model. By using neutral/ neutral two-dimensional agarose gel electrophoresis, they found that ribonucleotide patches are distributed randomly around the genome and replication initiates frequently at multiple sites downstream of O_H and followed by fork arrest at O_H. Thus, O_H acts not as the origin but as a terminus for mtDNA replication (26,27).

II. Proteins at the Mitochondrial DNA Replication Fork

1. Mitochondrial DNA Polymerase (Pol γ)

Pol γ represents less than 1% of the total cellular DNA polymerase activity and is the only DNA polymerase for mtDNA replication (28). Highly purified native pol γ can be isolated from *Drosophila* embryonic mitochondria (29) and recombinant pol γ can be purified from the baculovirus expression system (30). *Drosophila* pol γ is a heterodimer comprising two subunits of 125 and 35 kDa (29,31) that are encoded by nuclear genes (32,33). In a model for the overall architecture of the *Drosophila* pol γ holoenzyme, the catalytic and accessory subunits each consist of three regions, pol-spacer-exo in pol γ-α, and N-M-C in pol γ-β. Multiple physical contacts occur between the catalytic subunit and the accessory subunit (34).

i. Structure of Family A DNA Polymerases

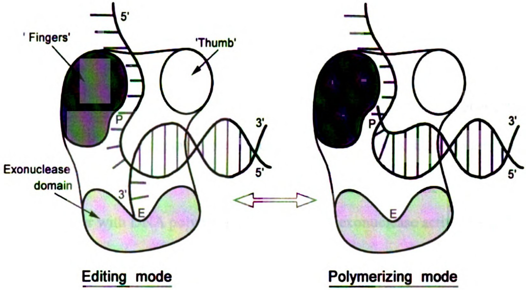
DNA polymerases have been classified based on their primary structure similarities. An alignment of the pol γ-α sequence with that of the *E. coli* pol I Klenow fragment indicated a high degree of amino acid sequence conservation in each of three DNA polymerase and three 3'-5' exonuclease domains (33,35-38). Therefore, mitochondrial DNA polymerase belongs to family A DNA polymerase.

Family A DNA polymerases are sensitive to dideoxynucleotide inhibitors, but are resistant to aphidicolin. *E. coli* Pol I is the prototype of family A DNA polymerases (39). Kinetic studies of Klenow fragment suggested that the active sites of the DNA polymerase and the 3'-5' exonuclease activities are separated on Pol I (40). The crystal structure analysis also indicates that the fragment is folded into two distinct domains. Deoxynucleoside monophosphate binds to the smaller 3'-5' exonuclease domain, with its 5'-phosphate interacting with a tightly bound metal ion. The right hand-like shape of the large polymerase domain forms a binding site for DNA substrate of polymerase reactions (41). There are three subdomains in the polymerase domain: the palm subdomain forms the base of the polymerase cleft, and contains important active site residues; the fingers subdomain also contributes side chains to the active site, especially those involved in nucleotide recognition; and the thumb subdomain interacts with the template-primer duplex upstream of the site of nucleotide addition (42). In the cocrystal structure of the Klenow fragment with duplex DNA, the template-primer duplex DNA was bound in a second cleft formed between two domains. The axes of these two clefts in the polymerase are roughly orthogonal to each other (43). In this case, the primer strand would approach the polymerase catalytic site from the direction of the exonuclease domain and the cleft in the polymerase domain would bind the single-stranded template strand beyond the site of DNA synthesis. The 3'-end of the primer/nascent DNA strand shuttles between two domains during DNA synthesis (Figure 1.2) (44).

More recent structures of the related DNA polymerases from *Thermus aquaticus* (*Taq* DNA polymerase) (45-47), *Bacillus stearothermophilus* (*Bst* DNA polymerase) (48), and bacteriophage T7 (T7 DNA polymerase) (49) complexed to DNA and/or nucleotide substrates indicate that these family A DNA polymerases share similar structures and DNA polymerases change between an open and a closed state (50). A wide minor groove allows duplex DNA access to protein side chains. Part of the DNA bound to the polymerase site shares a common binding site with DNA bound to the exonuclease site (47). Mismatched base pairs are accommodated in duplex DNA by the rotation of one or both bases into the major groove, which would disrupt minor groove interactions with polymerase and possibly trigger the switch from DNA synthesis to proofreading (49,50).

ii. Activities of the Catalytic Subunit

Both DNA polymerase and 3'-5' exonuclease activity are located in the 125 kDa catalytic subunit of *Drosophila* pol γ (33,51). A third catalytic activity, 5'-deoxyribose phosphate lyase, has also been shown in pol γ and other family-A polymerases (52-54). A photochemical crosslinking analysis shows that the catalytic subunit, instead of the



Editing mode

Polymerizing mode

Figure 1.2. **Direction of DNA synthesis and relative location of Pol and Exo active site in Klenow fragment**. "P" indicates the polymerase domain and "E" indicates the 3'-5' exonuclease domain. Double-stranded DNA binds to the cleft between two domains and the single-stranded template DNA binds to the cleft within the polymerase domain. 3' prime end of the primer or nascent DNA can shuttle between the 3'-5' exonuclease domain (in editing mode) and the polymerase domain (in polymerizing mode) without dissociating from the enzyme.

accessory subunit, makes contact with DNA (55).

Compared to other animal cell DNA polymerases, pol γ displays a remarkable ability to utilize different template-primer substrates. To distinguish pol γ from other nuclear DNA polymerases, DNA polymerase activity of pol γ has been assayed on either DNase I-activated calf thymus DNA or poly(rA):oligo(dT) during enzyme purification. In both cases, pol γ shows a broad pH optimum and high affinity for dNTPs (28). Recent studies indicate that pol γ catalyzes reverse transcription with a slightly higher efficiency than HIV-1 reverse transcriptase; proofreading activity with an RNA template is also observed in pol γ (56).

Together with DNA polymerase activity, 3'-5' exonuclease activity of pol γ is generally stimulated by moderate to high salt, exhibits a wide pH optimum and is dependent on a divalent metal cation (28). Like other DNA polymerases from both prokaryotic and eukaryotic sources, the proofreading exonuclease in pol γ is inhibited by 5'-monophosphates and the mispair extension can be promoted with an imbalanced nucleotide pool (57). The mispair specificity of the 3'-5' exonuclease activity was examined on M13 template-primers containing different mispair terminus, and the results indicates that the exonucleolytic hydrolysis is highly specific but equally efficient on different mispairs (57).

iii. Contributions of the Accessory Subunit

Based on the sequence-structure alignment from fold recognition and the sequence alignment between the C-terminal domain of pol-β and the anticodon-binding domain of class II aminoacyl-tRNA synthetases (aaRSs), a 3D structure model of the C-terminal domain of the accessory subunit was built using the anticodon-binding domain of *T.thermophilus* ProRS as a template (58). Even though the mouse accessory subunit was found to be a dimer (59), the crystal structure of the mouse accessory subunit agreed with the modeled structure of the *Drosophila* homolog because the *Drosophila* homolog lacks residues critical for dimerization of the mammalian accessory subunit (28). The anticodon-binding domain of class II aaRSs has been shown to be involved in tRNA binding. Therefore, the accessory subunit may participate in primer recognition. Indeed, a UV crosslinking method shows that the accessory subunit binds RNA in a complex of *Drosophila* pol γ with a synthetic template-primer (34).

In addition, despite lack of amino acid sequence homology, the accessory subunit model shows structural homology with thioredoxin, the accessory subunit of T7 DNA polymerase (58). Thioredoxin binds to the tip of the thumb in T7 DNA polymerase structure. Biochemical studies indicate that this association results in a ~80-fold increases in the affinity of T7 DNA polymerase for primer terminus and significantly increase the processivity of T7 DNA polymerase (60). The results indicate that the accessory subunit of polymerase γ also plays a role in enhancing pol γ processivity.

Overexpressed *Drosophila* catalytic subunits in either bacterial or baculorvirus expression systems are insoluble and carry very low catalytic activity (30). However, the recombinant holoenzyme from co-expression of both subunits in baculovirus-infected cells exhibits activity as high as that of the native enzyme (30). Biochemical studies demonstrate that the accessory subunit contributes to the structural and functional integrity of the catalytic subunit, including increasing the template-primer binding affinity, catalytic efficiency and processivity of the holoenzyme. This perhaps results from enhanced DNA binding and increased nucleotide binding of the holoenzyme.

iv. Processivity and Fidelity of mtDNA Replication

In the absence of other factors, pol γ has a high processivity (>1000nt) at low ionic strength. Biochemical studies demonstrate that the host protein thioredoxin increases the processivity of bacteriophage T7 DNA polymerase by stabilizing the complexes of T7 pol and its primed-template (60). The presence of the pol γ accessory subunit also increases the holoenzyme processivity. However, the kinetic assay of polymerase activity of the human pol γ catalytic subunit alone and of the holoenzyme indicates that the release of DNA from the enzyme is minimally affected by the accessory subunit. Instead, the accessory protein may alter the structure of the catalytic subunit and allow it to bind nucleotide and DNA more tightly and to catalyze faster polymerization (61).

Pol γ is highly accurate in nucleotide polymerization, with an *in vitro* error rate of only 1 misincorporation per ~500, 000 bases polymerized (31,62). However, the proofreading of pol γ is not equally active against all mispairs and lower fidelity is observed using different conditions (62).

Replication fidelity depends on both selection and incorporation of the incoming nucleotide and exonucleolytic editing of the mispaired nucleotide. In general, the former mechanism is the rate-limiting step and contributes 100-1000 fold more than the latter to replication fidelity (63). Random mutagenesis of yeast pol γ identifies ten mutator alleles, which exhibit much higher mutation frequencies above the spontaneous level. They map among 3'-5'exonuclease domain, polymerase domain, and conserved amino acids among family A DNA polymerases (64). Some deleterious mutations that result in human autosomal PEO are also identified and they map within the polymerase domain close to the corresponding mutator alleles in yeast (19,65).

2. Mitochondrial Single-Stranded Binding Protein (mtSSB)

SSBs play critical roles in DNA replication, repair, and recombination. They stabilize the single-stranded DNA (ssDNA) and stimulate the activity of DNA polymerase by enhancing both the processivity and the fidelity of nucleotide polymerization.

mtSSBs have been purified from yeast and various animal sources. It has been shown that mtSSB is a homotetramer in solution with each polypeptide protomer of 13-15 kDa. Multiple sequence alignments and the crystal structure data demonstrate a high similarity between mtSSBs and the *E.coli* SSB, which has a high affinity for ssDNA. mtSSB binds to ssDNA, coating the displaced ssDNA that is the template for lagging strand synthesis in mtDNA replication, and preventing it from renaturation (24). DNase I footprinting of pol γ:DNA complexes and initial rate measurements show that mtSSB enhances primer recognition and binding, and stimulates about 30 fold the rate of initiation of DNA strands of *Drosophila* pol γ. At the same time, mtSSB stimulates exonuclease activity to a similar extent. Moreover, mtSSB increases the processivity of *Drosophila* pol γ several fold in DNA strand elongation (66).

A *Drosophila* P element insertion that disrupts the mtSSB gene interferes with the development of the adult visual system, and represents the first mtSSB mutation identified in animals (*low power*, *lopo*). The *lopo* mutant shows the depletion of mtDNA and hence indicates a mitochondrial dysfunction. *lopo* is also defective in cell proliferation and development resulting in the lethality during the larval and pupal stages (67).

3. Other Proteins

Besides pol γ and mtSSB, other proteins are also required for proper replication and maintenance of mtDNA. They include mtDNA helicase, mtDNA topoisomerase, priming enzymes, RNase H-like enzymes, and mtDNA ligase (28).

Human mtDNA helicase, Twinkle, was identified in individuals with autosomal dominant PEO (adPEO) associated with multiple mtDNA deletions (68). Twinkle shares a similar amino acid sequence and protein structure to phage T7 gene 4 primase/ helicase and other hexameric ring helicases and co-localizes with mtDNA in mitochondrion. Purified recombinant Twinkle has 5' - 3' DNA helicase activity and is specifically stimulated by human mtSSB (69).

Instead of requiring a primase, mitochondrial RNA polymerase is thought to provide primers in the initiation of mtDNA replication. Pol γ can catalyze reverse transcription like HIV-1 reverse transcriptase (RT) (56), which uses tRNAs as primers. The accessory subunit of pol γ shares a similar structure to the anticodon-binding domain of aaRSs (58) and is demonstrated to bind to RNA with tRNA-like structure (34). Therefore, it is possible that pol γ uses mitchondrial tRNAs as primers and RNase H-like enzymes may be required to remove primers much as in HIV-1 RT mediated DNA metabolism (70). The majority of RNase H1 is in the nucleus and a fraction is targeted to mitochondria. RNase H1 knockout mice present mtDNA depletion and apoptotic cell death (71). This result links RNase H1 to generation of mtDNA and provides direct support for the strand-coupled mechanism of mtDNA replication (26,27).

Finally, mtDNA ligase (72) and mtDNA topoisomerase (73) have also been identified. *In vitro* and *in vivo* data indicate that they function with pol γ or helicase in mtDNA replication and repair at the replication fork.

HIV AND HIV INHIBITORS

AIDS in humans is caused by two lentiviruses, HIV-1 and HIV-2, which entered the human population by zoonotic transmissions from at least two different African primate species (74). A genetic comparison of HIV-1 and HIV-2 reveals a significant difference in amino acid sequences. For instance, the two viruses share only about 60% of predicted amino acid sequence for the entire *pol* gene. However, despite these genetic differences, HIV-1 and HIV-2 reverse transcriptase (RT) proteins are similar in overall structure and functionality. Nucleoside reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs), which are used most often for HIV-1 treatment, can be effective in dual and triple antiretroviral regimens. Their use can decrease HIV-2 viral load, increase CD(4)(+)T cell counts, and improve AIDS-related symptoms (75).

I. HIV-1 Viral Replication Cycle

Like other retroviruses, HIV encodes three structural genes *gag*, *pol*, and *env*. Besides regulatory proteins, three structural enzymes – protease (PR), reverse

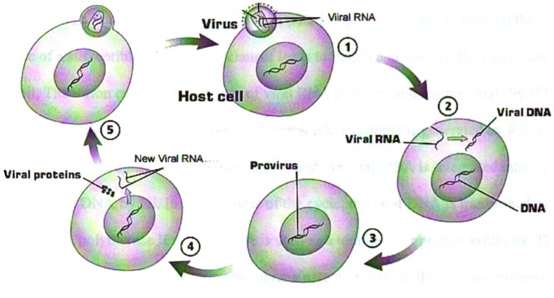


Figure 1.3. **HIV viral replication cycle.** 1) Membranes of the virus and the host cell fuse, and viral RNA and reverse transcriptase enter the host's cytoplasm. 2) Reverse transcriptase allows viral RNA to be copied to DNA. 3) Viral DNA is incorporated into the host chromosome as provirus by integrase. 4) Transcription and translation of viral proteins: viral RNA becomes incorporated into viral particles and is transcribed by cellular RNA polymerase II. 5) Viral particles bud out of the host cell, acquiring an envelope in the process. The polyprotein precursors are specifically cleaved by viral protease to generate mature, infectious viruses

transcriptase (RT), and integrase (IN) – are encoded in HIV and catalyze essential steps in viral infectious cycle. PR is required for proteolytic maturation of the viral particle, RT is required for reverse transcription of the viral RNA into DNA, and IN is required for integration of viral DNA into the host-cell chromosome (Figure 1.3).

HIV-1 virus is captured by interaction with receptor-envelope proteins on the surface of a susceptible cell. The attachment leads to fusion and entry of the virion into the cell. The virion contains two copies of viral RNA genome, and mature viral PR, RT, and IN. A double-stranded DNA copy of the viral RNA genome is generated by RT. The proviral genome is transported into the nucleus and the viral DNA is integrated into cell chromosomal DNA by IN. In a later phase of the cycle, the viral DNA is transcribed by cellular RNA polymerase II and the RNA is used as a template for protein synthesis. The viral proteins are delivered to plasma membrane assembly sites in the form of polyprotein precursors and assembled with progeny RNA. After assembly, the immature, noninfectious viruses are released by budding. Finally, the polyprotein precursors are specifically cleaved by viral PR to generate mature, infectious viruses (70).

II. HIV Replication Enzymes

1. Reverse Transcriptase (RT)

HIV-1 RT possesses three enzymatic activities: an RNA-dependent DNA polymerase activity to produce minus strand DNA from the plus strand viral RNA genome, ribonuclease H activity to remove the RNA template, and a DNA-dependent DNA polymerase activity to synthesize the plus strand DNA using the minus strand DNA as template. It has been assumed that the same polymerase active site is used for both

RNA and DNA dependent synthesis. By these activities, RT produces a double-stranded DNA copy from the plus-strand viral genomic RNA in the early phase of the viral replication cycle.

Kinetic studies with HIV-1 RT indicate an ordered reaction pathway similar to that of other polymerases, with binding of template-primer as the first step followed by binding of dNTP substrates (76). Because of the absence of an editing function in RT, HIV retroviral genomes mutate at a much higher rate than cellular genes. In addition, the viral DNA is transcribed by cellular RNA polymerase II in the late replication cycle for protein synthesis, and RNA polymerase II also lacks editing function. Therefore, a genetically diverse population of viruses is found in HIV patients and the rapid appearance of drug resistant mutants is common in clinical trails.

The HIV-1 RT is an asymmetric heterodimer composed of the 66 kDa subunit (p66) and the 51 kDa subunit (p51). The p51 subunit is generated by protease-mediated cleavage of the RNase H domain of the p66 molecule. The structures of the p51 and p66 subunits are totally different, even thought they share common amino acid sequence. The X-ray crystal structures indicated that the HIV-RT p66 subunit contains both the polymerase and the RNase H domains. There are four subdomains in the polymerase domain: fingers, palm, thumb, and connection subdomains. The first three subdomains construct a general right hand shape common to all polymerases, even though the subdomain arrangement shows limited homology to the polymerase region of the Klenow fragment of *E.coli* DNA polymerase I and to the bacteriophage T7 DNA polymerase. The connection subdomain lies between the RNase H domain and other subdomains, and the major contacts between the p66 and p51 subunits occur within this subdomain. Both the palm and the thumb are involved in template binding (77), and it has been proposed that the p51 subunit may bind tRNA primer as well as the template (78).

Processivity of HIV-1 RT varies depending on the template. Processivity is greater than 300 nt on poly(rA), and is low on natural templates or homopolymers other than poly(rA) as compared with other replicative polymerases (79). The RNase H domain may contribute to the processivity of the polymerase (80).

2. Protease (PR)

HIV-1 retroviral translation products are long polyprotein precursors. The precursors are specifically cleaved to release mature proteins by the viral PR. Because HIV-1 relies on its own PR for maturation, PR is an attractive target for antiviral drugs. HIV PR belongs to the aspartyl protease class, which includes pepsin, cathepsin D, chymosin, and renin. The active form of PR is a dimer and each PR monomer contains a single conserved active site triplet Asp-Thr/ Ser-Gly (70). There is no unique primary sequence of amino acids within a substrate polyprotein as cleavage sites of PR, but the PR target sequences are usually quite hydrophobic (81). Crystallographic analyses indicate that substrate target sequences must be located in regions that are flexible or extended in order to be accessible to the enzyme (70). Small synthetic peptides could function as substrates or inhibitors to HIV PR. The minimal peptide length required for recognition and cleavage is seven amino acids. Some peptides of six amino acids or fewer, which are not cleaved, can act as competitive inhibitors. Many renin inhibitors bind tightly to the active site of HIV PR and peptide-like active-site inhibitors are in clinical for treatment of AIDS.

3. Integrase (IN)

Integration ensures a stable association between viral DNA and the host cell chromosome. This step is site-specific with respect to viral DNA because it occurs at the linear DNA ends; however, it is essentially random with respect to host DNA. The reaction requires IN and the viral *cis*-acting DNA sequences at the ends of the LTRs. The integration reaction is also highly coordinated -- two viral linear DNA ends, separated by 7-10 kb, must be juxtaposed at the host target site in a pairwise manner (70).

The product of reverse transcription of viral RNA is a blunt-ended DNA duplex with terminal sequences 5'-TG...CA-3' for HIV-1. The TG...CA repeat is conserved throughout the retrovirus and retrotransposon families. Next, the linear duplex is nicked by IN on the 3' side of the CA to produce new 3' hydroxyl ends (82). IN monomers or multimers bind to each end of viral DNA and the ends are held together through protein-protein interactions to coordinate processing of both ends. After the subviral complex enters the nucleus, IN binds to host DNA in a sequence independent manner, and a 4-6 bp staggered cleavage of the target host DNA and the ligation of processed CA-OH-3' viral DNA ends to the 5' phosphate ends of the target DNA are stimulated by IN. The joining reaction produces a gapped intermediate and the gaps are repaired (perhaps by host cell enzymes) to produce flanking direct repeats of host DNA. *In vitro* studies show that IN alone can carry out the joining reaction (70).

III. Antiretroviral Drugs and Drug Resistant HIV Viruses

Highly active antiretroviral therapy (HAART), in which two nucleoside reverse transcriptase inhibitors (NRTIs) are often combined with either non-nucleoside reverse transcriptase inhibitors (NNRTIs) or PI, has led to a significant reduction in AIDS-related morbidity and mortality. However, HIV-specific immune responses may decline instead of being reconstituted by HAART. Reservoirs of virus-infected cells cannot be eliminated by HAART, and interruption of therapy usually leads to viral load rebound (83). In addition, significant adverse effects are associated with prolonged exposure to antiretroviral drugs, including lipodystrophy syndrome and lactic acidosis associated with NRTIs mitochondrial toxicity (84), emergence of drug resistant mutations (85), and prohibitive costs. In addition to improving the currently available drugs, researchers are developing both existing classes (NRTIs, NNRTIs, and PIs) and new classes (entry inhibitors and integrase inhibitors) of antiviral medication (86).

The high rate of HIV replication and virus production (10^{10} virions per day) drives the development of genetic variation (87). HIV is diploid and contains two genomic RNA molecules per virion. Cells infected with two different strains of HIV may produce heterozygous virions because of recombination during reverse transcription (74), so sequence diversity may be further obtained. In addition, the viral RT has no proofreading activity and mutations can be introduced into the viral genome easily during viral cDNA synthesis. Therefore, HIV exists as quasispecies or microvariants within each infected individual. The characteristics of HIV make it evolve rapidly, and this permits HIV to escape host immune surveillance, enables the establishment of drug resistant variants, and presents the greatest challenge to the development of an effective HIV vaccine.

IV. Therapeutic Vaccination

Vaccines are one of the most effective clinical interventions for controlling infectious diseases. The most commonly used immunogens are live attenuated or inactivated viruses, in which a nonpathogenic attenuated virus elicits an immune response that cross-reacts with the virulent virus. However, in the case of HIV infection, natural protective immunity does not essentially exist. In addition, HIV mutates rapidly and displays an unusually high level of genetic diversity among different strains. These traits make the development of a universally effective vaccine particularly challenging (88). As opposed to empirical strategies, molecular genetic engineering technologies have made significant progress in HIV vaccine development.

Gene based vectors are used to introduce pathogen-derived genes. For example, replication-defective viral vectors unrelated to the pathogen with the pathogen-derived gene can induce both humoral and cellular immune responses to the antigens *in vivo* by mimicking microbial infection (89). Another novel approach is to use plasmid DNA containing a gene encoding a viral immunogen with a eukaryotic enhancer/promoter region and a polyadenylation signal to confer appropriate expression of the viral immunogen (90). Upon injection, plasmids are introduced into cells at the injection site and transported to the nucleus for transcription, translation, and appropriate post-translational modification. Host cells synthesize large quantities of viral proteins that stimulate immune responses. Proteins expressed from a DNA vaccine are potentially more effective antigens than recombinant protein vaccines produced in bacteria or yeast because they are more likely to assume a native conformation. DNA vaccines could also circumvent some possibility of pathogenic infection like live-attenuated virus vaccines (89,91).

The HIV viral envelope is the part of the virus that is most accessible to the immune system, but it has evolved under the most intense selective pressure to evade immune detection (91). DNA vectors containing altered forms of HIV-1 *env* gene have been designed and evaluated for the immunogen production. These plasmid DNA vectors were injected intramuscularly into mice, and *in vitro* assays were used to assess the antibody and CTL responses. Mutant constructs to disrupt structures that are relevant to viral fusion can simultaneously elicit strong antibody and CTL responses equal to that induced by wild-type Env (92). Similar methods were used to assess humoral and CTL responses to DNA vectors encoding other major HIV proteins, Gag and Pol. The Gag-Pol fusion protein elicited the broadest and most potent CTL and antibody responses against both Gag and Pol as compared to Gag or Pol alone (93).

DermaVir is a novel DNA vaccine developed for immunotherapeutic intervention. The active ingredient of DermaVir is a plasmid DNA encoding the majority of HIV-1 genes, and it closely mimics the expression of wild-type virus. DermaVir is formulated to target epidermal Langerhans cells (LC). Once loaded with DermaVir, LC will migrate to the lymph nodes and mature to dendtritic cells (DC), which are a kind of professional antigen-presenting cells. DC then express the DNA that encodes HIV proteins and presents these DNA products to native T cells, ultimately inducing HIV-specific cytotoxic T cells. These T cells are capable of eliminating HIV-infected cells in the body, even in viral reservoirs, which have proven to be elusive to the effects of HAART (83).

DROSOPHILA AS ANIMAL MODELS IN GENETICS STUDIES

Drosophila is one of the best animal models. Besides a short life cycle and easy and low maintenance, *Drosophila* has only 4 chromosomes and "polytene" chromosomes in the salivary gland. Polytene chromosomes have a reproducible banding pattern that reveals an unparalleled chromosomal cytology, allowing a physical map to be linked with a genetic map (94). In 1910, Morgan and his students started to use *Drosophila* and formulated a revolutionary chromosome theory of heredity (95). Since Morgan, the fly community has grown stronger and stronger. The genetic and physical map, balancers, chromosomal deletions and duplications, and P-element mediated germline transformation are still routinely used. In 2000, the complete euchromatic *Drosophila* genome sequence was released (96). Combined with gene mapping and function study by transgenesis, scientists are able to study the relationships between genotype and phenotype (94).

I. Transposon mutagenesis

P-element mediated germline transformation is an efficient method to transfer exogenous genetic information into *Drosophila*. Transposable P-elements are engineered so that their transposition can be controlled by crossing into a fly line or background harboring an active transposase, and in the next generation crossing out the transposase to stabilize the integrated transposon (97,98). Besides the routine use of P-element vectors to ectopically overexpress a gene of interest, the availability of stable, single-copy, integrative transgenesis enabled a range of powerful techniques to be developed in *Drosophila*. These methods include enhancer traps, large-scale insertional mutagenesis with engineered transposable elements, site-specific recombination for generating chromosomal rearrangements, and two-component systems for controlling ectopic gene expression (95). Because transposition is almost random and P-element insertion often disrupts the neighboring gene, large-scale insertional mutagenesis with engineered transposable elements can be used to disrupt a gene without prediction of phenotype (99). Unlike conventional mutagenesis by chemicals or ionizing radiation, P-element mutations are sequence "tagged", so it is easier to identify the hits during the screen. Enhancer trapping involves use of a reporter coupled with a weak promoter that cannot normally drive gene expression in the absence of a transcriptional enhancer. When this construct is inserted close to endogenous transcriptional enhancers in a genome, the reporter gene expression will be activated. Therefore, it is an efficient way to screen for genes based on the gene expression pattern (100). The two-component GAL4-UAS inducible system allows precise control of gene expression in specific cell types (101). The two components are 2 different P-element transformed lines: one expresses GAL4 under the control of a tissue-specific promoter, the other carries the target gene downstream of GAL4-UAS site. A cross between the two lines leads to overexpression of the UAS-linked gene in a tissue-specific manner in the progeny.

II. Targeted Gene Disruption in Flies

Reverse genetics, which disrupts a gene by its position or DNA sequence instead of by its phenotype, has been used successfully in yeast and mice. However, a universal way for gene replacement and targeted gene disruption in *Drosophila* had been unavailable for a long time (102). Site-selected insertional mutagenesis, the PCR-based screen of the randomly inserted P-elements, is a principal strategy for gene targeting in *Drosophila* (103), but only P-element inserts lying within a few hundred base pairs of the second primer would permit amplification. "Inverse PCR", which amplifies of flanking DNA from all inserts, significantly improves the site-selected insertion approach (104,105), but the method is labor-intensive and is efficient only when several genes are screened simultaneously. Gene replacement is an option for cases in which a P-element lies within about 2 kb of the target (106). Excision of the element makes a DNA double-strand break (DSB) and repair of the DSB results in replacement of flanking sequences with an *in vitro* modified version. However, it is not always possible to find a P-element close enough to the targeted site (102).

A method of creating gene knockout phenotypes is the post-transcriptional RNA interference (RNAi) technique. RNAi is based on a process in which specific dsRNAs can stimulate the degradation of the homologous single strand RNA. A transgenic RNAi strategy is to design transgenic vectors that produce hairpin-loop RNAs corresponding to a gene region. The inherited transgene confers specific interference of gene expression (107). However, many laboratories have had difficulty in routine knockdown with RNAi transgenic strategies.

Rong and Golic developed a new technique to knock out the endogenous wild-type gene as well as insert an exogenous modified copy by homologous recombination. In their approach, most of the work is done by a pair of site-specific DNA-modifying enzymes – site-specific recombinase FLP and I-*Sce*I endonuclease. The selection of transgenic flies is not dependent on the known phenotypes of the gene (108,109). The generality and effectiveness of this technique has been demonstrated by disrupting several genes in different locations (110).

III. *Drosophila* as Animal Models in Chemical Genetics and Pharmacology

Because of their power in genetics, transgenesis and genomics, transgenic *Drosophila* disease models have been established to study human neurological diseases. Although the structure of the *Drosophila* brain does not resemble the human central nervous system, quite a few studies show that the molecular and behavioral patterns of the responses to psychoactive drugs in *Drosophila* are essentially indistinguishable from those in mammals (111). Genes between flies and humans are highly conserved (112), and *Drosophila* has a complex nervous system and displays complex behaviors such as learning and memory, making it a particularly attractive system for study of neuronal dysfunction that proceeds from neurodegenerative diseases (113).

Drugs or reagents can be easily delivered to flies by feeding them with food mixed with drugs or chemicals. Thompson and colleagues constructed transgenic flies as Huntington's disease models. By feeding the flies with drugged food, they observed the reduced level of degeneration and partially rescued young adult from death (114). Mutations in *Drosophila* orthologs of various human genes have also been demonstrated to be responsive to various pharmacological agents (115). Recently, more and more studies demonstrated the versatility of the use of *Drosophila* in genetic and pharmacological aspects of biomedical research and/or preventive medicine. For example, feeding flies with different DNA replication inhibitors is used to check the effects of various DNA-damaging and anti-cancer agents in the developing multicellular organism (116). Feeding flies with drugged food is also used to induce specific behavior changes in *Drosophila* (116,117). It has been reported that feeding *Drosophila* with 4-phenylbutyrate for 12 days (early or late in the life of adult flies) significantly increases their lifespan (118).

In conclusion, *Drosophila* research offers a mammalian relevant system where orthologous proteins can be studied by mutagenesis and other genetic tricks to modify functional pathways to reveal their function. Advances in *Drosophila* research allow the combination of genome sequence information, gene expression profiles, and genome-wide mutations in an unprecedented dissection of a complex organism.

CHAPTER II

ROLE OF THE SPACER REGION IN THE CATALYTIC SUBUNIT OF MITOCHONDRIAL DNA POLYMERASE

PART I

MUTATIONS IN THE SPACER REGION OF *DROSOPHILA* MITOCHONDRIAL DNA POLYMERASE AFFECT DNA BINDING, PROCESSIVITY, AND THE BALANCE BETWEEN POL AND EXO FUNCTION

INTRODUCTION

The mitochondrion is the eukaryotic organelle that carries out oxidative phosphorylation, fulfilling cellular requirements for energy production. Disruption of mitochondrial energy metabolism can occur by genetic or biochemical mechanisms, and is associated with human disorders including degenerative diseases, cancer and aging (119). Mutations in both the mitochondrial genome and in nuclear genes whose products have mitochondrial functions are linked to mitochondrial disease syndromes. Nuclear genes include those encoding the adenine nucleotide translocator 1 (ANT1; (120)), thymidine phosphorylase (121), mitochondrial DNA helicase (Twinkle; (68)) and the catalytic subunit of mitochondrial DNA polymerase (pol γ; (65)). Pol γ has also been shown to be a target of oxidative damage, which reduces DNA polymerase activity in the mitochondrial matrix (122).

Pol γ is the only DNA polymerase known to be required for mitochondrial DNA (mtDNA) replication in animals (28). It is a member of the family A DNA polymerase group (35,36), of which *E.coli* pol I is the prototype (39). The crystal structures of numerous family A DNA polymerases have been solved, revealing a high degree of structural similarity among its members (41,45-50). The interaction between DNA polymerase and template-primer DNA has also been revealed in molecular detail by structural determination of pol: DNA co-crystals, and in biochemical studies. The polymerase (pol) domain comprises palm, fingers and thumb subdomains that are separated from the 3'-5' exonuclease (exo) domain by a cleft that binds the template-primer. Whereas the template strand lies along the cleft between the two domains, the 3'-end of the primer (or nascent DNA strand) shuttles between them during proofreading DNA synthesis (42,44).

Drosophila pol γ is a heterodimeric protein comprising catalytic and accessory subunits (29,55). The catalytic subunit contains the DNA polymerase and 3'-5' exonuclease activities (33), and the accessory subunit contributes to both the structural integrity and catalytic efficiency of the holoenzyme (30,55). Mitochondrial DNA polymerases have a conserved interdomain region, that we have termed the spacer region (28), that is not conserved in *E. coli* pol I or other members of the family A group such as bacteriophage T7 DNA polymerase. The spacer region, which includes four γ-specific conserved sequence elements, is found in pol γs from yeast to man (Figure 2.1). Although the specific function of the conserved spacer sequences is not known, a missense mutation in the spacer region of *Drosophila* pol γ-α causes mitochondrial and nervous system dysfunction, and developmental lethality in the larval third instar (123). Furthermore, hereditary progressive external ophthalmoplegia (PEO) is associated with mutations in human pol γ-α, some of which map to the spacer region (19,65,124).

Here we examine the consequences of site-directed mutagenesis of conserved amino acid sequences in the spacer region of *Drosophila* pol γ-α. Mutant holoenzymes were expressed from baculovirus constructs in insect cells, purified to near-homogeneity and characterized biochemically. Our results show that mutations in conserved amino acid sequences within the spacer region alter enzyme activity, processivity and DNA binding affinity. In addition, several mutations affect differentially DNA polymerase and exonuclease (exo) activity, and/ or functional interactions with mitochondrial single-stranded DNA-binding protein, mtSSB.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids- Unlabeled deoxy- and ribonucleotides were purchased from Amersham Biosciences; for use at concentrations above 30 μM, nucleotide solutions were adjusted to pH 7.5 with Tris base (Research Organics). [^{3}H]dTTP, [α-^{32}P]dATP and [γ-^{32}P]ATP were purchased from ICN Biochemicals. Calf thymus DNA (highly polymerized Type I) was purchased from Sigma and was activated by partial digestion with DNase I (Boehringer Mannheim) as described by Fansler and Loeb (125). Poly(rA).p(dT) was purchased from P-L Biochemicals, and contained adenine and thymine in a molar ratio of 20:1, such that the average single-stranded DNA region between primers was 200 nucleotides.

Recombinant and wild type M13 DNAs (10,650 and 6407 nt, respectively) were prepared by standard laboratory methods for use in DNA polymerase and processivity assays. Synthetic oligodeoxynucleotides were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer. Primers (15 nt) for DNA polymerase and processivity assays were complementary to the M13 DNAs. The sequences of the 21-mer primer and the 45-mer template used in the gel electrophoretic mobility shift assay are 5'- GACCCGATCTGATCCGATTCG- 3' and
3'- AACTGCTGGGCTAGACTAGGCTAAGCTATGCTGCGCTCCAACCTA- 5'.

Baculovirus transfer vector pVL1392/1393 and linearized, modified baculovirus AcMNPV DNA (BaculoGold) were purchased from PharMingen. The pol γ-α and pol γ-β cDNAs were cloned previously into pVL1393/1392 and designated as pVLGA and pVLGB, respectively (30).

Enzymes and Proteins - Restriction enzymes were purchased from Life Technologies and New England BioLabs. *Pfu* DNA polymerase for PCR was purchased from Stratagene. Rabbit antisera raised against recombinant pol γ-α and pol γ-β expressed in bacteria were prepared as described by Wang *et al.* (32). Recombinant *Drosophila* mtSSB was prepared as described by Farr *et al.* (66). T4 polynucleotide kinase was purchased from Boehringer Mannheim.

Bacterial Strains - *E. coli* XL-1 Blue (*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac*, (*F'proAB, lacIqZ M15, Tn10* (tet^r)) was used for bacterial cloning.

Insect Cells and Tissue Culture Medium- Sf9 (*Spodoptera frugiperda*) cells were obtained from Dr. Suzanne Thiem. TC-100 insect cell culture medium was from United States Biological and fetal bovine serum was from Life Technologies, Inc. Insect cell transfection buffers were from Orbigen.

Chemicals - Sodium metabisulfite and leupeptin were purchased from the J. T. Baker Chemical Co. and the Peptide Institute (Minoh-Shi, Japan), respectively. Amphotericin, penicillin-G, streptomycin, and phenylmethylsulfonyl fluoride were purchased from Sigma. Nickel-nitriloacetic acid-agarose (Ni-NTA agarose) was purchased from Qiagen.

Methods

Construction of Recombinant Baculoviruses- Baculovirus transfer vectors carrying various pol γ-α mutants were prepared by QuickChange mutagenesis with *Pfu*

DNA polymerase (Stratagene) according to the manufacturer's recommendations. A typical PCR was carried out in a 50 µl reaction mixture with 50 ng of DNA template (pVLGA or His-pVLGA) and 2 units of *Pfu* DNA polymerase. A specific primer pair was used for each mutant as follows.

Primer pairs for deletions or triple alanine substitutions were:

pol γ-αΔ(413-470) (Δγ1)- 5'-

CCAACTGGGAGCGGTACATA^AAGCCTCTTCCTACAGTGG-3'and 3'-

GGTTGACCCTCGCCATGTAT^TTCGGAGAAGGATGTCACC-5';

pol γ-αΔ(483-533) (Δγ2)- 5'-

GGAGATTAAGGATTCTGGA^CTAGAGGATGACGAAGAGCCG-3' and 3'-

CCTCTAATTCCTAAGACCT^GATCTCCTACTGCTTCTCGGC-5';

pol γ-αΔ(536-581) (Δγ3)- 5'-

GGGAAGATGAAATTCTAGAG^TTTCGCAGCGATTCAGAAGG-3' and 3'-

CCCTTCTACTTTAAGATCTC^AAAGCGTCGCTAAGTCTTCC-5';

pol γ-αΔ(666-742) (Δγ4)- 5'-

ACAAAGTACTGGATGATTGC^AATGAATTCACTGGCGAAAA-3' and 3'-

TGTTTCATGACCTACTAACG^TTACTTAAGTGACCGCTTTT-5';

pol γ-αY419A/E420A/D421A (YED)- 5'-

GCAGCTCACC**G**(t)**C**(a)CG**C**(a)GG**C**(a)CCTCAGCATCGA-3' and 3'-

CGTCGAGTGG**C**(a)**G**(t)GC**G**(t)CC**G**(t)GGAGTCGTAGCT-5';

pol γ-αP556A/K557A/L558A (PKL)- 5'-

GCAGATTGCC**G**(c)CC**G**(a)**C**(a)G**G**(c)**C**(t)GCTTTCCCTC-3' and 3'-

CGTCTAACGG**C**(g)GG**C**(t)**G**(t)C**C**(g)**G**(a)CGAAAGGGAG-5';

pol γ-αG575A/W576A/F578A (GWF)- 5'-AGGGAGCAAG**C**(g)C**G**(t)**C**(g)GGGC**G**(t)**C**(t)CCTGGTTCCA-3' and 3'-TCCCTCGTTC**G**(c)G**C**(a)**G**(c)CCCG**C**(a)**G**(a)GGACCAAGGT-5';

pol γ-αK687A/D688A/F689A (KDF)- 5'-CCCTATCC**G**(a)**C**(a)GG**C**(a)C**G**(t)**C**(t)CCTCAACAAG-3' and 3'-GGGATAGG**C**(t)**G**(t)CC**G**(t)G**C**(a)**G**(a)GGAGTTGTTC-5'; and

pol γ-αS719A/Y720A/W721A (SYW)- 5'-ACGCATGATG**G**(t)CC**G**(t)**C**(a)**G**(c)**G**(t)**C**(g)GCGCAACAATA-3' and 3'-TGCGTACTAC**C**(a)GG**C**(a)**G**(t)**C**(g)**C**(a)**G**(c)CGCGTTGTTAT-5'.

Single alanine substitutions were constructed by PCR as described above with pol γ-αPKL or pol γ-αGWF as DNA template. Primer pairs for single alanine substitutions were:

pol γ-αP556A- 5'-GCAGATTGCC**G**(c)CCAAGCTGCTTTCCCTC-3' and 3'-CGTCTAACGG**C**(g)GGTTCGACGAAAGGGAG-5';

pol γ-αK557A- 5'-GCAGATTGCCCC**G**(a)**C**(a)GCTGCTTTCCCTC-3' and 3'-CGTCTAACGGGG**C**(t)**G**(t)CGACGAAAGGGAG-5';

pol γ-αL558A- 5'-GCAGATTGCCCCAAG**G**(c)**C**(t)GCTTTCCCTC-3' and 3'-CGTCTAACGGGGGTTC**C**(g)**G**(a)CGAAAGGGAG-5';

pol γ-αG575A- 5'-AGGGAGCAAG**C**(g)CTGGGGCTTCCTGGTTCCA-3' and 3'-TCCCTCGTTC**G**(c)GACCCCGAAGGACCAAGGT-5';

pol γ-αW576A- 5'-AGGGAGCAAGGC**G**(t)**C**(g)GGGCTTCCTGGTTCCA-3' and 3'-TCCCTCGTTCCG**C**(a)**G**(c)CCCGAAGGACCAAGGT-5'; and pol γ-αF578A- 5’-AGGGAGCAAGGCTGGGGC**G**(t)**C**(t)CCTGGTT-3’ and 3’-TCCCTCGTTCCGACCCCG**C**(a)**G**(a)GGACCAA-5’.

"^" indicates the position of deleted nucleotide residues, bold letters indicate nucleotides that replace those in the wild-type pol γ-α as indicated in parentheses with lower case letters. Restriction endonuclease cleavage sites that are either introduced or removed are indicated by underscore.

The DNA template was first denatured at 95°C for 45 sec, followed by 20-25 three-step cycles of 95°C for 45 sec, 50°C for 45 sec, and 68°C for 2 min per kbp DNA template. The reaction mixture was then digested with 10 units of *Dpn*I for 2 h at 37°C to eliminate the methylated parental DNA template. A 2 µl aliquot was used for transformation of competent *E. coli* XL-1 Blue cells by electroporation using an *E. coli* Pulser (BioRad). Plasmid DNAs extracted from transformed cells were screened by restriction endonuclease digestion for the introduced/ removed cleavage sites provided in each primer pair. DNA sequence analysis of the various plasmid constructs was performed to confirm their structure and sequence integrity.

Transfer vectors encoding mutant pol γ-α and wild-type pol γ-β were purified, and baculoviruses prepared as described by Wang and Kaguni (30).

Purification of Recombinant Drosophila pol γ from the Cytoplasm of Sf9 Cells- Soluble extracts from 500 ml cultures of Sf9 cells (~ 5 x 10^8) coinfected with mutant pol γ-α and wild-type pol γ-β recombinant baculoviruses were prepared, and recombinant pol γ was purified by phosphocellulose chromatography, ammonium sulfate fractionation, Ni-NTA agarose affinity chromatography, and glycerol gradient sedimentation as described by Farr and Kaguni (126).

DNA Polymerase Assay- DNA polymerase activity was assayed on DNase I-activated calf thymus DNA, poly(rA).p(dT), and singly primed M13 DNA as described by Wernette and Kaguni (29) and Farr *et al.* (66), respectively. Specific modifications are indicated in the figure legends. One unit of standard activity is that amount that catalyzes the incorporation of 1 nmol deoxyribonucleoside triphosphate into acid insoluble material in 60 min at 30°C using DNase I-activated calf thymus DNA as the substrate.

Analysis of Products of Processive DNA Synthesis by Gel Electrophoresis- Reaction mixtures (60 μl) contained 50 mM Tris-HCl pH 8.5, 4 mM $MgCl_2$, 10 mM dithiothreitol, 30 mM KCl, 400 μg/ml bovine serum albumin, 30 μM each of dTTP, dCTP, dGTP and 10 μM [α-^{32}P]dATP (2×10^4 cpm/pmol), 20 μM singly primed M13 DNA, and 20 ng of pol γ. Incubation was for 8 min at 30°C. Products were made 1% in SDS and 10 mM in EDTA, heated for 4 min at 80°C, and precipitated with ethanol in the presence of 0.5 μg tRNA as carrier. The ethanol precipitates were resuspended in 80% formamide and 90 mM Tris borate. Aliquots were denatured for 2 min at 100°C and electrophoresed in a 6% polyacrylamide slab gel (13 × 30 × 0.15 cm) containing 7M urea in 90 mM Tris-borate pH 8.3 and 25 mM EDTA. Approximately equal amounts of radioactivity were loaded in each lane. Gels were washed in distilled water for 20 min, dried under vacuum, and exposed to a Phosphor Screen (Molecular Dynamics). The data were analyzed by scanning the Phosphor Screen using the Storm 820 Scanner (Molecular Dynamics), and the volume of the bands was determined by computer integration analysis using ImageQuant version 5.2 software (Molecular Dynamics); the volume of the bands was normalized to the nucleotide level to correct for the uniform labeling of the DNA products.

Preparation of 5'-[^{32}P]-Labeled DNA Substrates for Product Analysis- The 5'-[^{32}P]-labeled DNA substrate for 3'-5' exonuclease assay was prepared as described by Farr *et al.* (66). The synthetic 21-mer oligonucleotide primer in the 45-mer: 21-mer DNA substrate for DNA binding analysis was 5'-end labeled. The kinase reaction (40 µl) contained 50 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1 mM spermidine, [γ-^{32}P]ATP (0.2 µM, 4500 Ci/mmol), 28 pmol (as nt) of oligonucleotide, and 10 units of T4 polynucleotide kinase. Incubation was for 30 min at 37 °C, followed by fractionation in a Micro Bio-Spin30 chromatography column (BIO RAD) to remove free [γ-^{32}P]ATP. The DNA was precipitated with ethanol and the pellet was resuspended in a buffer (0.1 ml) containing 10 mM Tris-HCl pH 8.0, 0.3 M NaCl, and 0.03 M sodium citrate. The 45-mer template was added in 6-fold molar excess over the 21-mer primer. The DNA mixture was incubated at 85°C for 3 min and slowly cooled to room temperature for 3 h in order to anneal the primer to the template.

3'-5' Exonuclease Assay- Reaction mixtures (50 µl) contained 50 mM Tris-HCl pH 8.5, 4 mM $MgCl_2$, 10 mM dithiothreitol, 30 mM KCl, 400 µg/ml bovine serum albumin, 4 µM 5'-end labeled singly-primed recombinant M13 DNA containing a 3'-terminal mispair, and 5 ng of pol γ. Incubation was for 30 min at 30 °C. Samples were then made 1% in SDS and 10 mM in EDTA, heated for 10 min at 65 °C and precipitated with ethanol in the presence of 1 µg sonicated salmon sperm DNA as carrier. The ethanol precipitates were resuspended in 80% formamide and 90 mM Tris-borate. Aliquots were denatured for 2 min at 100 °C, chilled on ice and electrophoresed in an 18% polyacrylamide slab gel (13 × 30 × 0.075 cm) containing 7 M urea in 90 mM Tris-borate pH 8.3 and 25 mM EDTA. After electrophoresis, the gel was washed in 15%

glycerol for 20 min and exposed to a Phosphor Screen. The data were analyzed by scanning the Phosphor Screen using the Storm 820 Scanner, and the volume of the bands was determined by computer integration analysis using ImageQuant version 5.2 software.

Gel Electrophoretic Mobility Shift Assay- DNA binding affinity was assayed by gel electrophoretic mobility shift assay. Pol γ (10 ng) was incubated with 5'-[^{32}P]-labeled template-primer (0.05 pmol) for 10 min at 30°C in a reaction buffer (20 μl) containing 50 mM Tris·HCl pH 8.5, 4 mM $MgCl_2$, 5 mM dithiothreitol, and 30 mM KCl. Bromophenol blue and glycerol were added to 0.01 and 5%, respectively, and the samples were electrophoresed in a 6% native polyacrylamide gel (13 × 30 × 0.075 cm) in 45 mM Tris-borate pH 8.3 and 1 mM EDTA. After electrophoresis, the gel was dried under vacuum, and exposed to a Phosphor Screen. The data were analyzed using the ImageQuant version 5.2a software as above.

Other Methods- Protein concentrations were determined by the method of Bradford (127) with bovine serum albumin as the standard. Silver staining of SDS-polyacrylamide gels was performed by the method of Wray *et al.* (128). SDS-PAGE, protein transfer and immunoblotting were performed as described by Olson and Kaguni (55).

RESULTS

Amino acid residues important for DNA polymerase activity are distributed within the spacer region of pol γ-α

To examine the role of the four conserved sequence elements, γ1-γ4, in the spacer region of the catalytic subunit of *Drosophila* DNA polymerase γ, we began by producing recombinant proteins carrying amino acid deletions in each of the conserved elements (Figure 2.1). Mutant pol γ-α variants were co-expressed with wild-type pol γ-β in the baculovirus system, and the reconstituted holoenzymes were purified by phosphocellulose chromatography. Proteins were characterized by immunoblot analysis, and DNA polymerase activity was assayed on DNase I-activated calf thymus DNA and on poly(rA)·oligo(dT) (Figure 2.2). Pol γ-αΔ413-470 (Δγ1) and pol γ-αΔ666-742 (Δγ4) were obtained in small amounts due to low solubility, and these mutants had barely detectable DNA polymerase activity. In contrast, pol γ-αΔ483-533 (Δγ2) and pol γ-αΔ536-581 (Δγ3) were expressed efficiently and were purified as soluble holoenzyme complexes associated with wild-type pol γ-β. The DNA polymerase activity of the Δγ3 holoenzyme was reduced greatly as compared to wild type, but that of Δγ2 was >80% of wild-type holoenzyme.

Both biochemical and structural studies of family A DNA polymerases support the possibility that amino acid residues required for enzyme function lie outside the catalytic active site regions (42,129-132). Because structural information on pol γ-α is very limited, a secondary structure prediction algorithm (PredictProtein) was used to identify the positions of potential α-helices and β-strands in the spacer region, and this

Figure 2.1. **Multiple sequence alignment of the spacer regions of the catalytic subunits of mitochondrial DNA polymerases.** Amino acid sequences were aligned with ClustalW 1.8 (*http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html*), and secondary structural elements were predicted with PredictProtein (*http://cubic.bioc.columbia.edu/predictprotein*). Conserved γ-specific sequences are indicated by underscore, and are labeled γ1, γ2, γ3, and γ4. Predicted α helices and β strands are represented by open or filled boxes above the sequence, respectively. The positions of triple or single alanine substitution mutants are indicated with "Δ" below the sequences.

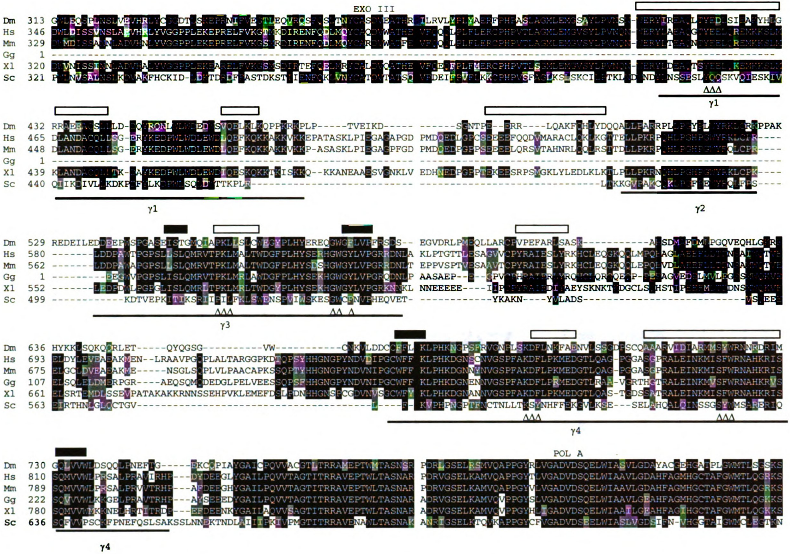


Figure 2.1. **Multiple sequence alignment of the spacer regions of the catalytic subunits of mitochondrial DNA polymerases.**

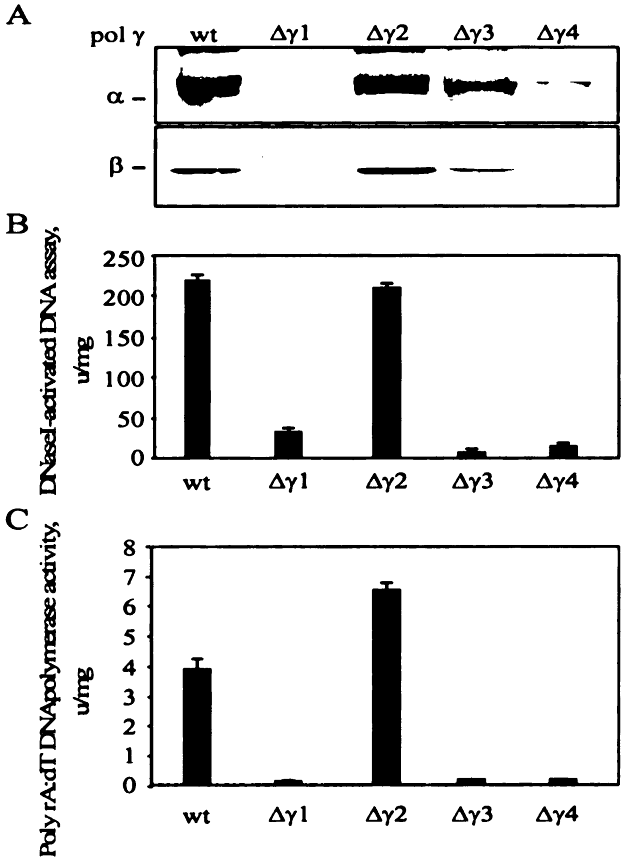


Figure 2.2. **Conserved sequence elements in the spacer region of *Drosophila* pol γ-α are essential for holoenzyme assembly and activity.** Deletion-mutant constructs were generated for each γ-specific element, spanning the following amino acid residues: Δγ1, Δ413-470; Δγ2, Δ483-533; Δγ3, Δ536-583; and Δγ4, Δ666-742. The mutant pol γ-α proteins were produced with pol γ-α in the baculovirus expression system, and the reconstituted pol γ was purified by phosphocellulose chromatography as described in *Methods*. *A*, Proteins (25 μg Fraction IIb) were analyzed by 10% SDS-PAGE followed by immunoblotting with subunit-specific antisera against *Dm* pol γ-α and -β. DNA polymerase activity of wild type and mutant pol γ was measured on DNase I-activated calf thymus DNA (*B*) and on poly (rA)·(dT) (*C*) and is presented in terms of specific activity. One unit of activity is that amount that catalyzes the incorporation of 1 nmol of deoxyribonucleoside monophosphate into acid-insoluble material in 60 min at 30°C.

information was taken into consideration in the generation of triple and nested single alanine mutations, that were introduced as substitutions for highly conserved residues in the γ1, γ3, and γ4 elements (Figure 2.1). Mutant proteins were co-expressed with wild-type pol γ-β in the baculovirus system, and the reconstituted holoenzymes were purified to near homogeneity by phosphocellulose chromatography, followed by Ni-NTA affinity chromatography and velocity gradient sedimentation. Proteins were characterized by SDS-PAGE, DNA polymerase activity was assayed on DNase I-activated calf thymus DNA, and specific activities were determined (Figure 2.3). The P556A/K557A/L558A (PKL) and G575A/W576A/F578A (GWF) mutations in γ3 and the S719A/Y720A/W721A (SYW) mutation in γ4 reduced DNA polymerase activity as much as 100 fold. In contrast, the Y419A/E420A/D421A (YED) mutation in γ1 and the K687A/D688A/F689A (KDF) mutation in γ4 resulted in DNA polymerase activities that were 60-80% of wild-type pol γ. Single alanine substitutions within the PKL and GWF triplets showed that the W576A mutant was nearly inactive, whereas the L558A and F578A mutants retained ~50% of wild-type DNA polymerase activity, and the P556A, K557A and G575A mutants had nearly normal activity. In composite, our deletion and amino acid substitution mutagenesis data indicate that amino acids important for DNA polymerase activity are distributed over a large portion of the pol γ-α spacer region.

Spacer-region mutants with reduced processivity

Standard gap-filling assays revealed that mutations in the pol γ-α spacer region have differential effects on DNA polymerase specific activity. To examine the products of DNA synthesis by the mutant proteins in more detail, we assessed the relative

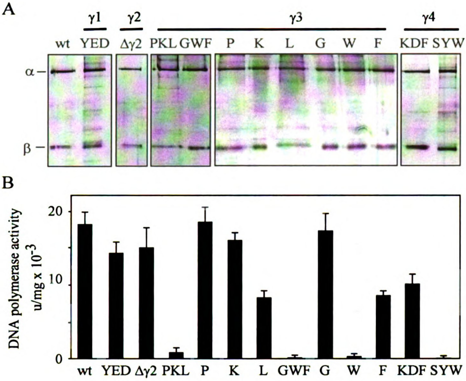


Figure 2.3. **Baculovirus reconstitution of spacer-region mutants of *Drosophila* pol γ.** Conserved amino acid residues were selected for alanine substitution mutagenesis of pol γ-α based on the multiple sequence alignment and secondary structure prediction shown in Figure 2.1. The mutant pol γ-α proteins were produced with pol γ-α in the baculovirus expression system, and the reconstituted pol γ was purified to near-homogeneity by phosphocellulose chromatography and Ni-NTA affinity chromatography, followed by glycerol gradient sedimentation as described in *Methods*. *A*, Proteins (100 ng) were analyzed by 10% SDS-PAGE followed by silver staining. Arrows indicate the positions of the catalytic (α) and accessory (β) subunits of reconstituted pol γ. *B*, DNA polymerase activity of wild type and mutant pol γ was measured on DNase I-activated calf thymus DNA and is presented in terms of specific activity. The pol γ-α mutations are designated as YED, Y419A/ E420A/ D421A; Δγ2, Δ483-533; PKL, P556A/ K557A/ L558A; GWF, G575A/ W576A/ F578A; P, P556A; K, K557A; L, L558A; G, G575A; W, W576A; F, F578A; KDF, K687A/ D688A/ F689A; and SYW, S719A/ Y720A/ W721A.

Table 2.1. Summary of the Enzymatic Assays

	DNA polymerase			SSB stimulation	Processivity	Exonuclease	Pol/Exo	DNA binding affinity
	CT-DNA (u/mg)*	M13-DNA (u/mg)**				M13 DNA (u/mg)***	relative ratio M13 DNA	EMSA
		-SSB ($\times 10^{-17}$)	+SSB ($\times 10^{-17}$)	(fold)	(nt)	-SSB ($\times 10^{-14}$)	-SSB	(% of bound)
Wild type	$(1.8\pm0.2)\times10^4$	2.3±0.2	69±11	31	165	2.8±0.5	1	0.42±0.05
Δγ1 (413-470)								
YED	$(1.4\pm0.2)\times10^4$	1.8±0.1	41±11	23	91	3.8±0.4	0.6	0.36±0.11
Δγ2 (483-533)								
	$(1.5\pm0.3)\times10^4$	1.5±0.6	60±5	41	126	2.0±0.5	0.9	0.46±0.03
Δγ3 (536-581)								
PKL	$(9.4\pm3.9)\times10^2$	<1.0	<1.0	ND	ND	2.7±1.0	<0.5	ND
P	$(1.9\pm0.2)\times10^4$	2.6±0.4	54±8	21	143	2.6±0.9	1.2	0.38±0.06
K	$(1.6\pm0.1)\times10^4$	2.7±0.3	44±6	16	131	3.9±0.8	0.8	0.36±0.06
L	$(8.2\pm0.1)\times10^3$	1.3±0.0	8±1	6	99	2.1±0.4	0.8	0.06±0.01
GWF	$(2.3\pm0.3)\times10^2$	<1.0	<1.0	ND	ND	2.0±0.4	<0.6	ND
G	$(1.7\pm0.4)\times10^4$	2.5±0.5	39±11	15	135	3.6±0.6	0.8	0.49±0.06
W	$(4.1\pm2.4)\times10^2$	<1.0	<1.0	ND	ND	3.1±0.3	<0.4	ND
F	$(8.6\pm0.5)\times10^3$	3.1±0.5	21±5	7	102	2.9±0.6	1.3	0.11±0.07
Δγ4 (666-742)								
KDF	$(1.0\pm0.1)\times10^4$	2.3±0.3	59±6	26	103	2.8±0.7	1.0	0.30±0.15
SYW	$(1.4\pm1.7)\times10^2$	<1.0	<1.0	ND	ND	8.4±1.5	<0.1	0.09±0.02

ND – Not Detectable

* 1u is the amount that catalyzed the incorporation of 1 nmol of dNMP into acid insoluble material in 60 min at 30°C

** 1u is the amount that catalyzed the incorporation of 1 nucleotide into acid insoluble material in 60 min at 30°C

*** 1u is the amount that catalyzed the hydrolysis of 1 nucleotide from the primer DNA in 30 min at 30°C processivity of the mutant polymerases. The processivity assay is carried out with excess oligonucleotide-primed M13 DNA under conditions that limit the initiation-dissociation cycle to one per holoenzyme molecule, and DNA product strand length is measured by denaturing gel electrophoresis. Mutants with very weak DNA polymerase activity on gapped DNA could not be evaluated in this assay. Among the mutants with measurable activity, pol γ-α L558A, F578A, YED and KDF exhibited ~60% of wild-type processivity, whereas that of mutants P556A, K557A, G575A and Δγ2 was nearly equal to that of wild-type holoenzyme (Figure 2.4).

Spacer-region mutants with altered pol/exo activity ratios

DNA polymerase and 3'-5' exonuclease activities were measured on oligonucleotide-primed M13 DNA in the presence or absence of a 3'-terminal mispair, respectively. The ratio of polymerase to exonuclease activity was compared for wild-type and mutant pol γ holoenzymes (Figure 2.5). Most of the mutant pol γs exhibited a lower pol/exo ratio than wild-type holoenzyme, suggesting that mutations in the spacer region have a larger negative impact on DNA polymerase activity than on 3'-5' exonuclease activity. For example, the YED, PKL, L558A, GWF, W576A, F578A and KDF mutants demonstrated strong or moderate reduction of DNA polymerase activity and/ or processivity, but they retained a nearly wild-type level of 3'-5' exonuclease activity. The Δγ2, P556A, K557A and G575A mutants demonstrated little or no reduction in either DNA polymerase or 3'-5' exonuclease activity. However, the SYW mutant within γ4 retains barely-detectable DNA polymerase activity (<1%), yet exhibits a 3-fold higher level of 3'-5' exonuclease activity than wild-type holoenzyme.

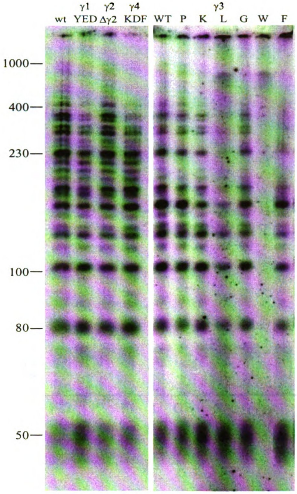


Figure 2.4. **Processivity of spacer-region mutants of *Drosophila* pol γ** DNA synthesis by mutant pol γ was measured at 30 mM KCl on singly primed M13 DNA as described in *Methods*. DNA product strands were isolated, denatured and electrophoresed in a 6% denaturing polyacrylamide gel, and the gel was exposed to a Phosphor Screen. Data derived from two independent experiments were quantitated as described in *Methods*, and yielded the following processivity values (expressed as average processivity units, apu): wt, 165 nt; YED, 91 nt; Δγ2, 126 nt; KDF, 103 nt; P, 143 nt; K, 131 nt; L, 99 nt; G, 135 nt; W, not determined: F, 102 nt.

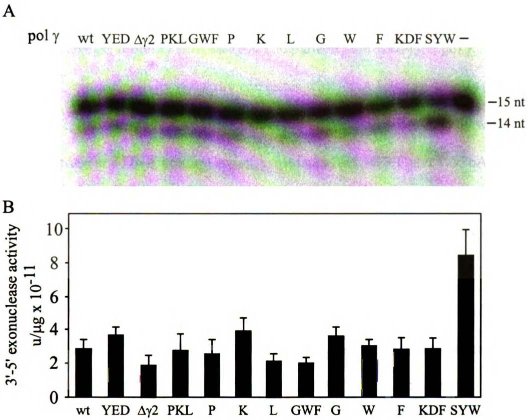


Figure 2.5. **3'-5' exonuclease activity of spacer-region mutants of *Drosophila* pol γ.** *A*, 3'-5' exonuclease activity of mutant pol γ was measured at 30mM KCl using oligonucleotide-primed M13 DNA containing a 3'-terminal mispair. DNA product strands were isolated, denatured and electrophoresed in an 18% denaturing polyacrylamide gel, and the gel was exposed to a Phosphor Screen. The positions of the 5'-end-labeled mispaired primer (15 nt) and the hydrolyzed product (14 nt) are indicated. *B*, Data derived from six independent experiments were quantitated as described in *Methods*. One unit of activity is that amount that catalyzes the hydrolysis of 1 nucleotide from the mispaired primer in 30 min at 30°C.

Spacer-region mutants with reduced DNA binding affinity

The effect of spacer-region mutations on template-primer DNA binding was examined by gel mobility shift assay in comparison with wild-type pol γ. We find that DNA polymerase activity generally correlates with DNA binding activity (Figure 2.6). The Δγ2, P556A, K557A and G575A mutant holoenzymes have nearly wild type DNA polymerase and DNA binding activity. Mutants PKL, GWF and W576A with very low DNA polymerase activity showed no detectable DNA binding activity. However, among the mutants with substantial DNA polymerase activity, L558A and F578A showed significantly lower relative DNA binding activity, whereas YED and KDF showed little apparent reduction in DNA binding as compared to wild-type pol γ. Interestingly, although the SYW mutant shows a 3-fold higher 3'-5' exonuclease activity than wild-type holoenzyme, it exhibits a relatively low DNA binding activity.

Defects in functional interactions between spacer-region mutants and mtSSB

Mitochondrial single-stranded DNA-binding protein stimulates initiation and elongation of DNA strands by wild-type pol γ, and increases pol γ processivity (66,133). Functional interaction between spacer-region mutants and mtSSB were evaluated by assaying DNA polymerase activity in the presence or absence of mtSSB using oligonucleotide-primed M13 DNA as the substrate. The mutants PKL, GWF, W576A and SYW that show strong defects in DNA polymerase function were not stimulated by mtSSB to a detectable level (Figure 2.7). Mutants L558A and F578A with moderate defects in DNA polymerase function were stimulated moderately by mtSSB (4-5 fold lower stimulation than wild-type holoenzyme). The remaining mutants with nearly wild-

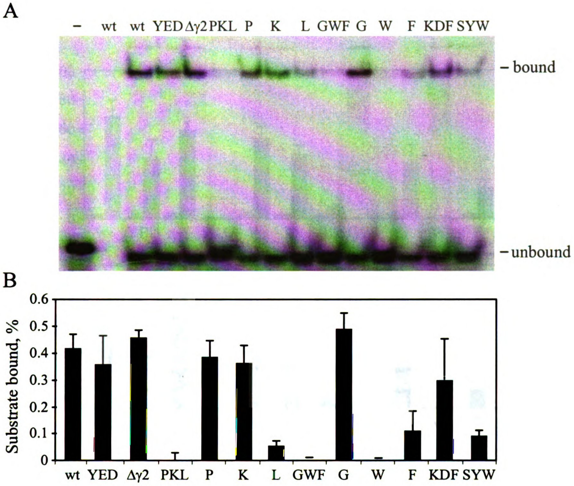


Figure 2.6. **DNA binding affinity of spacer-region mutants of *Drosophila* pol γ.** DNA binding affinity of mutant pol γ was determined by electrophoretic gel mobility shift assay (EMSA) using a radiolabeled oligonucleotide template-primer as described in *Methods*. *A*, Protein-DNA complexes were electrophoresed in a 6% native polyacrylamide gel, and the gel was exposed to a Phosphor Screen. The first two lanes represent DNA substrate only and pol γ only controls, respectively, and the adjacent lanes contain both the DNA substrate and the indicated forms of pol γ. *B*, Bound and unbound DNA substrate bands were quantitated as described in *Methods*. The data were derived from three independent experiments.

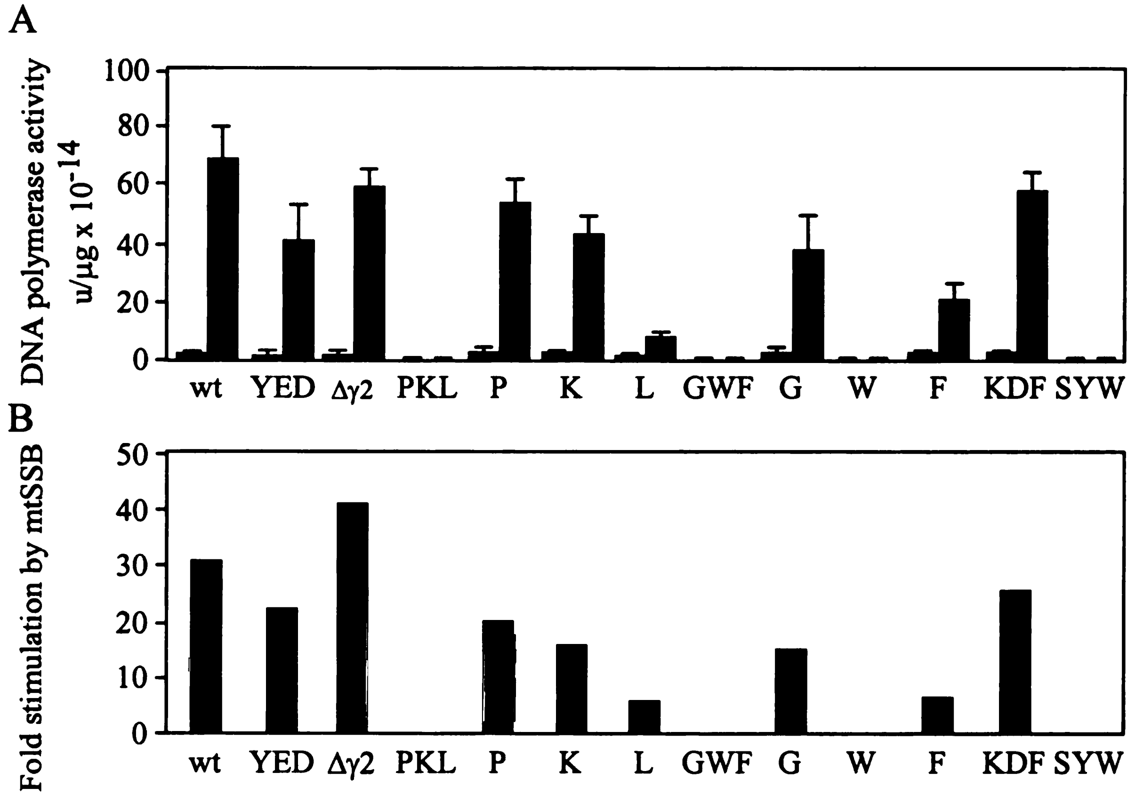


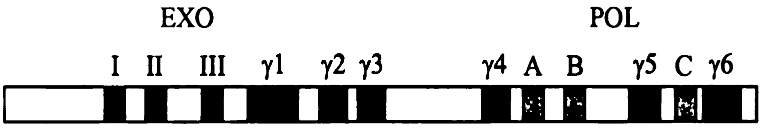
Figure 2.7. **Stimulation of spacer-region mutants of *Drosophila* pol γ by mtSSB.** *A*, DNA synthesis by wild type and mutant pol γ-α was measured at 30 mM KCl on singly primed M13 DNA in the presence (*black bars*) or absence (*gray bars*) of mtSSB (0.8 μg) as described in *Methods*. *B*, Fold stimulation of pol γ-α by mtSSB based on the data from *A*. One unit of activity is that amount that catalyzes the incorporation of 1 nucleotide into acid insoluble material in 60 min at 30°C.

type DNA polymerase activity, including YED and KDF with reduced processivity, were stimulated by mtSSB to approximately the same extent as wild-type pol γ.

DISCUSSION

In this study we examined *Drosophila* pol γ-α variants with deletion and base substitution mutations in each of the four conserved sequence elements in the interdomain spacer region. The mutants displayed widely varying biochemical characteristics (Figure 2.8), with functional defects that affect predominantly the DNA polymerase activity of the holoenzyme, suggesting that the spacer comprises a functionally significant part of the polymerase catalytic domain. Furthermore, our results suggest that the conserved γ3 sequence element spanning amino acids 536-581 contributes substantially to pol function and may play a role in stable DNA binding during DNA synthesis. In contrast, no functional requirement for the conserved γ2 sequence element was identified, whereas the γ1 and γ4 elements were found to contribute differentially to pol versus exo function.

In initial efforts to express and purify pol γ-α variants, it was evident that deletion mutants Δγ1 and Δγ4 had limited solubility when overexpressed in insect cells. In contrast, the remaining pol γ-α mutants in each of the four conserved elements were expressed efficiently and were reconstituted readily into pol γ holoenzyme complexes, including the 51 amino acid deletion mutant Δγ2. Thus it appears that the γ1 and γ4 elements may either contribute some of the multiple sequence contacts that we have shown previously are involved in subunit interactions in the *Drosophila* pol γ holoenzyme (34), or they contain structural motifs that are important for the folding and/ or the conformational stability of the catalytic subunit. Notably, the YED and KDF triple-alanine substitutions in the γ1 and γ4 elements, respectively, show reduced



Weak interaction with pol γ–β	Δγ1			Δγ4
Low DNA polymerase	Δγ1	PKL	Δγ3 GWF W	Δγ4 SYW
Reduced processivity	YED	L	F	KDF
Low DNA binding		PKL L	GWF W,F	SYW
Reduced stimulation by mtSSB		L	F	
Increased 3'-5' exonuclease				SYW

Figure 2.8. **Summary of the biochemical properties of spacer-region mutants of *Drosophila* pol γ.** The upper schematic diagram represents the catalytic subunit of *Drosophila* pol γ. The three exonuclease active site motifs are indicated in dark gray, the three polymerase active site motifs in light gray, and the four γ-specific sequences are shown in black. The biochemical properties of individual mutants described in the text are shown below.

processivity while they retain normal pol and exo function, and exhibit normal levels of stimulation by mtSSB. This may suggest altered interactions between the catalytic and accessory subunits, such that the enhanced processivity contributed by the accessory subunit is diminished.

pol γ-α mutants L558A and F578A within the conserved γ3 sequence element also show reduced processivity on a single-stranded DNA substrate. We would suggest that this derives from a different mechanism, because both these and the parent deletion mutant Δγ3 retain strong subunit interactions that are comparable to wild-type pol γ. One possibility is that the reduced processivity of the L558A and F578A mutants is related to defective interaction with mtSSB, a factor that increases strongly both the activity and processivity of wild-type DNA pol γ (25,133). Notably, the stimulation of DNA polymerase activity on M13 DNA by mtSSB is reduced 4-5 fold in the L558A and F578A mutant holoenzymes as compared to wild type (6-7 versus 30 fold stimulation).

In general, mutations in the pol γ spacer region that lowered significantly DNA polymerase activity also lowered DNA binding activity. However, the loss of DNA binding activity occurred without significant loss of exonuclease activity. Biochemical studies suggest that 5-8 base pairs of duplex DNA are covered by *E. coli* Klenow fragment when the primer terminus is in the pol active site (129). In the co-crystal structure of an editing complex, the 3'-terminus is bound in the exo active site, whereas the duplex portion of the template-primer occupies the cleft between exo and pol domains similar to binding in the pol mode (43,44). Importantly, the extent of single-stranded DNA binding in the 3'-5' exo active site dictates that three or four base pairs of the duplex DNA must be melted before binding occurs (134,135). A recent study demonstrates that a duplex DNA substrate binds much more tightly to the pol versus exo active site, and the presence of mismatched base pairs increases the binding of DNA to the exonuclease site (136). Though we show that our polymerase-defective mutants PKL, GWF, and G576A bind the duplex oligonucleotide substrate poorly, binding to the mismatched DNA substrate is clearly sufficient for these holoenzymes to hydrolyze efficiently a 3'-terminal mispaired nucleotide. The earlier study showed that the tip of the thumb subdomain in Klenow is important for template-primer DNA binding and enzyme processivity (129); side chain changes in the thumb also affect the fractional occupancies of template-primer into the two active sites (137). Likewise, amino acid substitutions in the spacer region of the catalytic subunit of pol γ may increase the partitioning of the 3'-terminus to the 3'-5' exonuclease active site. In particular, the SYW mutant lies within the γ4 conserved sequence element that maps immediately adjacent to the pol A active site motif, a position comparable to the thumb subdomain of Klenow. The SYW holoenzyme retains only ~1% of DNA synthetic activity and 30% residual DNA binding activity, yet its exonuclease activity is reproducibly 3-fold higher than that of wild-type pol $\tilde{\gamma}$. Given the relevance of mitochondrial mutagenesis to aging (28,138), it will be of substantial interest to evaluate the fidelity of single-substitution variants within the γ4 element that retain higher DNA polymerase activity, as potential anti-mutator enzymes.

PART II

(The work described in this part was carried out in collaboration with Carol L. Farr.)

POLGA SPACER MUTATIONS IN DOMINANT ATAXIA: GENETIC AND BIOCHEMICAL CHARACTERIZATION

INTRODUCTION

Mitochondrial DNA is replicated by a minimal group of enzymes and proteins in an asymmetric fashion (139). With 1% of the total cellular DNA polymerase activity, pol γ is the only DNA polymerase in mitochondria. Human pol γ is a heterodimer with a 140 kDa catalytic subunit (α) and a 55 kDa accessory subunit (β). Pol γ-α carries both polymerase and 3'-5' exonuclease activities, whereas the β subunit stimulates these activities and increases the DNA binding affinity and processivity of the holoenzyme (140). Recently, pol γ was detected as a target of oxidative damage in the mitochondrial matrix, with a resulting significant decline in DNA polymerase activity (122).

Progressive external ophthalmoplegia (PEO) is a Mendelian disorder characterized by the accumulation of multiple deletions in human mitochondrial DNA (mtDNA) (141). The disease has been linked to several nuclear genes controlling replication and maintenance of mtDNA. These genes are: adenine nucleotide translocator 1 (ANT1) at locus 4q34-35 encoding the heart/muscle isoform of the ATP/ADP translocator (120); thymidine phosphorylase at locus 22q13.32 (121); a putative mitochondrial helicase (Twinkle) that is a protein homologous with the primase/helicase encoded by T7 gene 4 at locus 10q24 (68); and the catalytic subunit of mitochondrial DNA polymerase (POLGA) at locus 15q25 (65).

Both autosomal dominant/recessive (ad/ar) and sporadic PEO have been found to be associated with POLGA mutations and the PEO patients typically suffer ptosis and skeletal muscle weakness (65,124,142). These mutations map not only to the polymerase and 3'-5' exonuclease domains, but also to the spacer region in human pol γ-α. Four γ-specific conserved sequence blocks in species varying from yeast to man are located within the spacer region of pol γ-α. Biochemical analysis showed that essential amino acids in the conserved spacer region are apparently critical to maintain enzyme function by several mechanisms. Enzyme activity, processivity, DNA binding affinity, and the balance of the polymerase and exonuclease activities are affected differentially among the various spacer region mutants (Luo and Kaguni, unpublished results).

In order to study the mechanisms of the multiple deletions or mutations in mtDNA of PEO patients with POLGA mutations, we constructed several human spacer region mutants (A467T, R627W, R627Q, Q1236H, and R627Q/Q1236H), which are associated with PEO, by site-directed mutagenesis. A467T maps in the $\gamma 1$ conserved element and has been reported to be a recessive mutation that is present in ~0.6% of Belgians (65,142) and it also shows a dominant phenotype (Dr. Wartiovaara, unpublished results). R627W maps in $\gamma 3$ conserved element and has been reported as a recessive mutation, while R627Q shows a dominant phenotype (Dr. Wartiovaara, unpublished results). R627Q is also found in combination with Q1236H, which maps in the C-terminal end of pol γ-α (Dr. Wartiovaara, unpublished results). Mutant pol γ-α constructs were overexpressed in the baculovirus system and purified to near homogeneity. *In vitro* enzymatic assays indicate that the A467T mutation results in significantly reduced DNA polymerase activity, processivity, and DNA binding affinity, whereas the rest of the mutations displayed normal, if not higher, activities.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids- Unlabeled deoxy- and ribonucleotides were purchased from Pharmacia BioTech; for use at concentrations above 30 μM, ATP, GTP, TTP, and CTP solutions were adjusted to pH 7.5 with Tris base (Research Organics). $[^3H]$dTTP, $[\alpha\text{-}^{32}P]$dATP and $[\gamma\text{-}^{32}P]$ATP were purchased from ICN Biochemicals.

Recombinant and wild type viral M13 DNAs (10,650 and 6407 nt, respectively) were prepared by standard laboratory methods for use in DNA polymerase and processivity assays. Synthetic oligodeoxynucleotides were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer. Primers for DNA polymerase and processivity assays were 15 nt complementary to the M13 DNAs. The sequence of the 45 mer template and the 21 mer primer used in the gel electrophoretic mobility shift assay are 5'-ATCCAACCTCGCGTCGTATCGAATCGGATCAGATCGGGTCGTCAA- 3' and 5'-GACCCGATCTGATCCGATTCG- 3'.

Baculovirus transfer vector PVL1393 harboring human α_{His} exo(-) and bacterial vector pQE9 harboring human β_{His} were the gifts of Dr. William Copeland (NIEHS). Linearized baculovirus AcMNPV DNA (BaculoGold) was purchased from PharMingen. Synthetic oligodeoxynucleotides used for spacer region site-directed amino-acid mutations were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer.

Enzymes and Proteins - Restriction enzymes were purchased from Life Technologies and New England BioLabs. *Pfu* DNA polymerases for PCR were purchased from Stratagene. Rabbit antisera raised against recombinant pol γ-α and pol γ-β expressed in bacteria were prepared as described by Wang et al (32). Bovine pancreatic DNase I (Type IV) and T4 polynucleotide kinase were purchased from Sigma and Boehringer Mannheim, respectively. Anti-6xHis monoclonal antibody was from Clontech.

Bacterial Strains - E. coli XL-1 Blue (*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac*, (*F'proAB, lacIqZ M15, Tn10* (tetr)) was used for bacterial cloning. *E. coli* BL21 (λDE3) (Novagen) was used for the expression of human recombinant pol γ-β.

Insect cells and Tissue culture medium- Sf9 (Spodoptera frugiperda) cells were the gift of Dr. Suzanne Thiem. TC-100 insect cell culture medium was from United States Biological and Fetal bovine serum was from Life Technologies, Inc. Insect cell transfection buffers were from Orbigen.

Chemicals- Amphotericin, penicillin-G, streptomycin, sodium metabisulfite and phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Leupeptin was purchased from the Peptide Institute (Minoh-Shi, Japan).

Methods

Construction of Recombinant Baculoviruses viruses- Baculovirus transfer vectors carrying various pol γ-αexo(-) mutants were prepared by QuickChange mutagenesis with *Pfu* DNA polymerase (Stratagene) according to the manufacturer's recommendations. A typical PCR was carried out in a 50 μl reaction mixture with 50 ng of DNA template (His-pVLGAexo(-)) and 2 units of *Pfu* DNA polymerase. A specific primer pair was used for each mutant as follows.

Pol γ-αexo(-)-A467T: 5’- AGTCGTTGATGGA**G**(t)CT**C**(g)A**C**(g)CAATGATGCCTGCCA -3’ and 5’- TGGCAGGCATCATTG**G**(c)T**G**(c)AG**C**(a)TCCATCAACGACT -3’;

Pol γ-α exo(-)-R627W: 5’- CTACTTGGT**A**(g)CCTGGGCGG**T**(c)GGGACAACCT -3’ and 5’- AGGTTGTCCC**A**(g)CCGCCCAGG**T**(c)ACCAAGTAG -3’;

Pol γ-α exo(-)-R627Q 5’- CTACTTGGT**A**(g)CCTGGGCGGC**A**(g)GGACAACCT -3’ and 5’- AGGTTGTCC**T**(c)GCCGCCCAGG**T**(c)ACCAAGTAG -3’;

Pol γ-α exo(-)-Q1236H: 5’- AACGAAGCCA**T**(g)CC**C**(t)GG**G**(a)CCATAGCACT 3’ and 5’- AGTGCTATGG**C**(t)CC**G**(a)GG**A**(c)TGGCTTCGTT -3’.

Bold letters indicate nucleotides that replace those in the wild-type pol γ-α as indicated in parentheses with lower case letters. The introduced/removed endonuclease cleavage sites are indicated by underscore.

The DNA template was first denatured at 95 °C for 45 sec, followed by 20-25 three-step cycles of 95 °C for 45 sec, 50 °C for 1 min, and 68 °C for 2 min per kbp DNA template. The reaction mixture was then digested with 10 units of *Dpn*I for 2 h at 37 °C to eliminate the methylated parental DNA template. A 2 μl aliquot was used for transformation of competent *E. coli* XL-1 Blue cells by electroporation using an *E. coli* Pulser (BioRad). The transformed colonies were screened with proper endonuclease to the introduced/removed cleavage sites in each primer pair. DNA sequence analysis of the various plasmid constructs was performed to confirm their structure and sequence integrity.

Transfer vectors encoding the pol γ-α(exo-)mutants were purified and baculoviruses prepared as described by Wang and Kaguni (30).

Cell Culture and Production of Recombinant α Subunit- Sf9 cells were grown in TC-100 insect cell culture medium containing 10% fetal bovine serum at 27°C and infected with recombinant virus at a multiplicity of infection (MOI) of 5. For protein analysis of whole cells lysates cells were collected by centrifugation at (3000 x g) at a density of 3 x 10^7 cells/ml and lysed in Laemmli gel loading buffer and stored at (-)20°C.

Purification of Recombinant Human β Subunit - All operations were performed at 4°C. Recombinant plasmid-containing BL21(λDE3) cells (2 liter) were grown at 37°C with aeration in Luria broth containing 100 μg/ml ampicillin. When the optical density at 600 nm reached 0.6, isopropylthio-β-D-galactoside was added to 0.3 mM, and the culture was incubated for an additional 2 h. Cells were harvested by centrifugation at 6000 x *g* for 5 minutes, and cell pellets were washed with 20 ml Tris-Sucrose and collected by centrifugation at 12000 x *g* for 5 minutes. Cell pellets were either stored at -80 °C or were processed for Ni-NTA agarose affinity purification. Cell pellets were suspended in 50 ml TNIX (35 mM Tris-HCl pH 7.5, 150 mM NaCl, 25 mM imidazole, 0.1%tritone X-100, 10 mM sodium bisulfite, 1 mM PMSF, and 2 μg/ml leupeptin). Cells were lysed by sonicate suspension with the tip at 50% strength (20 pulses) for three times. The lysate was collected by centrifugation at 12,000 x *g* for 15 min at 4 °C, and loaded onto a Ni-NTA agarose column at a flow rate of 2 column volumes (cV)/hr. The column was washed and eluted with 3 cV buffer containing 35 mM Tris-HCl pH 7.5, 500 mM NaCl, 80 mM imidazole, 10 mM sodium bisulfite, 1 mM PMSF, and 2 μg/ml leupeptin, and successively eluted with the same buffer containing 140 mM (3 cV) , 180 mM (3 cV) and 250 mM imidazole (1 cV). Fractions were pooled according to immunoblot results, and loaded onto 12-30 % glycerol gradients as described by Wernette and Kaguni (29). Final fractions were pooled according to silver-staining results, and stabilized by the addition of glycerol to 45% and stored at –20°C or frozen in liquid nitrogen and stored at –80°C.

Purification of Recombinant Human α from the cytoplasm of Sf9 cells- All operations were performed at 0-4°C. *Sf9* cells (600 ml) were grown as above to a cell density of 2 x 10^6 cells/ml, diluted to a density of 1 x 10^6 cells/ml (1200 ml total), and infected with recombinant baculovirus at MOI 5 and harvested 48h post infection. The cells were pelleted and washed with an equal volume of cold phosphate buffered saline (PBS) buffer. The cell pellet (~1.2 x 10^9 cells) was resuspended in 36 ml of homogenization buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM EDTA, 7 mM mercaptoethanol, 10 mM sodium bisulfite, 1 mM PMSF and 2 μg/ml leupeptin) and lysed by 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1,000 x g for 7 min. The resulting pellet was twice resuspended in 12 ml of homogenization buffer and rehomogenized and centrifuged as above. The combined supernatant fractions were centrifuged at 8,000 x *g* for 15 min to pellet mitochondria, and the resulting supernatant was centrifuged at 100,000-x g for 30 min to obtain the cytoplasmic soluble fraction (Fraction I).

Purification of recombinant pol γ-α exo(-) mutants was performed as described by Wernette and Kaguni (29) with the following alterations. Fraction I (140-240 mg protein) was adjusted to 80 mM potassium phosphate and loaded onto a phosphocellulose column equilibrated with 80 mM potassium phosphate buffer (80 mM potassium phosphate, pH 7.6, 20% glycerol, 7 mM 2-mercaptoethanol, 1 mM PMSF, 10 mM sodium metabisulfite and 2 μg/ml leupeptin) at a packing ratio of 6 mg protein per packed ml of resin and at a flow rate of 12 ml/h. The column was washed and fraction were analyzed and processed through to Fraction IIb as described in Wang and Kaguni (30). Fraction IIb (10-20 mg protein) was dialyzed when necessary in 10 mM potassium phosphate buffer in a collodion bag (molecular mass cut-of 10,000 kDa) until an ionic strength of ~300-400 mM KCl was reached and loaded onto Ni-NTA resin at a ratio of ~7.5 mg protein per ml of packed resin at a flow rate of 1-2 cV per hour. The resin was washed with 2 cV of buffer containing 20 mM Tris-HCl, pH 7.5, 500 mM KCl, 8% glycerol, 5 mM imidazole, 0.1% Tx-100, 7 mM 2-mercaptoethanol, 1 mM PMSF, 10 mM sodium metabisulfite and 2 µg/ml leupeptin and successively eluted with the same buffer containing 25 mM (2 cV) , 250 mM (2 cV) and 500 mM imidazole (2 cV). Active fractions were pooled (Fraction III, 1.6-2.3 ml) and loaded onto two 12-30 % glycerol gradients as described by Wernette and Kaguni (29). Active fractions were pooled (Fraction IV, 32-300 µg of protein, 3000 – 28000 units/ mg for various mutants, see Table I), stabilized by the addition of glycerol to 45% and stored at –20°C or frozen in liquid nitrogen and stored at –80°C.

Quantitative Silver-stain SDS-PAGE Analysis of Purified pol γ-α exo(-) Mutants- Equivalent units of Fraction IV enzymes were electrophoresed and stained using silver nitrate or electrophoresed and immunoblotted as described by Wang *et al.* (32) using anti-6xHis monoclonal antibody.

DNA polymerase assay- DNA polymerase activity was assayed on DNase I-activated calf thymus DNA and singly primed M13 DNA as described by Wernette and Kaguni (29) and Farr *et al.* (66), respectively. Specific modifications are indicated in the Figure legends. One unit of activity is that amount that catalyzes the incorporation of 1 nmol dNMP into acid insoluble material in 60 min at 30 °C using DNase I-activated calf thymus DNA as the substrate.

Analysis of products of processive DNA synthesis by gel electrophoresis- Reactions mixtures (50 μl) contained 50 mM Tris-HCl pH 8.0, 4 mM $MgCl_2$, 10 mM DTT, 100 mM KCl, 400 μg/ml bovine serum albumin, 30 μM each of dTTP, dCTP, dGTP and 10 μM dATP and [α-^{32}P]dATP (2×10^4 cpm/pmol), 10 μM singly primed M13 DNA, and 20 ng of purified enzyme. Incubation was for 10 min at 30 °C. Products were made 1% in SDS and 10 mM in EDTA, heated for 4 min at 80 °C, and precipitated with ethanol in the presence of 0.5 μg tRNA as carrier. The ethanol precipitates were resuspended in 80% formamide and 90 mM Tris borate. Aliquots were denatured for 2 min at 100 °C and electrophoresed in a 6% polyacrylamide slab gel (13×30×0.15 cm) containing 7M urea in 90 mM Tris-borate (pH 8.3) and 25 mM EDTA. Approximately equal amounts of radioactivity were loaded in each lane. Gels were washed in distilled water for 20 min, dried under vacuum, and exposed to a PhosphorImager screen (Molecular Dynamics). The data were analyzed using the ImageQuant version 5.2a software.

Gel electrophoretic mobility shift assay- DNA binding by the wild type and mutant pol γ-αexo(-)s was assayed by gel electrophoretic mobility shift assay. Purified pol γ-α(exo) (20ng) was incubated with the [γ-^{32}P]ATP-labeled template-primer substrate (0.1 pmol) for 5 min at 30°C in standard reaction buffer containing 50 mM Tris·HCl (pH 8.0), 4 mM $MgCl_2$, 10 mM dithiothreitol, and 30 mM KCl (without pol γ-β) or 100 mM KCl (with pol γ-β), followed by the addition of bromophenol blue and glycerol to 0.01 and 5%, respectively, and electrophoresis in a 6% native polyacrylamide gel

(13×30×0.075 cm) in 45 mM Tris-borate (pH 8.3) and 1 mM EDTA. After electrophoresis, the gel was dried under vacuum, and exposed to a PhosphorImager screen (Molecular Dynamics). The data were analyzed using the ImageQuant version 5.2a software.

Other Methods- Protein concentration was determined by the method of Bradford (127) with bovine serum albumin as the standard. Immunoblotting was performed as described by Wang *et al.* (32).

RESULTS

Overexpression and Purification of Recombinant Human pol γ-α PEO Mutants-

Recombinant human pol γ-α mutants containing the amino acid substitutions A467T, R627W, R627Q, R627Q/Q1236H and Q1236H were purified to near homogeneity from the soluble cytoplasmic fraction of Sf9 cells that were infected with baculoviruses encoding the complete coding sequence of the catalytic subunit as described in "Experimental Procedures". The mutations in the catalytic-subunit constructs represent those found in patients presenting with PEO, and localize to conserved sequence elements within the spacer region and the C-terminus of pol γ-α. Sequential chromatography was performed on phosphocellulose and Ni-NTA resins, followed by velocity sedimentation (see "Experimental Procedures"). The enzymes were purified 55-130 fold with an average yield of 6%, and producing ~0.15 mg of pol γ-α protein per liter of cultured cells. The chromatographic profiles were similar among the mutant constructs and as compared to the control enzyme, and all yielded symmetrical peaks at the same position as the control enzyme upon velocity sedimentation. Thus, we found no evidence for structural variation and/ or instability among the mutant enzymes. SDS-PAGE in conjunction with immunoblot analyses demonstrated the presence of full-length protein in each chromatographic step, though modest proteolytic degradation occurs throughout the purification in each preparation (data not shown). SDS-PAGE analysis of the final preparations shows that all of the preparations are of similar purity (Figure 2.9). Specific activities (expressed as units/mg) on a gapped double-stranded DNA substrate, DNase I-activated DNA, were as follows: *Hs* pol γ-α, 16,836; αA467T,

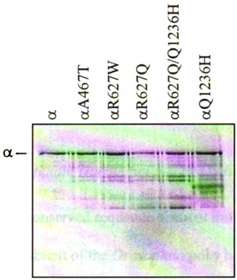


Figure 2.9. **SDS-polyacrylamide gel electrophoresis of mutant human pol γ-α.** Near-homogeneous fractions of baculovirus-expressed and purified recombinant pol γ-α (200ng, as indicated) were denatured and electrophoresed in a 10% SDS-polyacrylamide gel, and the proteins were stained with silver.

3,090; αR627W, 15,497; αR627Q, 13,000; αR627Q/Q1236H, 25,554; and αQ1236H, 19,760. Thus, among the PEO mutant enzymes, only the A467T mutation exhibits substantially reduced DNA synthetic activity, 5.5-fold lower than the pol γ-α control enzyme. The somewhat higher specific activity observed with the αR627Q/Q1236H enzyme falls outside of the range of ~8,000 -18,000 units/mg found in multiple previous purifications of the pol γ-α control, although the significance of this finding is unclear (see below).

DNA Binding Affinity of Recombinant Human pol γ-α PEO Mutants-

Our recent studies of conserved sequence element mutants document that the spacer region in the catalytic subunit of the *Drosophila* pol γ holoenzyme is involved in the template-primer binding (Luo and Kaguni, unpublished results). We performed a similar gel mobility shift assay using an oligonucleotide template-primer to examine DNA binding by the human pol γ-α mutants, in the presence and absence of the accessory subunit, pol γ-α at 100 mM KCl or 30 mM KCl, respectively (see "Experimental Procedures"). Whereas the other mutants show statistically similar DNA binding affinities as compared to the pol γ-α control enzyme in the presence or absence of pol γ-α, pol γ-αA467T alone shows weak DNA binding affinity, ~14% of the control (Figure 2.10). Notably, addition of the accessory subunit enhances DNA binding 18 fold by pol γ-αA467T to 70% of the control enzyme, as compared to an average of 2.2 fold for the other mutant enzymes, which showed somewhat higher activity overall than the pol γ-α control (albeit within the likely range of the control enzyme among various

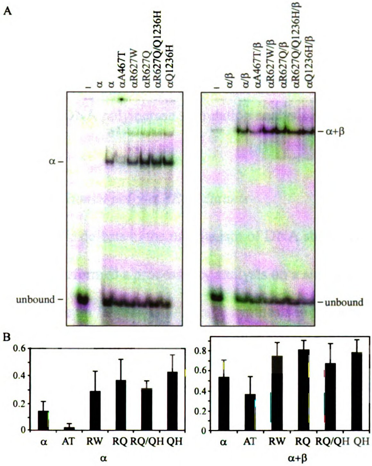


Figure 2.10. **DNA binding affinity of mutant human pol γ-α.** DNA binding affinity of mutant pol γ-α was determined by electrophoretic gel mobility shift assay (EMSA) using a radiolabeled oligonucleotide template-primer as described in *Methods*. A, Protein-DNA complexes formed by pol γ-α in the presence (*right*) or absence (*left*) of pol γ-α were electrophoresed in a 6% native polyacrylamide gel, and the gel was exposed to a Phosphor Screen. The first two lanes in each panel represent DNA substrate only and pol γ-α only controls, respectively, and the adjacent lanes contain both the DNA substrate and the indicated forms of pol γ-α. B, Bound and unbound DNA substrate bands were quantitated as described in *Methods*. The data in each panel were derived from duplicate assays in three independent experiments.

preparations). The DNA binding data argue that subunit interactions in the holoenzyme are not altered by the A467T mutation; rather, the intrinsic activity of the core is diminished, and this defect is compensated partially in the holoenzyme form. The latter is substantiated further in the DNA polymerase stimulation analysis reported below.

Processivity of Recombinant Human pol γ-α PEO Mutants-

To investigate possible defects in the mechanism of DNA strand synthesis by the human pol γ-α mutants, we performed a processivity analysis on a singly primed M13 DNA that mimics the substrate for lagging DNA strand synthesis in mitochondrial DNA replication. Assays were performed in the presence and absence of the accessory subunit at 100 mM KCl or 30 mM KCl, respectively (see "Experimental Procedures"). All of the mutant catalytic subunits show similar processivities as compared to the pol γ-α control enzyme, including pol γ-αA467T (Figure 2.11). This would suggest that its reduced DNA polymerase and DNA binding activities do not result from an intrinsic alteration in the mechanism of DNA strand synthesis, but in its ability to bind and initiate nucleotide incorporation at a primer terminus. Interestingly, addition of the accessory subunit enhances processivity of each of the mutant cores, though to a significantly lower extent for pol γ-αA467T: processivity increases ~7 fold for the other mutants as with the control pol γ-α, and only 2 fold for pol γ-αA467T (Figure 2.12).

Differential Stimulation of Recombinant Human pol γ-α PEO Mutants by pol γ-β-

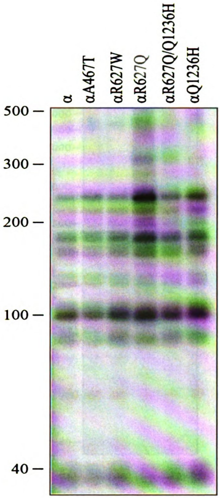


Figure 2.11. **Processivity of mutant human pol γ-α** DNA synthesis by mutant pol γ-α was measured at 30 mM KCl on singly primed M13 DNA as described in *Methods*. DNA product strands were isolated, denatured and electrophoresed in a 6% denaturing polyacrylamide gel, and the gel was exposed to a Phosphor Screen. Data derived from two independent experiments were quantitated as described in *Methods*, and yielded the following processivity values (expressed as average processivity units, apu): α, 63 nt; αA467T, 69 nt; αR627W, 70 nt; αR627Q, 108 nt; αR627Q/Q1236H, 77 nt; αQ1236H, 81 nt.

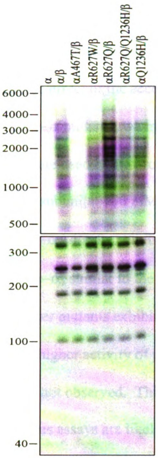


Figure 2.12. **Processivity of reconstituted mutant pol γ.** Human pol γ was reconstituted using a 3-fold molar excess of pol γ-β over pol γ-α, and DNA synthesis was measured at 100 mM KCl on singly primed M13 DNA as described in *Methods*. DNA product strands were isolated, denatured and electrophoresed in denaturing 1.5% agarose (*upper panel*) and 6% polyacrylamide (*lower panel*) gels, and the gels were exposed to a Phosphor Screen. Data derived from two independent experiments were quantitated as described in *Methods*, and yielded the following processivity values (expressed as average processivity units, apu): α, 42 nt; α/β, 437 nt; αA467T/β, 145 nt; αR627W/β, 358 nt; αR627Q/β, 558 nt; αR627Q/Q1236H/β, 416 nt; αQ1236H/β, 444 nt.

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The accessory subunit of pol γ has been shown to enhance the catalytic efficiency, and increase the DNA binding affinity and processivity of the holoenzyme (30,61,140,143,144). To investigate the effect of the accessory subunit on the DNA polymerase activity of the human pol γ–α mutants, we measured nucleotide incorporation on the singly primed M13 DNA substrate used in the processivity analysis, in the presence and absence of the accessory subunit at 100 mM KCl (Figure 2.13). In the absence of the accessory subunit, pol γ–αA467T exhibits only ~20% of the DNA polymerase activity of the control pol γ–α, similar to the 5.5-fold decrease in its activity on the gapped DNA substrate. The other mutants exhibit activities similar to the control enzyme, and in this assay, the slightly higher activity of pol γ–αR627Q/Q1236H that was found on the gapped DNA substrate is not observed. This would argue that 2-fold variations among enzymes in the various assays are likely not significant. Notably however, the accessory subunit stimulates ~20 fold the activity of pol γ–αA467T, as compared to ~6 fold for the other mutants and the control pol γ–α, to yield a holoenzyme activity of nearly 60% of the control holoenzyme. As with the DNA-binding and processivity analyses, it is apparent that interaction with pol γ–α mitigates partially the functional defects of the A467T mutation.

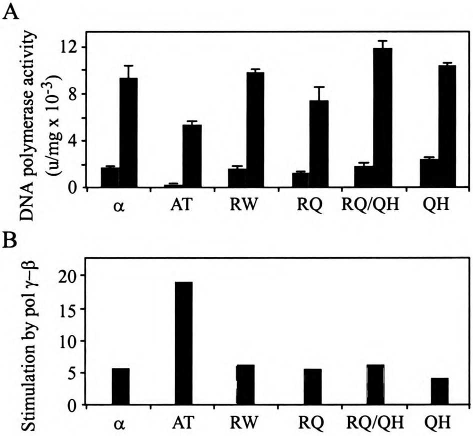


Figure 2.13. **Differential stimulation of mutant human pol γ-α by pol γ-β.** A, DNA synthesis by mutant pol γ-α was measured at 100 mM KCl on singly primed M13 DNA in the presence (*black bars*) or absence (*gray bars*) of pol γ-β as described in *Methods*. B, Fold stimulation of mutant human pol γ-α by pol γ-β based on the data from A.

DISCUSSION

Deletions and substitutions in mtDNA result in mitochondrial genetic diseases. Recently, several mutations in the catalytic subunit of human pol γ have been found to be associated with autosomal dominant/ recessive PEO, a mitochondrial disorder with multiple mtDNA deletions. This result is not surprising because pol γ is the sole DNA polymerase involved in mtDNA replication, repair, and recombination. These mutations map everywhere in pol γ-α, including the polymerase domain, 3'-5' exonuclease domain, and the spacer region in between. Interestingly, most of the dominant mutations map within the polymerase domain while recessive mutations map elsewhere (145). The mutant amino acids in the polymerase domain are conserved in pol γ-α, and the structural data from the corresponding T7 DNA pol homology suggest that these dominant mutations may alter DNA replication fidelity of the pol γ by altering base selection (145). Biochemical analysis of pol γ-α-Y955C indicated that this mutant polymerase has a 45-fold decrease in affinity to dNTPs (146). The authors predicted that Y955C may promote deletions between direct repeats in mtDNA through a slipped mispairing. Other mutations found in PEO have not been studied *in vitro*.

Biochemical study of the spacer region in *Drosophila* pol γ-α suggested that the spacer region affects enzyme activity, processivity, DNA binding affinity, and the balance between the pol and exo activities (Luo and Kaguni, unpublished results). Human pol γ-α-A467T maps within the γ1 conserved element, which is involved in subunit assembly in *Drosophila*. This mutation has been reported to be present in about 0.6% Belgians as a recessive mutation. A recent study suggested that A467T can also be a

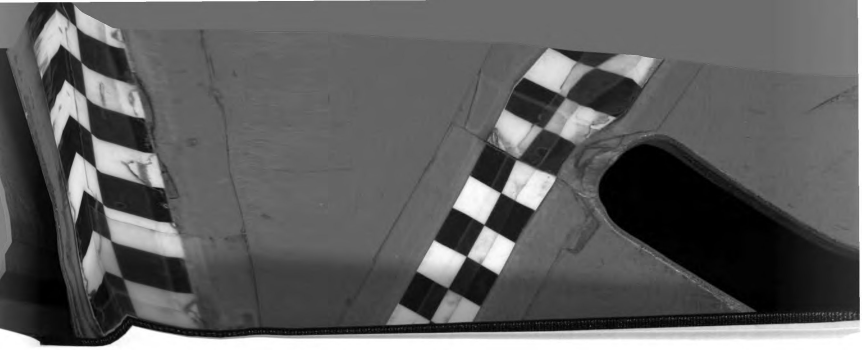
dominant mutation because carriers have an associated muscle disease. Results in this study demonstrate that human pol γ-α-A467T has significantly reduced catalytic activity, processivity, and DNA binding affinity. In the absence of pol γ-β, A467T has no detectable defect in processivity. However, the processivity of A467T is significantly lower than the control enzyme in the presence of pol γ-β. This result is consistent with the previous finding in *Drosophila* γ1 mutant YED (Luo and Kaguni, unpublished results). Interestingly, both the DNA polymerase activity and DNA binding affinity of A467T was stimulated by pol γ-β near 20 fold, which is 3-4 times higher than the control (5-6 fold). The increased stimulation of A467T by pol γ-β suggests an altered functional interaction between two subunits.

R627 maps within the γ3 conserved element. R627W is reported as a recessive mutation causing a neuromuscular disorder and R627Q yields a dominant phenotype and may also combine with the C-terminal mutation Q1236H. However, biochemical studies demonstrate that all of these mutants exhibit normal, if not higher, catalytic activity, processivity, and DNA binding affinity. Interestingly, it is reported that a missense mutation (E595A) in the spacer region of *Dm* pol γ-α causes mitochondrial and nervous system dysfunction, and E595A is lethal to the developing animal in the larval third instar (123). The corresponding *Dm* E595 in human is E640. It maps just outside of the γ3 element and is very close to R627. Biochemical analyses of *Dm* pol γ-α-E595A indicate normal specific activity, processivity, and DNA binding affinity (data not shown). A unique loop in the DNA binding crevice of T7 DNA polymerase has been demonstrated to interact with T7 gene 4 helicase/ primase and this interaction is required for the initiation of DNA synthesis (132). We predict that the observed mitochondrial disorders

in the human mutations (R627W/Q) and the lethal phenotype in *Dm* pol γ-α-E595A probably result from the defective interactions between the catalytic subunit of pol γ and other replicative proteins in mitochondria, such as mtSSB, mitochondrial helicase (Twinkle in human), and/ or mitochondrial transcription factor A (TFAM).

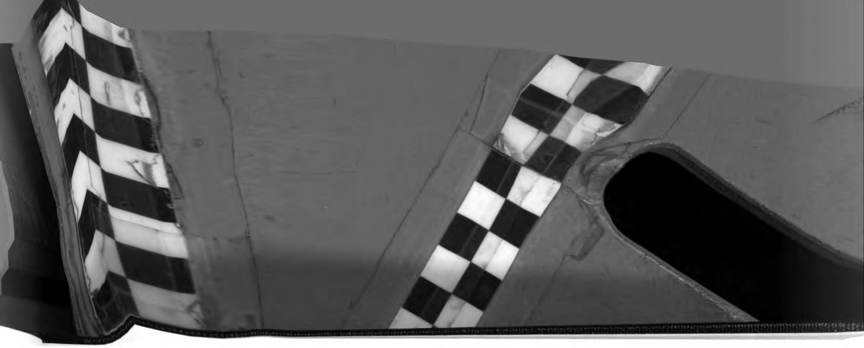
Recently, Dr. Spelbrink's group sequenced regions of mtDNA of PEO patients with mutations in either POLGA or Twinkle. The results showed increased mutation levels in the mtDNA control region but not the cyt b coding region in the patients compared with controls of similar ages. The majority of mtDNA mutations accumulated close to the heavy strand replication origin (O_H) and in conserved sequence block I (CSB I) in POLGA/PEO patients, whereas Twinkle/PEO patients showed more mutation accumulation in the region containing the L-strand and major H-strand promoter. These results suggest a mtDNA deletion mechanism in PEO patients created by stalling at regions of difficulty for DNA polymerase (147). Previous study of pol γ-α-Y955C indicated that the reduced fidelity results in adPEO (146). Biochemical studies on the spacer region in *Drosophila* pol γ-α demonstrated different deficiencies in various mutants. It is surprising to know that mutations in the polymerase domain (Y955C) and exonuclease/spacer region (R3P/A467T) in POLGA cause the same results -- high mutation accumulation at O_H region. However, if Dr. Spelbrink's prediction is true, the different biochemical defects *in vitro* may not be the reason, or at least not the only reason, to explain the mtDNA deletions in PEO patients. If there exists a common mechanism to explain the mtDNA deletions in the POLGA/PEO patients, it could be possible that pol γ-α mutants, including R627W, R627Q, and R627Q/Q1236H, carry normal function *in vitro* as we determined here. On the other hand, major mutations that

result in significant biochemical defects may be lethal to cells and do not cause disease. The phenotypes in the patients carrying R627W or R627Q may result from the accumulation of mitochondrial dysfunction from the subtle mutations.



CHAPTER III

GENETIC AND MOLECULAR APPROACHES TO MITOCHONDRIAL DNA REPLICATION PROTEIN FUNCTION



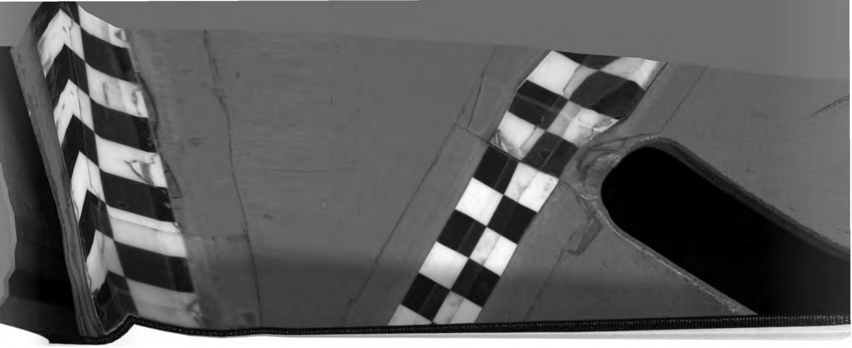
INTRODUCTION

Mitochondrial biogenesis is a key process in animal cell proliferation, and mtDNA replication is an essential component of that process. Unlike the DNA replication in bacterial and nuclear systems, mtDNA replication is much simpler. Only a minimal group of critical proteins are required in the mtDNA replication process. Biochemical studies have documented the major proteins at the mtDNA replication fork and pol γ is the only DNA polymerase for mtDNA replication (28).

Drosophila pol γ is a heterodimer comprising two subunits of 125 and 35 kDa (29,31) that are encoded by nuclear genes (32,33). Pol γ belongs to the family A DNA polymerase group and displays high processivity and fidelity during mtDNA synthesis. Both DNA polymerase activity and 3'-5' exonuclease activity are located in the 125 kDa catalytic subunit of *Dm* pol γ (33,51). The accessory subunit contributes to the structural and functional integrity of the catalytic subunit, including increasing the template-primer binding affinity, catalytic efficiency and processivity of the holoenzyme. mtSSB binds to ssDNA, coating the displaced ssDNA, which is the template for lagging DNA strand synthesis in mtDNA replication, and prevents it from renaturation (24). Biochemical studies demonstrated that mtSSB enhances primer recognition and binding, stimulates the rate of initiation of DNA strands and exonuclease activity of *Drosophila* pol γ, and increases the processivity of *Drosophila* pol γ in DNA strand elongation (66)

Here we constructed several *Drosophila* animal models to evaluate the function of these key proteins that are involved in mtDNA replication *in vivo*. Chemical mutagenesis and phenotypic screening was used to disrupt pol γ-β; standard P-element mediated

transformation was used to overexpress mutant forms of mtSSB and exonuclease-deficient pol γ-α; and targeted mutagenesis by homologous recombination was used to generate different fly lines with specific mutations in pol γ. The data we show here demonstrates that the integrity of the key proteins at the mtDNA replication fork is essential to maintain the normal function of the animal. In addition, *Drosophila* research offers powerful approaches to modify functional pathways of different proteins. Advances in *Drosophila* research are facilitated by the combination of genome sequence information, gene expression profiles, and generation of genome-wide mutations in this organism.



EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids- [α-^{32}P]dATP was purchased from ICN Biochemicals. Plasmid DNAs were prepared by standard laboratory methods. Synthetic oligodeoxynucleotides were synthesized in Applied Biosystems Model 477 oligonucleotide synthesizer.

The modified Bluescript II KS vector containing a *Not*I site and the injection vector pTV2 for targeted mutagenesis in *Drosophila* are the gifts of Dr. Kent Golic (University of Utah).

Bacterial Strains - E. coli XL-1 Blue (*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac,* (*F'proAB, lacIqZ M15, Tn10* (tetr)) was used for bacterial cloning. *Drosophila* genomic P1 clone DS00941 was obtained from Berkeley *Drosophila* Genome Project (BDGP).

Fly stocks- Transgenic flies carry both the *70FLP* and *70I-SceI* transgenes on chromosome 2 or carry *70I-CreI* transgene are the gifts of Dr. Kent Golic (University of Utah). All flies carried either the w^{1} or the w^{1118} null alleles on their *X* chromosomes.

Enzymes and Proteins- E. coli DNA polymerase I and its Klenow fragment were purchased from New England Biolabs. T4 polynucleotide kinase, RNase-free DNase I, and T7 RNA polymerase were from Boehringer Mannheim. T4 ligase and AMV reverse transcriptase were from GIBCO-BRL and Promega, respectively. Restriction enzymes were purchased from Life Technologies and New England BioLabs. *Pfu* DNA polymerase for PCR was purchased from Stratagene.

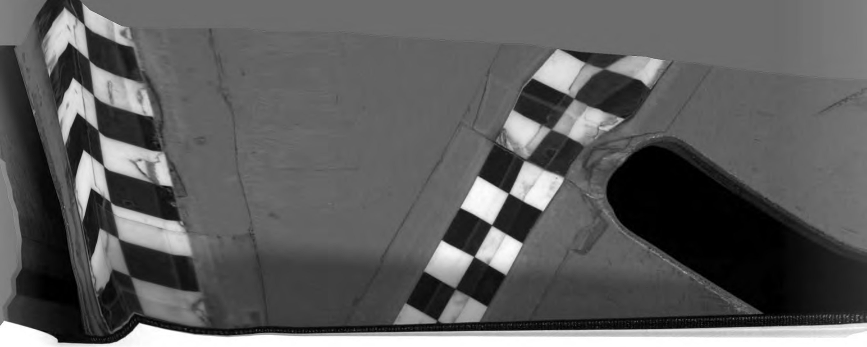
Chemicals- Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Research Organics, Inc. RNase inhibitor was from Boehringer Mannheim.

Methods

Southern blotting- Genomic DNA was purified from third instar larvae or adults by standard methods. DNA (3 μg) was cleaved with *Xho*I which cuts mtDNA once, fractioned in a 0.8% agarose gel/TBE and transferred to nylon membrane (Amersham Pharmacia Biotech). Hybridization was carried out for 16 h at 65°C in 10 mM sodium phosphate pH7.4/0.5% SDS. Filters were washed three times for 10 min at room temperature with 4 × SSC containing 0.1% SDS, once for 30 min at 65°C with 0.1 × SSC containing 0.1% SDS. Blots were probed with radiolabeled DNAs for the mitochondrial gene ATPase6 and the nuclear histone gene cluster.

RNA in situ hybridization- Embryos were collected overnight and dechorionated in 50% bleach for 2.5 min and transferred to a tube containing 3 ml of fixing buffer (phosphate buffered saline pH 8, 66 mM EGTA), 1 ml of 37% formaldehyde and 4 ml of heptane. After vigorous shaking the tube for 25 min and aspirating the lower aqueous phase, methanol was added to remove the vitelline membrane, followed by vigorous agitation for 60 sec. The solution was then aspirated, and the embryos were washed several times with methanol and stored under methanol at -20 °C.

Antisense mtSSB riboprobe was prepared by *in vitro* transcription using pBluescript containing the mtSSB cDNA linearized by *Sal*I digestion as template, and DIG-labeled UTP (Roche Molecular Biochemicals). The transcription reaction (10 μl)



containing 1 × transcription buffer and DIG RNA labeling mix, DNA (1 μg), 40 units RNase inhibitor and 20 units of T7 RNA polymerase was incubated for 2 h at 37 °C. Water (15 μl) was added followed by 25 μl of 2 × carbonate buffer (200mM $NaCO_3$, pH 10.2), and the RNA was partially hydrolyzed to shorten the probe length by heating for 40 min at 65 °C. The reaction was terminated with the addition of 0.1 M NaAc, pH 6.0 (50 μl). The RNA probe was ethanol-precipitated by the addition of LiCl to 0.4 M, 100 μg of *E.coli* tRNA and 2 volumes of ethanol. The pellet was dissolved in 150 μl of hybridization buffer and stored at -20 °C. The riboprobe was heated for 3 min at 80 °C before use. Hybridization was carried out overnight at 55 °C in a buffer containing 50% deionized formamide, 5 × SSC, 100 μg/ml of sonicated salmon sperm DNA, 50 μg/ml of heparin and 0.1% Tween 80. The digoxigenin-labeled mtSSB probe was detected using antidig-U antibody (Roche Molecular Biochemicals) coupled to alkaline phosphatase, and the reaction was visualized with nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate.

Plasmids construction- ~12kb *Drosophila* genomic DNA containing both the pol γ-α and -β genes was PCR-amplified using the following primers with genomic P1 clone DS00941 as template. Both primer 1 (5'-AA*GCGGCCGC*TTTAATGCCAAGCCGCTGATTATCTTCACCT- 3') and primer 2 (5'- CC*GCGGCCGC*GATTCCAGTGCGCCAGATATTTTATGCCGAG -3') contain *Not*I site and the PCR product was gel purified and cloned into a modified Bluescript II KS vector through *Not*I site.

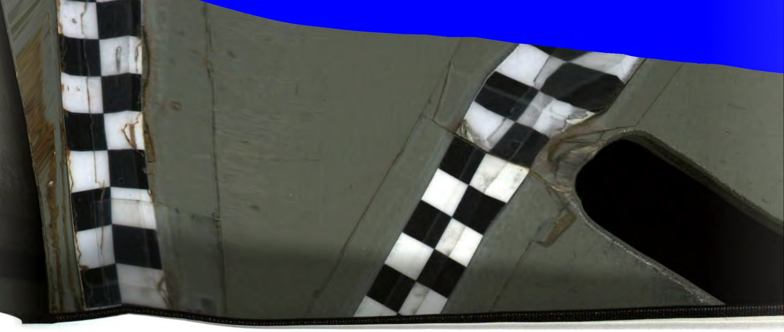
An I-*Sce*I site was introduced between pol γ-α and -β genes by PCR amplification with *Pfu* DNA polymerase (Stratagene) using primers (5'-

TACCAGTGCTCTTACGCACGTCAGCTGTAGGGATAACAGGGT*AAT*TTGGCGCCATTTCAATGGTC -3' and 5'-GACCATTGAAATGGCGCCAA*A*TTACCCTGTTATCCCTACAGCTGACGTGCGTAACAGCACTGGTA -3') containing the I-*Sce*I site indicated by underscore. Pol γ-α and -β mutants were constructed by QuickChange mutagenesis with *Pfu* DNA polymerase (Stratagene) according to the manufacturer's recommendations. A typical PCR was carried out in a 50 μl reaction mixture with 100 ng of DNA template and 2 units of *Pfu* DNA polymerase. A specific primer pair was used for each mutant as follows. Pol γ-α-Y873C, 5'- TATGCCCGGATCT**G**(a)CGG**C**(a)GC**C**(t)GGTCAATTGT -3' and 5'-ACAATTGACC**G**(a)GC**G**(t)CCG**C**(t)AGATCCGGGCATA -3'; Pol γ-α-Y869F, 5'-CCAAAGTGAT**T**(c)AACT**T**(a)TGCCCGGATCTA -3' and 5'-TAGATCCGGGCA**A**(t)AGTT**A**(g)ATCACTTTGG -3'; Pol γ-α-A434T, 5' –ATTTGGGCCGCAGA**A**(g)CTGAGGAAGC**T**(g)TGCTCCTTGCT -3' and 5' –AGCAAGGAGCA**A**(c)GCTTCCTCAG**T**(c)TCTGCGGCCCAAAT -3'; Pol γ-α-F578A, 5' -AGGGAGCAAGGCTGGGGC**G**(t)**C**(t)CCTGGTT-3' and 5'-AACCAGG**G**(a)**C**(a)GCCCCAGCCTTGCTCCCT -3'; Pol γ-α-D185A, 5'-AAGGGACTTGTCTTCG**C**(a)TGTGGAGGTGTGCGTCA -3' and 5'-TGACGCACACCTCCACA**T**(g)CGAAGACAAGTCCCTT -3'; Pol γ-α-D263A 5'-ACAATGTCTCCTACG**C**(a)**T**(c)**C**(a)G**A**(g)GCGCGACTGAAGGA -3' and 5'-TCCTTCAGTCGCGC**T**(c)C**G**(t)**A**(g)**G**(t)CGTAGGAGACATTGT -3'; Pol γ-β-D313A, 5'- AGCACTTGCT**C**(g)GA**G**(a)ACGG**C**(a)TATGCTGGGCATA -3' and 5' TATGCCCAGCATA**G**(t)CCGT**C**(t)TC**G**(c)AGCAAGTGCT -3'. Bold letters indicate nucleotides that replace those in the wild-type pol γ-α as indicated in parentheses with

lower case letters. The introduced/removed endonuclease cleavage sites are indicated by underscore. The DNA template was first denatured at 95°C for 45 s, followed by 20 three-step cycles of 95°C for 45 s, 50°C for 45 s, and 68°C for 30 min (2 min/kbp DNA template). The reaction mixture was then digested with 10 units of *Dpn*I for 3-4 h at 37°C to eliminate the methylated parental DNA template. A 2 µl aliquot was used for transformation of competent *E. coli* XL-1 Blue cells by electroporation using an *E. coli* Pulser (Bio-Rad). The introduced I-*Sce*I site was confirmed by I-*Sce*I (New England) digestion and mutageneses were confirmed by DNA sequence analysis. The engineered genes were then cloned into pTV2 vector through *Not*I restriction site. The orientation of the insertion was confirmed by *Kpn*I digestion.

Bulk cloning and sequencing of mtDNA from flies- Total DNA was isolated from y/w or transgenic flies with or without heatshock for 6 generations by standard laboratory method. Two regions spanning the cyt-b-tRNASer-ND1 gene or the ND5-tRNAHis-ND4 gene of mtDNA that each span 1.7 kb were PCR-amplified by *Pfu* DNA polymerase using total DNA as template with primer pairs 5'- CGCTACCAGCTCCAATTAAT -3' / 5'CACTGTGGCTCAGACTATTT -3' and 5'- GACCACCAGCTGTAACTAAT -3' / 5'- CTCAGCCAGAACGTTTACAA -3'. Blunt-ended PCR fragments were cloned into the *Sma*I site of pUC1193 and transformed into *E. coli* XL-1 Blue. After blue-white screening, plasmid DNAs containing the expected fly DNA inserts were sequenced. At least two total DNA samples from each fly line were used as template and each DNA sample was PCR-amplified twice before cloning in to the pUC1193. 20-30 colonies from each transformation were picked and > 100 kb of the inserted DNA fragments were

automatically sequenced for each fly line. The sequences were aligned and compared by computer program Pile-Up.



RESULTS

A. The accessory subunit of DNA polymerase γ is essential for mitochondrial DNA maintenance and development in Drosophila melanogaster

Biochemical studies have demonstrated that the accessory subunit is essential for the catalytic efficiency and the high processivity of the holoenzyme (30,61,140,143,144). It is likely involved in maintaining the structural integrity of the holoenzyme through multiple contacts with the catalytic subunit (34). In addition, structural modeling and functional studies suggested the role of the accessory subunit in primer recognition and in processivity (34,58,59). Therefore, it was important to test mtDNA content and integrity in pol γ-β mutants.

To evaluate the role of the accessory subunit in mtDNA synthesis and in mitochondrial function *in vivo*, mutations in the nuclear gene encoding pol γ-β were identified. The mutant allele pol γ-β^{1} is an EMS-induced mutation resulting in a glutamic acid substitution of a highly conserved glycine residue (G31E) in the N-terminal domain of the accessory subunit. The allele pol γ-β^{2} is a spontaneous mutation caused by an in-frame 74-bp insertion at T113 that creates a premature stop (148). Flies carrying either pol γ-β^{1} or pol γ-β^{2} are lethal at the early pupal stage.

To evaluate mtDNA content and integrity in the pol γ-β mutants, quantitative Southern blot analysis was pursued. Total DNA was isolated from wild-type and mutant pol γ-β larvae late in the third instar and digested with *Xho*I, which cuts *Drosophila* mtDNA once. A mtDNA probe encoding the ATPase6 gene was used to determine

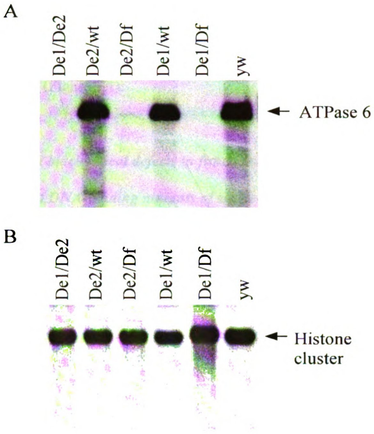


Figure 3.1. **Mitochondrial DNA is absent in mutants of the accessory subunit of *Drosophila* Pol γ.** Three micrograms of total DNA that was isolated from third instar larvae was digested with *Xho*I, which cleaves mtDNA once; duplicate samples of the DNAs (1 μg) were electrophoresed in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled DNA probes from the ATPase 6 gene of mtDNA and a repeated histone gene cluster as a nuclear DNA control.

mtDNA copy number and integrity, and a multiple-copy genomic probe of the histone cluster was used as a DNA loading control. Multiple analyses of mtDNA content demonstrated severe mtDNA depletion in pol γ-β mutants; no mtDNA was detected in mutant pol γ-β larvae (Figure 3.1).

B. Physiological and biochemical defects in functional interactions of mitochondrial DNA polymerase and DNA-binding mutants of single-stranded DNA-binding protein

Several mutant mtSSBs were constructed by site-directed mutagenesis of the DNA-binding domain that was identified in *E. coli* SSB and the recombinant proteins were overexpressed in the bacterial expression system and purified to homogeneity (149). Biochemical studies indicated that even modest DNA-binding defects in mtSSB significantly affect DNA synthesis by pol γ. Under conditions of reduced endogenous wild-type mtSSB, mtDNA depletion develops in cultured cells. This phenotype is rescued by production of exogenous wild-type but not mutant mtSSB.

To assess the impact on mitochondrial DNA metabolism of reduced DNA binding by mtSSB and loss of functional interactions with pol γ *in vivo*, we constructed transgenic flies that express either the wild-type or mutant forms of mtSSB under the control of the *Drosophila hsp70* promoter by standard P-element mediated transformation. RNA *in situ* hybridization of embryos with an antisense mtSSB probe revealed that the exogenous mtSSB was overexpressed throughout embryogenesis in both wild-type and mutant mtSSB transgenic lines (Figure 3.2). Compared with the wild type control, the staining of the transgenic embryos was much stronger and the staining patterns were entirely different at each stage of embryogenesis examined. A high steady-state level of mtSSB

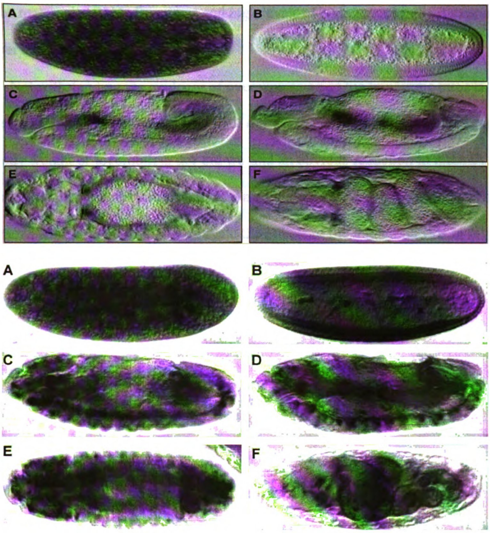


Figure 3.2. ***mtSSB* gene expression during *Drosophila* embryogenesis in *y/w* (upper panel) and mtSSB transgenic flies (lower panel).** The temporal and spatial distribution of *Drosophila mtSSB* mRNA was examined by whole-mount *in situ* hybridization with digoxigenin-labeled antisense RNA as described under "Experimental Procedures." The staining pattern of embryos is shown at different developmental stages. In all views, anterior is to the *left*. *A-D*, lateral views, with dorsal at the top: *A*, an early embryo in the syncytial blastoderm; *B*, cellular blastoderm; *C*, stage 12; *D*, stage 13; *E* and *F*, ventral views: stages 14 and 17, respectively.

mRNA was maintained in the transgenic embryos even at the cellular blastoderm stage, when the maternal mRNA disappears and high levels of endogenous mtSSB expression no longer occur (150).

Not withstanding the evidence of overexpression of the transgenic mtSSB, we observed no aberrant phenotypes in terms of developmental timing, eclosion frequency, adult body weight or locomotion, nor did we find an obvious reduction in mtDNA copy number by Southern analysis (data not shown). This suggested that the high-level of endogenous wild-type mtSSB may mitigate the effects of the mutant mtSSB in *Drosophila*.

C. Mitochondrial DNA replication fidelity, aging and human disease: Targeted mutagenesis in a fly model

To examine whether *Drosophila* is a good animal model to study human mitochondrial diseases, we introduced the *Drosophila* version of several pol γ-α mutations that are associated with progressive external ophthalmoplegia (PEO) in humans. Among different mutations that have been identified in the human pol γ-α gene, we selected the A467T (A434T in *Drosophila*) and Y955C (Y873C in *Drosophila*) mutations. In addition, we constructed several injection vectors with different mutations that map to different locations in *Drosophila* pol γ-α (D186A/D263A, F578A, and Y869F) and -β (D313A). The biochemical defects of these latter mutants have been characterized, but the disease phenotypes have not yet been reported in humans.

P-element mediated transformation represents a powerful approach to introduce exogenous genes into flies. However, potential problems may result from developmental

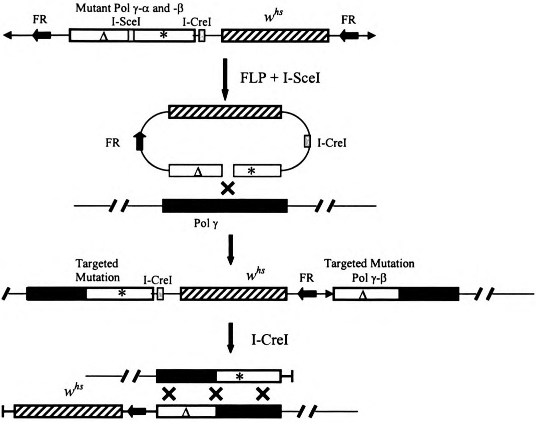


Figure 3.3. **The targeting scheme.** The donor construct is diagrammed at the top. FLP-mediated excision and I-SceI-mediated cutting produce the extrachromosomal targeting molecule shown. A 12-kb fragment of the γ-cluster genomic gene was used to construct the donor. This targeting molecule is expected to recombine with the endogenous γ-cluster locus, as shown, to produce a tandem duplication. After the second homologous recombination stimulated by I-CreI digestion, only one mutant copy (either pol γ-α mutation or pol γ-β mutation) remains in the genomic DNA. Arrowheads at the donor site represent *P*-element ends.

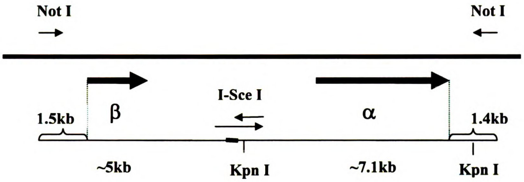


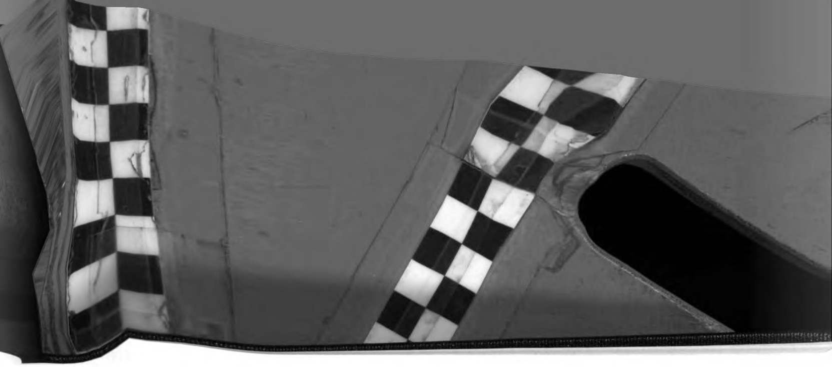
Figure 3.4. **The diagram of donor construct.** A ~12 kb genomic DNA containing both the pol γ-α and -β genes was PCR-amplified from genomic P1 clone DS00941. An I-*Sce*I site was introduced between the pol γ-α and -β genes and specific amino acid substitutions in the pol γ-α and/ or -β genes were introduced into a single recombinant donor construct as described in "Experimental Procedures". Modified *Dm* genomic DNA was cloned into pTV2 vector through the *Not*I site. An extra endonuclease site was introduced together with each mutation and will be used in Southern analysis for the subsequent mutagenesis screen (see text). The introduced endonuclease sites were confirmed by *in vitro* digestion and the mutation sites were confirmed by sequence analysis. The orientation of the inserted DNA was confirmed by *Kpn*I digestion.

and spatial mis-expression of a transgene from a standard P-element mediated transformation. In addition, from our experience in the mtSSB project (Chapter III-B), expression of the wild-type endogenous gene may interfere with phenotypic analysis of the introduced mutant copy. A recent, yet well-documented approach of targeted gene replacement by homologous recombination was used for mutagenesis in *Drosophila* (Figure 3.3) (108,109).

A P-element vector, pTV2, was used as a general transformation vector for gene targeting. The donor construct contains w^{hs}, FRTs and exogenous gene copy (genomic DNA) containing an I-*Sce*I site. w^{hs} is a hypomorphic allele of the *white* gene and serves as a w^+ marker for transformation and for the recovery of targeting events. FRT is a FLP recognition target. FLP is a site specific recombinase, which mediates recombination between the FRTs. I-*Sce*I is an intron-homing endonuclease from yeast. It recognizes and cuts a specific 18-bp sequence. Subsequent I-*Sce*I cutting generates the recombinogenic targeting molecule. Homologous recombination between this molecule and the resident gene locus is expected to carry the w^{hs} gene. Because the extent of donor:target homology influences the frequency of homologous recombination, at least 5 kb donor homology (including the pol γ-α gene) should be constructed into the recombinant plasmid (109). In *Drosophila,* both pol γ-α and -β map within a compact gene cluster (151). To take advantage of this specific genome structure, ~12 kb genomic DNA containing both the pol γ-α and -β genes was PCR-amplified from genomic P1 clone DS00941 to make the donor homology. An I-*Sce*I site was introduced between the two subunit genes (Figure 3.4). Specific amino acid(s) substitutions in the pol γ-α or -β genes were introduced together into a single recombinant donor construct by QuickChange mutagenesis twice

with two primer pairs containing expected mutations in either pol γ-α or -β. After homologous recombination, the expected product would be a tandem partial duplication of the target genome sequence, with one copy containing desired pol γ-α mutation and another copy containing the desired pol γ-β mutation. Another rare-cutting endonuclease (I-*Cre*I) is placed in the injection vector pTV-2. After the second homologous recombination, only one mutant copy (either pol γ-α mutation or pol γ-β mutation) remains in the genomic DNA.

The donor constructs were transformed into flies by standard P-element mediated transformation. The obtained transgenic flies that carry a donor construct on chromosome *X* or *3* will be crossed with transgenic flies that carry both the heat-inducible *70FLP* and *70I-Sce*I transgenes. Crosses will generate flies with a single copy donor element and *70FLP* and *70I-SceI*. The progeny will be heat shocked and test crossed to flies carrying the w^- mutation. When offspring with pigmented eyes are observed, the w^{hs} will be mapped to detect its mobilization to chromosome *2* (to which pol γ maps). These flies will carry a tandem partial duplication of the target genome sequence, with one copy containing the desired pol γ-α mutation and another copy containing the desired pol γ-β mutation. To reduce the extra copy in the flies, we will cross these flies with the targeted allele to flies with the *70I-CreI* transgene, and then heat shock their progeny. After heat shock, about 30% loss of w^+ will be seen in the male germline, and most of these are either single pol γ-α or -β mutant copy reduction alleles. The single copy alleles can then be screened by Southern blot because new restriction sites were introduced into/ removed from the construct along with each point mutation in pol γ-α or -β (see *Methods*).



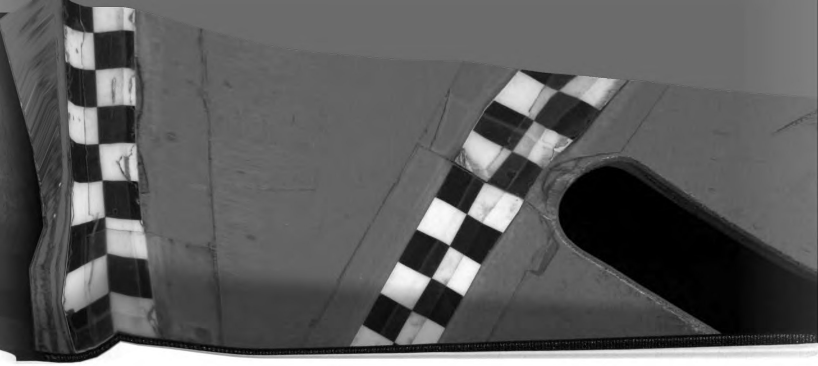
At this point, the target gene constructs have all been generated. The introduced I-*SceI* site was confirmed by endonuclease digestion and the mutagenesis was confirmed by DNA sequence analysis (see *Methods*). Further molecular or phenotypic analysis of the knock-in flies will be discussed in the DISCUSSION and FUTURE PLANS.

D. A mitochondrial mutator limits life span and causes developmental defects, delays and arrest in Drosophila melanogaster

Pol γ purified from different sources is found to be highly accurate in DNA replication (31,152,153). A mutational assay of human recombinant pol γ indicated that the high fidelity derives from both high nucleotide selectivity and exonucleolytic proofreading (154,155). The essential amino acid residues in governing fidelity map to each of the three 3'-5' exonuclease and three polymerase motifs (28). The exonuclease activity of mutant yeast pol γ with a double mutation D171G/D230A within exonuclease motifs I and II, respectively, was dramatically decreased and the mutation frequency in the mutant yeast was significantly increased (156). The corresponding amino acids in *Drosophila* are Asp^{186} and Asp^{263}.

To evaluate defective exonucleolytic proofreading in flies, we developed a mutator pol γ transgenic fly line carrying the D186A/D263A double substitution by standard P-element mediated transformation. The transgenic flies showed several altered phenotypes including reduced reproductive capacity, larval hatching and pupal eclosion rate, and life span. Immunobot assay showed a 2-3 fold reduction of the mitochondrial gene products, ND1 and CO I proteins. However, Southern analysis indicated that the copy number and the integrity of the mtDNA did not change, and Northern analysis

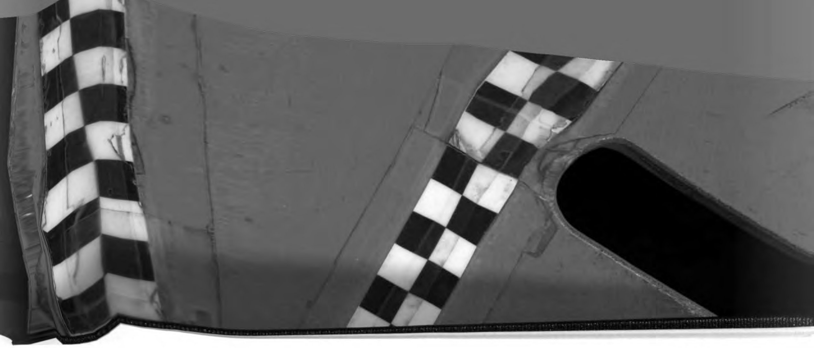
showed that the expression of mitochondrial genes stayed at the wild-type level in transgenic flies with long-term overexpression of the exonuclease-deficient pol γ-α. To link base substitution mutations to the expression of the exonuclease-deficient pol γ-α transgene, we pursued the bulk cloning and sequencing strategy (see *Methods*) and the result suggested that there was no significantly increased mutation rate in the exonuclease-deficient fly line that we had expected to observe.



DISCUSSION

Biochemical studies demonstrated that the pol γ-β subunit contributes to the structural and functional integrity of the catalytic subunit, including increasing the template-primer binding affinity, catalytic efficiency and processivity of the holoenzyme. These results suggested that the accessory subunit would be essential for animal mtDNA replication. Indeed, we found that mutations that disrupt the pol γ-β gene cause lethality in the early pupal stage. On the molecular level, mtDNA depletion occurred when the pol γ-β gene was disrupted. Biochemical study demonstrated that N-terminal region deletion mutants caused reduced DNA binding affinity and DNA polymerase activity (34), which is consistent with our *in vivo* observation. The biochemical consequence of the G31E mutation in the accessory subunit that we described has not yet been elucidated. We anticipate that *Drosophila* will provide a valuable model to carry out *in vivo* analysis by means of genetic and phenotypic characterization.

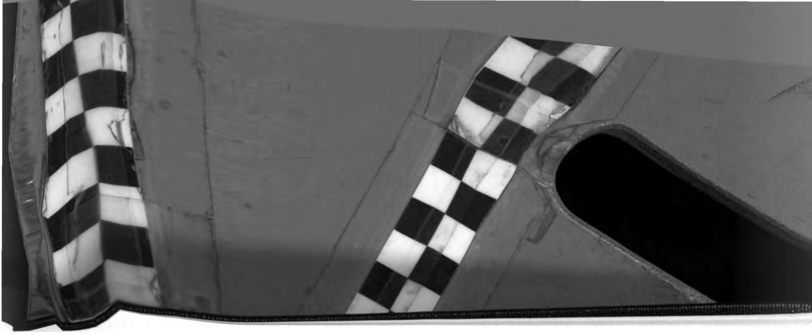
To evaluate the defects of mutant mtSSB *in vivo*, we introduced the mutant copy into both cultured cells and flies. Transgenic *Drosophila* strains that express either the wild-type or mutant forms of mtSSB under the control of the *Drosophila hsp70* promoter were constructed by standard P-element mediated transformation. RNA *in situ* hybridization with an antisense mtSSB probe showed that the gene expression intensity was significantly increased and the gene expression pattern was entirely changed in the transgenic embryos. These results proved that the exogenous mtSSB gene was highly expressed under the different promoter (*hsp70*) from the endogenous gene copy in the transgenic flies. However, unlike the obvious defects in DNA binding and DNA synthesis



of the mutant mtSSB from the *in vitro* data, no obvious changes were observed on mtDNA copy number after six-generation overexpression of the mutant mtSSB in the transgenic flies. Similarly, we observed that the growth rate of cells was not affected by overexpressing either wild-type or mutant mtSSB (data not shown).

mtSSB exists as a tetramer in solution (157) and the N-terminal portion of SSB is responsible for tetramerization and ssDNA binding (158). By equilibrium sucrose gradient centrifugation and immunoprecipitation, Williams' group showed that the mtSSB heterotetramers with both wild-type and mutant protomers displayed significantly decreased ssDNA binding affinity compared to the wild-type homotetramer. However, the ssDNA binding of wild-type mtSSB was not depleted but increased after adding excess of mutant mtSSB to the reaction system (159). There exists abundant endogenous wild-type mtSSB in *Drosophila* (67,133). Therefore, the overexpressed mutant mtSSB in the transgenic flies may assemble with the endogenous wild-type mtSSB and the formed heterotetramers in the animal may provide enough ssDNA binding ability to stabilize the ssDNA during mtDNA synthesis.

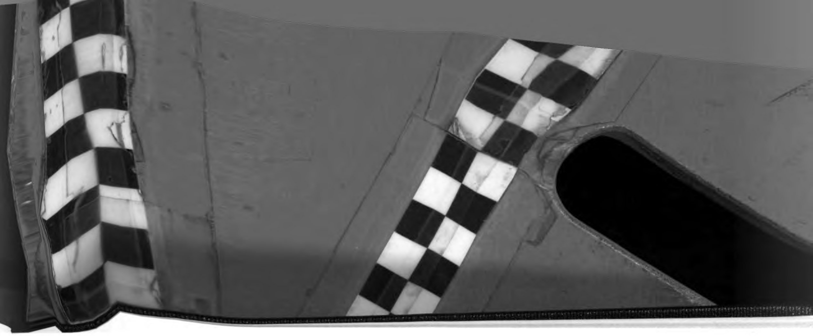
To further evaluate the effect of the mutant mtSSB *in vivo*, the endogenous mtSSB gene was disrupted through RNA interference while the exogenous mtSSB was overexpressed in the cell culture. Substantial mtDNA depletion and reduced cell growth rate were observed in the cell lines transfected with mutant but not wild-type mtSSB (149). A newly developed method for targeted modification of the *Drosophila* genome through homologous recombination opens avenues for the development of new *Drosophila* animal models in which induced mtDNA depletion may be manipulated by



controlling exogenous mutant transgene and removing the endogenous gene copy at the same time (see Chapter III-C).

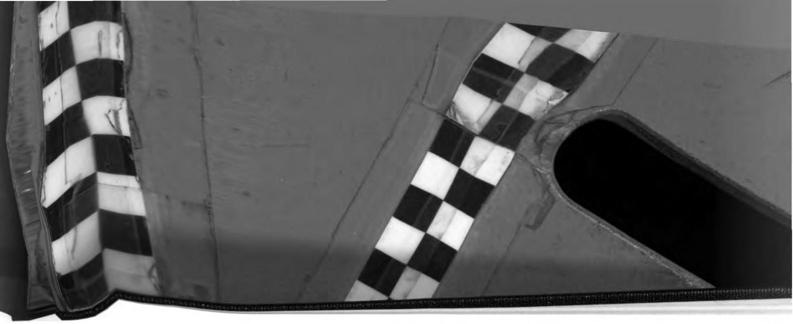
A possible explanation for the negative results of the Southern/ Northern and the bulk cloning and sequencing analysis of the exonuclease-deficient strain is that mutant DNA in our transgenic lines may be present only in some tissues or cells, and result nevertheless in the phenotypes observed. First, mitochondria are distributed unevenly among tissues or organs because different tissues and organs rely on mitochondrial energy generation to different extents. Tissues or organs like the central nervous system, heart and skeletal muscle that have a high energy demand more mitochondria (119). Also, proteins required for mtDNA replication are not expressed uniformly in all tissues (67) and mitochondria differ substantially in their mtDNA copy number (22). Therefore, the expressed exonuclease-deficient pol γ in the transgenic flies may have highly variable effects in different tissues and cell types. We used entire adult flies to prepare the DNA/ RNA samples for all the assays and this may mitigate both the Southern/ Northern and the sequence analysis results. Second, the endogenous wild-type pol γ-α still contributes normal mtDNA in nuclease-deficient flies. Thus, the chance of picking normal mtDNA by PCR increases during the bulk cloning. To circumvent these problems, we have initiated a new approach to generate exonuclease-deficient knock-in flies, in which the endogenous wild-type pol γ-α is removed (see Chapter III-C). Meanwhile, we plan to measure the mutational load and oxidative function in specific tissues of transgenic flies.

*Drosophil*a has been used widely as an animal model for genetic studies since the time of Morgan. Besides the features of short life cycle and easy and low maintenance, the genetic and physical map, balancers, chromosomal deletions and duplications, and P-

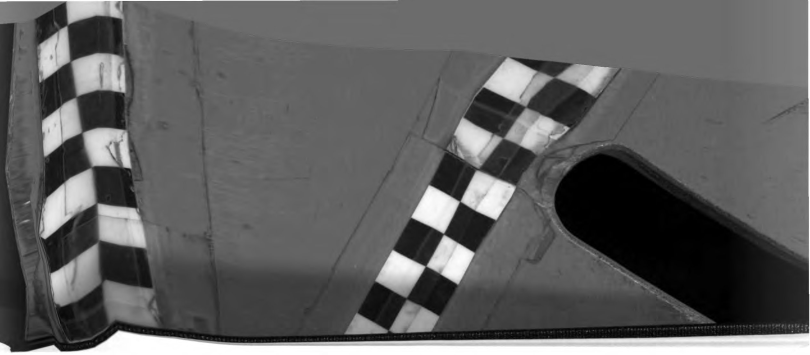


element mediated germline transformation are routinely used. With the release of the complete euchromatic *Drosophila* genome sequence (96), studies of the relationships between genotype and phenotype are made possible. A new technique to replace the endogenous wild-type gene by an exogenous modified construct by homologous recombination has been developed (108,109). No foreign DNA is left behind at the target locus except the introduced mutations after two steps of crosses in this approach. This fully ensures that a gene's regulation is not altered. In addition, there is no wild-type endogenous gene left as a background, which may interfere with the introduced mutant gene and mitigate the potential mutant phenotype. The generality and effectiveness of this technique has been demonstrated by disrupting several genes in different locations (110).

Here we adopted the technique to construct several transgenic fly lines with different mutations in *Drosophila* pol γ. Pol γ-α-D186A/D263A is a double mutation in exonuclease motif I and II, respectively. Standard P-element mediated transformation with this mutation displayed phenotypical changes, but evidence of a molecular defect at the DNA sequence is absent in transgenic flies. The endogenous pol γ-α gene that may mitigate the results of the mutation in transgenic flies is removed in knock-in flies. Thus, positive results at the molecular level in the new flies are expected. Pol γ-α-A434T (A467T in human) maps within the conserved γ1 element in the spacer region. A467T has been found to be associated with PEO. Our biochemical studies demonstrated reduced polymerase activity, processivity, and DNA binding affinity for this mutant (see Chapter II). Phenotypic analysis of Pol γ-α-A434T knock-in flies will provide a direct connection between the *Drosophila* model and human PEO. Pol γ-α-F578A is a single



substitution in the γ3 element of the spacer region with reduced polymerase activity and processivity. It is stimulated by mtSSB at a lower level (~5 fold) than the wild-type holoenzyme (~30 fold) (see Chapter II). Further phenotypic analysis of this mutant may suggest potential Pol γ-α mutations causing human mitochondrial diseases. Pol γ-α-Y873C (Y955C in human) maps in the Pol active site motif B. Y955C has been found to be associated with human PEO. The conserved tyrosine residue contributes to the selection of incoming dNTPs (49,160,161) and displays a modest pol mutator phenotype *in vitro* (146). Pol γ-α-Y869F corresponds to Y951F in humans. Human pol γ-α-Y951F showed a >1000 fold increase in discrimination against ddNTPs and D4T-TP (162). A similar local network has been found in HIV-RT-Q151M that is found to be a HIV-resistant virus mutation. Therefore, pol γ-α-Y869F knock-in flies will be a good animal model for the study of mitochondrial toxicity of NRTIs (see Chapter IV).



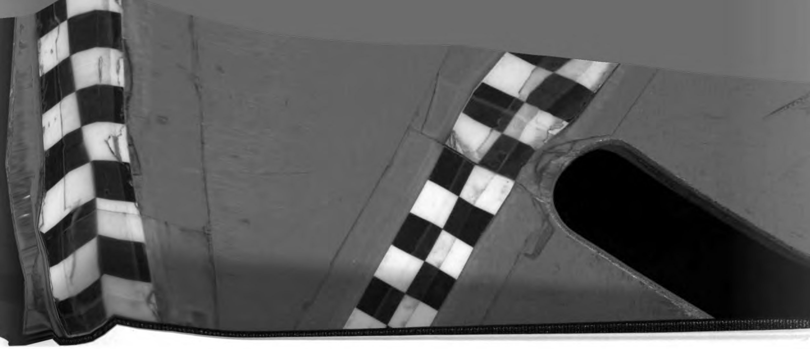
FUTURE PLANS

At this point, we have generated the target gene constructs and the corresponding transgenic flies that are now in the two-step crosses. After recovery of the single-gene knock-in lines, we will maintain them as heterozygous stocks using the appropriate balancing chromosomes, and then cross them to homozygosity to evaluate whether they are dominant or recessive mutations in *Drosophila*. We will evaluate development and life span of the obtained fly lines as described for the exonuclease mutator line. We will also characterize the physiological, biochemical, and molecular consequences of the introduced mutations as follows.

1. mtDNA analysis

Only a minimal group of critical proteins are required in the mtDNA replication process. One of the most common results after disrupting these genes is the depletion of mtDNA (67,123,148). PEO is a mitochondrial disease characterized by multiple mtDNA deletions in the patients. Recently, several mutations in pol γ-α have been found to be associated with PEO. Therefore, mtDNA is very likely to be impaired in the knock-in flies with mutant pol γ-α.

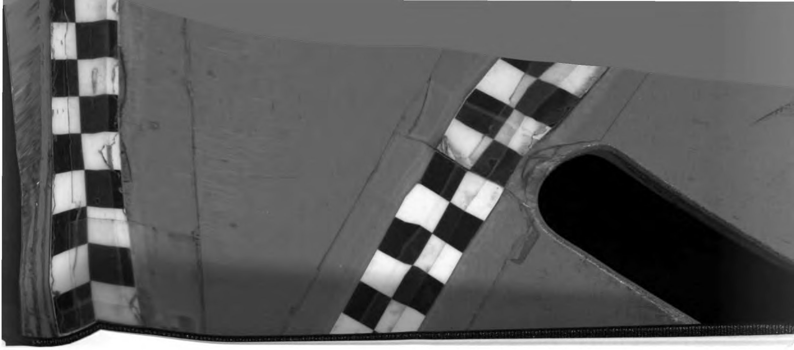
The quantity and integrity of mtDNA in these knock-in flies will be evaluated by Southern analysis. For short deletions or base mutations that are not detectable by Southern analysis, long PCR or sequencing analysis may be applied. The mutation load of the mtDNA will be specifically tested in the D185A/D263A and Y873C knock-in lines.



2. *Respiratory chain complex activity*

The fundamental role of mitochondria is in cellular energy metabolism, which includes fatty acid β oxidation, the urea cycle, and the final common pathway for ATP production – the respiratory chain (3). The mitochondrial respiratory chain is a group of five enzyme complexes and each of them is composed of multiple subunits encoded by nuclear and/or mitochondrial DNA. Reduced cofactors (NADH and $FADH_2$) generated from the intermediary metabolism of carbohydrates, proteins, and fats donate electrons to complex I and II. These electrons flow between the complexes and electrochemical gradient, shuttled by complexes III and IV and by two mobile electron carriers, ubiquinone and cytochrome *c*. The liberated energy is used by complexes I, III, and IV to pump protons out of the mitochondrial matrix into the intermembrane space. This proton gradient, which generates the bulk of the mitochondrial membrane potential, is harnessed by complex V to synthesis ATP from ADP. ATP must be released from the mitochondrion in exchange for cytosolic ADP. The OXPHOS capacity is strictly regulated during embryonic development and in different physiological situations. In many mitochondrial diseases, OXPHOS capacity is impaired. Therefore, respiratory chain complex activity is a good index to evaluate the function of mitochondria.

We will assess oxidative function directly by measuring the activities of the respiratory chain complexes: NADH-coenzyme Q1 reductase (complex I), succinate dehydrogenase (complex II), ubiquinol cytochrome c (complex III), cytochrome c oxidase (complex IV), ATP synthase (complex V) and citrate synthase (to normalize the mitochondrial mass).



3. *Mitochondrial distribution and function*

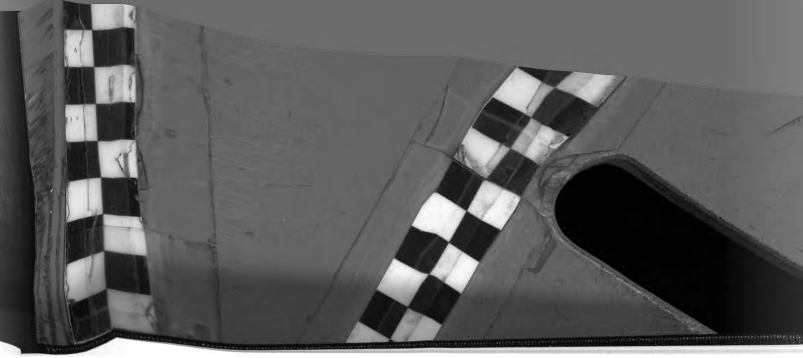
Different tissues and organs rely on mitochondrial energy generation to different extents. The central nervous system has the highest bio-energetic expression threshold (119). Many mitochondrial diseases present with neurological symptoms and signs.

We will evaluate the distribution and segregation of mitochondria by *in situ* hybridization to assess mitochondrial biogenesis. Specific antibodies to probe mesoderm (anti-twist) and the peripheral and central nervous system (anti-elav and –22C10) will be used to assess possible alterations in myogenesis and neurogenesis, respectively, during embryonic development.

4. *Behavior assays*

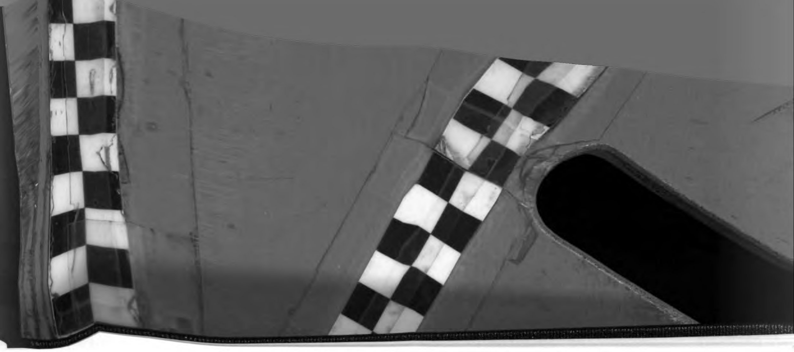
Several EMS-induced *Drosophila* recessive lethal lines with mutations in the catalytic subunit of pol γ displayed locomotory and phototactic deficits. Besides the three mutations that disrupt *Dm* pol γ-α, a single missense mutation at a non-active site residue also showed aberrant behavioral phenotypes (123). The developmental and behavioral phenotypes are concomitant with mtDNA depletion in moribund third instar larvae (Figure 5). Similar nervous system-related phenotypes are expected in the knock-in flies with mutations in the catalytic subunit of pol γ. Therefore, similar assays to those used by Iyengar and Campos will be carried out in our knock-in lines.

Based on the larval reaction to a light stimulus, the time required for larvae to move from a dark to a light quadrant will be measured to detect decreases in locomotion in larvae with defective pol γ-α (123). Also, locomotory behavior will be assayed under a



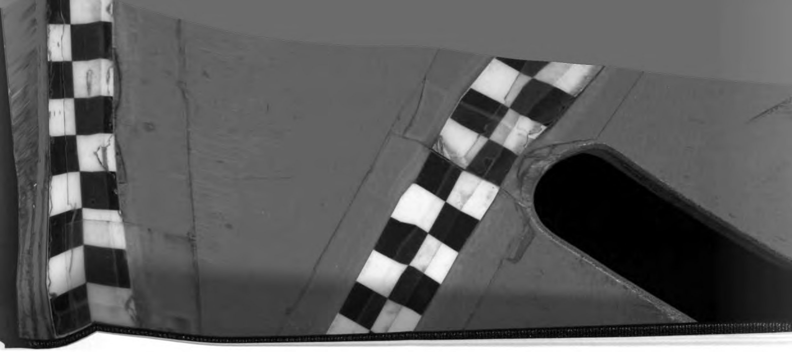
safe-light by measuring the distance traveled by the larva in 30 seconds using a semiautomatic tracking system (148).

To study adult locomotor activity, only female flies were used because the basal levels of locomotor activity differ significantly between male and female flies (163). A single fly will be aspirated into a 60-mm Petri dish with a transparent lid and a circular grid of 1-cm squares affixed to the lower surface of the dish. Each fly is allowed to recover for 30 s. Locomotor activity is observed for two consecutive periods for a total of 2 min each (0-2 min; 13-15min). The number of grid lines crossed during each observation period is recorded. A total of 20-50 flies will be assayed per experimental dose. The responses will be statistically evaluated by one way analysis of variance (ANOVA).



CHAPTER IV

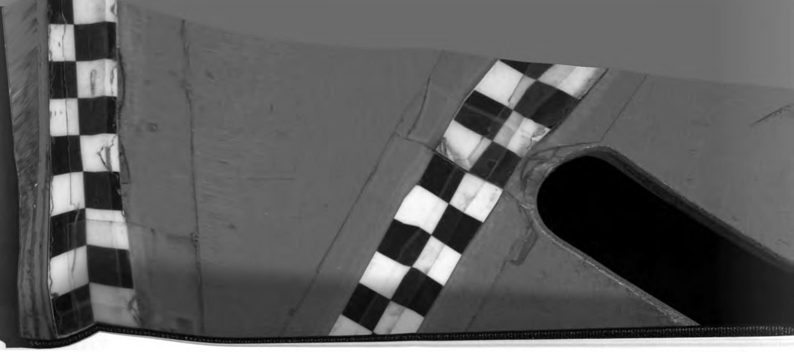
DEVELOPMENT OF A *DROSOPHILA* MODEL OF MITOCHONDRIAL TOXICITY IN ANTIVIRAL THERAPY



INTRODUCTION

The established nucleoside reverse transcriptase inhibitors (NRTIs) are a group of nucleoside analogs. They are one of the most popular treatments for the management of HIV in infected individuals (164). NRTIs resemble the natural nucleosides and can be incorporated into the viral genome by HIV-RT. They do not have a free 3' hydroxyl group; therefore, once they are added to a growing DNA chain, termination occurs. The first nucleoside analog to be approved for anti-HIV therapy was AZT, with the 3'-OH of ddTTP replaced by $-N_3$. Later, the dideoxynucleoside analogs ddI and ddC were also approved. However, the extensive use of NRTIs causes serious side effects. *In vitro* study has shown that one or more DNA polymerases may be inhibited by NRTIs, and Pol γ is the most sensitive of all DNA polymerases to NRTIs during the HIV drug treatment (164,165). mtDNA depletion and typical mitochondrial genetic diseases, including peripheral neuropathy, cardiac and skeletal muscle myopathy, pancreatitis, and bone marrow suppression, become key clinical observations after long-term NRTIs treatment. The inhibition of mtDNA replication by NRTIs triphosphates was also confirmed in transgenic AIDS mice (166). The acquired mitochondrial toxicity during HIV treatment may be caused by cellular uptake, transport, metabolic activation, incorporation/ removal from DNA, and/ or degradation from the system (167).

Further studies showed that NRTIs are substrates for human DNA pol γ as well as for HIV-RT (166). Different analogs inhibit pol γ to different extents. *In vitro* studies demonstrated that ddCTP and D4T-TP have a greater ability to inhibit pol γ and are more easily incorporated into DNA by pol γ. AZT-TP, 3TC-TP, and CBV-TP inhibit pol γ to a

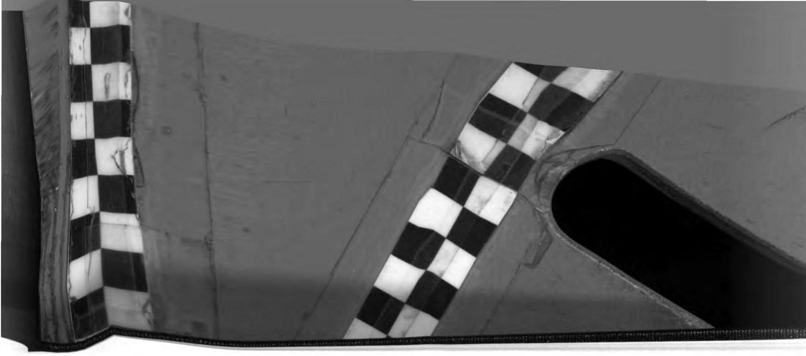


lesser, but still significant, degree and are only moderately incorporated. Pol γ does not remove other analogs except for 3TC as efficiently as the corresponding deoxynucleotides (168,169). The toxic side effects of NRTIs are correlated with the kinetics of incorporation by pol γ, varying over six orders of magnitude in the sequence zalcitabine (ddC) > didanosine (ddI/ddA) > stavudine (d4T) >> lamivudine (3TC) > tenofovir (PMPA) > zidovudine (AZT) > abacavir/carbovir (CBV) (169).

Direct analysis of mitochondrial toxicity of ddI was done by using human cell cultures. After exposing cell cultures with drugged media for four weeks, mitochondrial dysfunction was observed in pancreatic cells instead of hepatic cells (170). The result is consistent with the observation that the toxicity is cell-, tissue-, and organ-specific (166).

In vitro assays may easily guide the screening and search for new compounds, or eliminate the compounds with a high potential for toxicity during drug discovery processes (169). However, the action of drugs in vivo may differ from that observed in vitro because of differences of drugs in the rates of uptake, transport, metabolic activation, incorporation, and degradation *in vivo*. Therefore, extended toxicity studies in animal model systems are necessary for early drug discovery and screening. After long-term exposure to AZT, a dosage dependent decrease of mtDNA was observed in patas monkeys (171). However, the limited sample size makes the result less convincing, and the long life cycle of the animal slows down the whole process.

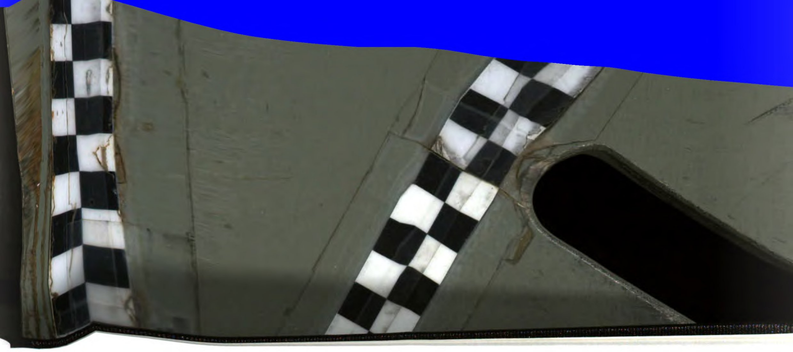
Drosophila is an excellent animal model for genetic studies because of its short lifetime, observable genetic markers, and ready-to-use techniques. Although the structure of the *Drosophila* brain does not resemble that of the human central nervous system, many studies show that molecular and behavioral patterns of responding to psychoactive



drugs in *Drosophila* are essentially indistinguishable from those in mammals (111). Drugs or reagents can be easily delivered to flies by feeding them food mixed with drugs or chemicals. Feeding flies with different DNA replication inhibitors was used to check the effects of various DNA-damaging and anti-cancer agents in the developing multicellular organisms (116). Feeding flies with drugged food was used for specific behavior changes in *Drosophila* (116,117). It has also been reported that feeding *Drosophila* 4-phenylbutyrate for 12 days significantly increases their lifespan (118). Therefore, *Drosophila* represents a convenient and applicable model organism for neuropharmacological research.

Cell energy production mainly occurs in mitochondria. When mitochondrial function is damaged, energy output may fall below the minimum requirement for normal tissue function. Because the central nervous system demands the most energy and is most sensitive to endogenous reactive oxygen species produced by mitochondria in the body, neuro-ophthalmologic defects become one of the most common observations in mitochondria dysfunction. Therefore, *Drosophila* may also serve as a good animal model for mitochondrial dysfunction studies. In fact, a direct test of the mitochondrial pathology was performed by using a *Drosophila* model (172).

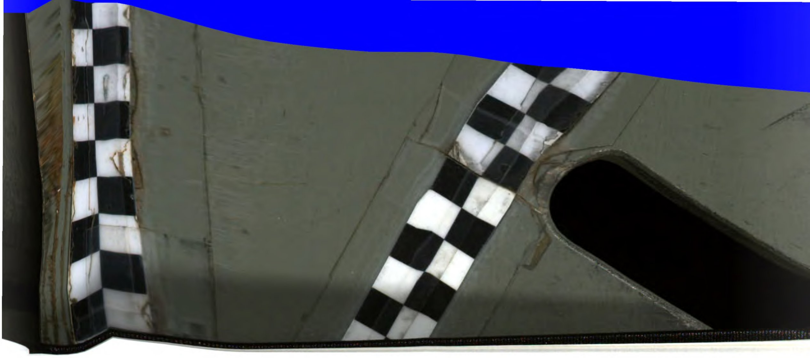
Pol γ is the sole DNA polymerase in mitochondria and the integrity of pol γ is essential for normal mitochondrial function. Mutations in human pol γ-α are reported to cause progressive external ophthalmoplegia (PEO), which is a group of mitochondrial disorders characterized by multiple mtDNA deletions (173). Flies with defects in pol γ-α present energy-depletion phenotypes and significantly reduced mtDNA content (Figure 4.1) (123).

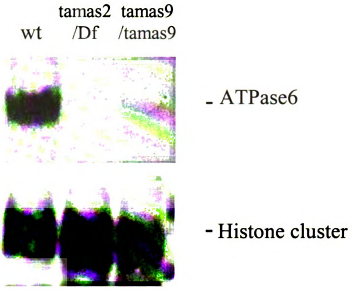


Incubation of cultured cells with media containing ddI/HU (hydroxyurea) caused obvious mitochondrial dysfunction (170). To test if *Drosophila* is sensitive to NRTIs, we delivered ddI/HU throughout *y/w* larvae and continued to feed the adult flies for four weeks. The mtDNA content in the treated flies was quantitated by Southern analysis and no significant mtDNA reduction was found after ddI/HU treatment. This may be because ddI is instable at low pH conditions. In late 2000, a capsule containing enteric-coated beadlets started to be used in clinics. In addition, ddI is suggested to be taken on an empty stomach, one hour before or two hours after a meal (*www.hivinsite.ucsf.edu*).

Compared with other nucleoside analogs, ddC appears to be a relatively strong inhibitor of pol γ *in vitro* (168,169). In addition, Q151M in HIV-RT is one of the major mutations associated with the resistance to multiple nucleoside analogs, including ddC (174). Therefore, ddC was used to study mitochondrial toxicity of NRTIs in *Drosophila*.

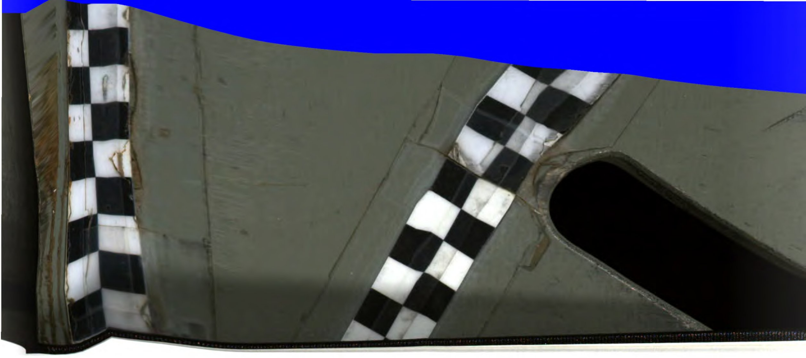
Approximately 100 embryos were collected overnight from *y/w* flies and incubated in 2 ml standard corn meal agar medium with 0, 1, 10, 100, and 500 μM ddC, respectively. The hatched larvae were maintained at 25°C with a light/dark cycle until new adult flies eclosed. Newly eclosed flies were transferred to fresh vials every 2-3 days, and adult flies from each treatment were collected every week for three weeks. Meanwhile, newly eclosed adult flies were fed with drugged medium containing 0, 1, 10, 100, and 500 μM ddC directly without the feeding of the larval period. Total DNA was prepared from approximately 10-20 flies from each treatment, and then digested with *Xho*I. DNA was hybridized with a mtDNA probe (ATPase6), and with a multiple-copy genomic probe (histone cluster) as a control (Figure 4.2). Multiple analyses of mtDNA content from each treatment indicated that mtDNA content decreases with increasing ddC





tamas9 - spacer region, E595A
tamas2 - pol region, E814V

Figure 4.1. **Mitochondrial DNA is absent in mutant of the catalytic subunit of *Drosophila* Pol γ.** Three micrograms of total DNA that was isolated from third instar larvae was digested with *Xho*I, which cleaves mtDNA once; duplicate samples of the DNAs (1 μg) were electrophoresed in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled DNA probes from the ATPase 6 gene of mtDNA and a repeated histone gene cluster as a nuclear DNA control.



dosage.

In clinics, the prolonged use of NRTIs has also resulted in the emergence of NRTIs-resistant viruses (85). For example, a set of six specific substitutions on HIV-RT gave rise to high levels of AZT resistance and this mutant RT was able to catalyze a primer-unblocking reaction related to pyrophosphorolysis for removing the chain-terminating AZTMP. Mutant HIV-RT with single substitution Q151M also exhibited multidrug resistance. The resistance of this mutant HIV-RT is because of an altered recognition of incoming nucleotide analogs. Nucleotide analogs with a modification on their α-phosphate by a borano (BH_3^-) group could overcome the resistance of HIV-RT-Q151M, and recover its sensitivity to these NRTIs (175).

Human pol γ-α-Y951 is critically important for the selection of dideoxynucleotides and D4T-TP by pol γ (162). The mutation of Y951 to F showed a >1000 fold increase in discrimination against ddNTP and D4T-TP (162). Although HIV-RT shares limited conservation with pol γ, a similar local hydrogen bonding network that promotes discrimination of ddNTP by HIV-RT, harboring the Q151M alteration, has been suggested (175).

Human Y951 is located in the second polymerase motif in pol γ-α and is conserved among different organisms. In *Drosophila*, the corresponding site is Y869. Knock-in *Drosophila* with Y869F is being generated as described in Chapter III-C. Our prediction is that the Y869F knock-in flies would be an excellent animal model to study the resistance of mitochondrial toxicity of NRTIs. Similar to the resistance of the HIV virus carrying Q151M in RT, the incorporation of ddC to mtDNA by pol γ-αY869F will

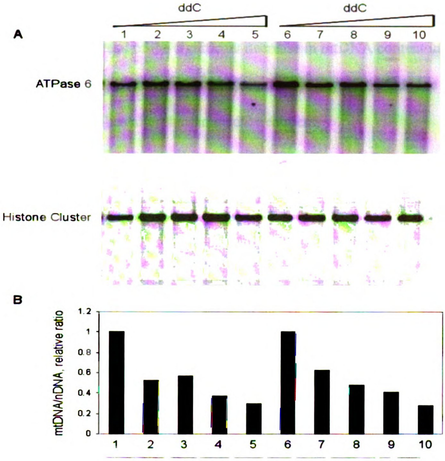
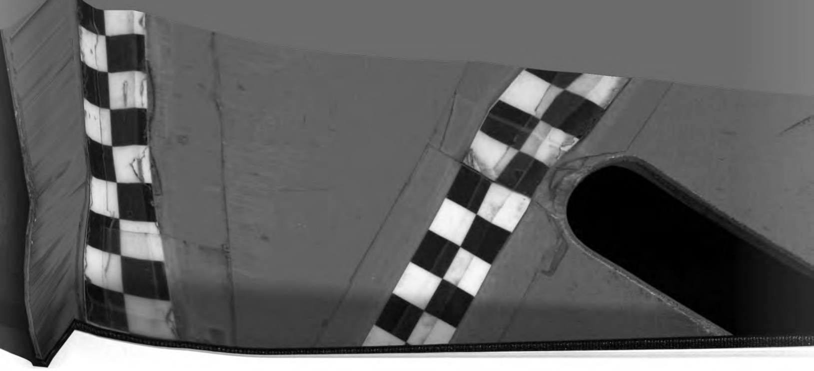
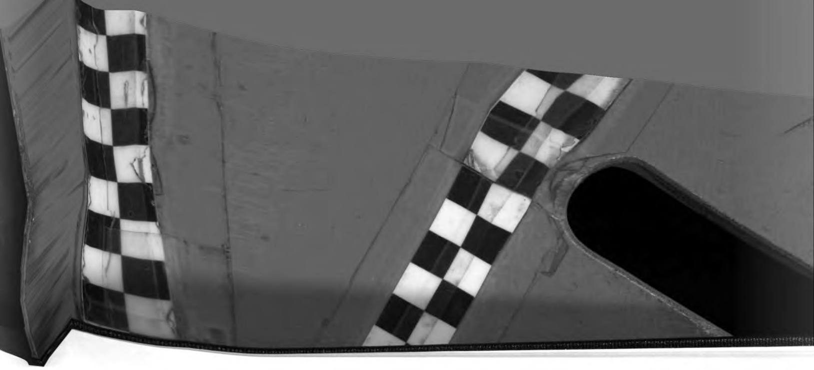


Figure 4.2. **Mitochondrial DNA content is reduced in *Drosophila* after ddC treatment.** A, three micrograms of total DNA was isolated from flies that were fed with drugged medium containing 0, 1, 10, 100, and 500 µM ddC, respectively, as described in the text. DNA was digested with *Xho*I and electrophoresed in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P-labeled DNA probes from the ATPase 6 gene of mtDNA and a repeated histone gene cluster as a nuclear DNA control. B, quantification data from panel A, the value for the flies without ddC treatment was taken as 1. *Lane1-5*, flies were fed throughout larval period and the adult flies were continually fed for three weeks; *lane6-10*, adult flies were fed for 3 weeks directly.



be significantly reduced in the knock-in flies. Therefore, the generated mutant mtDNA in the Y869F knock-in flies may be much less than that in the *y/w* control flies after long-term ddC treatment. Instead of the significant decrease in mtDNA content in *y/w* flies as seen in Figure 4.2, subtle decrease in mtDNA content, if any, is predicted to be observed in the Y869F knock-in flies after the same ddC treatment.



FUTURE PLANS

The development and life span of *y/w* and Y869F knock-in fly lines with ddC treatment will be evaluated. Also, the physiological, biochemical, and molecular consequences of the ddC treatment and the introduced mutation Y869F will be characterized as follows.

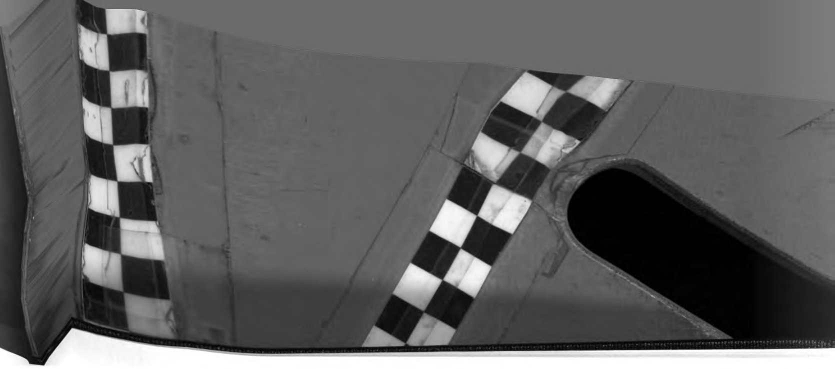
1. Feeding flies with drugged food

Approximately 100 embryos will be collected overnight from *y/w* flies and incubated in 2 ml standard corn meal agar medium with 0, 1, 10, 100, and 500 μM ddC, respectively. The hatched larvae will be maintained at 25°C with a light/dark cycle until the eclose of new flies. Newly eclosed flies will be transferred to fresh vials every 2-3 days, and adult flies from each treatment will be collected every week for three weeks.

To examine the effect of ddC in adult flies, newly eclosed flies will be collected and maintained in 500 μl standard corn meal agar medium containing 0, 1, 10, 100, and 500 μM ddC, respectively, at 25°C with a light/dark cycle. Flies will be transferred to fresh vials every 2-3 days, and flies from each treatment will be collected every week for three weeks.

2. Southern blot analysis

Approximately 10-20 flies from each sample will be used to prepare total DNA, and Southern blots will be performed to measure the mtDNA content in flies from each



treatment. After digestion with *Xho*I, DNA will be hybridized with a mtDNA probe (ATPase6), and with a multiple-copy genomic probe (histone cluster) as a control.

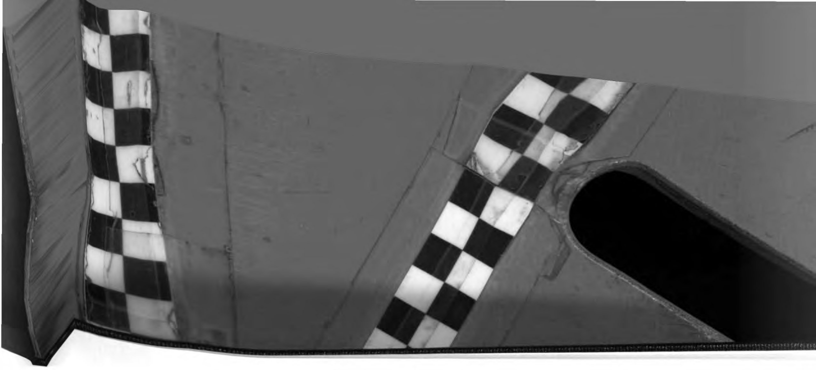
According to the results from Southern blot analysis, the standard treatment with fixed drug concentration (e.g., 100 μM) and feeding period (e.g., two weeks) will be determined for the rest of the assays.

3. Longevity assay

Newly eclosed flies will be fed as described above throughout their lifetime with standard fly media with or without ddC (e.g., 100 μM). For each test group, 10 vials, each containing 20 flies, will be maintained at 25°C with a light/dark cycle and transferred to fresh vials every 2-3 days. The number of dead flies will be counted every day (118).

4. Climbing assays

Approximately 20-30 flies will be placed into the first chamber, and the chamber will be tapped to keep the flies at the bottom, then the flies will be given 30 s to climb. Flies that successfully climb 10 cm or higher in 30 s will be transferred to a new chamber, and both sets of flies are given another opportunity to climb the 10-cm distance. This procedure is repeated a total of five times. The number of flies in each chamber will be counted. The climbing index will be calculated. At least 60 flies will be used for each experimental group (172). Statistical analysis, such as Student's *t* test or χ^2 test, will be used to compare the control group (without ddC treatment) and the test group (with ddC treatment).



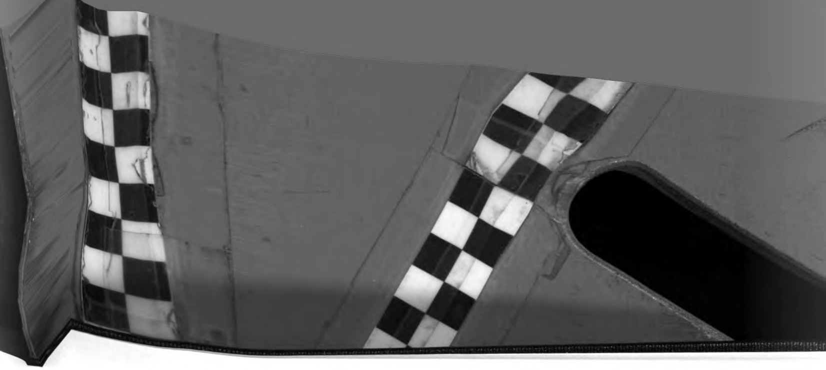
5. *Locomotor activity assay*

Due to the larval reaction to a light stimulus, the time required for larvae to move from a dark to a light quadrant will be measured. The decreases in locomotion in larvae with defective pol γ-α are expected (123). Also, locomotory behavior will be analyzed under a safe-light by measuring the distance traveled by larvae in 30 s using a semiautomatic tracking system (148).

To study locomotor activity of adult flies, only female or male flies will be used because the basal levels of locomotor activity differ significantly between male and female flies (163). A single fly will be aspirated into a 60-mm Petri dish with a transparent lid and a circular grid of 1-cm squares affixed to the lower surface of the dish. Each fly is allowed to recover for 30 s. Locomotor activity is observed for two consecutive periods, for a total of 2 min each (0-2nd min and 13-15th min). The number of grid lines crossed during each observation period is recorded. A total of 20-50 flies will be analyzed from each ddC treatment group. The responses will be statistically evaluated by one way analysis of variance (ANOVA).

6. *Mitochondrial staining*

The pattern of distribution of mitochondria in the central nervous system is reported to be severely disrupted in *Drosophila* carrying a pol γ-α mutation (123). To visualize mitochondrial mass in drug-treated *Drosophila*, a similar experiment will be performed. A mitotracker probe (Mitotracker Red CMXRos) will be made at 100 nM in PBS. The *Drosophila* brains will be dissected in PBS, then incubated in the staining

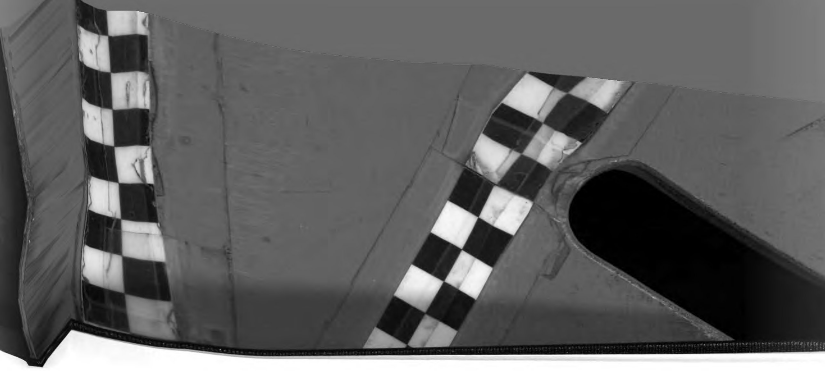


solution for 10 min at room temperature and mounted for observation using fluorescein filters (123).

7. *Oxygen consumption assays*

Oxygen consumption will be analyzed using the isolated mitochondria from ddC treated flies to detect if there is any mitochondrial functional change. Enzyme and protein assays for the specific activities of OXPHOS enzymes Complexes I, II, III, and IV will also be performed to check if the affect is at any specific stage(s).

There are several comparisons to be made in this project: 1) comparison of *y/w* flies with and without ddC treatment; 2) comparison of Y869F knock-in flies with and without ddC treatment; 3) comparison of *y/w* and Y869F knock-in flies without ddC treatment; 4) comparison of *y/w* and Y869F knock-in flies with ddC treatment. In all above cases, appropriate statistical analysis such as student's t test and χ^2 test will be applied.



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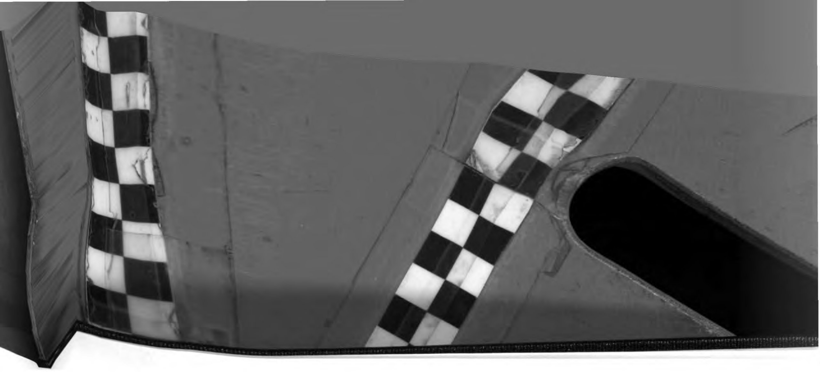

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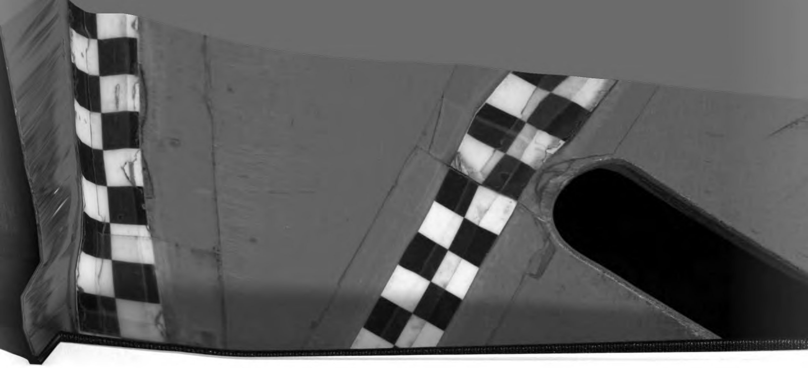

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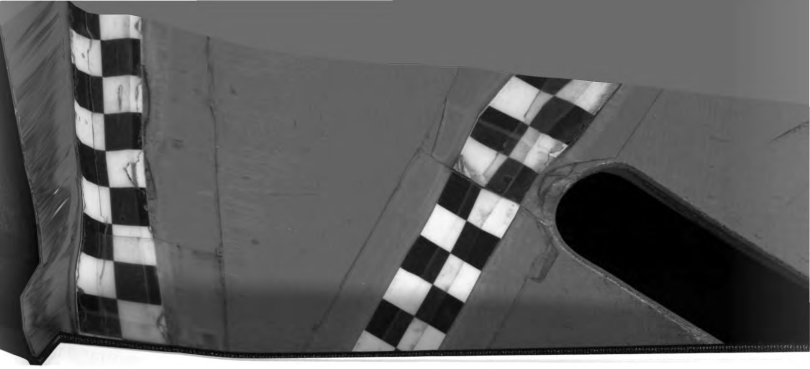

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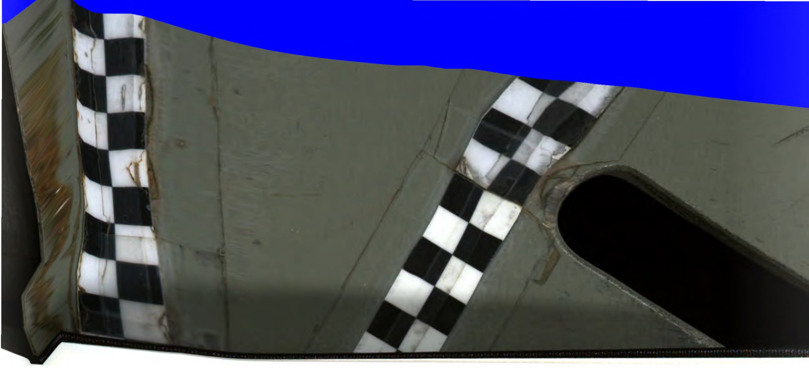

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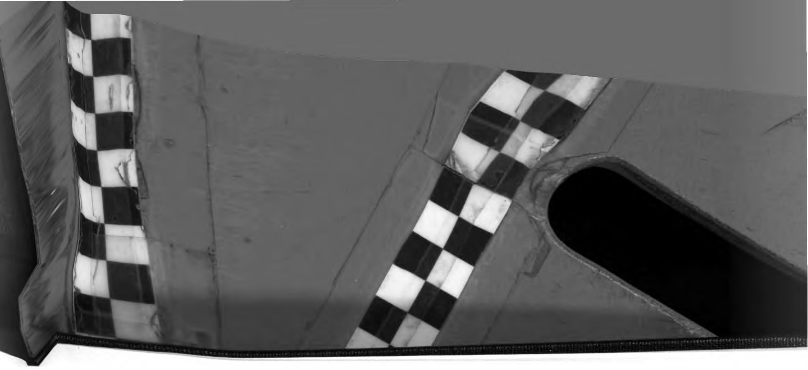

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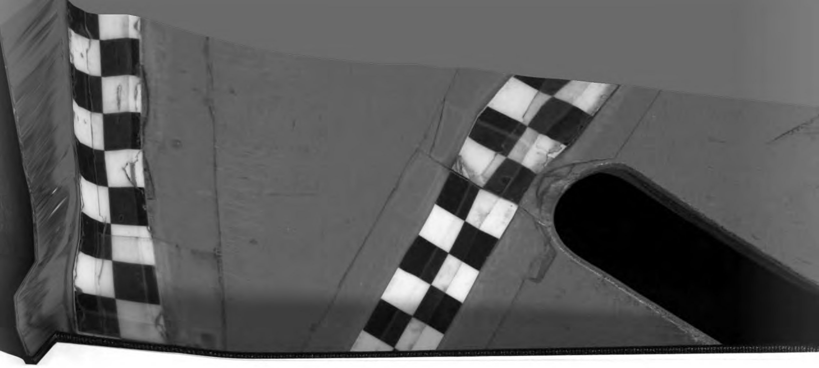

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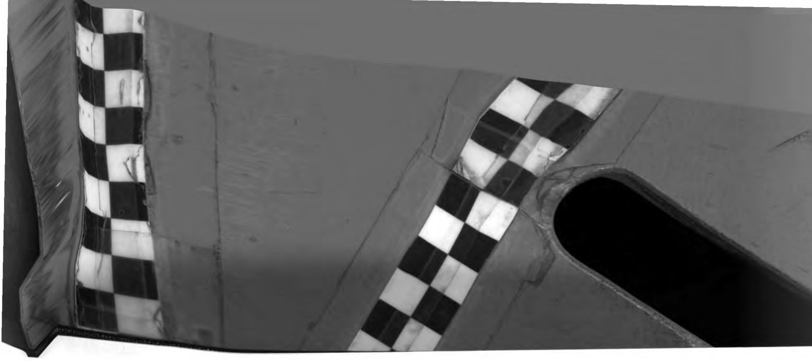

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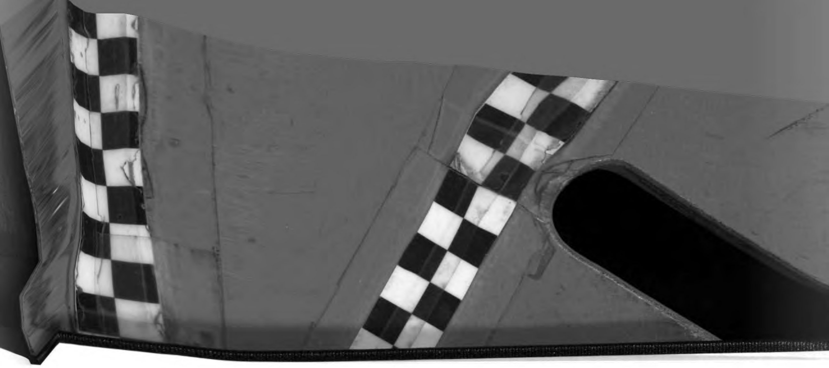

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