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DROSOPHILA POL γ AND ITS RECOMBINANT CATALYTIC AND ACCESSORY SUBUNITS: EFFECTS OF REACTION CONDITIONS AND *DROSOPHILA* MITOCHONDRIAL SINGLE-STRANDED DNA-BINDING PROTEIN

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ABSTRACT

DROSOPHILA POL γ AND ITS RECOMBINANT CATALYTIC AND ACCESSORY SUBUNITS: EFFECTS OF REACTION CONDITIONS AND *DROSOPHILA* MITOCHONDRIAL SINGLE-STRANDED DNA-BINDING PROTEIN

by

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We have purified the mitochondrial single-stranded DNA-binding protein (mtSSB) from *Drosophila* embryos and as an active recombinant protein overexpressed in bacteria. The identity of the mtSSB was confirmed by its isolation from a mitochondrial fraction, N-terminal amino acid sequencing and by comparison of primary amino acid sequences, that show *Drosophila* mtSSB shares a high degree of amino acid sequence similarity with other known mtSSBs . We examined the effects of mtSSB on DNA synthesis by *Drosophila* mitochondrial DNA polymerase (Pol γ). We found that mtSSB stimulates the overall rate of DNA synthesis by Pol γ, by increasing primer recognition and binding and by destabilizing DNA secondary structures. Template-primer binding and enzyme idling experiments revealed dramatic stabilization of Pol γ:DNA interactions at low ionic strength in the presence or absence of mtSSB.

In addition, we purified the recombinant catalytic (α) and accessory (β) subunits of Pol γ overexpressed in bacteria. We found that both 5' → 3' DNA polymerase and 3' → 5' exonuclease activities cosediment with the catalytic polypeptide in glycerol gradient sedimentation analyses. Using subunit-specific antisera in immunoprecipitation analyses, we demonstrated that the Pol γ holoenzyme is composed of both the α and β subunits.

To Alexis and Jasmine.

"To doubt everything or to believe everything are two equally convenient solutions; both dispense with the necessity of reflection."
Jules Henri Poincaré

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Mitochondria

The mitochondrion is the energy producing organelle of the eukaryotic cell. According to the endosymbiotic hypothesis, the mitochondrion originated with the formation of permanent symbiosis between a bacterum and the primitive eukaryotic cell (1). The mitochondrion is the major source of energy for the eukaryotic cell. Mitochondria produce energy through the complete oxidation of carbohydrates, fatty acids and amino acids releasing this energy in the chemical form of ATP, through the process called oxidative phosphorylation. Like the nucleus, the mitochondrion is surrounded by a double membrane and its assembly is an intricate multistage process (2). The outer mitochondrial membrane resembles the outer membrane of the endoplasmic reticulum while the inner mitochondrial membrane folds into tightly packed invaginations called cristae.

The mitochondrion is the only extranuclear organelle in animal cells that contains multiple copies of its own genome, which encodes many proteins necessary for oxidative phosphorylation and mitochondrial protein synthesis. Biogenesis of mitochondria depends on the coordinated expression of nuclear and mitochondrial DNAs (3) and during cell proliferation mitochodrial DNA (mt DNA), mitochondrial membranes and mitochondrial proteins must be reduplicated. Moreover, fluctuations in metabolite concentrations within the mitochondrion can dramatically effect mitochondrial functions.

Mitochondrial DNA

The mitochondrial genome (mt genome) is separate from that of the nucleus, in that it may be replicated often during S and early G2 phases of the cell cycle (3), while the nuclear genome undergoes one round of replication in S phase per mitotic cell division. Metazoan mitochondrial DNAs vary in size from 14 to 39 kb (4). The complete sequence of the mt genomes for various mammalian species including human, gorilla, rat, mouse, cow, horse, fin whale, blue whale, harbor seal, grey seal and American opposum (5-10); for an amphibian (11); for three fish (12-14); one bird (15); three sea urchins (16); a sea lamprey (17); an earthworm (18); two nematodes (19); a chiton (20); a crustacean (21); and for six insects, a locust (22); three fruit flies (23-25); a bumble bee (26) and two mosquitoes (27) have been determined.

The mt genome is a compact structure with a single non-coding region. It contains the origin of replication, the promoters for transcription, and encodes thirteen polypeptides required for electron transport and oxidative phosphorylation. In addition, the mt genome encodes twenty-two tRNAs and two rRNAs required for mitochondrial protein synthesis (25). Efficient expression of these genes is essential for proper mitochondrial function and though the order of mt genes in insects varies somewhat, the total gene content is invariant (23-28).

The mt genomes of the *Drosophila* subfamily of insects vary in size from 14 to 19 kb, with the largest, at 19,517 bp, from *Drosophila melanogaster* (25). Most of the sequence length variation in mitochondrial DNA arises from variations in the length of the unique non-coding A + T-rich region (A + T region). The A + T region, so named due to its >95% A + T content (29) is the only non-coding region in the *Drosophila* mt genome. It contains both the origin of replication and presumably the promoters for transcription. Variations in the A + T region of *Drosophila* arose from the repetition of direct or inverted repeats of consereved DNA sequence elements (29).

Though replication of the mt genome is independent of nuclear genome replication, maintenance and expression of the mt genome are dependent upon nuclear encoded proteins to carry out replication, transcription and translation. Only 5–15% of the total mitochondrial protein is synthesized on mitochondrial ribosomes, while most of the mitochondrial proteins are synthesized on cytoplasmic ribosomes and co-translationally or post-translationally imported into the mitochondrion (2). Primary mutations in the mt genome, which impair respiration, are responsible for over 100 human diseases (30). Of these 100, seven syndromes have contributed the most to the understanding of mitochondrial medicine: KSS, Kearns-Sayers syndrome; MERRF, myoclonus epilepsy and ragged red fibers (syndrome); LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke like episodes; Leigh syndrome; CPEO, chronic progressive external opthalmoplegia; Alper syndrome (30). In addition, mitochondrial DNA mutations have also been implicated in aging and in many age-related diseases such as Parkinson, Alzheimer and Huntington's diseases, amyotrophic lateral sclerosis and cardiomyopathies, athero-sclerosis, and diabetes milletus (30). Therefore, the study of the mechanism of replication, transcription and translation of mitochondrial genes is essential for understanding the development, maintenance and proliferation of mutations in the mt genome.

DNA Replication

Faithful and efficient duplication of nuclear and mitochondrial genomes is essential for cell proliferation and energy metabolism in all eukaryotic cells. Fundamental studies in prokaryotes, such as *Escherichia coli* (*E. coli*), and in bacteriophage and viruses have contributed to understanding the mechanisms of nuclear and mitochondrial DNA replication in eukaryotes. Characterization of the biochemical processes of DNA replication is an

essential component of understanding the growth and development of organisms and in understanding various disease states.

The general mechanism of replication is conserved from the prokaryotic bacteria to eukaryotic organisms, including humans, and has the same general requirements (for reviews see (31-33)). Replication minimally requires recognition of an origin of replication, initiation of replication through the synthesis of primers, elongation of primers to duplicate the genome, termination of replication and separation of the daughter molecules. Replication of chromosomal and mitochondrial DNAs requires many protein-protein and protein-DNA interactions.

Origins of replication are recognized as repeated DNA sequence elements and are characterized as regions of high deoxyadenylate and thymidylate content allowing easy melting of the duplex DNA strands. Sequence specific initiation proteins recognize and bind the chromosomal origin of replication inducing localized unwinding of the duplex DNA strands. Sequence specific initiation proteins, such as those found in the nucleus of bacteria, have not yet been identified in mitochondria, though the origin of mitochondrial DNA replication has been mapped (23) and repeated sequence elements are present.

Unwinding of the origin allows DNA helicase to bind and unwind the duplex DNA strands. Unwound single strands of DNA are then prevented from reassociating and protected from nucleolytic degradation by the coating action of single-stranded DNA-binding protein (SSB). The open complex allows the loading of a DNA primase, alone or in association with a DNA polymerase, which catalyzes the synthesis of a short ribo- or deoxyribo oligonucleotide containing a 3'-OH tail. The replicative DNA polymerase, which cannot initiate *de novo* DNA synthesis, then uses this short oligonucleotide primer to initiate DNA synthesis of the genome. Elongation by the replicative DNA polymerase proceeds in the 5' $\rightarrow$ 3' direction with the incorporation of deoxyribonucleoside monophosphates. Deoxyribonucleoside monophosphate incorporation proceeds through the formation of a phosphodiester bond, by attack of the primer strand 3'-OH on the 5'-α-

phosphate of an incoming deoxyribonucleoside triphosphate and pairing of a nucleotide base complementary to that on the SSB coated single-stranded DNA template.

Leading strand replication proceeds continuously along the DNA template following the advancing replication fork(s) until the entire genome has been replicated, while lagging strand replication (Okazaki fragment replication) proceeds discontinuously in the direction opposite to movement of the replication fork. As replication proceeds the duplex DNA is unwound at the replication fork by the action of DNA helicase and torsional strain introduced ahead of the replication fork is alleviated by the action of DNA topoisomerases. Replication is terminated with the recognition of termination sequences or by the completion of the genome. The replicative DNA polymerase dissociates and the daughter molecules are completed with the removal of oligonucleotide primers to complete the newly replicated strands. Concatenated strands are separated into two new daughter strands with the aide of topoisomerases and cell division, virus or bateriophage packaging or mitochondrial division occurs.

Replication of nuclear and mitochondrial genomes follows the same general mechanism. Replication can be conservative, with the new molecules resembling the parental strand exactly, as in viral or bacteriophage DNA replication or it may be semiconservative, as in replication of double-stranded DNA templates with the completed molecules representing a combination of parental and daughter strands. Three major modes of replication are: continuous strand synthesis, with initiation at a single site and proceeding uni-directionally through multiple rounds as in rolling circle replication; semi discontinuous, proceeding uni- or bidirectionally with long stretches of leading strand synthesis and short stretches of lagging (Okazaki) strand synthesis and terminating with the duplication of each parental strand; or discontinuous with short stretches of leading and lagging (Okazaki) strand synthesis and terminating with the duplication of each parental strand.

Replication by DNA-dependent DNA polymerases in prokaryotes and eukaryotes follows the general mechanism of nucleotide incorporation and chain elongation described above, but variations in the replicative DNA polymerases and their accessory proteins account for a variety of models for DNA replication. Ito and Braithwaite have divided the greater than forty DNA polymerases, with deduced amino acid sequences reported, into three families: A, B and C, based on amino acid sequence homologies with *E. coli* DNA polymerases I, II and III, respectively (34). Family A includes not only the type I bacterial DNA polymerases, but also the T-odd bacteriophage DNA polymerases and all mitochondrial DNA polymerases (34). All of the family A DNA polymerases are sensitive to dideoxynucleotide inhibitors and all are resistant to aphidicolin. Family B DNA polymerases are extensive in number and variety with most but not all sensitive to aphidicolin and resistant to dideoxynucleotide inhibitors. Family C DNA polymerases are the major bacterial replicative DNA polymerases, which do not have appreciable homology with those of the family A or B DNA polymerases.

DNA Replication Proteins

Though understanding of the mechanisms of nuclear and mitochondrial genome duplication is incomplete, much has been learned from studies of bacterial and bacteriophage replication systems, especially in *E. coli* and T4 and T7 bacteriophage. Prokaryotic *E. coli* DNA polymerase I, bacteriophage T4 (gene 43) DNA polymerase and bacteriophage T7 (gene 5) DNA polymerase, are perhaps the best understood due to the large foundation of work on these systems.

Chromosomal replication in *E. coli* is carried out by *E. coli* DNA polymerase I, the first DNA polymerase discovered (33), and *E. coli* DNA polymerase III holoenzyme. *E. coli* DNA polymerase I (Pol I) is the 103 kDa single polypeptide gene product of the pol A gene and it contains three catalytic activities: 5' $\rightarrow$ 3' DNA polymerase, for

deoxyribonucleoside monophosphate incorporation; 3' → 5' exonuclease activity for proofreading misincorporated nucleotides during chain elongation; and 5' → 3' exonuclease activity for the removal of oligonucleotide primers or for repair of damaged or distorted sections of DNA (33, 35). A striking feature of the family A polymerase Pol I is its ability to promote strand displacement replication in the absence of accessory proteins (33). Other polymerases known to advance the replicating fork require accessory proteins to bind the template DNA, unwind duplex or bind the unwound ssDNA. Careful studies of mutants of the pol A gene revealed that Pol I functions not only in excision and replicative repair of DNA lesions, but it is also essential for the removal of Okazaki fragments created during discontinuous semiconservative replication of the *E. coli* chromosome (33).

E. coli DNA polymerase I can be cleaved, by trypsinolysis, into two distinct polypeptides: a 68 kDa polypeptide containing the 5' → 3' DNA polymerase and 3' → 5' exonuclease activities, known as the Klenow fragment and a 35 kDa polypeptide containing only the 5' → 3' exonuclease activity (36, 37). The three-dimensional crystal structure of *E. coli* DNA Pol I Klenow fragment has been solved to high resolution (35, 38, 39). Crystal structure data shows that the enzyme is folded into two distinct structural domains, an amino-terminal domain containing the 3' → 5' exonuclease active site and a larger carboxy-terminal polymerase domain. Studies of mutations in the exonuclease domains of Pol I Klenow fragment have identified critically important aspartate residues involved in contacting the two Mg^{2+} ion cofactors and a phenylalanine residue important for deoxyribonucleoside triphosphate selection (40), all of which are conserved in family A DNA polymerases.

T4 bacteriophage DNA polymerase (T4 DNA Pol) belongs to the family B DNA polymerases and it is responsible for the replication of the linear T4 bacteriophage double-stranded DNA (dsDNA) genome (33). T4 DNA Pol is the 104 kDa polypeptide product of the T4 gene 43 and has both 5' → 3' DNA polymerase and 3' → 5' proofreading exonuclease activities. Unlike *E. coli* Pol I, T4 DNA Pol cannot catalyze strand

displacement DNA synthesis on nicked dsDNA in the absence of its accessory proteins, it prefers instead to utilize primed single-stranded DNA (ssDNA) templates (33).

T4 DNA polymerase holoenzyme can be divided into three components: the gene 43 DNA polymerase, the 77 kDa gene 45 DNA polymerase processivity factor, and the 164 kDa gene 44/62 DNA polymerase accessory protein complex (41). The gene 45 product is similar to the β-subunit of *E. coli* DNA polymerase III holoenzyme (42) and the eukaryotic proliferating cell nuclear antigen (PCNA) and tethers the T4 DNA Pol to the template. The gene 44/62 complex is similar to the γ complex of *E. coli* DNA polymerase III holoenzyme and the replication factor C (RF-C) complex in yeast and human DNA replication systems and it contains a potent DNA dependent ATP-ase activity (42, 43). The gene 44/62 accessory complex only interacts with T4 DNA Pol in the presence of the gene 45 protein to form the holoenzyme (42). T4 DNA Pol holoenzyme is barely stimulated in the presence of the T4 gene 32 product, a single-stranded DNA-binding protein (T4 SSB), which coats the single strand displaced during replication of the leading strand (44-47).

Bacteriophage T7 DNA polymerase (T7 DNA Pol) differs from T4 DNA Pol in that it is a family A DNA polymerase sharing sequence similarity with *E. coli* Pol I (34, 35) and it contains both 5' → 3' DNA polymerase and 3' → 5' exonuclease activities. T7 DNA Pol is unique in that it requires the host protein thioredoxin for its full activation (48, 49). T7 DNA Pol is a heterodimer, composed of an 80 kDa catalytic polypeptide (T7 gene 5 product) in 1:1 stoichiometry with its 12 kDa processivity factor (*E. coli* thioredoxin) (34, 50-52), that catalyzes bi-directional DNA synthesis on the linear bacteriophage T7 DNA (52).

T7 DNA Pol resembles T4 DNA polymerase in that it cannot perform strand displacement replication in the absence of the T7 gene 4 product, which acts similarly to the 44/62 accessory protein complex of T4 DNA polymerase to allow strand displacement when T7 DNA polymerase is presented with a nicked dsDNA (34). While both *E. coli* DNA Pol I and T4 DNA Pol are stimulated weakly by their cognate SSBs, primer

extension by T7 DNA Pol is stimulated in the presence of *E. coli* SSB. In addition mutants in the T7 gene 2.5 (T7 SSB) do not grow on *E. coli* strains that lack *E. coli* SSB suggesting the presence of an active SSB is required for the growth of the virus (53).

Eukaryotic DNA polymerases are of two classes, nuclear and mitochondrial. Nuclear DNA polymerases α, δ and ε (Pol α, Pol δ and Pol ε) are required for duplication of the chromosomal DNA, while DNA polymerase β (Pol β) and Pol ε have been implicated in DNA repair (34, 54). Mitochondrial DNA polymerase (Pol γ) is the only known DNA polymerase involved in the replication of the mt genome (68).

The eukaryotic nuclear DNA polymerase α is a member of the family B DNA polymerases and it exists as a four-subunit complex composed of the 180 kDa catalytic subunit containing both the DNA polymerase and 3' → 5' exonuclease activities in tight association with the 48 kDa DNA primase subunit. The functions of the 58 and 70 kDa subunits are unknown. (55). Pol α with its tightly associated DNA primase activity is required for synthesis and extension of primers for leading and lagging strand DNA synthesis in the nucleus.

Elongation of the leading and lagging strands is completed by the action of the Pol δ replication complex. Human Pol δ is isolated as a 170 kDa catalytic subunit and contains both a 5' → 3' DNA polymerase and proofreading 3' → 5' exonuclease activities (56). Pol δ has also been isolated from calf thymus as a single 170 kDa polypeptide (57) and as a heterodimer from *Drosophila* (185 kDa) and mouse (175 kDa) (58, 59). The heterodimeric Pol δ combines with its processivity factor PCNA and RF-C to form the complex that functions in replication of leading strand DNA synthesis and completion of Okazaki fragments initiated by Pol α-primase (60).

Pol ε is a ~200 kDa single polypeptide isolated from human and calf thymus and it contains both a highly processive DNA polymerase activity and a 3' → 5' exonuclease activity. It has been shown to potentially play a role in both DNA replication (61) and DNA repair (62).

DNA polymerase β isolated from calf thymus, cultured human cells and rat is a low molecular weight DNA polymerase of ~40 kDa (34, 60), while Pol β from *Drosophila* is a 110 kDa single polypeptide (61) . Both forms of Pol β contain a non-processive DNA polymerase activity. Exonuclease activity has been shown only in fractions isolated from rodent liver, which contain a 12.5 kDa protein with both 3' → 5' and 5' → 3' exonuclease activities (34).

Of the five DNA polymerases identified in eukaryotic cells Pol α, β, δ, ε and γ, Pol γ is the least abundant and most poorly characterized. Pol γ comprises less than 1% of the total DNA polymerase activity in the cell (63) and it is differentiated from the other eukaryotic DNA polymerases by its sensitivity to both dideoxynucleoside triphosphates and the sulfhydryl-group blocking-agent N-ethylmaleimide (NEM) (63). Pol γ has been identified in many species varying from Baker's yeast (*S. cerevisiae*) to humans (64-71) and though it has been partially purified from a few sources including frog, pig, chick and human it has been highly purified from only a single source, *Drosophila melanogaster* (63).

Pol γ from *Drosophila* embryos is a 160 kDa heterodimer comprised of a 125 kDa catalytic α subunit and a 35 kDa β subunit of undetermined function (63) . Both the 5' → 3' DNA polymerase activity, determined by *in situ* DNA polymerase assay (72) and *in vitro* assay of the recombinant α-subunit (69), and 3' → 5' proofreading exonuclease activity (73) , confirmed by sequence comparisons with yeast, frog and mouse Pol γs and *in vitro* assays with the recombinant α subunit, have been assigned to the catalytic subunit.

Deduced amino acid sequences are now available for four mitochondrial DNA polymerase catalytic subunits (66, 68, 69, 74) and in comparisons with amino acid sequences of other family A DNA polymerases, especially Pol I, it is clear that three conserved sequences exist in both the polymerase and exonuclease domains. These conserved sequences in the exonuclease domain include three completely conserved aspartate residues and an isoleucine residue, in place of the phenylalanine of Pol I, that are necessary for a functional DNA polymerase. The three aspartate residues have been

mutagenized in *S. cerevisiae* Pol γ and are required for its exonuclease activity (71) . There are similar conserved sequences in the polymerase domain of the family A DNA polymerases located near the active site, that contain conserved residues important for polymerase activity.

mtDNA comprises less than 1% of the total cellular DNA, but its replication has been studied extensively *in vivo* and *in vitro* (75) . Replication of the mt genome offers a simple model for the study of replication systems *in vitro*. Replication of the mt genome originates at a single site in the A + T region and proceeds in an asymmetric unidirectional manner. In *Drosophila*, mtDNA synthesis proceeds around the double-stranded circular genome looping out large regions of ssDNA (termed the D-loop in vertebrate mtDNA) until > 95% (~60% in vertebrate mtDNA) of the leading strand is completed (75) . Initiation of lagging strand DNA synthesis initiates in the same non-coding A + T region and continues until the genome is duplicated. This highly asymmetric mode of DNA synthesis places constraints on the mitochondrial DNA polymerase requiring efficient polymerization on both ssDNA and dsDNA templates. The origin of mtDNA replication is among the most thoroughly studied initiation regions isolated from eukaryotes and Pol γ is the only known mitochondrial polymerase. These above properties, along with the similarity of mtDNA and mitochondrial replication proteins of various species and the availability of sequences containing mtDNA replication origins, present an ideal model system for the establishment of an *in vitro* replication system. Such a model system may allow insight into the cause of various mitochondrial and perhaps genetic diseases.

As in nuclear DNA replication, the mitochondrial DNA replication machinery includes primase, helicase, topoisomerases and single-stranded DNA-binding protein in addition to DNA polymerase to carry out duplication of the mt genome. To date mt primase, mt helicase, mt topoisomerase and mitochondrial SSBs have been described (76-84). Mitochondrial SSB is perhaps the best characterized of these mt replication accessory

proteins in terms of its structure and function using genetics, biochemistry and electron microscopy.

Drosophila melanogaster mtSSB (*Dm* mtSSB) as determined by amino acid sequence comparisons is closely related to *E. coli* SSB and other eukaryotic mtSSBs with an average identity of 25% and an average similarity of 38%. mtSSBs function analogously to nuclear SSBs in coating the ssDNA displaced during replication, but they share no sequence or structural homology with the latter. *E. coli* SSB and mtSSBs are homotetrameric proteins composed of 13-16 kDa protomers, that assume a tetrahedral structure determined by electron microscopy (85).

SSBs perform important functions in the cell not only in DNA replication, but also in DNA repair and recombination by protecting ssDNA intermediates from nucleolytic degradation. In *S. cerevisiae* mtSSB was shown to be required for maintenance of the mtDNA (78) and in *E. coli* deletion of the *ssb* gene was shown to be lethal unless complemented by an *ssb* harboring plasmid (84). *E. coli* SSB has also been shown to enhance the fidelity of DNA synthesis and is required for specificity in priming the DNA template (84) . Conversely, mtSSB has been shown to inhibit the action of mtDNA primase presumably by blocking access to potential primase binding sites (79) and the effects of mtSSB on its cognate DNA polymerases in rat and frog are unclear (79, 86). *E. coli* SSB and mtSSBs have been shown to enhance the binding of DNA polymerase to the template and to increase both the processivity and overall synthesis rate of DNA polymerase (79, 81, 83, 85). Both *E. coli* SSB and mtSSBs have a strong preference for ssDNA versus dsDNA and under varying conditions the binding to ssDNA can be highly cooperative (79, 81, 83, 84).

Research Project Goals

The goals of this thesis included the following. First, we identified and purified the mitochondrial single-stranded DNA-binding protein from *Drosophila* embryos. Isolation of the *Drosophila* mtSSB allowed characterization of its mechanism of action in DNA synthesis by *Drosophila* mitochondrial DNA polymerase. Second, we explored the effects of *Dm* mtSSB on the DNA synthetic and proofreading activities of Pol γ. These experiments demonstrated a direct effect of mtSSB on DNA synthesis catalyzed by Pol γ. In addition, these experiments demonstrated that mtSSB plays a role in mtDNA replication and likely also plays significant roles in mtDNA repair and recombination similarly to *E. coli* SSB in the analogous bacterial processes. Third, we attempted isolation of active recombinant catalytic (α) and accessory (β) subunits of mitochondrial DNA polymerase γ from overexpressing bacterial strains, to determine the effects of reaction conditions and mtSSB on catalytic functions in the absence or presence of the β subunit.

This thesis describes the purification of mtSSB from *Drosophila melanogaster* embryos, and as an active recombinant protein overexpressed in bacteria. Characterization of *Dm* mtSSB shows it shares a high degree of amino acid sequence similarity to *E. coli* SSB and other mitochondrial SSBs. In addition, we have proposed a mechanism for*Dm* mtSSB stimulation of the polymerase and 3' → 5' exonuclease of its cognate DNA polymerase *Drosophila* Pol γ. Furthermore, we have partially purified the recombinant catalytic (α) and accessory (β) subunits of *Dm* Pol γ from bacteria. We describe the catalytic activities of the α subunit and show that the β subunit is a *bona fide* component of the mitochondrial DNA polymerase holoenzyme.

DROSOPHILA MITOCHONDRIAL SINGLE-STRANDED DNA-BINDING PROTEIN: PURIFICATION, PHYSICAL CHARACTERIZATION AND BACTERIAL OVEREXPRESSION

Overview

Drosophila mitochondrial DNA polymerase, Pol γ, is responsible for replication of the mitochondrial genome, and its DNA synthetic and proofreading activities have been well defined in our laboratory (63, 72, 73, 87-90). Further definition of mtDNA replication requires the identification and isolation of mitochondrial replication accessory proteins, that either directly or indirectly affect DNA polymerase γ activities. To this end, we have identified and purified the mitochondrial single-stranded DNA-binding protein from *Drosophila* embryos and purified the active recombinant protein overexpressed in bacteria.

Experimental Procedures

Materials

Chemicals— Leupeptin was purchased from the Peptide Institute, Minoh-Shi, Japan. Sodium metabisulfite was purchased from J. T. Baker Chemical Company. Dithiothreitol (DTT), Isopropyl-β-D-thio-galactoside (IPTG), nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma. Phenylmethylsulfonyl fluoride (PMSF) was purchased from Research Organics. Goat anti-Rabbit IgG alkaline phosphatase conjugate was purchase from BioRad.

Nucleic Acids— PCR primers 5'-GCCGGCACATATGGCAACAACAACAA CGG-3' (forward) and 5'-GTTTGGTCATATGGGCTTAAATTTTAGTT-3' (reverse), for subcloning the mtSSB cDNA, were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer.

Enzymes— *Drosophila* mtSSB was prepared as described in "*Methods*" from either *Drosophila* embryos or *E. coli* cells expressing recombinant mtSSB.

Chromatography Resins and Buffers— Single-stranded DNA cellulose (ssDNA cellulose) and Cibacron Blue agarose were as described by Wernette and Kaguni (63). All buffers described contained 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, 2 μg/ml leupeptin and 2 mM EDTA. The ionic strength of buffers was determined using a Radiometer conductivity meter.

Bacterial Strains— The bacterial strain *E. coli* BL21 (λDE3) *omp*T, r_B^- M_B^- (Novagen) was used for the expression of pET-11a / mtSSB constructs.

Methods

Purification of Dm mtSSB— All operations were performed at 0-4°C. Mitochondrial extracts from starting material of 150-200 g embryos were prepared as described by Wernette and Kaguni (63) except that the extracts were filtered through 0.22 µm nitrocellulose membranes and adjusted to 0.5 M NaCl prior to ssDNA cellulose chromatography. The mitochondrial extract (Fraction I) was loaded onto a 3.0 ml ssDNA cellulose column (2.5 cm × 1.3 cm ; ~50 mg protein/packed ml resin) equilibrated with 30 mM Tris-HCl buffer pH 7.5 containing 10% glycerol and 0.5 M NaCl at a flow rate of 3 ml/hr. The column was washed with 12 ml of equilibration buffer at 6 ml/hr followed by 12 ml of the same buffer containing 0.75 M NaCl. *Dm* mtSSB eluted with the application of 9 ml of 30 mM Tris-HCl pH 7.5 buffer containing 1.5 M NaCl and 50% ethylene glycol. Column fractions were examined by silver staining SDS-polyacrylamide gels following SDS-polyacrylamide gel electrophoesis (SDS-PAGE) following the method of Laemmli (91) and fractions containing *Dm* mtSSB were pooled (Fraction II).

Glycerol Gradient Sedimentation— Fraction II was dialyzed against 2 × 1L 1 × glycerol gradient buffer containing 50 mM KPO_4 pH 7.6, 200 mM $(NH_4)_2SO_4$, 0.015% Triton X-100, 2 mM DTT, 2 mM EDTA, 1 mM PMSF, 10 mM sodium metabisulfite, 2 µg/ml leupeptin in a collodion bag (Schleicher and Schuell; MWcutoff, 10 kDa) for a total of 4 hours at 4°C. Dialyzed Fraction II was layered onto 3 preformed 12-30% glycerol gradients as described by Wernette and Kaguni (63). Fractions containing *Dm* mtSSB, as determined by silver-stained SDS-PAGE, were pooled then concentrated in a Centricon-30 spin concentrator (Amicon), treated with Tween 20 as described by Amicon, into a final buffer containing: 20 mM KPO_4 pH 7.6, 150 mM NaCl, 50% glycerol, protease inhibitors and DTT.

Production of Dm mtSSB Antiserum— *Dm* mtSSB protein derived from ssDNA cellulose column chromatography was diluted with PBS buffer (10 mM $NaPO_4$ pH 7.0

and 154 mM NaCl) to 0.5 ml final volume, then emulsified with an equal volume of Freund's complete adjuvant. Immunizations of virgin female New Zealand White rabbits were performed by subcutaneous injections of 15 µg of the purified protein. Booster immunizations were administered in Freund's incomplete adjuvant at 4 week intervals.

Bacterial overexpression of Dm mtSSB— The cDNA clone of *Dm* mtSSB was generously provided by Dr. Peter Tolias (Public Health Research Institute, New York, NY). The cDNA was subcloned by PCR to generate 5'- and 3'-end flanking *Nde*I restriction sites for ease of cloning into the bacteriophage T7 promoter-based expression vector pET-11a. The *E. coli* strain BL21 (λDE3) was used for transformation, and ampicillin-resistant plasmid-containing cells were screened, by Julie Ebels, for insert size and orientation of recombinant DNAs by *Bam*HI restriction analysis.

For overexpression of the recombinant mtSSB (r-mtSSB) protein, plasmid-containing BL21 (λ DE3) cells were grown at 37°C with aeration, in L broth containing 100 µg/ml ampicillin. When the bacterial cells reached an optical density of ~0.6 at 595 nm, IPTG was added to 0.3 mM, and the culture was incubated an additional 2 hours to obtain maximum overexpression. Cells were harvested by centrifugation, washed in 50 mM Tris-HCl pH 7.5 / 10% sucrose, recentrifuged and cell pellets were frozen in liquid nitrogen and stored at -80°C.

For preparation of cell extracts, frozen cells were thawed on ice and remaining steps were perfomed at 0-4°C. Cells were suspended in 1/50 volume of original cell culture in 50 mM Tris-HCl pH 7.5 / 10 % sucrose / 2 mM EDTA / 5 mM DTT / 1 mM / 10 mM sodium metabisulfite / 2 µg/ml leupeptin buffer, and lysed by incubation, on ice, for 30 min in the presence of 0.3 mg/ml final concentration of lysozyme and 0.25 M NaCl. Cell extracts were cleared by centrifugation at 16,000 x *g* for 30 min and supernatant fluid containing soluble r-mtSSB (Fraction I) was retained for Cibacron Blue agarose chromatography.

Purification of recombinant Dm mtSSB— Cibacron Blue agarose resin was used to purify r-mtSSB from the soluble extract obtained from 1L of induced cells. Soluble extract containing on the order of 1-1.5 mg r-mtSSB was loaded onto a 2.5 ml Blue agarose column equilibrated with buffer containing 30 mM Tris-HCl pH 7.5 / 10 % glycerol / 100 mM NaCl / 2mM EDTA / 1 mM PMSF / 10 mM sodium metabisulfite / 2 µg/ml leupeptin. The column was washed with the same buffer containing 800 mM NaCl and the protein was eluted using buffers containing the 0.5 and 1.5 M NaSCN. Fractions containing thr r-mtSSB were pooled, dialyzed against buffer containing 50 mM KPO_4 pH 7.6 / 200 mM $(NH_4)_2SO_4$ / 2 mM EDTA / 0.015% Triton X-100 / 1 mM PMSF / 10 mM sodium metabisulfite / 2 µg/ml leupeptin and loaded onto preformed 12 - 30% glycerol gradients essentially as described by Wernette and Kaguni (63). Fractions containing r-mtSSB were pooled and concentrated in a Centricon-30 spin concentrator as described above.

Results

Identification and purification of mtSSB from *Drosophila* embryos

Drosophila mtSSB was purified from embryonic mitochondrial extracts, prepared as described by Wernette and Kaguni (63), following a modified procedure for the purification of the yeast mtSSB (78) as described under "Methods." Chromatography of mitochondrial extracts on ssDNA cellulose under stringent binding conditions (0.5 M NaCl), followed by elution of the putative mtSSB with ethylene glycol and high salt (1.5 M NaCl), enriched for ~4 polypeptides ranging from 10-20 kDa in size. Figure 1A shows an SDS-polyacrylamide gel of pooled column fractions from a typical purification, which yields ~0.4 μg *Dm* mtSSB per gram of embryos. In order to purify further the putative mtSSB from contaminating small molecular weight polypeptides and to define its subunit structure, mtSSB-containing ssDNA cellulose fractions were pooled, dialyzed and sedimented on 12-30% glycerol gradients following the method described by Wernette and Kaguni (63). Analysis by SDS-PAGE of glycerol gradient fractions reveals a single polypeptide migrating at ~18 kDa (Figure 1), that is well separated from contaminants. In comparison with marker gradients, run in parallel, the putative mtSSB has a sedimentation coefficient of 4.04 ± 0.34 suggesting that it is a tetramer in solution, with an approximate molecular weight of 56 kDa.

To confirm the identity of the putative mtSSB, the amino-terminal sequence of the purified protein was determined by automated sequential Edman degradation. Sequence comparison of the resulting 29-mer, ATTTTAAAPAKVEKTVNTVTILGRVXADP, with the deduced amino-acid sequence attributed to *Dm* mtSSB (80) shows 96% identity between the two sequences and reveals a 16 amino acid mitochondrial signal peptide in the latter sequence (Figure 2). Furthermore, analysis of the purified 18 kDa mtSSB monomer by MALDI-MS revealed a molecular mass of 13,845 ± 14 Da, which corresponds closely

Figure 1. **Purification of mitochondrial single-stranded DNA-binding protein from Drosophila embryos.** Protein fractions were denatured and electrophoresed in a 17% SDS-polyacrylamide gel. Proteins were detected by silver staining (A) or by immunoblotting using the goat anti-rabbit alkaline phosphatase method with rabbit antiserum against Dm mtSSB at a 1:1000 dilution (B). Lane 1, *E. coli* SSB (0.25 μg); lane 2, mitochondrial extract; lane 3, ssDNA cellulose pool; lane 4, glycerol gradient pool.

Figure 1. **Purification of mitochondrial single-stranded DNA-binding protein from *Drosophila* embryos.** Protein fractions were denatured and electrophoresed in a 17% SDS-polyacrylamide gel. Proteins were detected by silver staining (A) or by immunoblotting using the goat anti-rabbit alkaline phosphatase method with rabbit antiserum against *Dm* mtSSB at a 1:1000 dilution (B). Lane 1, *E. coli* SSB (0.25 μg); lane 2, mitochondrial extract; lane 3, ssDNA cellulose pool; lane 4, glycerol gradient pool.

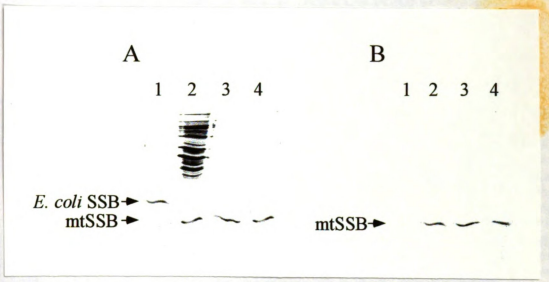


Figure 1. Purification of mitochondrial single-stranded DNA-binding protein from *Drosophila* embryos.

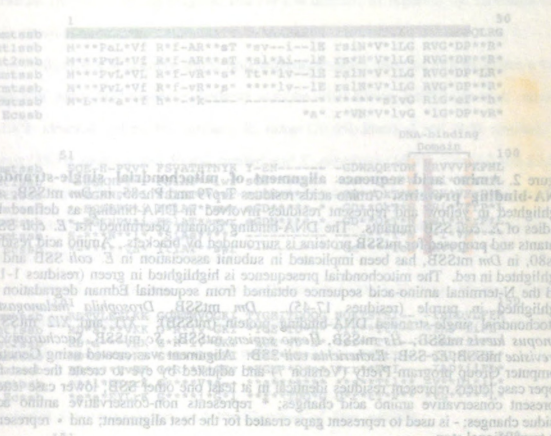


Figure 2. Amino acid sequence alignment of mitochondrial single-stranded DNA-binding proteins.

Figure 2. **Amino acid sequence alignment of mitochondrial single-stranded DNA-binding proteins.** Amino acids residues Trp79 and Phe85, in *Dm* mtSSB, are highlighted in yellow and represent residues involved in DNA-binding as defined by studies of *E. coli* SSB mutants. The DNA-binding domain determined for *E. coli* SSB mutants and proposed for mtSSB proteins is surrounded by brackets. Amino acid residue His80, in *Dm* mtSSB, has been implicated in subunit association in *E. coli* SSB and is highlighted in red. The mitochondrial presequence is highlighted in green (residues 1-16) and the N-terminal amino-acid sequence obtained from sequential Edman degradation is highlighted in purple (residues 17-45). *Dm* mtSSB, *Drosophila melanogaster* mitochondrial single-stranded DNA-binding protein (mtSSB); *Xl*1 and *Xl*2 mtSSB, *Xenopus laevis* mtSSB; *Hs* mtSSB, *Homo sapiens* mtSSB; *Sc* mtSSB, *Saccharomyces cerevisiae* mtSSB; *Ec* SSB, *Escherichia coli* SSB. Alignment was created using Genetics Computer Group program Pretty (Version 7) and adjusted by eye to create the best fit. Upper case letters represent residues identical in at least one other SSB; lower case letters represent conservative amino acid changes; * represents non-conservative amino acid residue changes; - is used to represent gaps created for the best alignment; and • represents the translational stop.

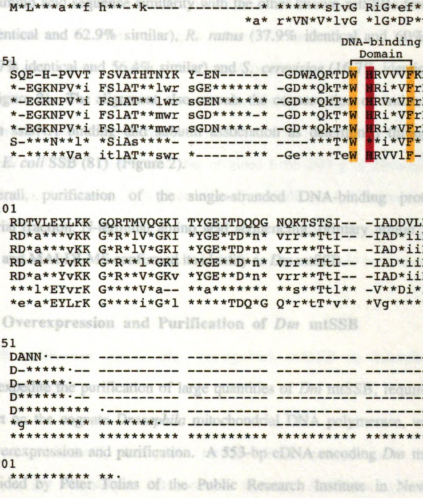


Figure 2. Amino acid sequence alignment of mitochondrial single-stranded DNA-binding proteins.

to the predicted 13,832 Da for the mature 124 amino acid polypeptide. It is also comparable to the deduced molecular masses of the mtSSBs isolated from rat, human, *Xenopus* and yeast (77, 78, 92). In addition, characterization of the DNA-binding substrate specificity and binding-site size for *Dm* mtSSB, as reported by Thömmes *et al* (83), showed correlation amongst mtSSBs and *E. coli* SSB.

Alignment of primary amino acid sequences shows that *Dm* mtSSB shares a high degree of amino acid sequence similarity with the other known mtSSBs from *H. sapiens* (41.4% identical and 62.9% similar), *R. rattus* (37.9% identical and 60% similar), *X. laevis* (35.7% identical and 56.4% similar) and *S. cerevisiae* (16.7% identical and 27.5% similar) (Figure 2). The alignment also reveals the conservation of amino acid residues involved in ssDNA binding and subunit association as determined through studies of mutants of *E. coli* SSB (81) (Figure 2).

Overall, purification of the single-stranded DNA-binding protein from a mitochondrial fraction, N-terminal amino acid sequencing, primary amino acid sequence alignments and MALDI-MS confirmed its identity as *Dm* mtSSB.

Bacterial Overexpression and Purification of *Dm* mtSSB

To expedite the purification of large quantities of *Dm* mtSSB, required for studies of the effect on the cognate *Drosophila* mitochondrial DNA polymerase, we pursued its bacterial overexpression and purification. A 553-bp cDNA encoding *Dm* mtSSB (generously provided by Peter Tolias of the Public Research Institute in New York) was subcloned into the bacteriophage T7 promoter-based expression vector pET-11a. A PCR strategy was designed to remove the 16 amino acid mitochondrial targeting sequence and provide a DNA fragment suitable for subcloning into the unique *Nde*I site of the pET-11a vector. PCR primers, as described under "Materials" and "Methods," were designed to create flanking *Nde*I restriction sites, to insert an ATG initiation codon one amino acid

upstream of the N-terminal alanine of the mature mtSSB, and to maintain the original UAA termination codon.

Optimal overexpression upon isopropylthio-β-D-galactoside induction of plasmid-containing BL21 (λDE3) cells yields ~16 μg of r-mtSSB polypeptide per ml of cell culture. Fractionation of induced cells revealed that ~60% of the *Dm* r-mtSSB is soluble. The soluble fraction was subjected to chromatography on Cibacron Blue agarose and fractions containing r-mtSSB were pooled and concentrated in a Centricon-30 spin concentrator. SDS-PAGE followed by silver staining or immunoblot analysis of cell lysate and purified fractions (Figure 3) reveals a single 18 kDa polypeptide is purified to near homogeneity from bacterial cells in a single step, that is specifically recognized by the *Dm* mtSSB antiserum (see below). Overall, the yield of *Dm* mtSSB from 400 ml of overexpressing bacterial cells is 12-fold greater than that obtained from 200 g of *Drosophila* embryos. Sedimentation analysis on 12-30 % glycerol gradients indicates that the recombinant protein maintains its tetrameric structure and in comparison with mtSSB from *Drosophila* embryos it exhibits similar stimulation of mitochondrial DNA polymerase in *in vitro* assays (data not shown).

_Dm_ mtSSB antiserum specifically recognizes mtSSB in mitochondrial and bacterial extracts.

We have isolated *Drosophila* mtSSB from embryos and from bacteria overexpressing the recombinant mitochondrial protein. Antiserum against the purified mtSSB from *Drosophila* was developed to confirm the overexpression of the recombinant mtSSB and for future biochemical studies.

Antiserum was obtained from a virgin female New Zealand White rabbit immunized with 15 μg injections of the purified mtSSB. Immunoblot analysis shows that the resulting antiserum specifically recognizes *Dm* mtSSB in both crude mitochondrial fractions from

Figure 3. Bacterial overexpression and purification of *Drosophila* msSSB.
Protein fractions were analyzed by electrophoresis in a 17% polyacrylamide gel. Proteins were detected by Blue staining (A) or by immunoblot using the goat anti-rabbit alkaline phosphatase-linked antibody with rabbit antiserum against msSSB as 1:1000 dilution (B). Lanes 1, [illegible] molecular weight markers of 45, 31, 21, 14 (faint), 14 and 6 kDa; lane 2, 2ce SSB (0.3 μg); lane 3, uninduced cells; lane 4, induced cells; lane 5, soluble extract; lanes 6 and 7, Chaetomium blue agarose pool (0.05 and 0.3 μg; respectively).

Figure 3. **Bacterial overexpression and purification of *Drosophila* mtSSB.** Protein fractions were denatured and electrophoresed in a 17% SDS-polyacrylamide gel. Proteins were detected by silver staining (A) or by immunoblotting using the goat anti-rabbit alkaline phosphatase method with rabbit antiserum against *Dm* mtSSB at a 1:1000 dilution (B). **A**. Lane 1, molecular weight markers of 45, 29 (faint), 18 (faint), 14 and 6 kDa; lane 2, *Eco* SSB (0.5 μg); lane 3, blank; lane 4, induced BL21 DE3 cells; lane 5, soluble extract; lanes 6 and 7, Cibacron blue agarose pool (0.25 and 0.5 μg, respectively). **B**. Lane 1, *Eco* SSB (0.25 μg); lane 2, *Dm* mtSSB (0.05 μg); lane 3, uninduced BL21 DE3 cells; lane 4, induced cells; lane 5, soluble extract; lanes 6 and 7 Cibacron blue agarose pool (0.05 and 0.3 μg, respectively).

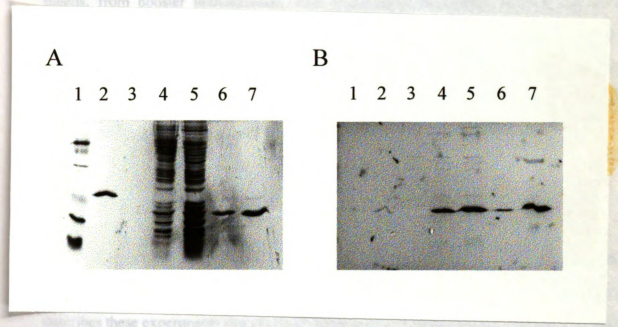


Figure 3. Bacterial overexpression and purification of *Drosophila* mtSSB.

Drosophila embryos, and in *E. coli* cell lysates expressing the recombinant protein (Figure 3B). Likewise, immunoprecipitations show that the antiserum recognizes native *Dm* mtSSB (data not shown). Initial bleeds, from antiserum prepared against native *Dm* mtSSB, showed limited crossreactivity with purified *E. coli* SSB protein, while later bleeds, from booster immunizations with lyophilized *Dm* mtSSB, showed no cross-reactivity with either *E. coli* SSB or yeast mtSSB (RIM 1 protein) suggesting common structural epitopes amongst the proteins. Antiserum preparations lacking crossreactivity with the *E. coli* SSB protein confirmed the identity of the purified recombinant mtSSB (Figure 3B).

Summary and Future Plans

The purification of *Dm* mtSSB from both flies and induced *E. coli* cells offered the opportunity to study its effect on the cognate mitochondrial DNA polymerase. Chapter 2 describes these experiments and evaluates the mechanism of action of *Dm* mtSSB in DNA synthesis. In conjunction with studies on native *Dm* Pol γ, the recent cloning and overexpression of the cDNA encoding the catalytic (α) subunit of Pol γ will allow examination of the effects of mtSSB on its DNA polymerase and proofreading 3' $\rightarrow$ 5' exonuclease activities. Future studies of the effect of mtSSB on the fidelity of native Pol γ and its catalytic subunit are necessary to understand the mechanism of mtSSB action in mtDNA replication, and important for explaining the relatively high mutation rates in mtDNA *in vivo*. Further, experiments to confirm the subcellular localization of mtSSB and to examine mitochondrial nucleoid fractions for the presence of mtSSB will be aided with the use of *Dm* mtSSB antiserum.

Mitochondrial SSBs have been shown to be associated with mtDNA replication intermediates (93), but the actual complexes formed by mitochondrial replication proteins have not been demonstrated. *E. coli* SSB, besides enhancing the fidelity of a variety of

prokaryotic and eukaryotic DNA polymerases, is implicated in stabilizing DNA replication origins and in promoting the assembly of the primosome (81). Experiments to show the association of *Dm* mtSSB with the *Dm* mitochondrial nucleoid *in vitro* and *in vivo* can be performed using the mtSSB specific antiserum by probing nucleoid fractions in immunoblot analyses and by examining isolated mitochondria in immunofluorescence studies, respectively. In conjunction with the probing of the mt nucleoid fractions, experiments to isolate other mitochondrial replication accessory proteins will contribute to the ultimate goal of constructing *in vivo* mtDNA replication models based on an *in vitro* mtDNA replication system.

Affinity chromatography using a mtSSB fusion protein may be useful in isolating mitochondrial replication accessory proteins. This technique has been used to isolate replication accessory proteins in the bacteriophage T4 system (94) and from *E. coli* (95). A 25 kDa *E. coli* protein isolated by such methods enhances the binding of *E. coli* DNA polymerase III core and holoenzyme to the chromosome (95).

The recent identification of a mtDNA helicase (96) from *Drosophila* now allows the study of the interactions between mtSSB and helicase, particularly to examine the enhancement of helix destabilization as shown for *E. coli* SSB (97). Studies of the action of mtSSB, helicase and mitochondrial DNA polymerase at the origin of mtDNA replication would also be of tremendous value toward deciphering the initial stages of leading strand DNA synthesis in mtDNA replication.

Determination of the three-dimensional structure of mtSSB mutants alone and in complex with DNA is of vital importance to confirm mutagenesis studies of *E. coli* SSB and to implicate the corresponding amino acids in similar functions in mtSSBs, (that is to identify amino acid residues directly involved in DNA-protein interactions and protein-protein interactions). The recent publication of a high resolution crystal structure of human mtSSB in the absence of DNA confirms the tetrahedral structure of the SSB tetramer, but it does not address the involvement of specific amino acid residues involved in DNA-

binding. Crystal structure information should also allow modeling of potential protein-protein interaction domains apart from SSB subunit association.

EFFECTS OF *DROSOPHILA* MITOCHONDRIAL SINGLE-STRANDED DNA-BINDING PROTEIN ON MITOCHONDRIAL DNA POLYMERASE

Introduction

Drosophila mitochondrial DNA polymerase is a heterodimeric DNA polymerase, comprising a 125 kDa catalytic α-subunit and a 35 kDa β-subunit of unknown function (63). Both the 5' → 3' DNA polymerase activity, determined by *in situ* DNA polymerase assay (89) and primary sequence alignments with family A DNA polymerases (34), and 3' → 5' proofreading exonuclease activity (73), also confirmed by sequence comparisons with family A DNA polymerases, have been assigned to the α-subunit.

The high asymmetry of mtDNA replication requires a polymerase that catalyzes efficient and faithful DNA synthesis on both single- and double-stranded DNA templates. Pol γ satisfies these requirements, catalyzing efficient DNA synthesis on both single- and double-stranded DNAs, though its activity varies greatly with reaction conditions. Pol γ exhibits optimal polymerase activity (standard activity) and moderate processivity (average processive unit of 145 nt) at moderate KCl concentrations (120 mM) on singly-primed ssDNA templates, with low activity (25% of standard activity) and high processivity (average processive unit of ~2000 nt) at low KCl concentrations (30 mM) (87). Variations in pH, ATP, GTP or ADP concentrations and polyethylene glycol or glycerol concentrations, to simulate metabolite fluctuations in the mitochondrial matrix, similarly revealed that optimal activity and processivity could not be achieved concurrently (87) in the absence of polymerase accessory proteins. Isolation and characterization of potential replication accessory proteins involved in mitochondrial DNA replication, that impart concurrent high activity and processivity to Pol γ, is a major goal in our laboratory. The study presented here reports the effects of the replication accessory protein mtSSB on Pol γ function.

Experimental Procedures

Materials

Nucleotides and Nucleic Acids— Unlabeled deoxy- and ribonucleotides were purchased from Pharmacia BioTech. [^{3}H]dTTP, [α-^{32}P]dATP and [γ-^{32}P]ATP were purchased from ICN Biochemicals.

Wild type and recombinant M13 DNAs (M13H2C2: 6407 nt, M13trx22: 10650 nt and M13mp7: 7238 nt) were prepared by standard laboratory methods. Oligonucleotides (listed below) complementary to the M13 viral DNAs were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer according to the manufacturer's protocols. The primers for primer extension and proofreading exonuclease assays were 17 nt in length, to produce a 3'-terminal base pair, and 15 nt in length, to produce a 3'-terminal mispair, respectively and annealed to M13trx22 DNA. The sequences of the 17 nt (LSK-8) and 15 nt (LSK-4) primers are 5'-AGGATCGCCCCGTCCGC-3' and 5'-GA TCGCCCCGTCCGG-3', respectively. The sequence of the 38 nt primer (LSK-75) used in Pol γ primer binding and idling experiments is 5'-GGGGGATGTGCTGCAAGGCG ATTAAGTTGGGTAACGCC-3', which is complementary to positions 6291-6329 in M13mp7 DNA.

Proteins— *Drosophila* DNA polymerase γ (Fraction VI) was prepared as described by Wernette and Kaguni (63). *Drosophila* mtSSB was prepared as described in Chapter 1 from either *Drosophila* embryos or *E. coli* cells expressing recombinant mtSSB. T4 polynucleotide kinase was purchased from Boehringer Mannheim.

Chemicals— All solutions were prepared in water and stored at either -20°C or 4°C. Chemicals were purchased from either Research Organics or Sigma Chemical unless otherwise noted. Kinase reaction buffer was provided with the T4 kinase as a 10 × concentrated stock from Boehringer Mannheim.

Methods

DNA polymerase γ assay— Reaction mixtures (0.05 ml) contained 50 mM Tris-HCl pH 8.5, 4 mM $MgCl_2$, 10 mM dithiothreitol, 0-180 mM KCl, 400 μg/ml bovine serum albumin, 20 μM each of dGTP, dATP, dCTP, and [^{3}H]dTTP (100 cpm/pmol), 10 μM (as nucleotides) singly primed recombinant M13 DNA, and 0.1 units of Fraction VI enzyme. Incubation was at 30°C for 30 min. Specific modifications are described in the figure legends. One unit of activity is that amount that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphate into acid insoluble material in 60 min at 30°C using DNase I-activated calf thymus DNA as the substrate.

Analysis of processive DNA synthesis products by gel electrophoresis— Reactions were performed as above, except that reactions mixtures contained 30 μM each of dGTP, dTTP, dCTP, and 10 μM [α-^{32}P]dATP (1000 cpm/pmol), 20 μM (as nucleotides) singly primed wild-type M13 DNA, and 0.02 unit of Fraction VI enzyme. Incubation was at 30°C for 8 min. Reaction products were made 1% in SDS and 10 mM in EDTA, heated 10 min at 65°C, extracted twice with phenol:chloroform (1:1) and precipitated with ethanol in the presence of 0.5 μg of tRNA as carrier. The ethanol precipitates were resuspended in 0.3 M NaOH and 20 mM EDTA. Aliquots of equivalent radioactivity (~4000 cpm) were denatured for 2 min at 100°C cooled on ice and electrophoresed on a 1.5% agarose slab gel (13 × 18 × 0.7 cm) containing 30 mM NaCl and 2 mM EDTA. The remaining aliquots were resuspended in 80% formamide and 90 mM Tris-borate, denatured as above and electrophoresed on a 6% polyacrylamide slab gel (13 × 30 × 0.15 cm) containing 7 M urea in 90 mM Tris-borate pH 8.3 and 25 mM EDTA. Equal sample volumes were loaded on each type of gel to allow comparative quantitation of products. Gels were washed in distilled water 20 min, dried under vacuum, and exposed at -80°C to Kodak X-Omat x-ray film using DuPont Quanta III intensifying screens. Quantitation of the data was performed by scanning autoradiographs using a Bio-Image Visage 110 digital imager. The area under

the peaks was determined by computer integration analysis and was normalized to the nucleotide level to correct for the uniform labeling of the DNA products. For the determination of processivity values, the length of the primer (15 nt) was subtracted from the DNA product strand lengths.

Preparation of 5'-^{32}P labeled M13 DNA substrates for primer extension, 3' → 5' exonuclease and primer binding assays— Synthetic oligodeoxyribonucleotides (15,17 or 38 nt) as described under "Materials" were 5'-end labeled. The kinase reaction (0.04 ml) contained 50 mM Tris-HCl pH 8.25, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM dithiothreitol and 0.1 mM spermidine), [γ-32P]ATP (0.2 μM, 4500 Ci/mmol), 28 or 84 pmol (as nucleotides) of oligonucleotide (15 and 17 or 38, respectively), and 10 units T4 polynucleotide kinase. Incubation was for 30 min at 37°C. M13 viral DNA (M13trx22 for 15 and 17 nt primers and M13mp7 for 38 nt primer) was added to a concentration of ~70 mM (as nucleotide, in 4-fold molar excess over homologous oligonucleotide), and the DNA mixture was precipitated with ethanol. The pellet was resuspended in a buffer (0.08-0.1 ml) containing 10 mM Tris-HCl pH 8.0, 0.3 M NaCl, and 0.03 M sodium citrate for a final concentration of 800 μM (as nt). This sample was incubated at 65°C for 1 hour, followed by incubation at 37°C for an additional hour in order to anneal the primer to the template.

Primer extension assay— Reaction mixtures (0.05 ml) 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 dGTP, dATP, dTTP, 20 μM (as nucleotides) 5'-end labeled singly primed recombinant M13 DNA, and 0.35 units of Fraction VI enzyme. Incubation was at 30°C for 8 min. Samples were made 1% in SDS and 10 mM in EDTA to terminate reactions, heated for 10 min at 65°C and ethanol precipitated in the presence of 1 μg salmon sperm DNA. 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 on an 18% polyacrylamide slab gel (13 × 24 × 0.075 cm) containing 7 M

urea in 90 mM Tris-borate pH 8.3 and 25 mM EDTA. Gels were washed in 15% glycerol for 20 min and exposed at -80°C to Kodak X-Omat x-ray film using DuPont Quanta III intensifying screens. Quantitation of the data was performed by scanning autoradiographs using a Bio-Image Visage 110 digital imager and integrating the area under the peaks using computer integration analysis.

3' → 5' exonuclease assay on M13 DNA substrates—The reaction mixtures (0.05 ml) contained 50 mM Tris-HCl pH 8.5, 4 mM $MgCl_2$, 10 mM dithiothreitol, 0-180 mM KCl, 400 μg/ml bovine serum albumin, 4 μM 5'-end labeled singly primed recombinant M13 DNA containing a 3'-terminal mispair, and 0.1 units of Fraction VI enzyme. Incubation was for 30 min at 30°C. Samples were made 1% in SDS and 10 mM in EDTA to terminate reactions, heated for 10 min at 65°C and ethanol precipitated in the presence of 1 μg salmon sperm DNA. Samples were electrophoresed, autoradiographs were obtained and data were quantitated as for primer extension reactions above.

Assay for the stability of Polymerase γ primer binding and enzyme idling— The experimental schemes shown in Figure 9 is described below. Reaction mixtures (0.05 ml/assay, in a 1.5 ml μfuge tube) contained 50 mM Tris-HCl pH 8.5, 4 mM $MgCl_2$, 10 mM dithiothreitol, 400 μg/ml bovine serum albumin, 30 or 120 mM KCl, 4 μM M13mp7/LSK-75 primer template (1:1 molar ratio). For experiments to examine the effect of mtSSB on the stability of primer binding, mtSSB protein (0.4 μg/reaction) was added to the bulk reaction mixture and incubated for 1 min at 30°C. After 1 min, DNA polymerase γ Fraction VI (0.17 units at 120 mM or 0.7 units at 30 mM) was added to the reaction mixture and incubated for 1 min at 30°C. After 1 min pre-incubation with Pol γ (or Pol γ and mtSSB (0.4 μg/pmol DNA)), a 50 μl aliquot was removed to 0.5 ml μfuge tube containing 1/10 volume stop solution (10% SDS / 100 mM EDTA), followed by the addition of either 180 μM (moderate salt) or 700 μM (low salt) DNase I-activated calf thymus DNA as the DNA trap. 50 μl aliquots were removed at varying times, after DNA trap addition, to 0.5 ml μfuge tubes containing 0.3 M dGTP, dATP and dTTP. Reactions

containing dNTPs were incubated an additional 2 min at 30°C to allow extension to the G-stop. Reactions were made 1% in SDS and 10 mM in EDTA to terminate reactions, heated for 10 min at 65°C and ethanol precipitated in the presence of 1 μg salmon sperm DNA. The ethanol precipitates were resuspended in 80% formamide and 90 mM Tris-borate and analyzed on 18% polyacrylamide / 7 M urea gels as for primer extension assays above.

Experiments to examine polymerase idling were conducted essentially as above. Pol γ Fraction VI with or without *Dm* mtSSB (0.4 μg/pmol DNA) was preincubated with the M13 substrate 1 min at 30°C. dGTP, dATP and dTTP were added to 0.5 μM and samples were incubated for 20 sec at 30°C, allowing extension to the first G position. Next, DNase I-activated calf thymus DNA was added (as above) and aliquots were removed at varying time points, following DNA trap addition and added to pre-warmed tubes containing 300 μM dGTP, dATP, dTTP and dCTP and incubated for an additional 2 min at 30°C for extension by polymerase molecules still associated with the template-primer. Reactions were terminated and analyzed as above for primer binding stability assay. Products extended past the hairpin in 30 mM KCl samples (+) mtSSB were analyzed on a denaturing 6% polyacrylamide / 7 M urea slab gel (13 × 30 × 0.15 cm).

Results

Drosophila mtSSB stimulates *Drosophila* Pol γ DNA polymerase and 3' → 5' exonuclease activities.

Studies of putative replication accessory proteins were begun by Andrea Williams, who examined the role of *E. coli* SSB in DNA synthesis by Pol γ. SSBs play an essential part in DNA replication in bacteria, bacteriophage, viruses and eukaryotic nuclear DNA replication, and they have been implicated in DNA repair and recombination processes. Furthermore, *E. coli* SSB is known to stimulate the activity of a variety of DNA polymerases from *E. coli* DNA polymerases II and III to viral and bacteriophage DNA polymerases (81). Though it is known that mtSSB is required for mtDNA maintenance in yeast (78), the function of mtSSB in mtDNA replication is unresolved, with experiments in rat and frog showing that mtSSB stimulates DNA synthesis by mitochondrial DNA polymerase containing fractions only under certain conditions (79, 85).

In the absence of the mitochondrial counterpart to *E. coli* SSB, its effects on DNA synthesis by Pol γ were examined. These studies revealed that over a broad range of KCl concentrations (0-180 mM) the overall rate of DNA synthesis by Pol γ is stimulated up to 40-fold at low salt (30 mM), to allow simultaneous achievement of high DNA polymerase activity and processivity (90). Moreover, it was shown that the resulting stimulation occurs mainly from an increase in the efficiency of template-primer recognition and binding (90). With the identification and purification of mtSSB from *Drosophila* embryos, these studies were repeated and extended to include the effect of mtSSB on Pol γ 3' → 5' exonuclease activity.

Experiments examining the effect of the highly purified *Dm* mtSSB on near homogenous Pol γ yield results similar to the *E. coli SSB* studies on Pol γ. *Dm* mtSSB stimulates the DNA polymerase activity of Pol γ on singly primed M13 DNA over a broad

Figure 4. ***Dm* mtSSB stimulates the rate of DNA synthesis by *Drosophila* Pol γ over a broad KCl range. A:** DNA synthesis was measured on singly primed M13 DNA (10 μM) under standard reaction conditions, in the presence of the indicated KCl concentrations and in the absence (closed circles) and presence (open circles) of *Dm* mtSSB (0.8 μg). **B:** The data from **A** were replotted to show the ratio of nucleotide incorporation by *Dm* Pol γ in the presence versus absence of *Dm* mtSSB at each KCl concentration.

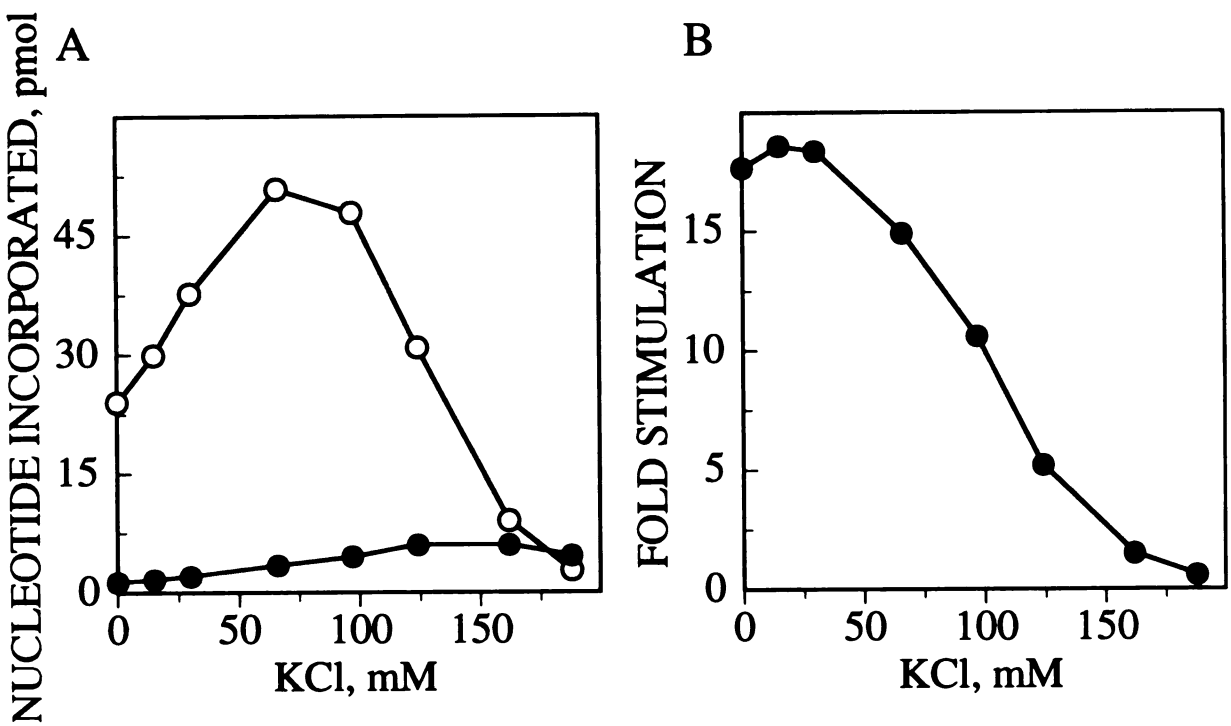


Figure 4. *Dm* mtSSB stimulates the rate of DNA synthesis by *Drosophila* Pol γ over a broad KCl range.

Figure 5. ***Dm* mtSSB stimulates mispair hydrolysis *Drosophila* Pol γ 3' → 5' exonuclease.** **A:** Mispair hydrolysis was measured using a singly primed 5'-end labeled M13 DNA with a 3'-terminal mispair (4 μM) in the presence (open circles) and the absence (closed circles) of *Dm* mtSSB (0.4 μg). DNA products were isolated, denatured and electrophoresed on an 18% polyacrylamide / 7 M urea gel (73). The products were normalized to the standard % hydrolysis by Pol γ 3' → 5'-exonuclease in the absence of *Dm* mtSSB and plotted accordingly (closed circles). **B:** The data from **A** were replotted to show the ratio of mispair hydrolysis by *Dm* Pol γ 3' → 5' exonuclease in the presence versus absence of *Dm* mtSSB at each KCl concentration.

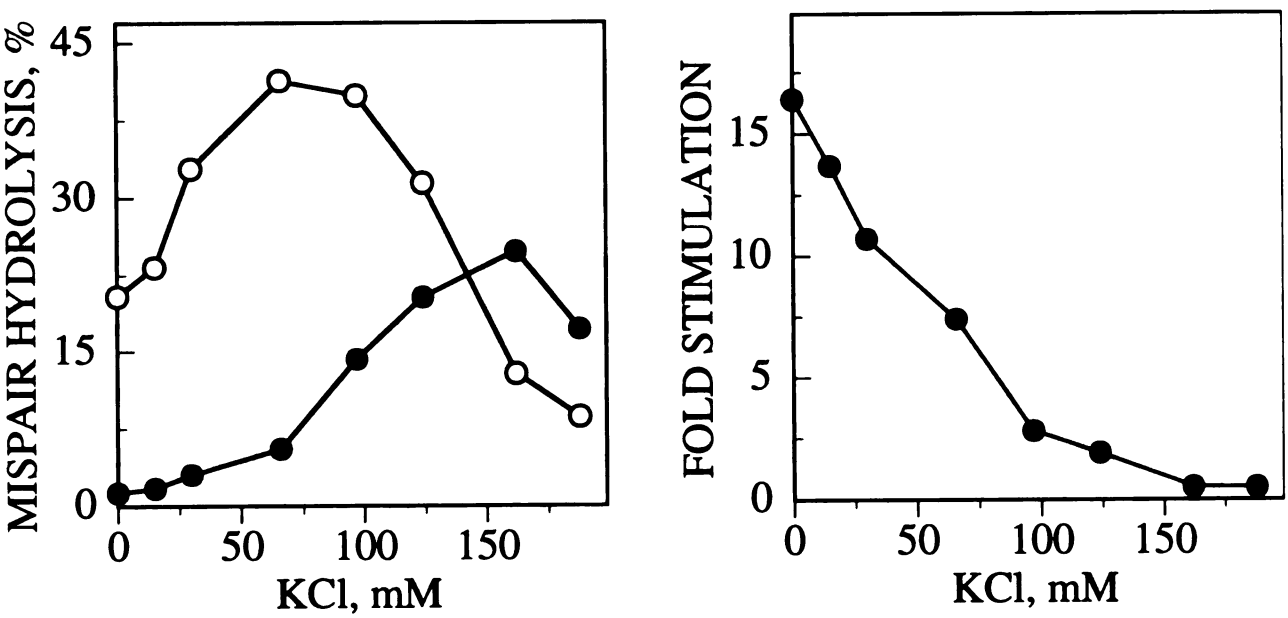


Figure 5. *Dm* mtSSB stimulates mispair hydrolysis *Drosophila* Pol γ 3'→5' exonuclease.

range of KCl concentrations, up to 18-fold at low salt (30 mM) (Figure 4). Optimal stimulation was observed when mtSSB was present in amounts sufficient to coat all of the single-stranded DNA present in the reaction (data not shown). Stimulation of Pol γ DNA polymerase activity by mtSSB is approximately two-fold lower than that by *E. coli* SSB. Differences in stimulation by mtSSB may result from the nature of the purified protein; that is, the use of the mild denaturant, ethylene glycol, in the purification may have caused some dissociation of the homotetramer causing reduced stimulation overall. Identical experiments with the purified recombinant and embryonic mtSSBs show the same stimulation of DNA polymerase activity (data not shown).

In addition to its DNA polymerase activity, Pol γ has an associated 3' → 5' proofreading exonuclease activity. This exonuclease activity copurifies with the DNA polymerase activity through all steps of the purification of native Pol γ and like the polymerase activity it is contained within the catalytic (α) subunit (69). *Dm* mtSSB stimulates the 3' → 5' exonuclease activity of Pol γ on singly-primed M13 DNA containing 3'-terminal mispairs over the same range of KCl concentrations used above, up to 16-fold at low salt (30 mM) (Figure 5). The similarity of the stimulation of Pol γ DNA polymerase and 3' → 5' exonuclease activities by mtSSB suggests coordination of the two activities, that are both facilitated by *Dm* mtSSB. In addition, this is the first demonstration of the stimulation of a 3' → 5' exonuclease activity by an SSB.

Dm mtSSB increases the processivity of mitochondrial DNA polymerase.

To investigate the mechanism of stimulation of *Dm* Pol γ by mtSSB, we determined the effects of mtSSB on the processivity of nucleotide incorporation by Pol γ. Processivity is defined as the number of nucleotides incorporated per polyermase binding event and is measured by calculating the average DNA product strand length. In the absence of *Dm* mtSSB, the processivity of Pol γ decreases on singly primed M13 DNA as

DNA polymerase activity increases with increasing KCl concentrations (87). Experiments conducted with *E. coli* SSB showed concurrent achievement of optimal DNA synthesis and processivity at low salt (30 mM), but an increase in processivity was not the major contributor to the stimulatory effect of *E. coli* SSB.

Dm mtSSB increases the processivity of *Dm* Pol γ. Processivity increases disproportionately to the stimulation of DNA polymerase activity at 30, 65, and 120 mM KCl. Pol γ processivity increases less than 2-fold at 30 mM KCl from an average DNA product strand length of ~2000 nt to 2300 nt , where the stimulation of DNA polymerase activity is 18-fold. This result is similar to that for Pol γ in the presence of *E. coli* SSB where processivity increased less than 2-fold at low salt. Pol γ processivity increases ~5-fold from an average DNA product strand length of ~145 nt to 780 nt at 120 mM KCl, where DNA polymerase stimulation is 5-fold. Though the increase in processivity is insufficient to account for the dramatic stimulation revealed in the standard polymerase and exonuclease assays, mtSSB does have a dramatic effect on eliminating DNA secondary structures. Comparing lanes 1-3 with lanes 4-6 in Figure 6 reveals that virtually all of the pause sites are eliminated in the presence of mtSSB at low salt allowing completion of DNA synthesis on the 6400 nt substrate.

Dm mtSSB dramatically increases initiation of DNA synthesis by DNA polymerase γ.

To investigate further the mechanism of *Dm* mtSSB stimulation of DNA polymerase γ, we examined the initiation of DNA synthesis by Pol γ in the absence and presence of *Dm* mtSSB. Experiments using *E. coli* SSB revealed the major role of SSB in DNA synthesis is to increase initiation of DNA synthesis by Pol γ (90). The similarity of the effect of *E. coli* SSB and *Dm* mtSSB on DNA polymerase γ processivity suggested that *Dm* mtSSB acts similarly to enhance initiation of DNA synthesis.

Figure 6. ***Dm* mtSSB increases the processivity of *Drosophila* Pol γ.** DNA synthesis was performed on singly primed M13 DNA (6407 nucleotides) under standard conditions, the uniformly labeled DNA product strands were isolated, denatured and electrophoresed on a 1.5% denaturing agarose gel (8). Reactions were performed in the absence (lanes 1-3) or presence (lanes 4-6) of *Dm* mtSSB (2 μg) and incubated at 30°C for 8 min. Lanes 1 and 4, 30 mM KCl; lanes 2 and 5, 65 mM KCl; lanes 3 and 6, 120 mM KCl. Numbers *at left* indicate the positions and sizes (in nt) of *Hin*dIII restriction fragments of λ-DNA and *Hpa* II fragments of M13*Gori*1 DNA that were electrophoresed in an adjacent lane. The reaction products were also electrophoresed in a denaturing 6% polyacrylamide / 7 M urea gel as described by Williams *et al.*(90), in order to quantitate product DNA strands of ≤ 500 nt (data not shown).

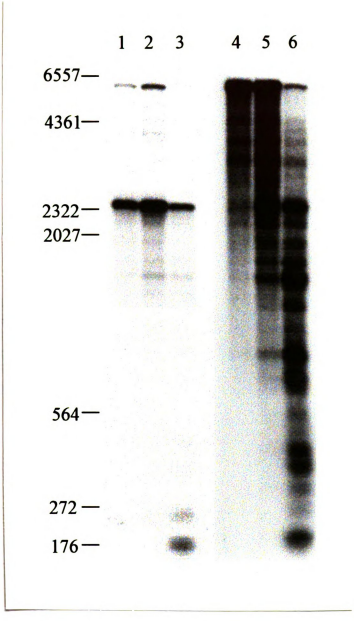


Figure 6. *Dm* mtSSB increases the processivity of *Drosophila* Pol γ.

Figure 7. ***Dm* mtSSB increases the rate of initiation of DNA synthesis by *Drosophila* Pol γ.** Primer extension was performed at 30 mM KCl in the absence of dCTP on singly primed 5'-end labeled M13 DNA under standard conditions. DNA product strands were isolated, denatured and electrophoresed on an 18% polyacrylamide gel (83). Reactions were performed in the absence (lane 2) or the presence of *Dm* mtSSB (2 μg, lane 3) or *E.coli* SSB (2 μg, lane 4). Lane 1 represents a control lacking both Pol γ and SSB.

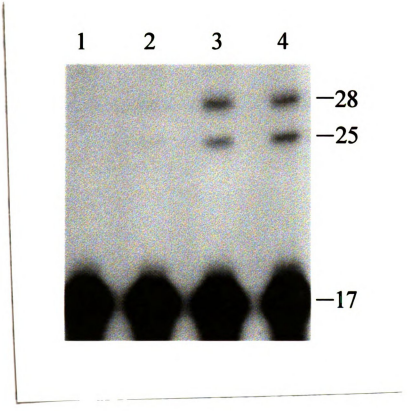


Figure 7. *Dm* mtSSB increases the rate of initiation of DNA synthesis by *Drosophila* Pol γ.

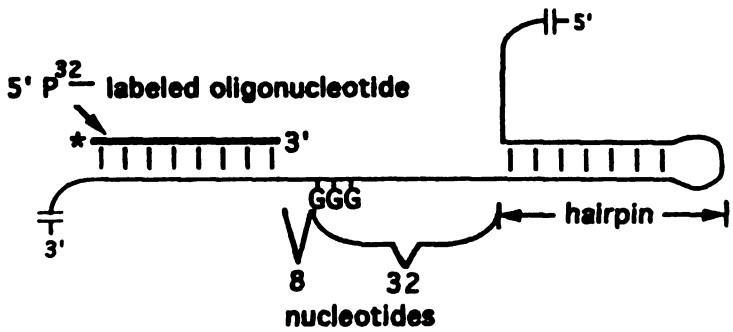
The effect of *Dm* mtSSB on initiation of DNA synthesis was determined using limited primer extension analysis. Limited DNA synthesis was performed on 5'-end labeled singly primed M13 DNA (17 nt primer, LSK-8) in the absence of dCTP, to yield DNA synthesis products terminated after the incorporation of either 8 or 11 nucleotides. Limiting the DNA synthesis to the initial incorporation events allows comparison of the extent of initiation under various reaction conditions. Quantitation of DNA synthesis products (Figure 7) shows initiation of DNA synthesis by Pol γ is increased 12-fold, corresponding closely to the stimulation observed for the overall rate of DNA synthesis by Pol γ in the presence of mtSSB at low salt. Thus, the mechanism of stimulation of Pol γ by mtSSB, parallels that for *E. coli* SSB, and can be attributed to an increase in the productive binding of Pol γ to the template-primer terminus. Such a mechanism is supported by the evidence that *Dm* mtSSB stimulates similarly both the DNA polymerase and 3' → 5' exonuclease activities of *Dm* Pol γ (see above). Finally, these *in vitro* data support the hypothesis that mtSSB plays an important role in *in vivo* replication of the mitochondrial genome.

An assay to measure the dissociation of Pol γ bound at the template-primer terminus, stalled by nucleotide exclusion or at a DNA hairpin helix.

Because we have shown that *Dm* mtSSB acts to stimulate the DNA polymerase and 3' → 5' exonuclease activities of Pol γ by increasing initiation of DNA synthesis, we continued to dissect the mode of *Dm* mtSSB action on polymerase interaction at the template-primer terminus. To do so, we used a novel assay developed by Kevin Hacker and Bruce Alberts (40) to examine the stability T4 DNA polymerase holoenzyme binding to the template-primer terminus (primer binding), paused by the omission of a single nucleotide (enzyme idling) or paused at a DNA hairpin helix (enzyme barrier). It is noteworthy to mention that Hacker and Alberts were unable to measure the stability of T4

Figure 8. **Template structure and experimental scheme for *Drosophila* Pol γ template-primer binding and enzyme idling.** A, The 5' ^{32}P-labeled oligonucleotide primer is 38 nucleotides long and complementary to map positions 6291-6329 of M13mp7. Downstream from the primer are 8 C, A or T nucleotides followed by three G nucleotides (GGG) and 32 nucleotides further downstream is a 22 bp DNA hairpin helix . B, When dCTP is omitted from the primer extension reactions the polymerase will stop just before the GGG in primer binding experiments. Polymerase idling is measured by monitoring the extension to the DNA hairpin helix upon the addition of dCTP to the reaction mixture.

A



B

Experimental Scheme

Primer binding: Pol γ + DNA +/- SSB → DNA Trap → various incubation times → dGTP dATP TTP

Enzyme idling: Pol γ + DNA +/- SSB + dGTP, dATP, TTP → DNA Trap → various incubation times → dCTP →

Figure 8. Template structure and experimental scheme for *Drosophila* Pol γ template-primer binding and enzyme idling.

DNA polymerase binding or idling in the absence of its holoenzyme accessory subunits, while we were able to measure the stability of the heterodimeric mitochondrial DNA polymerase in the absence of accessory proteins.

Using the 5' end-labeled singly primed viral M13mp7 DNA template shown in Figure 8, we examined the stability of Pol γ binding at the template-primer terminus at 30 and 120 mM salt, in the absence and presence of mtSSB, to elucidate further the mechanism for the stimulation of the mitochondrial DNA polymerase activities by mtSSB. We examined the stability of polymerase association with the template-primer terminus and polymerase idling in the absence of a single deoxyribonucleoside triphosphate using modified versions of the Hacker and Alberts assay. The important features of the assay are the following. First, the template is single-stranded and circular so it resembles lagging strand DNA synthesis in mtDNA replication. Second, the stop site in the absence of dCTP contains three consecutive dGMP residues (triG) in the template strand, which are less likely to be passed by the enzyme in the presence of low concentrations of contaminating dCTP in the dGTP, dATP and dTTP stocks. Third, a polymerase molecule that has been allowed to synthesize through the triG nucleotide stop will quickly encounter a 22 bp DNA hairpin helix, that acts to block DNA synthesis. Fourth, the 38 nt primer is 5' ^{32}P end-labeled, so the amount of radioactivity in each product is directly proportional to the number of polymerase molecules that have elongated the primer to either the triG stop or the hairpin position. Last, both the singly primed M13 DNA template and the DNase I-activated calf thymus DNA trap are in large excess relative to enzyme, to ensure a single binding / polymerizing event per template. Products of the primer extension reaction are resolved in 18% polyacrylamide / 7 M urea gels and visualized by autoradiography or phosphorimaging. Primers extended to the triG stop produce a single product of 46 nt, while those extended to the hairpin helix produce a single band at 76 nt. The basic assays used are diagrammed in Figure 8B to show the experimental strategy employed to examine enzyme binding stability and enzyme idling.

Pol γ dissociation from the template-primer terminus is substantially slowed at low salt.

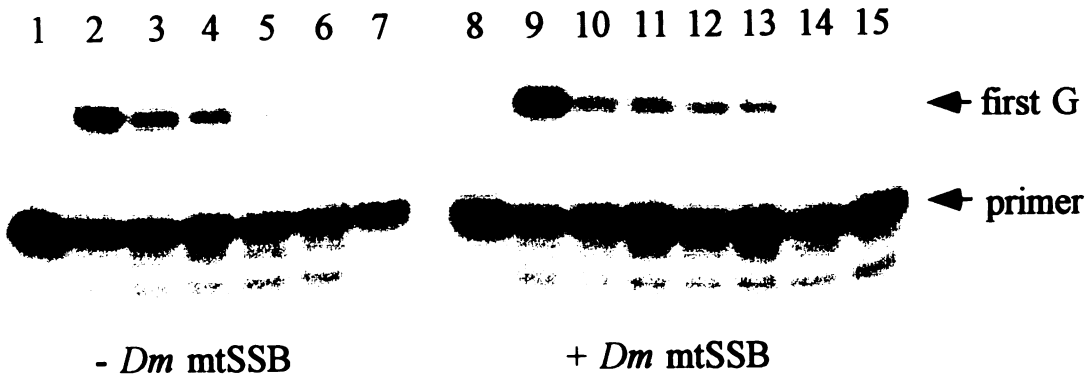
The association of Pol γ at the template-primer terminus was first examined to determine the effect of salt concentration on its stability. Previous experiments conducted by Andrea Williams, showed that Pol γ activity increases with salt concentration in parallel with a decrease in the processivity of nucleotide polymerization (87). If low salt concentrations stabilize protein-DNA interactions, then we expect to see an increase in the stability of Pol γ at the template-primer junction at low salt versus moderate salt. Similarly, DNase I footprinting and gel mobility shift experiments conducted by Yuxun Wang show that mitochondrial DNA polymerase binds the template-primer terminus more stably at low salt than at moderate salt (unpublished data).

The autoradiographs in Figures 9 and 10 show the products obtained in the primer binding stability assay described above. Lanes 1-7 in Figure 9A show the dissociation of Pol γ from the primer terminus at 30 mM KCl, while lanes 1-7 in Figure 10A show the dissociation of Pol γ at 120 mM KCl. Densitometric scanning of the autoradiographs followed by linear regression analysis of the data allowed the calculation of the half life of the dissociation ($t_{1/2}$) of Pol γ at the template-primer terminus. The decay rates obtained from time points following the addition of the DNA trap are nearly linear after trap addition (Figures 9B and 10B). The $t_{1/2}$ of Pol γ dissociation from the template-primer at 30 mM KCl is 13.2 ± 2.3 minutes, which is 15-fold greater than that at 120 mM KCl where the $t_{1/2}$ of dissociation is 0.87 ± 0.15 minutes. This substantial stabilization of Pol γ binding at low salt corroborates the results obtained by A. Williams and Y. Wang mentioned above.

Fluctuations in salt concentrations within the mitochondrion may alter DNA and protein structures and in turn affect molecular interactions. Low salt conditions likely promote high processivity by reducing the stability of DNA secondary structures that can

Figure 9. ***Drosophila* Pol γ template-primer binding stability at 30 mM KCl in the presence and absence of *Dm* mtSSB.** A, An autoradiograph of primer extension products electrophoresed on a 18% polyacrylamide / 7 M urea gel. *Lanes* 1 and 8, no enzyme controls; *lanes* 2 and 9, no DNA trap added; *lanes* 3-7, primer extension products obtained from 0.3, 0.67, 1, 2 and 4 min incubations using 0.7 units Pol γ Fraction VI and (-) *Dm* mtSSB; *lanes* 10-15, primer extension products obtained from 2, 4, 10, 20, 30 and 40 min incubations using 0.17 units Pol γ Fraction VI (+) *Dm* mtSSB. The position of the primer and the product terminated prior the first G are indicatred on the right. B, Quantitation of data in A is plotted to show the linear rate of of polymerase dissociation by plotting percent polymerase molecules remaining associated with the template versus time. *Open circles* represent reactions performed in the absence of *Dm* mtSSB, while *closed circles* represent reactions performed in the presence of *Dm* mtSSB.

A



B

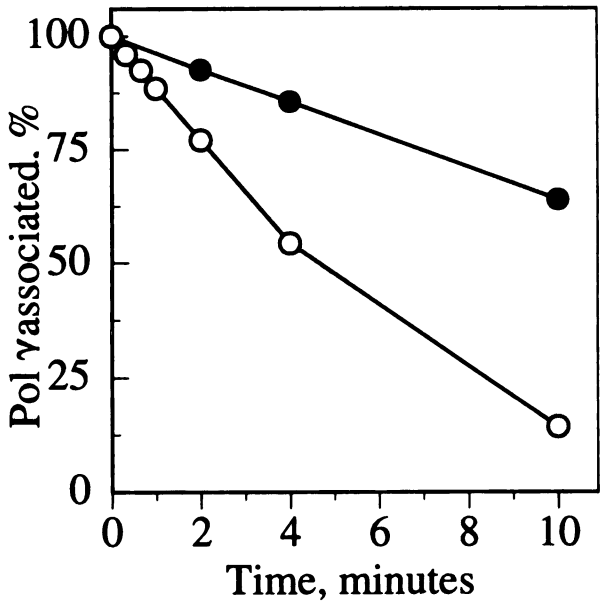
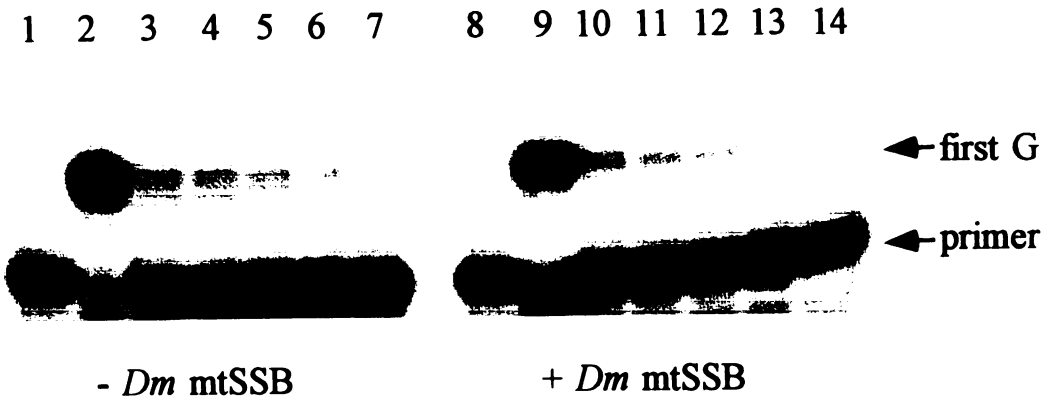


Figure 9. *Drosophila* Pol γ template-primer binding stability at 30 mM KCl in the presence and absence of *Dm* mtSSB.

Figure 10. ***Drosophila* Pol γ template-primer binding stability at 120 mM KCl in the presence and absence of *Dm* mtSSB.** A, An autoradiograph of primer extension products electrophoresed on a 18% polyacrylamide / 7 M urea gel. *Lanes* 1 and 8, no enzyme controls; *lanes* 2 and 9, no DNA trap added; *lanes* 3-7, primer extension products obtained from 0.2, 0.3, 0.67, 1 and 2 min incubations using 0.17 units Pol γ Fraction VI and no *Dm* mtSSB; *lanes* 10-15, primer extension products obtained over the same time course using 0.17 units Pol γ Fraction VI with *Dm* mtSSB (0.4 μg/assay). The position of the primer and the product terminated prior the first G are indicatred on the right. B, Quantitation of data in A is plotted to show the linear rate of of polymerase dissociation by plotting percent polymerase molecules remaining associated with the template versus time. *Open circles* represent reactions performed in the absence *Dm* mtSSB, while *closed circles* represent reactions performed in the presence of *Dm* mtSSB.

A



B

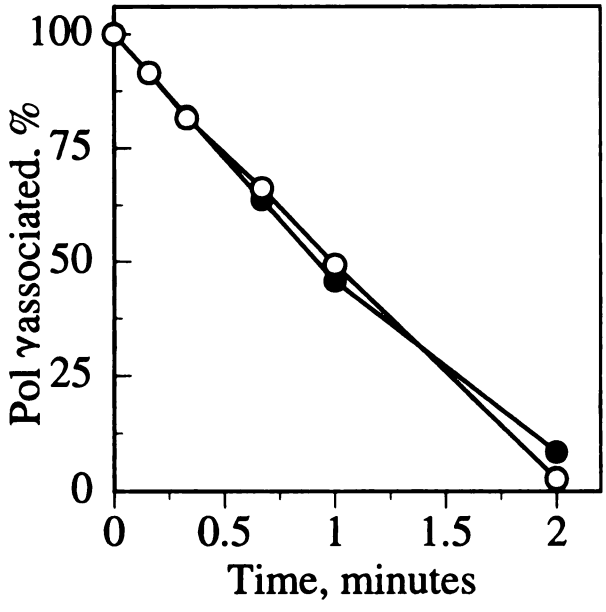


Figure 10. *Drosophila* Pol γ template-primer binding stability at 120 mM KCl in the presence and absence of *Dm* mtSSB.

impede polymerase movement along the ssDNA template. In contrast, moderate salt concentrations tend to stabilize DNA secondary structures (98) and destabilize protein-DNA interactions, likely promoting enzyme cycling and increasing the overall polymerase activity. These notions are supported by the template-primer binding stability experiments presented here, that show decreases in salt concentration stabilize protein-DNA interactions, and by experiments presented earlier, that show an increase in the overall polymerase activities with increases in salt concentration (Figures 4 and 5).

_Dm_ mtSSB has no effect on the stability of Pol γ template-primer binding at low salt.

To continue studying the mechanism of mtSSB action in stimulating mitochondrial DNA polymerase activity, we investigated the effect of adding saturating amounts of mtSSB to the template-primer binding stability assay. The assays were conducted as above, except that mtSSB was added to the reaction mix to coat the ssDNA template before the addition of Pol γ. Because mtSSB greatly stimulates Pol γ activity at low salt to achieve concurrent high activity and processivity, we expected to see a similar stabilization of Pol γ binding to the primer terminus.

In contrast to the expected results, linear regression analysis of the products in Figure 10 lanes 8-14 reveals no effect of mtSSB addition at 120 mM KCl with a $t_{1/2}$ of 0.82 ± 0.14 minutes. Surprisingly, analysis of the products in Figure 9 lanes 8-14, obtained at 30 mM KCl, actually reveals a decrease in the stability of template-primer binding stability to a $t_{1/2}$ of 4.6 ± 0.82 minutes, under conditions where the overall rate of DNA synthesis is stimulated 18-fold.

At first, these results appear to contradict the proposed mechanism of mtSSB stimulation through increasing primer recognition and binding, but careful examination reveals this is likely not the whole answer. We have only examined the stability of Pol γ

bound to the template-primer and not the actual binding event. Addition of mtSSB to the low salt reaction does increase initiation DNA synthesis by 12-fold as shown in Figure 7, and mtSSB also reduces the amount of Pol γ required to obtain a stable footprint at low salt (Y. Wang, unpublished data). These data imply that initial primer recognition and binding must be enhanced substantially by mtSSB, likely by excluding non-productive binding of Pol γ to the ssDNA template.

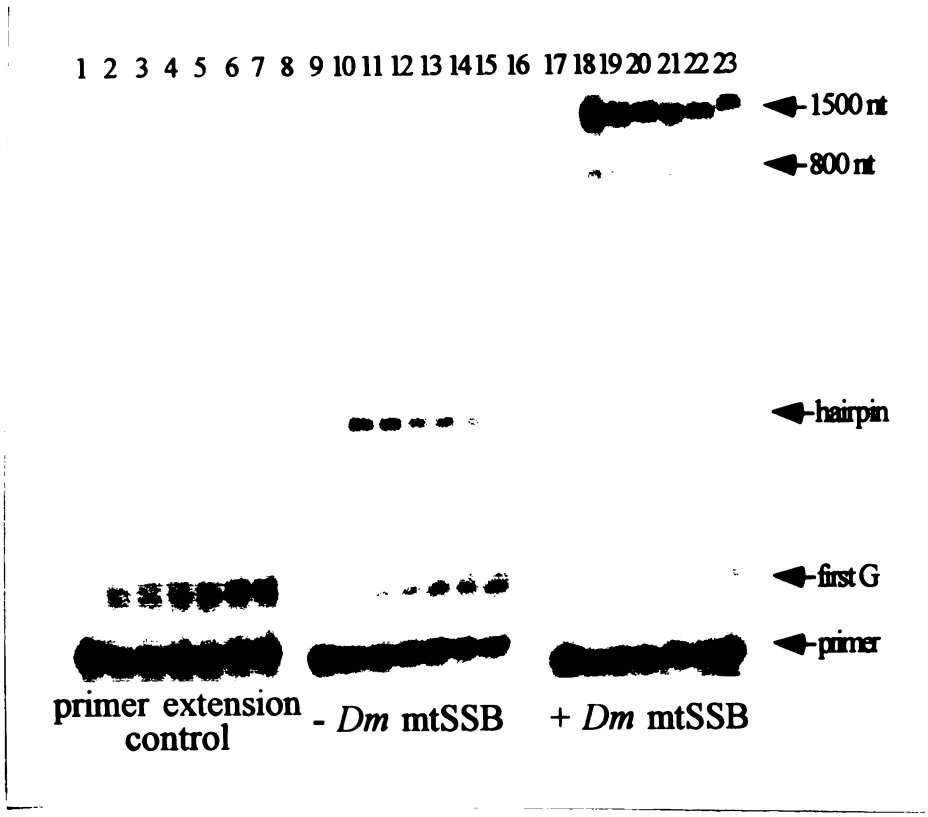
Dissociation of Pol γ paused by nucleotide omission is dramatically slowed at low salt

The autoradiographs in Figures 11 and 12 show the primer extension products obtained in experiments analyzing the stability of Pol γ idled at the template-primer terminus. Pol γ was stalled at the triG stop position, by omitting dCTP from the initial extension reaction, followed by addition of excess DNase I-activated calf thymus DNA, to trap dissociating polymerase molecules (Figure 11A lanes 1-7 and Figure 12A lanes 1-7). At varying times following the addition of the DNA trap, aliquots were incubated for an additional 2 min in the presence of dGTP, dATP, dTTP and dCTP in the presence or absence or mtSSB, allowing remaining polymerase molecules to extend to and possibly through the hairpin.

Linear regression analysis of primer extension products reveals the dissociation of Pol γ upon idling is 16-fold slower at 30 mM KCl, where the $t_{1/2}$ for dissociation of Pol γ is 17.9 ± 3.2 minutes at 30 mM KCl versus 1.08 ± 0.18 minutes at 120 mM KCl. Plotting the data reveals linear decay rates following the addition of the DNA trap (Figures 11B and 12B). This is within the same order of magnitude as for template-primer binding at low versus moderate salt, once more arguing in favor of stabilization of protein-DNA interactions at low salt, which may reduce enzyme cycling and thus the overall activity of Pol γ at low salt. The stability of Pol γ association upon template-primer binding or idling

Figure 11. ***Drosophila* Pol γ idling stability at 30 mM KCl in the presence and absence of *Dm* mtSSB.** A, An autoradiograph of primer extension products electrophoresed on a 18% polyacrylamide / 7 M urea gel, *lanes* 1-15, and a 6% polyacrylamide / 7 M urea gel, *lanes* 16-23. *Lanes* 1, 9 and 17, no enzyme controls; *lanes* 2, 10 and 18, no DNA trap added; *lanes* 8 and 16, blank; *lanes* 3-7, primer extension control; lanes 11-15 primer extension products obtained from 2, 4, 10, 20 and 40 min incubations using 0.7 units Pol γ Fraction VI and no *Dm* mtSSB; *lanes* 19-23, primer extension products obtained 2, 4, 10, 20, 40 and 60 min incubations using 0.17 units Pol γ Fraction VI with *Dm* mtSSB (0.4 μg/assay). The positions of the primer, products terminated at the first G and the hairpin are indicated on the right. The sizes of products extended through the hairpin in the presence of *Dm* mtSSB were determined by comparison with molecular weight markers run in a parallel lane. B, Quantitation of data in A is plotted to show the linear rate of of polymerase dissociation by plotting percent polymerase molecules remaining associated with the template versus time. *Open circles* represent reactions performed in the absence *Dm* mtSSB, while *closed circles* represent reactions performed in the presence of *Dm* mtSSB.

A



B

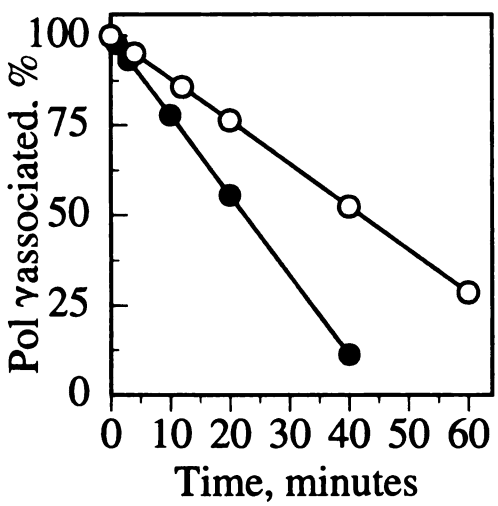
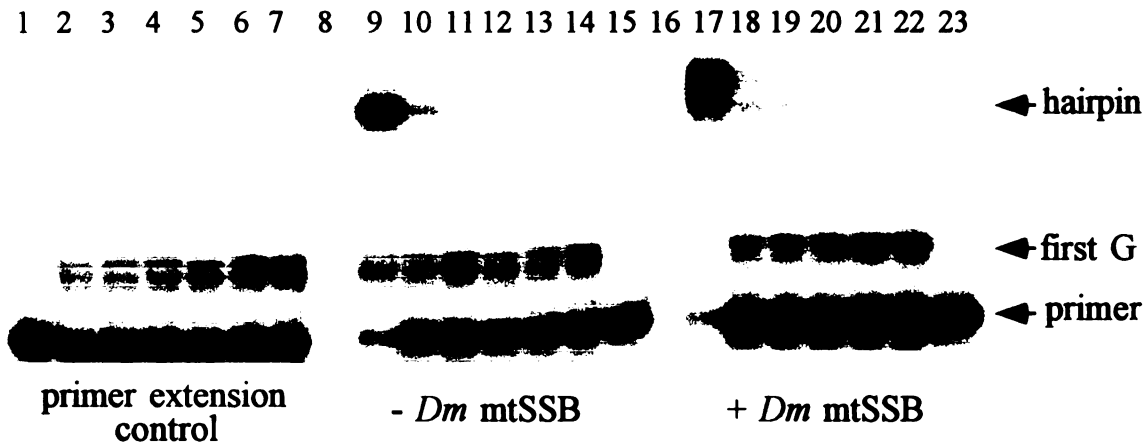


Figure 11. *Drosophila* Pol γ idling stability at 30 mM KCl in the presence and absence of *Dm* mtSSB.

Figure 12. ***Drosophila* Pol γ idling stability at 120 mM KCl in the presence and absence of *Dm* mtSSB.** A, An autoradiograph of primer extension products electrophoresed on a 18% polyacrylamide / 7 M urea gel. *Lanes* 1, 15 and 23, no enzyme controls; *lanes* 2, 9 and 17, no DNA trap added; *lanes* 8 and 16, blank; *lanes* 3-7, DNA trap control; lanes 10-14 primer extension products obtained from 0.3, 0.67, 1 and 2 min incubations using 0.17 units Pol γ Fraction VI and no *Dm* mtSSB; *lanes* 18-22, primer extension products obtained from the same time course using 0.17 units Pol γ Fraction VI and *Dm* mtSSB (0.4 μg/assay). The positions of the primer, products terminated at the first G and the hairpin are indicated on the right. B, Quantitation of data in A is plotted to show the linear rate of of polymerase dissociation by plotting percent polymerase molecules remaining associated with the template versus time. *Open circles* represent reactions performed in the absence *Dm* mtSSB, while *closed circles* represent reactions performed in the presence of *Dm* mtSSB.

A



B

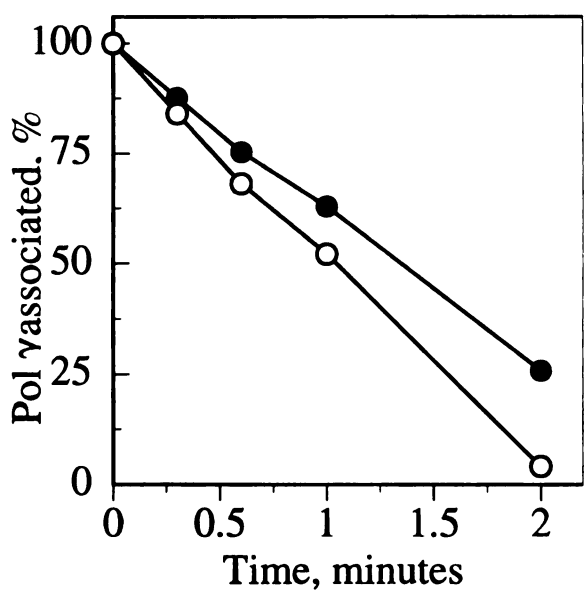


Figure 12. *Drosophila* Pol γ idling stability at 120 mM KCl in the presence and absence of *Dm* mtSSB.

is remarkable in the absence of replication accessory proteins. The Pol γ-DNA complex formed is ~5-fold more stable than the five protein T4 DNA polymerase holoenzyme complex formed under similar conditions.

_Dm_ mtSSB further stabilizes the association of Pol γ with the primer terminus when it is paused by nucleotide omission.

The autoradiographs in Figures 11A lanes 17-23 and 12A lanes 17-23 show primer extension products derived from idling experiments performed at 30 and 120 mM KCl in the presence of saturating amounts of *Dm* mtSSB. The idling assay was performed as above except that mtSSB was allowed to coat the ssDNA template prior to the addition of Pol γ.

Linear regression analysis of experimental data plotted in Figures 11B and 12B reveals a $t_{1/2}$ for polymerase dissociation of 17.9 ± 3.2 min at 30 mM KCl in the absence of mtSSB, that is stabilized 2-fold at 30 mM KCl in the presence of mtSSB yielding a $t_{1/2}$ of 33.8 ± 6.0 min. This stabilization of polymerase idling supports the argument for enhanced protein-DNA interactions at low salt. It also indicates that in the synthetic mode (binding in the presence of dNTPs), Pol γ binds the template-primer terminus more stably when mtSSB is included in the reaction mix.

Furthermore, DNA synthesis is stalled at the DNA hairpin helix only in the absence of mtSSB. In the presence of mtSSB at 120 mM ($t_{1/2}$ of 0.84 ± 0.15 min), DNA synthesis proceeds three nucleotides into the hairpin, while synthesis proceeds completely through the hairpin at 30 mM KCl. These results eliminated the need to perform the enzyme barrier studies, that examine the ability of mtSSB to destabilize DNA hairpin helices of varying stabilities. The 22 bp hairpin located 40 nucleotides downstream of the primer terminus impedes the progress of T4 DNA polymerase in the presence of the T4 gene 32 protein (T4 SSB) and causes near immediate dissociation of the polymerase ($t_{1/2}$ of 1 sec) at the hairpin

(99). This contrasts sharply with the *Dm* mtSSB effect at the same position, where the 22 bp hairpin is partially melted out at 120 mM KCl and completely melted out at 30 mM KCl, suggesting the mtSSB is more efficient in destabilizing DNA secondary structures than its bacteriophage counterpart.

Summary and Future Plans.

We have shown that *Dm* mtSSB stimulates DNA synthesis by DNA polymerase γ by increasing productive binding to the template-primer terminus (Figure 7) and stabilizes template-primer terminus interactions. Further, we can achieve optimal DNA synthetic activity and processivity with Pol γ in the presence of the mtDNA replication accessory protein mtSSB. Using the information provided by the above experiments we can continue efforts to define the function of DNA polymerase γ in mtDNA replication.

The most interesting experiments will be to determine the contribution of the mtSSB to the fidelity of DNA synthesis by Pol γ. Such experiments are important to explain the higher rate of mutations incurred in mtDNA molecules despite the apparent high fidelity of the polymerase in *in vitro* experiments (89). SSB protein has been shown to enhance the fidelity of a variety of prokaryotic and eukaryotic DNA polymerases, by at least an order of magnitude, on an assortment of DNA templates (81). The increase in fidelity may result from stimulation of 3' → 5' proofreading exonuclease activity, but polymerases lacking proofreading exonuclease show proportional increases in fidelity. Our data, demonstrating stimulation of DNA polymerase and 3' → 5' exonuclease activities, support a mechanism of mtSSB stimulation by increased primer recognition, through exclusion of nonproductive DNA-binding, and through increased stability of template-primer binding. Such a mechanism suggests that Pol γ fidelity at low salt in the presence of mtSSB may be lowered.

Two types of experiments should yield useful answers. First, the ϕX174 reversion assay of Kunkel (100), that allows the determination of the reversion frequency for copied DNA when compared to an uncopied control and allows the use of polymerases with an associated exonucleolytic activity, will be useful for comparisons with previous reversion frequencies determined for Pol γ (89). Second, a recently developed gel kinetic assay of Creighton and Goodman (101), specifically established to allow quantitation of primer extension products synthesized by exonuclease containing DNA polymerases, will allow quantitation of products derived from our standard primer extension assay under varying reaction conditions. Performing such experiments in the absence and presence of mtSSB will allow a quantitative determination of its effect on Pol γ fidelity.

In addition to studies of the fidelity of DNA synthesis we need to complete studies toward defining the ultimate mechanism of mtSSB stimulation. Available data are consistent with a mechanism involving enhanced primer recognition and stable primer binding. We can define further these roles by performing gel mobility shift assays on shorter radiolabeled DNA template-primers, allowing us to determine the apparent on rate for Pol γ binding in the presence and absence of mtSSB at low and moderate salt, in competition and DNA-binding time course experiments.

CATALYTIC (α) AND ACCESSORY (β) SUBUNITS OF DNA POLYMERASE γ: BACTERIAL OVEREXPRESSION, PURIFICATION AND ASSOCIATION OF THE TWO SUBUNITS IN THE NATIVE ENZYME

Overview

The mechanism and subunit structure of Pol γ purified from *D. melanogaster* embryos has been studied extensively (63, 69, 87, 88, 90). Pol γ consists of two polypeptides of 125 kDa (α) and 35 kDa (β) (89) and contains two catalytic activities. Both the 5' → 3' DNA polymerase and 3' → 5' proofreading exonuclease activities have been assigned to the 125 kDa catalytic subunit, while no function has yet been assigned to the 35 kDa accessory subunit. Attempts to separate the subunits of the native enzyme and probe structure and function relationships between the two subunits resulted in the loss of >95% activity prior to subunit dissociation (88). To extend these studies, cDNA clones of the two subunits isolated by David Lewis and Yuxun Wang were overexpressed in and purified from *E. coli.* The resulting protein fractions were assayed for polymerase and exonuclease activities and the subunit composition of native DNA polymerase γ was confirmed.

Experimental Procedures

Materials

Nucleotides and Nucleic Acids— Unlabeled deoxynucleotides were purchased from Pharmacia BioTech. [^{3}H]dTTP and [γ-^{32}P]ATP were purchased from ICN Biochemicals. M13, pET-16b and pET-11a (Novagen) DNAs were prepared by standard laboratory methods. Synthetic oligonucleotides as indicated in the text were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer according to the manufacturer's protocols.

Enzymes and Proteins— *Drosophila* DNA polymerase γ (Fraction VI) was prepared as described by Wernette and Kaguni (63). T4 polynucleotide kinase and *Escherichia coli* DNA polymerase I were purchased from New England Biolabs. Human serum albumin and bovine carbonic anhydrase were purchased from Worthington. Hen egg white lysozyme was purchased from Boehringer Mannheim.

Bacterial strains— *E. coli* BL21 (λDE3) *ompT*, r_B^- M_B^- (Novagen) was used for expression of pET-11a and pET-16b constructs.

Chemicals— Isopropylthio-β-D-galactoside (IPTG), nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate and imidazole were purchased from Sigma. Leupeptin was purchased from the Peptide Institute, Minoh-Shi, Japan. Phenylmethylsulfonyl fluoride (PMSF), ultra pure urea and dithiothreitol (DTT) were purchased from Research Organics. Sodium metabisulfite was purchased from J. T. Baker Chemical. Goat anti-Rabbit IgG alkaline phosphatase conjugate was purchase from BioRad.

Resins— Amberlite MB-3 ion exchange resin was purchased from Mallinckrodt. Phosphocellulose resin was purchased from Whatman. HisBind resin for Ni^{2+}-affinity chromatography was purchased from Novagen.

Methods

Cloning, sub-cloning and bacterial overexpression of the catalytic (α) and accessory β subunits of Pol γ.— Using peptide sequences obtained from 255 pmol of *Drosophila melanogaster* Pol γ, degenerate oligonucleotides primers were generated and used in PCR synthesis on an ovarian cDNA library derived from *D. melanogaster* ovarian mRNA as described by Lewis, *et al.* (69) for the α subunit and Wang, *et al.* (102) for the β subunit. The resulting cDNAs, encoding either the α (3435 bp) or β (1083 bp) subunit and engineered to contain *Nde* I restriction sites at the ends, were subcloned into the bacteriophage T7 promoter-based expression vectors pET-11a or pET-16b as described by Lewis, *et al.* (69) and Wang, *et al* (102) . The pET-16b vector encodes a 10-histidine N-terminal tag to facilitate purification using Ni^{2+}-affinity chromatography (described below). The *E. coli* strain BL21 (λ DE3) was used for transformation, and ampicillin-resistant plasmid-containing cells were screened for insert size and orientation of recombinant DNAs by restriction analyses (see Lewis, *et al* (69) and, Wang, *et al* (102)).

For overexpression of the catalytic α (γ125-11a or γ125–16b) and β (γ35-11a or γ35-16b) subunits, plasmid-containing BL21 (λ DE3) cells were grown at 37°C with aeration, in L broth containing 100 μg/ml ampicillin. When the bacterial cells reached an optical density of ~0.6 at 595 nm, IPTG was added to 0.3 mM, and the culture was incubated an additional 45 min to 2 hours to obtain maximum overexpression from various constructs. Cells were harvested by centrifugation, washed in 50 mM Tris-HCl pH 7.5 / 10% sucrose, recentrifuged and cell pellets were frozen in liquid nitrogen and stored at -80°C.

Catalytic subunit isolation from soluble and insoluble fractions.— For preparation of cell extracts and purification of recombinant catalytic subunit, frozen cells were thawed on ice and all further steps were performed at 0-4°C. Cells were suspended in 1/30 volume of original cell culture in 50 mM Tris-HCl pH 7.5 / 10% sucrose / 5 mM DTT / 2 mM EDTA / 1 mM PMSF / 10 mM sodium metabisulfite / 2 μg/ml leupeptin, and lysed by incubation for 30 min in the presence of 0.3 mg/ml final concentration of lysozyme and 0.25 M NaCl. The suspension was then sonicated with a Ultrasonic Processor model W-225 (Heat Systems—Ultrasonics, Inc.), for three rounds of 10 pulses using the microtip at maximum output and 50% usage, with cooling in an ice water-salt bath between each set. Soluble and insoluble fractions were separated by centrifugation for 15 min at 20,000 x *g*. Soluble protein was recovered in the supernatant fluid and set aside for phosphocellulose (γ125-11a) or Ni^{2+}-affinity (γ125-16b or γ35-16b) chromatography (see below). Insoluble protein was extracted from the pellet fraction using two different methods. First, a salt washing method modified from Xiao and O'Donnell (103) for the isolation of the ψ subunit of DNA Pol III holoenzyme was used. Pellet material was resuspended in 1/80 volume of original cell culture in buffer A (20 mM Tris-HCl pH 7.5 / 20% glycerol / 2 mM DTT / 0.5 mM EDTA / 1 mM PMSF / 10 mM sodium metabisulfite / 2 μg/ml leupeptin) containing 10 mM NaCl and sonicated once. The sample was then centrifuged at 20,000 x *g* for 15 min, and the resulting supernatant fluid was removed and discarded. The pellet was suspended in the same volume of buffer A containing 1M NaCl and recentrifuged. The previous step was repeated and the resulting pellet was washed by the same procedure in buffer A containing 10 mM NaCl. The final "salt-washed, insoluble pellet" was extracted by incubation for 2 hours in 1/300 volume of original cell culture in buffer A containing 2 M urea (deionized by stirring at 25°C for 20 min with 0.2 g Amberlite MB-3 per ml of solution, and then filtered). The extract was then centrifuged at 12,000 x *g* for 10 min and the supernatant fluid recovered.

The second method for extraction of insoluble protein utilized the detergent-washing procedure of Nagai and Thogersen (104). The cell extract was prepared as above with the following exceptions: cell lysis was performed in the presence of 2 mg/ml lysozyme final concentration and 0.1 M NaCl in 1/100 volume of original cell culture; prior to centrifugation 4.5 ml (equal volume) of detergent buffer (20 mM Tris-HCl pH 7.5 / 1% deoxycholic acid (w/v) / 1% Nonidet P-40 (v/v) / 2 mM EDTA) was added and the lysate was sonicated as above, then centrifuged for 10 min at 5,000 x *g*. The supernatant fluid is removed and the pellet is completely suspended in 0.5% Triton X-100, 1 mM EDTA and centrifuged for 10 min at 5,000 x *g*. The resulting supernatant fluid is removed and discarded and the Triton / EDTA wash is repeated three times. The final "detergent-washed, insoluble pellet" is suspended in buffer containing 7 M urea (deionized as above) incubated for ≥16 hours on ice and centrifuged at 100,000 x *g* for 30 min (S-100 spin).

Ni^{2+}-affinity chromatography— HisBind resin of Novagen was used to purify γ125-16b and γ35-16b from detergent-washed insoluble fractions (obtained from 400 ml cell culture) following protocols in the pET System Manual by Novagen (1993). Cell extracts were prepared as described for denaturing chromatography in the presence of 6M deionized urea. γ125-16b and γ35-16b detergent-washed insoluble fractions 0.3 mg/ml packed resin and 20 mg/ml packed resin, respectively were loaded onto 2.5 ml of His-Bind resin. Chromatography buffers (20 mM Tris-HCl pH 7.9 / 500 mM NaCl / 2 mM DTT / 1 mM PMSF / 10 mM sodium metabisulfite / 2 μg/ml leupeptin) containing 5 mM imidazole, 60 mM imidazole, 100 mM imidazole or 150 mM imidazole were used for binding target proteins to the resin and as washing buffers. Target proteins were eluted with 500 mM imidazole buffer and the resin was stripped of remaining proteins with 100 mM EDTA buffer.

Phosphocellulose chromatography— Phosphocellulose chromatography of the soluble extract from γ125-11a induced cells was essentially as described by Wernette and Kaguni (63), except that the linear gradient was from 150-600 mM potassium phosphate.

The catalytic subunit eluted at 400 mM potassium phosphate; fractions were pooled and adjusted with 80% sucrose to a final concentration of 10%. After addition of 1.2 volumes of saturated ammonium sulfate, pH 7.5, to achieve 55% saturation at 0°C, the suspension was incubated on ice for 2 hours. The precipitate was collected by centrifugation at 96,000 x *g* at 3°C, resuspended and stored at -20°C.

Glycerol Gradient Sedimentation— Glycerol gradient sedimentation of the urea-extracted salt-washed insoluble pellet (see above) was performed as described by Wernette and Kaguni (63) except that the gradients contained 2 M deionized urea and were centrifuged at 41,000 rpm for 96 hours. Other glycerol gradient sedimentations using putatively renatured α and β subunit fractions (see below) were performed as described by Wernette and Kaguni (63).

DNA polymerase and 3' → 5' Exonuclease Assays— DNA polymerase and 3' → 5' activities were assayed on Dnase I-activated calf thymus DNA at 200 mM KCl, and on M13 DNA, annealed to either a 15 nt primer (LSK-4) forming a 3'-terminal mispair or a 17 nt primer (LSK-8) forming a 3'-terminal base pair, at 120 mM KCl, respectively, as described by Olson and Kaguni (73).

Protein Gel Electrophoresis, Transfer and Immunoblotting— SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (91). Proteins were transferred to nitrocellulose membranes (BA85, Schleicher and Schuell) and probed by immunoblotting using 1/1000 dilutions of primary antiserum produced against native Pol γ and its isolated subunits (see below) and 1/3000 dilution of Goat anti-Rabbit IgG alkaline phosphatase conjugate secondary antibody as described in Olson, *et al.* (88) in place of protein A-alkaline phosphatase.

Immunoprecipitation of Drosophila DNA polymerase γ— Pol γ (Fraction VI) was first mixed with equal volumes of PBS buffer (10 mM $NaPO_4$ (pH 7.0) and 154 mM NaCl) and incubated at 65°C for 10 min and then incubated with preimmune serum or polyclonal subunit-specific antisera overnight on ice. Immunecomplexes were precipitated

by incubation with preswollen protein A agarose (50 µl of 50% slurry) for 2 hours with gentle inversion. The precipitates were collected by centrifugation and washed three times with PBS, suspended in Laemmli sample buffer (91), heated for 10 min at 85°C, and recentrifuged. The supernatant fractions were then subjected to immunoblotting as described above.

Production of DNA Polymerase γ Subuni-specific Antisera—

Recombinant α and β subunit derived from urea-extracted insoluble fractions were electrophoresed in 10% SDS-polyacrylamide gels, bands were detected by staining with Coomassie blue in water and the α- and β-subunit polypeptide bands were excised. The gel slices were minced in PBS buffer, then homogenized using a microcentrifuge tube pestle and emulsified with an equal volume of Freund's complete adjuvant. Immunizations of virgin female New Zealand White rabbits were performed by subcutaneous injections of 15-30 µg of the relevant protein. Booster immunizations were administered in Freund's incomplete adjuvant at 2-4 week intervals.

Renaturation of DNA polymerase γ α and β subunits — Renaturation of detergent-washed 7M urea-extracted insoluble α- and β-subunit fractions involved the slow removal of the denaturant urea by dialysis against buffers of decreasing urea concentration. All buffers containing urea were deionized using 2 g of Amberlite ion exchange resin per 10 ml of solution immediately prior to use and all buffers contained protease inhibitors (1 mM PMSF / 10 mM sodium metabisulfite / 2 µg/ml leupeptin) and DTT (2 mM DTT). Optimal renaturation, of the α subunit alone or combined in a 1:2 molar ratio with the β subunit, was achieved using the following conditions. Protein fractions were diluted to 25-50 ng per µl (800 µl final volume) in buffer containing 7 M urea / 0.2 M Tris-HCl pH 8.0 / 500 mM NaCl / 4 mM $MgCl_2$ / and 0.1% Triton X-100. The samples were incubated on ice for 30 min and then transferred to a collodion bags (Schleicher and Schuell, MW cutoff 25 kDa) and dialyzed. First, the samples were dialyzed for 6 hours at 4°C against 100 ml buffer containing 4 M urea / 0.2 M Tris-HCl pH 8.0 / 200 mM NaCl / 4 mM $MgCl_2$ / and

0.1% Triton X-100 with gentle stirring of the buffer. Next, samples were dialyzed for 6 hours at 4°C against 100 ml buffer containing 2 M urea / 0.1 M Tris-HCl pH 8.0 / 200 mM NaCl / 4 mM $MgCl_2$ / and 0.1% Triton X-100, then against 100 ml 1 M urea buffer in 50 mM Tris-HCl pH 7.5 / 200 mM NaCl / 4 mM $MgCl_2$ / and 0.1% Triton X-100. Last, samples were dialyzed into buffer containing 10% glycerol / 50 mM Tris-HCl pH 7.5 / 200 mM NaCl / 4 mM $MgCl_2$ / and 0.01% Triton X-100 for storage at 4 °C. The concentration of Triton X-100 was reduced to 0.01% in the final buffer to mimic Pol γ Fraction VI, which contains 0.015% Triton X-100, and to eliminate potential inhibition in polymerase activity assays. The final samples were spun at 100,000 x *g* for 1 hour (S-100) to remove any protein aggregates and the supernatant fluid was recovered. S-100 supernatant fraction were subjected to glycerol gradient sedimentation as described by Wernette and Kaguni (63) and fractions were examined by either silver stain or immunoblot analysis of SDS-polyacrylamide gels.

Results

Bacterial overexpression, purification and renaturation of the α and β subunits of *Drosophila* DNA Polymerase γ.

The coding sequences of the α (3435 bp) and β (1083 bp) subunits of Pol γ, containing PCR generated *Nde* I restriction sites, were subcloned (by D. Lewis and Y. Wang, respectively) into the unique *Nde* I site of either the pET-11a or pET-16b bacteriophage T7 promoter-based expression vectors (Novagen). Ampicillin-resistant plasmid-harboring *E. coli* BL21 (λDE3) strains were used for bacterial overexpression. Expression upon IPTG induction of the two subunits in either pET-11a or pET-16b followed the same procedure with variation only in the length of incubation following induction of target protein expression. IPTG induced overproduction yielded ~10 μg of α subunit and ~7 μg of β subunit per ml of cell culture. Despite the use of a variety of induction and coexpression conditions, only 10-20% of the α subunit and ~5% of β subunit remained in the soluble fraction following cell lysis.

Extracts of the α and β subunits obtained from optimal induction and lysis conditions were subjected to chromatography under both denaturing and non-denaturing conditions. Soluble extracts containing the α subunit were subjected to chromatography on phosphocellulose and fractions containing the recombinant protein were pooled and concentrated by ammonium sulfate precipitation. Concurrently, extracts of both the α and β subunits were derived from the insoluble inclusion body fraction using three different methods of insoluble protein extraction. First, we used the salt washing procedure of Xiao and O'Donnell (103) to recover α-subunit inclusion body fractions followed by extraction of the recombinant protein with 2 M urea. Second, we followed the Novagen manual for isolation and purification of insoluble histidine-fusion proteins under denaturing conditions to purify both the α and β subunits. Third, we used the detergent-washing procedure of

Nagai and Thogersen (104) to recover inclusion body fractions for both the α and β subunits which were then extracted with 7 M urea. The soluble and 2 M urea-extracted insoluble α-subunit fractions were assayed for DNA polymerase activity on DNase I-activated calf thymus DNA under standard conditions (89). Recombinant α-subunit DNA polymerase activity is stimulated several fold by 200 mM KCl and inhibited by the addition of ddTTP in keeping with.the characteristics of native Pol γ from *Drosophila* embryos (data not shown). Because the function of the β subunit is unknown, no activity assays were conducted. Alternatively, protein analysis by SDS-polyacrylamide gel electrophoresis of α- and β-subunit containing fractions followed by either silver staining or immunoblot analysis with *Drosophila* Pol γ antiserum, identifies the overexpressed polypeptides as intact components of the native protein (Figures 13A and B and Figures 14A and B, respectively). Smaller immunoreactive polypeptides are present in α-subunit containing fractions, that likely arise from *in vivo* proteolysis. Comparison of α-subunit whole cell extracts with soluble and urea-extracted insoluble fractions reveals the insolubility of the intact catalytic subunit is greater than that of the truncated polypeptides. The intact α subunit comprises 50% of the polypeptides in the urea-extracted insoluble fraction, but only 20% of α-related polypeptides in the soluble fraction. In addition, the specific activity of the isolated catalytic subunit is ~20-fold lower than that of native Pol γ and may suggest either a role for the β subunit in stabilizing enzyme-DNA interactions or it may reflect the composition of the fraction assayed, which contained a variety of polypeptides that may retain DNA-binding activity with no associated polymerase activity.

7 M urea extracts of α- and β-subunit inclusion body fractions chromatographed according to the Novagen manual for purification under denaturing conditions yielded highly purified proteins (data not shown). However, DNA polymerase assays of the his-tagged α subunit revealed, that the protein was inactive, which may be due to the conditions utilized during the purification. The HisBind chromatography protocol involves the use of both the denaturant urea and the metal chelating-agent imidazole to elute the

Figure 13. **Bacterial overexpression and purification of the catalytic subunit of *Drosophila* Pol γ.** Protein fractions were denatured and electrophoresed in a 10% SDS-Polyacrlyamide gel. Proteins were detected by silver staining (A) or by immunoblotting using the Goat anti-rabbit alkaline phosphatase method with rabbit antiserum against *D. melanogaster* Pol γ at a 1:1000 dilution (B). Lanes 1, *D. melanogaster* Pol γ Fraction VI (0.05 μg); lanes 2, uninduced α-subunit/BL21 (λDE3) cells; lanes 3, α-subunit-induced cells; lanes 4 α-subunit soluble extract; lanes 5 α-subunit phosphocellulose pool; lanes 6 α-subunit, urea-extracted insoluble fraction.

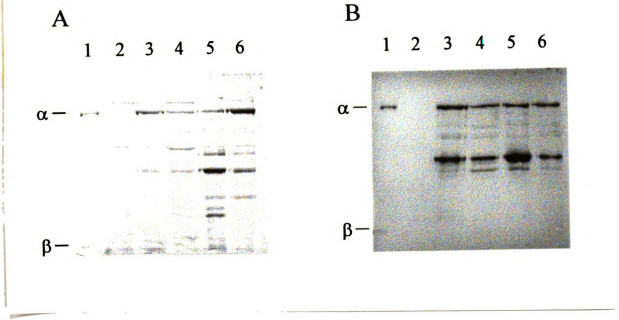


Figure 13. Bacterial overexpression and purification of the catalytic subunit of *Drosophila* Pol γ.

Figure 14. **Bacterial overexpression and purification of the β subunit of *Drosophila* Pol γ.** Protein fractions were denatured and electrophoresed in a 10% SDS-polyacrylamide gel. Proteins were detected by silver staining (A) or by immunoblotting using the goat anti-rabbit IgG-alkaline phosphate method with rabbit antiserum against *Drosophila* Pol γ at a 1:100 dilution (B). Lane 1, *Dm* Pol γ Fraction VI (0.05 μg); lane 2, uninduced β-subunit, urea-extracted insoluble fraction; lane 3, β-subunit induced cells; lane 4, β-subunit soluble extract; lane 5, β-subunit urea-extracted insoluble fraction. The protein fractions electrophoresed in lanes 2-5 were derived from 2 x 10^7 bacterial cells.

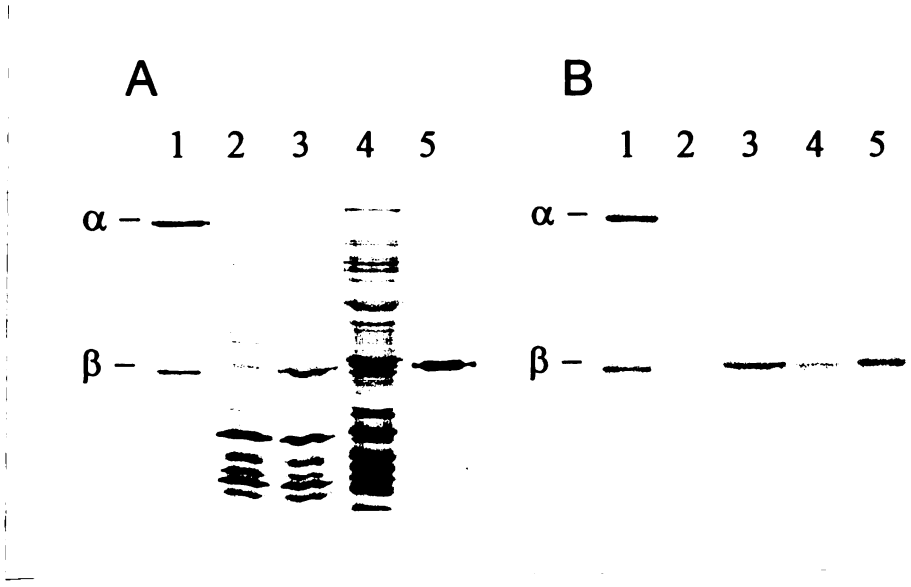


Figure 14. Bacterial overexpression and purification of the β subunit of *Drosophila* Pol γ.

tagged proteins. The use of either of these chemicals may have caused irreversible inactivation of the catalytic subunit, through complete denaturation and improper refolding or by stripping of the metal centers within the polymerase active site.

Renaturation of non-tagged detergent-washed urea-extracted insoluble α- and β-subunit fractions was attempted following the procedures described in *"Methods"* in an attempt to isolate highly purified and active α and β subunit. Despite multifarious attempts to isolate renatured and active catalytic subunit or to reconstitute native Pol γ *in vitro*, we were only able to renature a limited amount of the catalytic subunit. Potentially renatured fractions of the α subunit alone and in combination with the β subunit (2:1 molar ratio of β:α) were subjected to glycerol gradient sedimentation under standard conditions (63) and analyzed by three methods. First, aliquots were examined for protein content by immunoblot analysis following SDS-polyacrylamide gel electrophoresis, which revealed only partial renaturation of the catalytic subunit and other α-subunit related polypeptides (Figure 15). Similar analysis of simultaneously renatured α- and β-subunit fractions revealed only partial folding of α-subunit related polypeptides with no evidence for *in vitro* reconstitution of Pol γ (data not shown). Second, using a standard primer extension assay we tried to correlate polymerase activity with fractions containing the renatured catalytic subunit (Figure 16A). We found polymerase activity, but it did not correlate with the catalytic subunit. Third, together with the primer extension assay we examined DNA binding in a gel mobility shift assay (Figure 16B). This assay revealed DNA binding activity nearly coincident with the primer extension activity, which also failed to correlate with fractions containing the renatured catalytic subunit.

Figure 15. **Renaturation of detergent-washed urea-solubilized *Drosophila* Pol γ catalytic subunit.** Glycerol gradient fractions (fractions 3-35) of renatured recombinant catalytic subunit, sedimented as described under *Methods,* were denatured and electrophoresed on a 10% SDS-polyacrylamide gel and probed with α-subunit specific antiserum (1:1000 dilution). The positions of the full length (p125) and truncated ($p125_t$) catalytic subunit polypeptides are indicated on the left. Bracketed fractions indicate the expected position of intact folded p125 and $p125_t$.

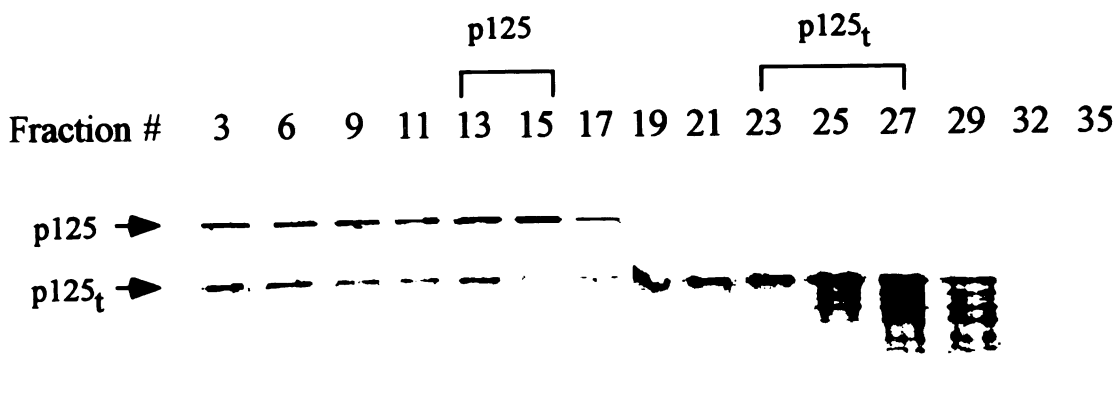


Figure 15. Renaturation of detergent-washed urea-solubilized *Drosophila* Pol γ catalytic subunit.

Figure 16. **Primer extension and DNA-binding assays of renatured *Drosophila* Pol γ recombinant catalytic subunit.** Glycerol gradient fractions (5 μl) shown in Figure 15 were assayed for DNA polymerase activity by primer extension assay (A) and for DNA-binding activity by gel mobility shift assay (B). The positions of the primer for DNA synthesis and free DNA in the gel shift assay are indicated on the right.

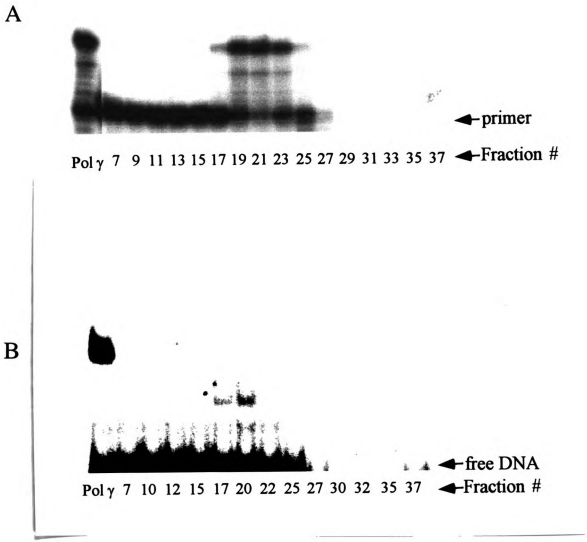


Figure 16. Primer extension and DNA-binding assays of renatured *Drosophila* Pol γ recombinant catalytic subunit.

The catalytic subunit exhibits both DNA polymerase and mispair specific 3' → 5' proofreading exonuclease activities.

To demonstrate the association of polymerase and exonuclease activities in the catalytic subunit, we subjected a 2 M urea-extracted insoluble fraction to glycerol gradient sedimentation in a 12-30% glycerol gradient containing 2 M urea. Analysis of glycerol gradient fractions showed cosedimentation of both DNA polymerase and 3' → 5' exonuclease activities coincident with the position of the intact catalytic subunit (Figure 17). Because of the limited amount of α-subunit polypeptide on the gradient, its position could not be determined by simple silver staining or immunoblot analysis of an SDS-polyacrylamide gel. Instead, Yuxun Wang was able to determine its position (p125 bracketed area in Figure 17) by photochemically crosslinking enzyme-DNA complexes (data not shown) to correlate the two activities with the α-subunit polypeptide. These experiments allowed the first biochemical demonstration of 3' → 5' exonuclease activity associated with the catalytic subunit of an animal mitochondrial DNA polymerase

To extend the characterization of the catalytic subunit proofreading exonuclease activity, we conducted experiments to compare its mispair specificity with that of the native holoenzyme. Previous work with native Pol γ using M13 DNA annealed to either 15 nucleotide primers to generate 3'-terminal dGMP:dGMP mispairs or 17 nucleotide primers to generate 3'-terminal dGMP:dCMP base pairs at the same position, showed that the rate of mispair hydrolysis was 15-fold greater than base pair hydrolysis at the salt optimum (120 mM) (73). Mispair hydrolysis was linear to 4 minutes, while base pair hydrolysis was not evident until after 20 minutes of incubation. Since the DNA polymerase and 3' → 5' exonuclease activities of Pol γ and its catalytic subunit vary with changes in the salt concentration, we investigated the mispair specificity of the 3' → 5' exonuclease of both native Pol γ and its catalytic subunit at salt concentrations optimal and sub-optimal for exonucleolytic activity. We found that the mispair specificity (mispair hydrolysis versus

Figure 17. **Cosedimentation of DNA polymerase and 3'→ 5' exonuclease in the catalytic subunit of *Drosophila* Pol γ.** Recombinant catalytic subunit (urea-extracted insoluble fraction, ~70% pure) was sedimented in a 12-30% glycerol gradient containing 2 M urea as described under "Methods" and they assayed for DNA polymerase (open circles) and 3'→ 5' exonuclease activity (closed circles) activity. The fractions bracketed as p125 represent the peak fractions containing catalytic subunit that was bound photochemically cross-linked to the template-primer DNA in an analysis performed as described by Lewis, et al. (69). Protein markers run in parallel gradients in the absence of urea were *E. coli* DNA polymerase I (Pol I, 5.5 S), human serum albumin (HSA, 4.6S) and bovine carbonic anhydrase (BSA, 3.2S).

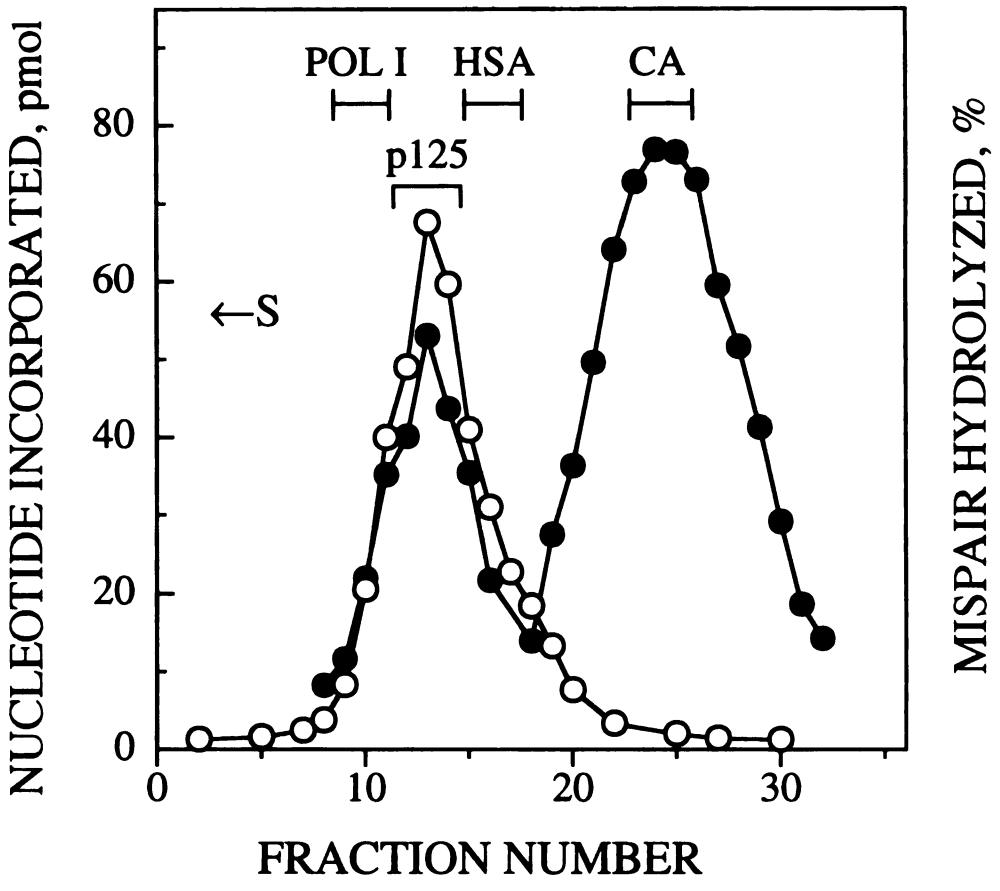


Figure 17. Cosedimentation of DNA polymerase and 3'→ 5' exonuclease in the catalytic subunit of *Drosophila* Pol γ.

base pair hydrolysis) of Pol γ is ~6-fold at low salt, where mispair hydrolysis is linear to 10 minutes and base pair hydrolysis is observable at 4 minutes of incubation (Figure 18). Thus, the overall specificity of mispair hydrolysis by Pol γ is 2.5-fold greater at 120 mM KCl where DNA polymerase and 3' → 5' exonuclease activity are optimal and enzyme processivity is moderate suggesting that the fidelity of DNA synthesis may be lower at low salt. Surprisingly, the specificity of mispair hydrolysis by the recombinant catalytic subunit of *Dm* Pol γ on the same mispaired and base paired substrates is at least 30-fold at both low and moderate salt. Mispair hydrolysis by the recombinant catalytic subunit is linear to 20 minutes at both KCl concentrations, and no base pair hydrolysis is discernible at any time point (Figures 19 and 20). This dramatic difference between the native and recombinant catalytic subunit activities suggests a role for the β subunit in modulating the 3' → 5' exonuclease activity associated with the catalytic subunit, perhaps through stabilizing DNA-protein interactions.

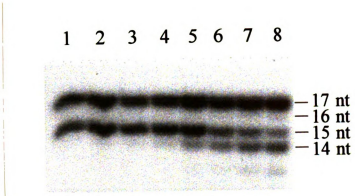
Immunoprecipitation with Pol γ subunit-specific antisera confirms the association of the β subunit in native Pol γ.

We have shown that the two enzymatic activities associated with DNA polymerase γ reside in the 125 kDa subunit. However, conclusive evidence of the physical association of the α and β subunit isolated from *Drosophila* embryos has been difficult. Olson *et al.* (88) showed that the α- and β-subunit polypeptides are intact and distinct components of Pol γ, but immunoprecipitation experiments using antiserum developed against native Pol γ could only suggest the physical association of the subunits.

Subunit-specific rabbit antisera were generated by injecting 15-30 μg of gel-purified α– or β–subunit recombinant polypeptides into virgin female New Zealand White rabbits. The resulting antisera, in an immunoblot analysis, exclusively recognize either the α or β subunit of *Drosophila* Pol γ in the Fraction III enzyme (Figure 21A), that is only ~7% pure.

Figure 18. **Specificity of exonucleolytic hydrolysis on M13 DNA by *Drosophila* Pol γ at 20 mM KCl.** A, DNA polymerase γ (Fraction VI) was assayed for 3' → 5' exonuclease activity on M13 DNA (6 μM) containing 3'-terminal mispaired primers (dGMP:dGMP, 15 nt) and base-paired primers (dGMP:dCMP, 17 nt). The position of the 3' terminus was the same for both primers; their 5'-end positions differed by 2 nt. Nucleotide excision was analyzed by denaturing gel electrophoresis and autoradiography. *Lane 1* represents no enzyme control; *lanes 2-8* correspond to reactions incubated for 0, 2, 4, 10, 20, 30, 40 min, respectively. B, quantitation of mispair (open circles) and base pair (closed circles) excision.

A


1 2 3 4 5 6 7 8
17 nt
16 nt
15 nt
14 nt

B

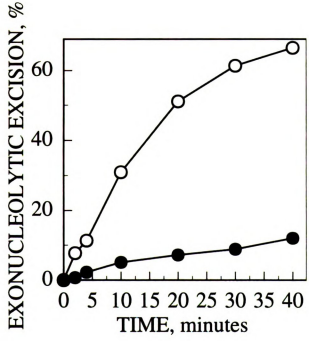
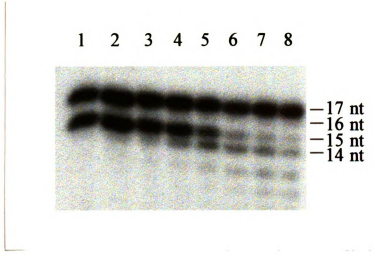

EXONUCLEOLYTIC EXCISION, %
0
20
40
60
0 5 10 15 20 25 30 35 40
TIME, minutes

Figure 19. **Specificity of exonucleolytic hydrolysis on M13 DNA by the catalytic subunit of *Drosophila* Pol γ at 120 mM KCl.** A, Salt-washedurea-extracted insoluble fraction (glycerol gradient pool) was assayed for 3' → 5' exo-nuclease activity on M13 DNA (4 μM) containing 3'-terminal mispaired primers (dGMP:dGMP, 15 nt) and base-paired primers (dGMP:dCMP, 17 nt). The position of the 3' terminus was the same for both primers; their 5'-end positions differed by 2 nt. Nucleotide excision was analyzed by denaturing gel electrophoresis and autoradiography. *Lane 1* represents no enzyme control; *lanes 2-8* correspond to reactions incubated for 0, 2, 4, 10, 20, 30, 40 min, respectively. B, quantitation of mispair (open circles) and base pair (closed circles) excision.

A


1 2 3 4 5 6 7 8
17 nt
16 nt
15 nt
14 nt

B

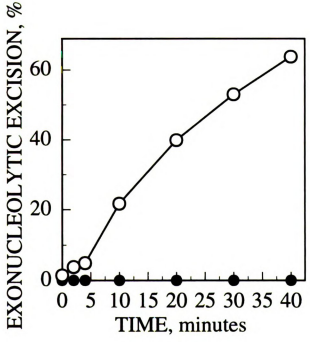
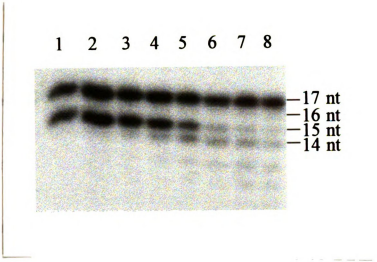
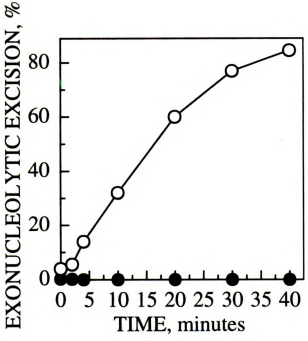

EXONUCLEOLYTIC EXCISION, %
60
40
20
0
0 5 10 15 20 25 30 35 40
TIME, minutes

Figure 20. **Specificity of exonucleolytic hydrolysis on M13 DNA by the catalytic subunit of *Drosophila* Pol γ at 30 mM KCl.** A, Salt-washed urea-extracted insoluble fraction (glycerol gradient pool) was assayed for 3' → 5' exo-nuclease activity on M13 DNA (4 μM) containing 3'-terminal mispaired primers (dGMP:dGMP, 15 nt) and base-paired primers (dGMP:dCMP, 17 nt). The position of the 3' terminus was the same for both primers; their 5'-end positions differed by 2 nt. Nucleotide excision was analyzed by denaturing gel electrophoresis and autoradiography. *Lane 1* represents no enzyme control; *lanes 2-8* correspond to reactions incubated for 0, 2, 4, 10, 20, 30, 40 min, respectively. B, quantitation of mispair (open circles) and base pair (closed circles) excision.

A


1 2 3 4 5 6 7 8
17 nt
16 nt
15 nt
14 nt

B


EXONUCLEOLYTIC EXCISION, %
0
20
40
60
80
0 5 10 15 20 25 30 35 40
TIME, minutes

An immunoprecipitation analysis was performed using these highly-specific antisera. Despite the fact that neither the α nor the β subunit-specific antiserum inhibits DNA polymerase activity, we were able to immunoprecipitate the native enzyme. Both the α and β subunit-specific antisera immunoprecipitate the same two polypeptides from Pol γ Fraction III (Figure 21B, lanes 3 and 4) demonstrating the physical association of the two subunits in native Pol γ. Moreover, these data are consistent with the subunit dissociation experiments, which showed separation of the α and β subunits of Pol γ could only be achieved upon partial denaturation of the enzyme (88).

Summary and Future Plans

We have only begun the potential analysis of the individual subunits of *Drosophila* mitochondrial DNA polymerase. Though bacterial overexpression of the α and β subunits of Pol γ has proven to be intractable, we have been able to make a few important conclusions regarding polymerase structure and function. First, the catalytic subunit contains both 5' → 3' DNA polymerase and 3' → 5' exonuclease activities. Second, the β subunit is a *bona fide* subunit of *Drosophila* mitochondrial DNA polymerase. Further attempts to express soluble and active recombinant α and β subunits in bacteria will not be pursued until innovative procedures are identified or developed.

Working to obtain *Drosophila* Pol γ in greater yield, while maintaining catalytic function, our laboratory continues the search to find an efficient system for recombinant mitochondrial protein expression. Yuxun Wang developed baculovirus expression vectors concurrently with the development of the bacterial expression system. Though tremendously more expensive and time consuming than bacterial overproduction, the baculovirus system has yielded more promising results. Yuxun has shown the formation of active Pol γ holoenzyme in mitochondria of baculovirus-infected insect cells. This system yields only limited success as the amount of recombinant protein imported into the

Figure 21. **Reactivity of subunit-specific rabbit antisera with *Drosophila* Pol γ.** A, immunoblot analysis of Pol γ Fraction III. Pol γ Fraction III in the amount of 25 units (~4 μg total protein, 7% pure) was denatured, electrophoresed in a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Immunoblotting with rabbit antiserum (1:1000 dilution) against *Drosophila* Pol γ (lane 1) or its recombinant α (lane 2) or β (lane 3) subunit was performed using the goat anti-rabbit alkaline phosphatase method. B, immunoprecipitation of Pol γ Fraction VI. Pol γ Fraction VI (~1 μg of near-homogeneous enzyme) was incubated with either rabbit preimmune serum (lane 2) or antiserum against recombinant α (lane 3) or β (lane 4) subunit. Immune complexes were precipitated and then electrophoresed in a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and detected by immunoblotting with *Drosophila* Pol γ antiserum. Lane 1 represents an immunoblotting control sample containing Pol γ Fraction III as in A.

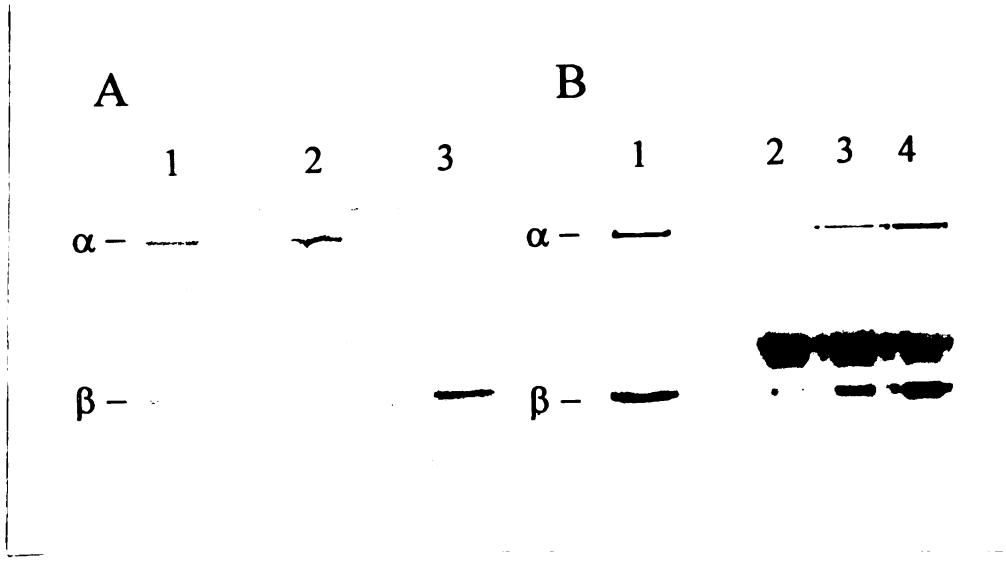


Figure 21. Reactivity of subunit-specific rabbit antisera with *Drosophila* Pol γ.

mitochondrion is limited by the import apparatus, and yields little more holoenzyme than that isolated from *Drosophila* embryos. However, this system may still prove useful for examining the assembly of α and β subunits. Li Fan has undertaken this project to map the α- and β-subunit interaction domains.

Development of a new method is underway to isolate active recombinant mitochondrial DNA polymerase. Anthony Lagina, in our laboratory, in collaboration with Inmaculada Ruiz de Mena from the laboratory of Dr. Raphael Garesse (Departamento de Bioquimica, Facultad de Medicina, Universidad Autonoma de Madrid, Spain) has developed transgenic fly lines. These lines contain either wild type or site-directed mutant polymerase subunits under control of weak- and strong-constitutive and heat-shock inducible promoters. Hopefully, analysis of these transgenic lines will provide flies overexpressing the two subunits. In addition to providing a potentially abundant source of both the individual subunits and the Pol γ holoenzyme, fly lines will be analyzed to evaluate the effects of wild type and mutator polymerase expression on mitochondrial function *in vivo*.

LIST OF PUBLICATIONS--CAROL L. FARR

Lewis, D.L., **Farr, C.L.**, Farquhar, A.L., and Kaguni, L.S. (1994) Sequence, organization and evolution of the A+T region of *Drosophila melanogaster* mitochondrial DNA. *Mol. Biol. Evol.* 11, 523-538.

Lewis, D.L., **Farr, C.L.**, and Kaguni, L.S. (1995) *Drosophila melanogaster* mitochondrial DNA: Completion of the nucleotide sequence and evolutionary comparisons. *Insect Mol. Biol.* 4, 263-278.

Thömmes, P., **Farr, C.L.**, Marton, R.F., Kaguni, L.S., and Cotterill, S. (1995) Mitochondrial single-stranded DNA-binding protein from *Drosophila* embryos: Physical and biochemical characterization. *J. Biol. Chem.* 270, 21137-21143.

Lewis, D.L., **Farr, C.L.**, Wang, Y., Lagina, A.T. III, and Kaguni, L.S. (1996) Catalytic subunit of mitochondrial DNA polymerase from *Drosophila* embryos: Cloning, bacterial overexpression and biochemical analysis. *J. Biol. Chem.* 271, 23389-23394.

Wang, Y., **Farr, C.L.**, and Kaguni, L.S. (1997) Accessory subunit of mitochondrial DNA polymerase from *Drosophila* embryos: Cloning, molecular analysis and association in the native enzyme. *J. Biol. Chem.* 272, 13640-13646.

Farr, C.L., and Kaguni, L.S. (1997) Template-primer binding and enzyme idling by *Drosophila* mitochondrial DNA polymerase: Influence of reaction conditions and mitochondrial single-stranded DNA-binding protein. In preparation for *J. Biol. Chem.*

Note: The first two publications resulted from research prior to entry into the Master's Program.

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