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PROCESSIVITY OF MITOCHONDRIAL DNA POLYMERASE FROM *DROSOPHILA* EMBRYOS: EFFECTS OF REACTION CONDITIONS, DNA-BINDING PROTEINS AND ENZYME PURITY

By

Andrea Jean VonTom

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ABSTRACT

PROCESSIVITY OF MITOCHONDRIAL DNA POLYMERASE FROM *DROSOPHILA* EMBRYOS: EFFECTS OF REACTION CONDITIONS, DNA-BINDING PROTEINS, AND ENZYME PURITY

By

Andrea Jean VonTom

Mitochondrial DNA polymerase from *Drosophila melanogaster* embryos was characterized with regard to its mechanism of DNA synthesis under the influence of a variety of compounds in moderate salt, where the enzyme is most active and only moderately processive, and in low salt, where it is less active yet most processive. Disparate activity and processivity optima were obtained in low salt in the presence of varying pH or $MgCl_2$ and ATP concentrations; in moderate salt, optimal activity and processivity were achieved coincidentally. Whereas no correlation between processivity and activity optima was observed upon addition of polyethylene glycol, the optima were coincident at both salt levels upon addition of glycerol. None of the reaction conditions examined allowed DNA polymerase γ to exhibit maximal activity and processivity concurrently; maximal activity was always achieved in moderate salt and highest processivity in low salt.

Addition of single-stranded DNA binding protein from *Escherichia coli* resulted in the concurrent achievement of maximal activity and processivity by pol γ in low salt, suggesting that γ polymerase, like other DNA polymerases, associates with accessory factors *in vivo* to catalyze efficient and processive DNA synthesis. The processivity of and intrinsic rate of nucleotide polymerization by mitochondrial DNA polymerase were increased only 3-fold by single-stranded DNA binding protein, while the rate of initiation of DNA synthesis was increased 25-fold. Maximal stimulation of γ polymerase was achieved following a lag time that correlated with the time required for DNA polymerase to productively bind DNA. The high degree of amino acid sequence similarity between

mitochondrial and *Escherichia coli* single-stranded DNA binding proteins suggests that mitochondrial single-stranded DNA binding protein may affect pol γ similarly.

The ability of mitochondrial DNA polymerase to copy singly-primed DNA was enhanced then diminished during the course of purification, suggesting the loss of an accessory factor. While stimulatory and DNA binding activities co-sedimented with a 145 kDa polypeptide, gel filtration chromatography resulted in their separation. While a potent endonuclease activity was responsible for DNA polymerase stimulatory activity, purification of the 145 kDa polypeptide by immunoblot confirmed its identity as an apparent template-primer specific DNA binding protein.

For

My Students

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I would like to thank Laurie Kaguni for her friendship, guidance, and perseverance over the last five years. If not for her persuasive arguments, I would have left graduate school years ago unable to fulfill my only wish upon enrolling: to teach college science. Laurie has had a huge influence on my perception of life and, at the risk of sounding maudlin, I will always remember the lessons that I have learned from her.

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PREFACE

Chapter II of this dissertation, along with figure 21 (chapter IV), was reproduced with permission from the *Journal of Biological Chemistry*.

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Chapter III of this dissertation, along with experiments examining the effects of *Escherichia coli* single-stranded DNA binding protein on the 3'→5' exonuclease activity of mitochondrial DNA polymerase, will be submitted to the *Journal of Biological Chemistry*.

> Williams, Andrea J., and Kaguni, Laurie S. (1994) Stimulation of Mitochondrial DNA Polymerase from *Drosophila* Embryos by Single-Stranded DNA Binding Proteins. *J. Biol. Chem.*

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LIST OF ABBREVIATIONS

Å	angstrom
apu	average processive unit
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
CAPS	3-(cyclohexylamino) propanesulfonic acid
dATP	deoxyadenosine triphosphate
DBP-145	145 kilodalton DNA-binding protein
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
D. melanogaster	*Drosophila melanogaster*
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNMP	deoxynucleoside monophosphate
dNTP	deoxynucleoside triphosphate
DSSB	*Drosophila* single-stranded DNA binding protein
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
gp	gene product
HSV-1	herpes simplex virus-1
kDa	kilodalton
mtDNA	mitochondrial DNA
mtSSB	mitochondrial SSB
NAD	nicotinamide adenine dinucleotide
nt	nucleotide

PCNA	proliferating nuclear cell antigen
PEG	polyethylene glycol
pfu	plaque forming unit(s)
pol α	DNA polymerase α
pol γ	DNA polymerase γ
pol δ	DNA polymerase δ
pol ε	DNA polymerase ε
pol III	DNA polymerase III
RF-A	replication factor A
RF-C	replication factor C
S. cerevisiae	*Saccharomyces cerevisiae*
SSB	single-stranded DNA binding protein
ssDNA	single-stranded DNA
SV40	simian virus 40
topo II	DNA topoisomerase II
X. laevis	*Xenopus laevis*

CHAPTER I

LITERATURE REVIEW

DNA REPLICATION

Duplication of cellular genomes is accomplished with great efficiency and fidelity. While it is not completely understood how this process is carried out, considerable insight has been derived from studies of bacterial, bacteriophage, and viral DNA replication systems. Detailed biochemical characterization of DNA replication and its regulation is essential for our understanding of the growth and development of living organisms, and should also allow increased understanding of the perturbations of growth that occur in various disease states.

Chromosomal replication in *Escherichia coli (E. coli)* has been studied in detail. The following general description of DNA replication draws upon our knowledge of this overall process, which appear to be evolutionarily conserved from bacteria to humans, although replication is initiated from a single origin in prokaryotes and multiple origins in eukaryotes (For reviews see Campbell, 1986; Huberman, 1987; Kelly, 1988; Kornberg, 1988; Hurwitz et al.,1990; Kornberg and Baker, 1992). The initiation of chromosomal replication requires specific DNA and protein participants. The origin of replication on duplex DNA is characterized by the presence of repeated elements serving as initiation protein binding sites, and regions rich in deoxyadenylate and thymidylate base pairs providing localized duplex unwinding sites. Initiation proteins recognize and bind the duplex origin and, with the aid of interacting proteins, promote structural conformation alteration and localized unwinding of the duplex DNA. This unwinding allows the entry of DNA helicase and single-stranded DNA binding protein (SSB), promoting further duplex unwinding and preventing reannealing and nuclease degradation of the DNA strands. Additional proteins aid the initiation process indirectly by suppressing other potential start sites to help ensure initiation at the chromosomal origin.

The unwound duplex is prepared for use by replicative DNA polymerase by DNA primase which catalyzes the synthesis of an oligo-deoxyribonucleotide or -ribonucleotide serving as a primer. Because DNA polymerase can extend only a pre-existing chain, this

priming (which generally requires no specific DNA sequence) is always required. The association of primase with DNA polymerase or helicase activities often increases the efficiency of the events involved in starting a DNA chain.

Replicative DNA polymerase extends the primer by catalyzing deoxyribonucleoside monophosphate (dNMP) incorporation complementary to the SSB coated single-stranded DNA template. This reaction involves the attack of the 3'-hydroxyl group of the primer strand on the α-phosphorous of the incoming deoxyribonucleoside triphosphate, resulting in addition of dNMP to the primer strand and the production of pyrophosphate. Replicative DNA polymerase typically utilizes accessory factors to achieve its characteristically high catalytic efficiency of dNMP incorporation and fidelity in selection of complementary moieties.

The replication fork advances ahead of DNA polymerase to continually supply the enzyme with single-stranded template DNA (see figure 1). As DNA helicase opens the duplex, topoisomerases relieve the positive supercoiling induced ahead of the replication fork, and exposed single strands are coated with SSB. Replication proceeds in a semidiscontinuous manner where the leading DNA strand (the strand that is synthesized with the movement of the replication fork) is synthesized continuously, while the lagging strand (the stand that is synthesized against the movement of the replication fork) is synthesized discontinuously. In continuous DNA synthesis, DNA polymerase extends the primer continuously without need for dissociation from the primer terminus. Discontinuous DNA synthesis involves joining together small segments of DNA that are repeatedly initiated with primers. Here, a single-stranded region of template DNA must be exposed (generally 200-1000 nucleotides), a primer is synthesized in the opposite direction of fork movement , and DNA polymerase extends this primer until it reaches the previous region of DNA (Okazaki fragment) which has been synthesized in the same way. Finally, the previously synthesized primer is removed and filled with DNA, allowing DNA ligase to seal the nick left between the extended chain and the DNA which replaced the primer.

Figure 1. *Advancing DNA replication fork of E. coli.* DNA replication is toward the top of the page. As DNA helicase unwinds the duplex DNA, DNA topoisomerase relieves positive supercoiling. Single-stranded DNA binding protein coats the single-stranded DNA which is the template used by DNA polymerase III holoenzyme for continuous leading strand synthesis and discontinuous lagging strand synthesis. The primosome, comprising dnaB-dnaC complex, dnaT, pri A, B, and C, and primase synthesize RNA/DNA primers for use in lagging strand synthesis. DNA polymerase I removes these primers and refills them with DNA. Ligase seals the nicks left between the pol I synthesized DNA and the DNA that is immediately 3' of it. From Kornberg and Baker (1992).

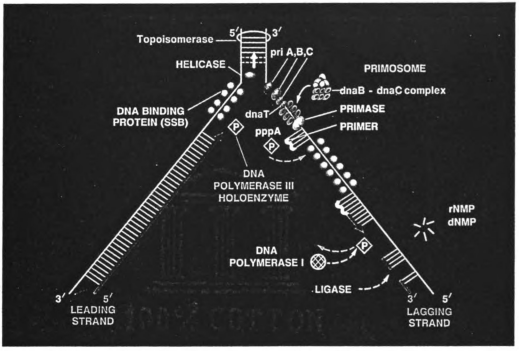
Topoisomerase
5′
3′
pri A,B,C
HELICASE
PRIMOSOME
dnaB - dnaC complex
DNA BINDING
PROTEIN (SSB)
dnaT
PRIMASE
PPPA
PRIMER
P
DNA
POLYMERASE III
HOLOENZYME
rNMP
dNMP
DNA
POLYMERASE I
LIGASE
3′
5′
LEADING
STRAND
3′
5′
LAGGING
STRAND

Figure 1

Semidiscontinuous DNA synthesis allows essentially concurrent replication of the two DNA strands; however, it requires that the priming of the lagging strand be integrated with continuous synthesis of the leading strand. A model proposed to achieve this coordination requires dimerization of DNA polymerase at the replication fork and looping of the lagging strand to endow it with the same orientation as the leading stand at the fork (Figure 2).

Although events leading to the termination of DNA replication are not well elucidated, specific DNA sequences and proteins appear to be involved in bacteria. In contrast, replication of viral DNA often terminates at the meeting place of two replication forks.

DNA SYNTHESIS IN *ESCHERICHIA COLI*

Prokaryotic organisms, and *E. coli* in particular, provide excellent models for the study of various biochemical functions. The simplicity of chromosomal organization and the single point of initiation in prokaryotic DNA replication contribute to its usefulness as an *in vitro* study system. In addition, both genetic and biochemical approaches can be utilized to provide corroborative data.

Replicative DNA polymerase

In general, the mechanistic characteristics of replicative DNA polymerase are well suited for the function it provides in DNA replication; for a review see Young et al., 1992. DNA polymerase must catalyze rapid and highly processive DNA synthesis to ensure that replication of the entire genome is completed on the time scale of the cell cycle. Because dNMP incorporation and polymerase translocation is more rapid than incorporation, dissociation and reassociation of DNA polymerase, DNA replication is most rapid when the polymerase does not dissociate from the DNA template during the replication cycle. In addition, efficient concurrent replication of leading and lagging DNA strands requires the coordination of a dimeric DNA polymerase. Because the leading strand DNA polymerase

Figure 2. *Looping model for coordinated DNA synthesis of the leading and lagging DNA strands.* DNA replication is toward the top of the page. As the replication fork progresses, the lagging DNA strand is looped out while DNA synthesis occurs on the Okazaki fragment which was looped out in the preceeding cycle of discontinuous DNA synthesis. From O'Donnell and Studwell (1990).

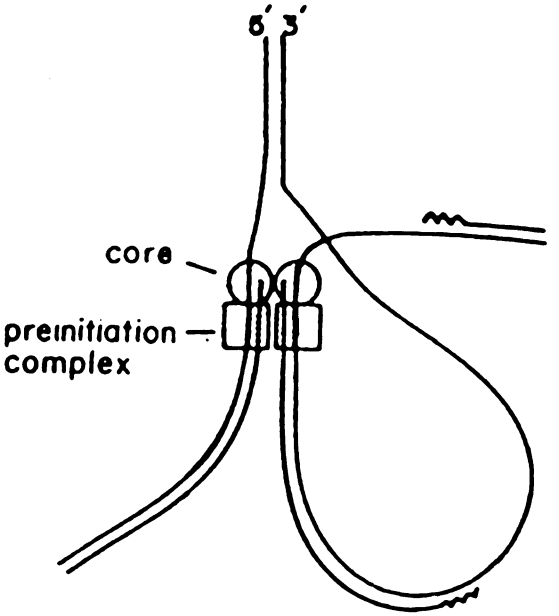

Figure 2

need not dissociate until the entire chromosome is replicated and the lagging strand polymerase must dissociate from completed products and reassociate with the next primer, these two polymerase activities must have different catalytic properties. Therefore, the polymerase complex may be capable of interacting with other proteins in the replication apparatus which alter the mechanistic characteristics of the two polymerase activities relative to each other. Finally, replicative DNA polymerase must exhibit high fidelity in nucleotide selection to ensure the viability of the daughter DNA molecules.

DNA polymerase III is the replicative DNA polymerase in *E. coli* (For reviews see McHenry, 1991; Kornberg and Baker, 1992). DNA polymerase III core (pol III core) comprises three subunits of 130 kDa (α), 27 kDa (ε), and 10 kDa (θ) which are arranged in a linear conformation such that the ε subunit modulates the interaction between the α and θ subunits (Studwell-Vaughan and O'Donnell, 1993). The α and ε subunits display 5'→3' DNA polymerase and 3'→5' exonuclease activities, respectively, while the θ subunit has no known function. However, θ may play a role in fidelity, as its presence stimulates the proofreading exonuclease activity of the ε subunit (Studwell-Vaughan and O'Donnell, 1993). DNA polymerase III holoenzyme (pol III holoenzyme) comprises at least seven subunits in addition to core, contributing to difficulties in the study of holoenzyme due to its instability during purification. However, the cloning, sequencing, and overexpression of all ten pol III subunits has alleviated these problems and facilitated careful studies of the individual subunits and their associated activities (Welch and McHenry, 1982; Cox and Horner, 1983; Echols et al., 1983; Scheuermann and Echols, 1984; Johanson et al., 1986; Blinkowa and Walker, 1990; Flower and McHenry, 1990; Tsuchihashi and Kornberg, 1990; Dong et al., 1993; Onrust and O'Donnell, 1993; Studwell-Vaughan and O'Donnell, 1993; Xiao et al., 1993a, b).

The processivity of a DNA polymerase is simply defined as the average number of nucleotides it incorporates per polymerase-DNA association event. Processivity and the intrinsic rate of nucleotide incorporation of DNA polymerase contribute to its catalytic

efficiency. The processivity of pol III core is only about 10 nucleotides (Fay et al., 1981), while that of pol III holoenzyme is greater than 5000 nucleotides and has been estimated to be as high as 150,000 nucleotides (Fay et al., 1981; Mok and Marians, 1987). Completely processive DNA synthesis of the leading DNA strand would require that pol III be processive for 4,700,000 nucleotides, the approximate length of the *E. coli* genome (Kornberg and Baker, 1992), while that of the lagging DNA strand would require a processivity of 1000-2000 nucleotides, the approximate length of an *E. coli* Okazaki fragment (Okazaki et al., 1973). The processivity of pol III holoenzyme appears to be characteristic of leading strand DNA polymerase, while some subassembly of holoenzyme is likely suited for lagging strand DNA polymerase since various holoenzyme subassemblies display processivity values ranging between that of core and holoenzyme (McHenry, 1991).

The propensity for replication error, or fidelity, of a DNA polymerase is typically characterized by the number of incorrect nucleotides it incorporates into the growing DNA chain. Because DNA is self propagating, uncorrected errors become permanently affixed into the genetic code; therefore, the fidelity of replicative DNA polymerase is expected to be high. Although the fidelity of pol III holoenzyme has not been carefully studied, DNA polymerase III core misincorporates 1 nucleotide per 3×10^4 base pairs replicated (Kunkel et al., 1983). The addition of SSB lowers this error rate ≈7-fold to 1 misincorporation in 2×10^5 base pairs replicated (Kunkel et al., 1983). Thus, holoenzyme may display the fidelity characteristic of a replicative DNA polymerase since following DNA replication and repair errors are accumulated *in vivo* at a rate of 1 in 10^9 - 10^{10} base pairs replicated (Drake et al., 1969).

The rate of DNA replication in *E. coli* has been estimated to be approximately 750 nucleotides per second *in vivo* (O'Donnell and Kornberg, 1985). Although pol III core is inert on natural replicative templates, holoenzyme copies these template DNAs at approximately the same rate *in vitro* as that observed *in vivo* (Godson, 1974; Bouché et al,

1975; Burgers and Kornberg, 1982a, b). Thus, DNA polymerase III holoenzyme appears to be uniquely suited as a replicative DNA polymerase in *E. coli*, exhibiting the catalytic efficiency and fidelity expected of an enzyme with this role.

DNA polymerase accessory factors

The rate, processivity, and fidelity of replicative DNA polymerase should be consistent with models of DNA replication *in vivo*. Although core DNA polymerase is capable of DNA synthesis, it is typically less efficient than expected, given its role *in vivo*. Therefore, replicative DNA polymerase often associates with SSB and accessory proteins involved in enhancing processivity and primer recognition to achieve its characteristically high catalytic efficiency. Core DNA polymerase in the presence of its accessory factors is regarded as a replisome or DNA polymerase holoenzyme.

Processivity factors, like the *E. coli* pol III β subunit, act to increase the tenacity of DNA polymerase-DNA associations. A 40 kDa monomer, the β subunit binds DNA as a dimer (Johanson and McHenry, 1980) by forming a ring around the DNA strand. X-ray crystallographic data (2.5 Å resolution) has revealed that β forms an internally symmetric star-shaped ring with 12 α-helices lining the 35 Å diameter inner surface and 6 β-sheets forming the 80 Å diameter outer surface (Kong et al., 1992). DNA binding is achieved by virtue of this topology which allows the β subunit to slide freely along duplex DNA, acting as a sliding clamp and tethering core to DNA via its interaction with the polymerizing (α) subunit (Stukenberg et al., 1991; Kong et al., 1992; O'Donnell et al., 1992). Thus, the β subunit can increase the processivity of DNA polymerase, but requires energy to undergo the conformational change which forms the closed ring structure encircling the DNA.

This energy is provided by the primer recognition complex which chaperones the processivity factor onto primed DNA, forming the pre-initiation complex and resulting in a highly processive DNA polymerase (Wickner, 1976; O'Donnell, 1987; Maki and Kornberg, 1988; Onrust et al., 1991). Five subunits of pol III holoenzyme compose the

primer recognition complex in *E. coli*: γ (47 kDa), δ (34 kDa), δ' (32 kDa), χ (16 kDa), and ψ (15 kDa). The δ and δ' subunits and χ and ψ subunits each form separate 1:1 complexes which associate with γ in a $\gamma_2\delta_1\delta'_1\chi_1\psi_1$ complex. The γ subunit contains DNA and ATP binding activities as well as a DNA-dependent ATPase activity which is stimulated ≈140-fold by δδ'. Interaction of the primer recognition complex with the processivity factor occurs through the δ subunit and stimulates the γ subunit ATPase activity a further 3-fold (Onrust et al., 1991; Onrust and O'Donnell, 1993; Xiao et al., 1993). These activities and subunit associations led to the following model for accessory factor function (although this model seems plausible, there is little evidence regarding the order of events): the γ subunit, in a complex with δ, δ', χ, and ψ, binds the primed DNA template allowing δ to guide in the β subunit. Subsequent ATP hydrolysis by the γ subunit is coupled to assemble the ring shaped β dimer around the DNA to form the pre-initiation complex to which pol III core binds through its association with the β subunit (Onrust et al., 1991; Stukenberg et al., 1991; Onrust and O'Donnell, 1993). Therefore, the β subunit is a sliding clamp, activated by the γδδ'χψ complex, which tethers DNA polymerase to the DNA, conferring highly processive DNA synthesis.

Although DNA polymerase III holoenzyme from *E. coli* catalyzes DNA synthesis at a high rate, fully efficient DNA synthesis requires SSB coated template DNA. As noted previously, the apparent general function of SSB is to coat single-stranded DNA to prevent nuclease degradation and reannealing of the duplex strands. Its stimulation of DNA polymerase activity suggests that it also plays a role in rendering the substrate suitable for efficient DNA replication. *E. coli* single-stranded DNA binding protein is a monomer of 18.5 kDa which binds DNA as a homotetramer (Weiner et al., 1975; Williams et al., 1983; Williams et al., 1984). Mutagenesis and crosslinking studies indicate that the amino terminal third of the monomer is involved in tetramerization and DNA binding, whereas protein-protein interactions occur at the carboxyl terminus (Chase et al., 1984; Merrill et al., 1984; Williams et al., 1984; Chase et al., 1985; Griepel et al., 1987; Meyer and Laine,

1990). SSB tetramers bind single-stranded DNA (ssDNA) cooperatively, and cover a 33 nucleotide region of DNA, which likely wraps around the protein complex to utilize all four DNA binding sites (Krauss et al., 1981; Meyer and Laine, 1990).

Many proteins that must bind ssDNA to carry out their functions are inhibited by SSB since they often have a lower affinity for ssDNA and cannot displace bound SSB. For example, transcription is inhibited by SSB (Niyogisk et al., 1977), as are most ssDNA nucleases (Molineux and Gefter, 1974) and DNA polymerases I and III core (Molineux et al., 1974; Fay et al., 1981, 1982). However, several enzymes involved in DNA replication, recombination and repair are stimulated by SSB: *E. coli* DNA helicase II (Matson and George, 1987), DNA topoisomerase I (Srivenugopal and Morris, 1986), DNA polymerase II (Bonner et al.,1988) and pol III holoenzyme (McHenry, 1988), as well as replicative DNA polymerases from bacteriophage T7 (Myers and Romano, 1988), herpes simplex virus (O'Donnell et al., 1987), and humans (Kenny et al., 1989). Whether the stimulatory capacity of SSB is due to its effects on template DNA or to direct protein-protein interactions is, in most cases, unknown. However, its ability to increase the fidelity of pol III holoenzyme is proposed to be due to its effects on template DNA conformation (Kunkel et al., 1979). The role of SSB in DNA replication appears to include some direct or indirect interaction with the other enzymes working at the replication fork in addition to coating and protecting the template ssDNA.

DNA replication in *E. coli* must be catalyzed with high processivity, rate and fidelity to ensure chromosomal duplication within the time scale of the cell cycle. DNA polymerase core is capable of such activity on SSB coated single-stranded DNA only in the presence of its accessory factors. In fact, the catalytic parameters of DNA polymerase III holoenzyme observed *in vitro* are similar to those expected from analysis of mechanisms of DNA replication *in vivo*. This similarity implies that the enzymatic characteristics of DNA polymerases responsible for chromosomal replication in other DNA replication systems

should correlate with models of *in vivo* DNA replication, but may require accessory factors to do so.

DNA SYNTHESIS IN BACTERIOPHAGE T4

Study of the bacteriophage T4 DNA replication apparatus has aided in, and often preceded, the understanding of analogous enzymes in *E. coli*. The simplicity of the T4 chromosome as well as the combined use of genetic and biochemical approaches allowed the proteins essential for T4 DNA replication fork movement to be identified, purified, and studied *in vitro* (Alberts et al., 1983; Cha and Alberts, 1989). Seven purified proteins encoded by the T4 chromosome generate efficient *in vitro* DNA elongation on nicked duplex DNA, resembling closely the *in vivo* T4 replication apparatus with regard to speed, geometry, and fidelity of DNA synthesis (Hubner and Alberts, 1980; Sinha et al., 1980; Nossal et al., 1983).

Replicative DNA polymerase

The monomeric 104 kDa protein product of bacteriophage gene 43 (gp43) is the replicative DNA polymerase of bacteriophage T4 (For a review see Young et al., 1992), exhibiting 5'→3' DNA polymerase and 3'→5' exonuclease activities with the respective active sites near its carboxyl and amino termini (Sinha et al., 1980; Reha-Krantz, 1988). Bacteriophage T4 DNA polymerase in association with three additional subunits (the protein products of genes 44, 45, and 62) composes the DNA polymerase holoenzyme which exhibits a processivity of 2000-3000 nucleotides, at least 20-fold more processive than gp43 by itself (Jarvis et al., 1991). Assuming fully processive DNA synthesis, the expected processivity of leading strand DNA polymerase is 166,000 nucleotides, the approximate length of the T4 genome (Kornberg and Baker, 1992), while that of the lagging strand DNA polymerase is 1200 nucleotides, the approximate length of a T4 Okazaki fragment (Alberts et al., 1983; Selick et al., 1987. Thus, the processivity of T4

DNA polymerase holoenzyme does not appear to be characteristic for leading strand polymerase, while some subassembly of holoenzyme (or holoenzyme itself) may be well suited for lagging strand DNA polymerase. In fact, calculation of the minimum time necessary to replicate the T4 genome at the observed processivity reveals that it is longer than the latent period of wild-type T4 phage (Jarvis et al., 1991; Young et al., 1992). Although this apparent discrepancy may be reconciled by the presence of a high concentration of DNA polymerase molecules and their multiple associations with the growing DNA strand, a putative interaction between T4 DNA helicase-primase and DNA polymerase may provide an increase in processivity sufficient to do so as well (Richardson and Nossal, 1989; Young et al., 1992; Spacciapoli and Nossal, 1994a, b).

T4 DNA polymerase misincorporates 1 nucleotide per 10^7 base pairs replicated (Loeb and Kunkel, 1982; Kunkel et al., 1984). The addition of gene products 44, 45, and 62 do not improve the fidelity of gp43 which is similar to that of T4 DNA replication *in vivo* (Drake, 1969; Liu et al., 1978; Sinha and Haimes, 1980) . The rate of DNA synthesis observed *in vivo* is approximately 500 nucleotides per second, a speed which is matched by holoenzyme *in vitro* (approximately 600 nucleotides per second) but not gp43 alone (250 nucleotides per second; McCarthy et al., 1976; Mace and Alberts, 1984a, b). Thus, T4 DNA polymerase holoenzyme maintains a fidelity and rate of DNA synthesis characteristic of replicative DNA polymerase, but is expected to exhibit a higher processivity to function as leading strand DNA polymerase.

DNA polymerase accessory factors

Bacteriophage T4 gene product 45 (gp45), a 25 kDa homodimer or homotrimer, is the processivity factor associated with T4 DNA polymerase (Spicer et al., 1982; Alberts et al., 1983; Formosa et al, 1983; Jarvis et al., 1989a; Rush et al, 1989; Reddy et al., 1993). This processivity factor has been shown by crosslinking and DNA footprinting analyses to bind DNA alone or in a complex with the primer recognition complex (see below;

Hockensmith et al., 1986; Capson et al., 1991; Munn and Alberts, 1991a), and is hypothesized to form a homotrimeric ring around duplex DNA, similar to the "sliding clamp" formed by the β subunit of *E. coli* DNA polymerase III (Alberts, 1984; Jarvis et al., 1989; O'Donnell and Studwell, 1990; Gogol et al., 1992). While gp45 exhibits no obvious amino acid sequence similarity to pol III β subunit, comparison of the gp45 predicted secondary structure with the tertiary structure of pol III β subunit derived from x-ray crystallographic studies does reveal a weak structural alignment (Kong et al., 1992). A more detailed comparison will depend on the determination of the three dimensional structure of T4 gp45.

As in *E. coli*, the primer recognition complex appears to provide the energy required to lock the processivity factor onto the DNA. The products of bacteriophage T4 genes 44 and 62 (gp44 and gp62) are 36 kDa and 21 kDa respectively, and exist as a tightly bound complex of four gp44 to one gp62 (gp44/62; Spicer et al., 1984; Jarvis et al., 1989a; Rush et al., 1989). Like the *E. coli* primer recognition complex, the gp44/62 complex exhibits an ATPase activity which is stimulated ≈20-fold by DNA and a further ≈100-fold by the T4 processivity factor (Mace and Alberts, 1984a; Jarvis et al., 1989b; Rush et al, 1989). Although the ATPase active site is located in gp44 (similar to *E. coli* γ subunit, to which it exhibits considerable homology), stimulation of this activity by gp45 is only slight in the absence of gp62, suggesting a role for gp62 as a mediator for the interaction between gp44 and gp45 (similar to *E. coli* δ subunit; Rush et al., 1989; O'Donnell et al., 1993).

The mode of action of T4 DNA polymerase and its accessory factors is likely to be similar to that of its *E. coli* counterparts, although direct proof has not yet been obtained. Assuming analogy to the model proposed for *E. coli*, the primer recognition complex (gp44/62) binds the primed template, allowing gp62 to guide in the processivity factor (gp45). Subsequent ATP hydrolysis by gp44 is coupled to assemble the ring shaped gp45 trimer around the DNA to form the preinitiation complex to which T4 DNA polymerase

binds through its association with gp45. Thus, gp45 appears to be a sliding clamp activated by the gp44/62 complex which tethers DNA polymerase to the DNA and confers upon it high processivity.

Fully efficient synthesis in bacteriophage T4 DNA replication, as in *E. coli* DNA replication, requires protein-coated DNA template. Bacteriophage single-stranded DNA binding protein, the product of the T4 gene 32 (gp32), is a 33.5 kDa monomer in dilute solution (Carroll et al., 1975; Kirsch and Allet, 1982), but dimer- and trimerizes at estimated physiological concentrations (von Hippel et al., 1982). Proteolysis experiments indicate that, as for *E. coli* SSB, this subunit interaction occurs through the amino terminal amino acids (Spicer et al., 1979; Williams et al., 1979; Hosoda et al., 1980; Lonberg et al., 1981; Williams and Konigsberg, 1981), while gp32 association with other proteins (see below) occurs through the carboxyl terminal amino acids (Burke et al., 1980; Chase and Williams, 1986). The DNA binding domain of gp32 is suspected to reside in the central region of the protein (Williams et al., 1980, 1981; Williams and Konigsburg, 1981; Chase and Williams, 1986), through which it binds cooperatively to ssDNA to an area spanning eight nucleotides (Alberts and Frey, 1970; Jensen et al., 1976; Newport et al., 1981; Prigodich et al., 1984). Although they are clearly functional homologs, sequence comparison of T4 gp32 and *E. coli* SSB reveals very little homology (Sancar et al., 1981; Williams et al., 1983).

Affinity chromatography indicates that T4 gp32 associates physically with both the T4 DNA polymerase and processivity factor, as well as several proteins involved in DNA recombination (Formosa et al., 1983). In addition, gp32 stimulates DNA polymerase core activity 5- to 10-fold (Huberman et al., 1971; Nossal and Peterlin, 1979), holoenzyme activity 100- to 200-fold (Nossal and Peterlin, 1979; Alberts et al., 1980), and increases DNA polymerase fidelity 15- to 20-fold, likely by increasing nucleotide specificity (Gillen and Nossal, 1976; Topal, and Sinha, 1983). Binding of gp32 to single-stranded DNA at the T4 replication fork, like binding of SSB at the *E. coli* replication fork, prevents

nuclease degradation and reannealing of duplex DNA. Its stimulation of DNA polymerase activity suggests that it also plays a role in rendering the substrate suitable for efficient DNA replication. *E. coli* SSB cannot substitute for gp32 in stimulation of T4 DNA polymerase activity or fidelity, suggesting that the two coating proteins impose different conformations onto the bound DNA or that direct protein-protein interactions are very important, or both (Sigal et al.,1972; Burke et al., 1980).

Bacteriophage T4 DNA polymerase utilizes accessory factors similar to those used by *E. coli* DNA polymerase III to catalyze efficient DNA synthesis. Although as yet unproven, the mechanisms employed by these accessory factors appear conserved between the two systems. These similarities support the hypothesis that DNA polymerases responsible for chromosomal replication in various DNA replication systems may require accessory factors to allow correlation of their enzymatic characteristics with models of *in vivo* DNA replication.

DNA SYNTHESIS IN SIMIAN VIRUS 40

The study of replication of eukaryotic genomes is complicated by initiation of DNA replication at multiple sites. Therefore, models of single initiation sites are studied and the results applied to overall replication events. Simian virus 40 (SV40) DNA replication occurs in the host (human) cell nucleus and represents a model of a single replicon in eukaryotic DNA replication (For a review see Kelly, 1988). A double-stranded circular 5000 base pair molecule, the viral genome is complexed with histones to form nucleoprotein structures similar to cellular chromatin and encodes only one protein required for its replication. The remainder of the DNA replication machinery, including DNA polymerase and accessory factors, is provided by the host cell.

Multiple DNA polymerases in eukaryotic DNA replication

While prokaryotic and bacteriophage genomes are copied by single DNA polymerases, eukaryotic genomes employ at least three DNA polymerases for efficient DNA replication. In contrast, *E. coli* utilizes three DNA polymerases in cellular function, but only DNA polymerase III is a replicative enzyme. DNA polymerase I is involved in DNA repair and gap filling, while DNA polymerase II appears to have a role in repair of damaged DNA (Tait et al., 1974; Bonner et al., 1988). Biochemical analysis of SV40 DNA replication *in vitro* indicates that DNA polymerases α (pol α) and δ (pol δ) are required to copy the viral genome efficiently (Murakami et al., 1986; Lee et al., 1989; Weinberg and Kelly, 1989). Further, genetic studies have revealed that DNA polymerases α, δ, and ε (pol ε) are required for replication of yeast DNA *in vivo* (Johnson et al., 1985; Morrison et al., 1990). The lack of ε polymerase requirement in SV40 DNA replication *in vitro* appears to indicate that either the replication of the viral genome differs from cellular chromosomal DNA replication (Linn, 1991), or that pol δ can substitute for pol ε *in vitro*. Nevertheless, considerable insight into the proteins involved in eukaryotic DNA replication has been gained through the study of the SV40 system.

Biochemical characterization of DNA polymerases α and δ seemed to indicate that they were suited for lagging and leading strand DNA synthesis, respectively, in SV40 DNA replication. However, the requirement of the processivity factor associated with pol δ in both leading and lagging strand DNA synthesis implicated pol δ as the sole replicative DNA polymerase (Downey et al., 1988; Prelich and Stillman, 1988; Bullock et al., 1991), leading to a hypothesized role for pol α in priming of DNA synthesis on both DNA strands with, perhaps, some limited role in primer extension. In yeast, pol δ was assigned the role of lagging strand DNA polymerase due to its conditional processivity while the highly processive pol ε was designated the leading strand DNA polymerase (Morrison et al., 1990). However, the data presently available does not allow conclusive assignment of DNA polymerases α, δ, or ε as leading or lagging strand DNA polymerases. For the

purposes of this report the prevailing opinions in the eukaryotic DNA replication literature will be observed: pol δ is the leading strand DNA polymerase, pol ε is the lagging strand DNA polymerase, and pol α primes DNA synthesis and partially extends primers for use by pols δ and ε.

Leading strand DNA polymerase

DNA polymerase δ is a heterodimer comprising 125 kDa and 50 kDa subunits and exhibiting 5'→3' DNA polymerase and 3'→5' exonuclease activities (Boulet et al., 1989; Downey et al., 1990; Syvaoja et al., 1990). The large subunit is the catalytic subunit while the function of the small subunit is unknown, but may mediate the interaction between the DNA polymerase and its processivity factor (Goulian et al., 1990; Syvaoja et al., 1990). DNA polymerase δ holoenzyme comprises pol δ and its processivity (proliferating cell nuclear antigen; PCNA) and primer recognition (replication factor C; RF-C) factors. The cDNAs encoding PCNA and the subunits of RF-C have been identified, sequenced and, in some cases, overexpressed, facilitating their study (Almendral et al, 1987; Chen et al., 1992a,b; Burbelo et al., 1993; O'Donnell et al., 1993; Howell et al., 1994).

The processivity of DNA polymerase δ core is 5 to 25 nucleotides, while that of pol δ in the presence of PCNA is greater than 2000 nucleotides (Crute et al., 1986; Prelich et al., 1987b; Sabatino et al., 1988; Syvaoja et al., 1990). Presumably, the processivity of δ polymerase in the presence of both PCNA and RF-C would be even higher. The expected processivity of a completely processive leading strand DNA polymerase is 5000 nucleotides in SV40, the length of the SV40 genome, but is 10,000 to 300,000 nucleotides in chromosomal DNA replication, the approximate range in lengths of single eukaryotic replicons (Kornberg and Baker, 1992). Processive lagging strand DNA replication requires a processivity of 135 to 250 nucleotides, the approximate length of an Okazaki fragment in eukaryotes (Anderson and DePamphilis, 1979; Nathanel and Kaufmann, 1990).

Although the fidelity of δ polymerase core cannot be determined due to its inefficient use of single-stranded DNA, the accuracy of pol δ in the presence of PCNA has been estimated to be at least 3-fold lower than that observed *in vivo* (Thomas et al., 1991). While pol δ holoenzyme polymerizes DNA at a speed of 110 nucleotides per second *in vitro*, DNA polymerase δ core synthesizes DNA at a rate of only 2 nucleotides per second (Burgers, 1991). In comparison, the rate of replication fork movement in eukaryotes *in vivo* is estimated to be between 50 and 60 nucleotides per second (Blumenthal et al., 1974; Rivin and Fangman, 1980). Thus, DNA polymerase δ holoenzyme exhibits a rate of DNA synthesis characteristic of replicative DNA polymerase and may exhibit processivity and fidelity values consistent with this role as well.

DNA polymerase accessory factors

The processivity factor associated with DNA polymerase δ (PCNA) is a 36 kDa homotrimer which is required for efficient SV40 DNA replication *in vitro* (Bravo et al., 1987; Prelich et al., 1987a; Bauer and Burgers, 1988). PCNA promotes binding of pol δ to the template-primer (Ng et al., 1993), stimulates pol δ activity 20- to 100-fold (Tan et al., 1986; Bauer and Burgers, 1988; Ng et al., 1990) and processivity approximately 10-fold (Kunkel et al., 1990), yet has no effect on α polymerase and little effect on ε polymerase (Hamatake et al., 1990). PCNA, like T4 gp45, is hypothesized to form a homotrimeric ring around duplex DNA, similar to the "sliding clamp" formed by the β subunit of *E. coli* DNA polymerase III. While PCNA, again like T4 gp45, exhibits no obvious amino acid sequence similarity to pol III β subunit, comparison of the predicted secondary structure for PCNA with the tertiary structure of pol III β subunit derived from x-ray crystallographic studies does reveal a weak structural alignment (Kong et al., 1992). In contrast, sequence comparison of PCNA to T4 gp45 reveals 30% to 50% amino acid similarity between the two processivity factors (Tsurimoto and Stillman, 1990), which are

likely more structurally similar to each other than to *E. coli* β subunit given their hypothesized trimeric binding conformation.

As in *E. coli* and bacteriophage T4, the primer recognition complex appears to provide the energy required to lock the processivity factor onto the DNA. Replication factor C comprises a large subunit of approximately 140 kDa, containing DNA binding activity, and four small subunits of approximately 40 kDa (Tsurimoto and Stillman, 1990; Lee et al., 1991; Tsurimoto and Stillman, 1991; Yoder and Burgers, 1991; Fien and Stillman, 1992). One of the small subunits contains an ATPase activity which, similar to the ATPase activities of *E. coli* and T4 primer recognition complexes, is stimulated ≈20-fold by DNA and a further ≈4-fold by its homologous processivity factor, suggestive of a PCNA:RF-C interaction (Tsurimoto and Stillman, 1990, 1991; Yoder and Burgers, 1991; Chen et al., 1992a). In addition, RF-C appears to be analogous to other primer recognition complexes in its ability to facilitate the binding of PCNA to template-primer (Tsurimoto and Stillman, 1991).

The genes for all of the subunits of RF-C have been identified and sequenced. The large (140 kDa) subunit of RF-C exhibits homology to the small subunits (27% identical and 48% similar over 186 amino acids), which exhibit homology to each other, to T4 gp44 (≈30% amino acid identity and ≈65% similarity), and to the *E. coli* γ subunit (≈20% amino acid identity and ≈70% similarity; Chen et al., 1992 a, b; O'Donnell et al., 1993). Crosslinking and photoaffinity labelling experiments indicate that in RF-C, only the 40 kDa subunit contains ATP binding activity even though the 140, 40, 37 and 36 kDa subunits, as well as T4 gp44 share an ATP binding motif which is nearly identical to the motif found in the *E. coli* γ subunit (Spicer et al., 1984; Chen et al., 1992a, b; Burbello et al., 1993; O'Donnell et al., 1993; Howell et al., 1994). The high degree of similarity of replication factor C with primer recognition complexes from T4 and *E.coli* provide support for its hypothesized analogous role in eukaryotic DNA replication.

The mode of action of DNA polymerase δ and its accessory factors is likely to be similar to that of its bacteriophage T4 and *E. coli* counterparts, although direct proof has not yet been obtained. Assuming analogy to the model proposed for *E. coli*, the primer recognition complex, replication factor C, binds the primed template through action of its 140 kDa subunit, thereby permitting association of the processivity factor, PCNA. Subsequent ATP hydrolysis by RF-C is coupled to assemble the ring shaped PCNA trimer around the DNA to form the preinitiation complex to which pol δ binds through the putative association of its small subunit with PCNA (Hurwitz et al., 1990). Therefore, PCNA appears to be a sliding clamp activated by the RF-C complex which tethers DNA polymerase to the DNA, facilitating processive DNA synthesis.

Lagging strand DNA polymerase

DNA polymerase δ or ε appear capable to serve as either the leading or lagging strand DNA polymerase. However, because pol ε is not required in SV40 DNA replication *in vitro* (Murakami et al., 1986; Lee et al., 1989; Weinberg and Kelly, 1989), it has been relegated to the role of lagging strand DNA synthesis in eukaryotic DNA replication in general. DNA polymerase ε comprises a large catalytic subunit of 200-260 kDa and possibly two additional subunits of 80 and 34 kDa (Budd et al., 1989; Syvaoja and Linn, 1989; Hamatake et al., 1990; Syvaoja et al., 1990). Both 5'→3' DNA polymerase and 3'→5' exonuclease activities are harbored by ε polymerase which is stimulated ≈7-fold by PCNA and RF-C, but is unaffected by either accessory factor acting on its own (Goslin and Byrnes, 1982; Lee et al., 1984; Bauer et al., 1988). The processivity of pol ε is greater than 1500 nucleotides and is unaffected by PCNA, although PCNA and RF-C together may alter it (Syvaoja and Linn, 1989; Syvaoja et al., 1990). This high processivity does not appear to be well suited for Okazaki fragment synthesis of only 135-250 nucleotides (Anderson and DePamphilis, 1979; Nathanel and Kaufmann, 1990). DNA polymerase ε misincorporates 1 nucleotide per 2 x 10^5 incorporated, an error rate

approximately 3-fold higher than that observed *in vivo* and similar to that of pol δ in the presence of PCNA (Kunkel et al., 1987; Thomas et al., 1991). The *in vivo* rate of replication fork movement of 50 to 60 nucleotides per second is matched by pol ε in the presence of PCNA and RF-C, but pol ε alone incorporates only 7 nucleotides per second (Blumenthal et al., 1974; Rivin and Fangman, 1980; Burgers, 1991). Thus, only the rate of DNA synthesis and perhaps the fidelity of nucleotide selection by pol ε appears suited for its putative role as a lagging strand DNA polymerase. The inherent high processivity of pol ε seems to favor a role in leading DNA strand synthesis, as suggested by Morrison and colleagues (1990). In fact, the low processivity of pol δ core implicates it, or a subassembly of pol δ holoenzyme, in lagging strand DNA synthesis; whereas, the PCNA requirement of leading strand DNA synthesis of SV40 DNA replication *in vitro* may reflect the ability of pol δ to substitute for pol ε in this system.

DNA polymerase-primase

DNA polymerase α appears to initiate leading strand DNA synthesis at the replication origin, prime Okazaki fragment synthesis on the lagging DNA strand, and catalyze some limited extension of these primers (Morrison et al., 1990). Required in SV40 DNA replication *in vitro*, pol α comprises four subunits including a 140-185 kDa subunit containing DNA polymerase activity, a 70 kDa subunit of unknown function, and 60 and 50 kDa subunits exhibiting DNA primase activity (Kaguni et al., 1983; Plevani et al., 1985; Suzuki et al., 1985; Murakami et al., 1986; Lee et al., 1989; Weinberg and Kelly, 1989). DNA primase synthesizes primers 24-28 nucleotides in length and is either unaffected or inhibited by the presence of DNA polymerase accessory factors (Conaway and Lehman, 1982; Badarocco et al., 1985; Singh et al., 1986; Cotterill et al., 1987). The association of DNA polymerase with primase limits the primer length to 12-14 nucleotides and appears to suppress multimeric primer synthesis (Conaway and Lehman, 1982; Badaracco et al., 1985).

DNA polymerase-primase is normally processive for 8-20 nucleotides, but manipulation of reaction conditions promotes processive DNA synthesis of up to 1000 nucleotides (Villani et al., G., 1981; Pritchard et al., 1983; Cotterill et al., 1987; Sabatino et al., 1988). Depending on the source of pol α, the polymerase misincorporates 1 nucleotide per 2 x 10^4 to 4 x 10^5 incorporated, 2- to 35-fold lower than the fidelity observed *in vivo* (Kaguni et al., 1984; Kunkel et al., 1987; Thomas et al., 1991). Finally, the rate of DNA synthesis by pol α is approximately 2 nucleotides per second, 25- to 30-fold slower than observed fork movement *in vivo* (Blumenthal et al., 1974; Banks et al., 1979; Rivin and Fangman, 1980; Hubscher et al., 1982). Thus, the enzymatic characteristics of DNA polymerase α do not correlate well with that of a replicative enzyme, but appear to suit its hypothesized role of priming and limited primer extension.

In summary, DNA polymerase α appears to be responsible for priming both the leading and lagging DNA strands, while polymerases δ and ε likely carry out the bulk of extension of these primers. It is unclear whether pol δ or pol ε is involved in leading strand DNA replication. DNA polymerase accessory factors appear to have the largest influence over pol δ; therefore, determination of the specific involvement of these accessory factors in DNA replication may allow more defined elucidation of the role of pol δ *in vivo*.

Single-stranded DNA binding protein

Eukaryotic single-stranded DNA binding protein (replication factor A; RF-A) is structurally dissimilar, but appears to be functionally analogous, to *E. coli* SSB and bacteriophage T4 gp32. Replication factor A coats single-stranded DNA, preventing nuclease degradation and reannealing of duplex, and is required for SV40 DNA replication *in vitro* (Fairman and Stillman, 1988). A heterotrimer comprising 70 kDa, 30 kDa, and 14 kDa subunits, RF-A binds DNA with low cooperativity and a site size of 22 nucleotides (Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988; Mitsis et al., 1993). DNA binding activity resides in the 70 kDa subunit (Wold et al., 1989), while the

30 kDa and 14 kDa subunits may be involved in protein-protein interactions (Erdile et al., 1990; Umbricht et al., 1993). RF-A interacts directly with pol α-primase and the SV40 T-antigen, and stimulates their polymerase and helicase activities, respectively, as well as the polymerase activity of pol δ (Stahl et al., 1986; Dean et al., 1987; Wold et al., 1987; Kenny et al., 1989; Tsurimoto and Stillman, 1989a; Dornreiter et al., 1992). Thus, in addition to its DNA coating function, RF-A appears to aid in promoting replication origin unwinding and priming by mediating the interaction between T-antigen (origin recognition and helicase activities) and pol α (priming activity; Melendy and Stillman, 1993).

Eukaryotic DNA replication, as modeled by SV40 DNA replication, is similar to prokaryotic and bacteriophage DNA replication in many respects. Semidiscontinuous DNA replication is carried out by a pair of DNA polymerases possessing differing catalytic properties. In *E. coli* and bacteriophage T4, replicative DNA polymerase appears to dimerize and the leading and lagging strand DNA polymerases achieve altered enzymatic characteristics by virtue of each monomer's association with different accessory factors, while in SV40 DNA replication two different DNA polymerase assemblies appear to be utilized. Rapid and processive synthesis catalyzed by leading strand DNA polymerase requires the presence of polymerase accessory factors which appear to be functionally conserved from bacteria to humans. Although no obvious amino acid sequence homology is shared between the bacterial processivity factor and its bacteriophage and eukaryotic homologs, they do appear to be functionally and structurally conserved. In addition, bacteriophage and eukaryotic processivity factors exhibit some amino acid sequence similarity, as do primer recognition factors from bacteria, bacteriophage and humans. Finally, single-stranded DNA binding proteins from the three systems are clearly functionally similar, although they share no amino acid sequence homology.

In summary, DNA polymerases throughout widely divergent DNA replication systems exhibit similar mechanisms of DNA synthesis and require accessory factors for efficient catalysis. In addition, the mechanistic characteristics of replicative DNA

polymerases are well suited for the function they provide in DNA replication. Thus, the correlation of the enzymatic characteristics of DNA polymerase with models of DNA replication appear to be indicative of either the lack of required polymerase accessory factors or the presence of additional DNA polymerases which are enzymatically more well suited for a particular cellular function.

MITOCHONDRIAL DNA REPLICATION

As energy producing organelles, mitochondria are required in virtually all eukaryotic cells. Mitochondrial function is dependent on the expression of its genome which encodes thirteen polypeptides required in electron transport and oxidative phosphorylation, two ribosomal RNAs, and twenty-two transfer RNAs (Tzagaloff and Myers, 1988). Mitochondrial DNA mutations can lead to disease and have been proposed to contribute to aging and common degenerative diseases including epilepsy, cardiac disease, Parkinson's disease, and Alzheimer's disease, as well as maturity onset diabetes (Wallace, 1992).

Many mutations in mitochondrial DNA are associated with ophthalmological and neurological disease. Disease resulting from point mutations in protein encoding sequences are most commonly found associated with mutations in NADH dehydrogenase subunits 1, 2, 4, and 5, cytochrome b, and ATP synthase subunit 6 genes (Singh et al., 1989; Holt et al., 1990; Howell et al., 1991a, b; Huoponen et al., 1991; Johns and Berman, 1991; Brown et al., 1992). Conditions resulting from point mutations in tRNA genes are most commonly associated with defects in complex I (NADH dehydrogenase complex) and IV (cytochrome oxidase complex), the two complexes for which mtDNA encodes the largest number of subunits (Shoffner et al., 1988; Goto et al., 1990; Hammans et al., 1991; Hess et al., 1991; Ino et al., 1991; Johns and Hurko, 1991; Lauber et al., 1991; Yoon et al, 1991). Deletions of mtDNA typically occur between the origin of replication and the initiation site of lagging strand DNA synthesis and are often flanked by 13 nucleotide long

direct repeats (Wallace, 1989; Wallace et al., 1991). Although there are several proposed mechanisms for the appearance of these deletions, the slip-replication mechanism appears to be the favored model (Shoffner et al., 1989; Mita et al., 1990). In this model, while the lagging strand is displaced during DNA replication, an upstream repeat on the lagging strand base pairs with a downstream repeat on the leading strand. A break in the lagging strand downstream of the base paired repeats serves as a primer for DNA synthesis, resulting in deletion of the region between the direct repeats. This slip-replication model has also been implicated in the proposed relationship between mitochondrial DNA damage and aging. Oxidative phosphorylation normally declines with age, apparently due to a time dependent increase in mtDNA damage caused by oxygen radicals (Linnane et al., 1989; Cortopassi and Arnheim, 1990; Corral-Debrinski et al., 1991). This damage leads to inhibition of replication and transcription which, by prolonging the time the DNA is triple stranded, fosters mitochondrial DNA deletions. Because the rate of mtDNA replication appears to be directly proportional to its length, deleted DNAs have a replicative advantage over normal DNAs and become enriched with time (Larsson et al., 1990). Elucidation of the mechanisms involved in the faithful duplication of the mitochondrial genome may provide us with a more detailed understanding of these genetic disorders.

Vertebrate mitochondrial DNA is a double-stranded circular molecule, ranging in size from 16.5-17.6 Kb (Gray, 1989). A single substantial non-coding region contains the origin of replication (Brown and Vinograd, 1974; Robberson et al., 1974; Tapper and Clayton, 1981) and promoters for transcription of both DNA strands (Figure 3; Clayton, 1991). Replication of vertebrate mitochondrial DNA appears to be initiated by RNA polymerase which synthesizes transcripts for use as primers for DNA synthesis (Chang and Clayton, 1985; Chang et al., 1989). Therefore, the initiation of DNA replication is described by the initiation of transcription at the light strand promoter since the light strand is the template for leading DNA strand synthesis. The light strand promoter is an approximately 50 base pair sequence located about 100 base pairs upstream of the origin of

Figure 3. *Representation of vertebrate mitochondrial DNA.* The shaded areas represent the 22 tRNA genes. The 12S and 16S rRNA genes and non-coding region (D-loop) are shown. O and I are the origin of replication and initiation site of lagging strand DNA synthesis, respectively. LSP and HSP are the respective promoters for transcripts copied from the leading and lagging DNA template strands. Arrows denote the direction of synthesis. The protein coding genes are: cytochrome c oxidase subunits I, II, and III (COI, COII, COIII); ATPase subunits 6 and 8 (ATP6, ATP8); cytochrome *b* (CYT *b*); and subunits 1, 2, 3, 4, 4L, 5, and 6 of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6). From Clayton (1991).

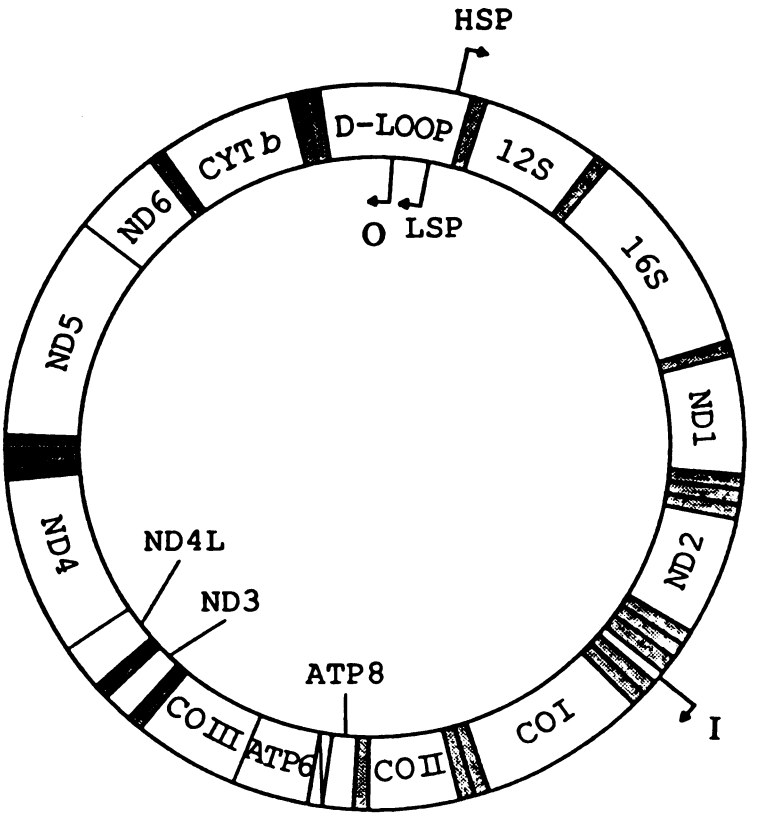
HSP
D-LOOP
12S
16S
ND1
ND2
I
COI
COII
ATP8
ATP6
COIII
ND3
ND4L
ND4
ND5
ND6
CYT b
O
LSP

Figure 3

DNA replication (Chang et al., 1987; Topper and Clayton, 1989). It comprises two functionally distinct domains: a short sequence near the transcriptional start site, and a sequence 25-35 base pairs upstream which is required to stimulate transcription (Chang and Clayton, 1984; Hixson and Clayton, 1985; Chang and Clayton, 1986a, b). This stimulation also requires the binding of a putative DNA packaging protein, mtTFA (Fisher et al., 1987; Fisher and Clayton, 1988; Parisi and Clayton, 1991; Parisi et al., 1993), and possibly an additional factor to ensure promoter specificity (Fisher et al., 1989). The transition of RNA priming to DNA synthesis occurs over a region of three short conserved sequence elements (Walberg and Clayton, 1981; Chang and Clayton, 1985; Chang et al., 1985) and may involve a ribonucleolytic mitochondrial RNA processing protein whose role is a subject of controversy: although this protein cleaves RNA transcripts between two of the three conserved sequences, the locations of these cleavage sites do not correlate exactly with the locations of the majority of RNA to DNA transitions (Chang and Clayton, 1987a). Further, the RNA component of the processing ribonucleoprotein is much more abundant in the nucleus than the mitochondria and appears to play a role in the processing of pre 5.8S ribosomal RNA in the nucleus (Chang and Clayton, 1987b; Schmitt and Clayton, 1993).

Biochemical and electron microscopic analyses of replication intermediates reveal that mitochondrial DNA synthesis is unidirectional and asymmetric (Figure 4), where synthesis of the lagging DNA strand is not initiated until synthesis of the leading DNA strand is 67% complete (Matsumoto et al., 1974). This asymmetry is due to the distant location of the lagging strand initiation site which must be exposed in single-stranded form by leading strand DNA synthesis in order to function (Wong and Clayton, 1985). In vertebrate mtDNA, this initiation site is an approximately 30 nucleotide region surrounded by several tRNA genes (Martens and Clayton, 1979) which, upon exposure, is capable of forming a stem-loop structure hypothesized to be required for its recognition. Primer formation is complementary to the T-rich loop and is proposed to be catalyzed by

Figure 4. *Model of mouse mitochondrial DNA replication.* The order of replication is clockwise starting with (A). O and I are the origin of replication and initiation site of lagging strand DNA synthesis, respectively. The double arrows between (A) and (G) reflect the metabolic instability of the D-loop strands and the consequent equilibrium between superhelical mtDNA (G) and the D-loop DNA (A). DNA synthesis from the replication origin is depicted prior to (B) and after the initiation of lagging strand DNA synthesis (C) and (D). Both daughter molecules (E) are converted to closed circles (F) which are negatively supercoiled (G) to form the substrate for D-loop strand synthesis. From Clayton (1982).

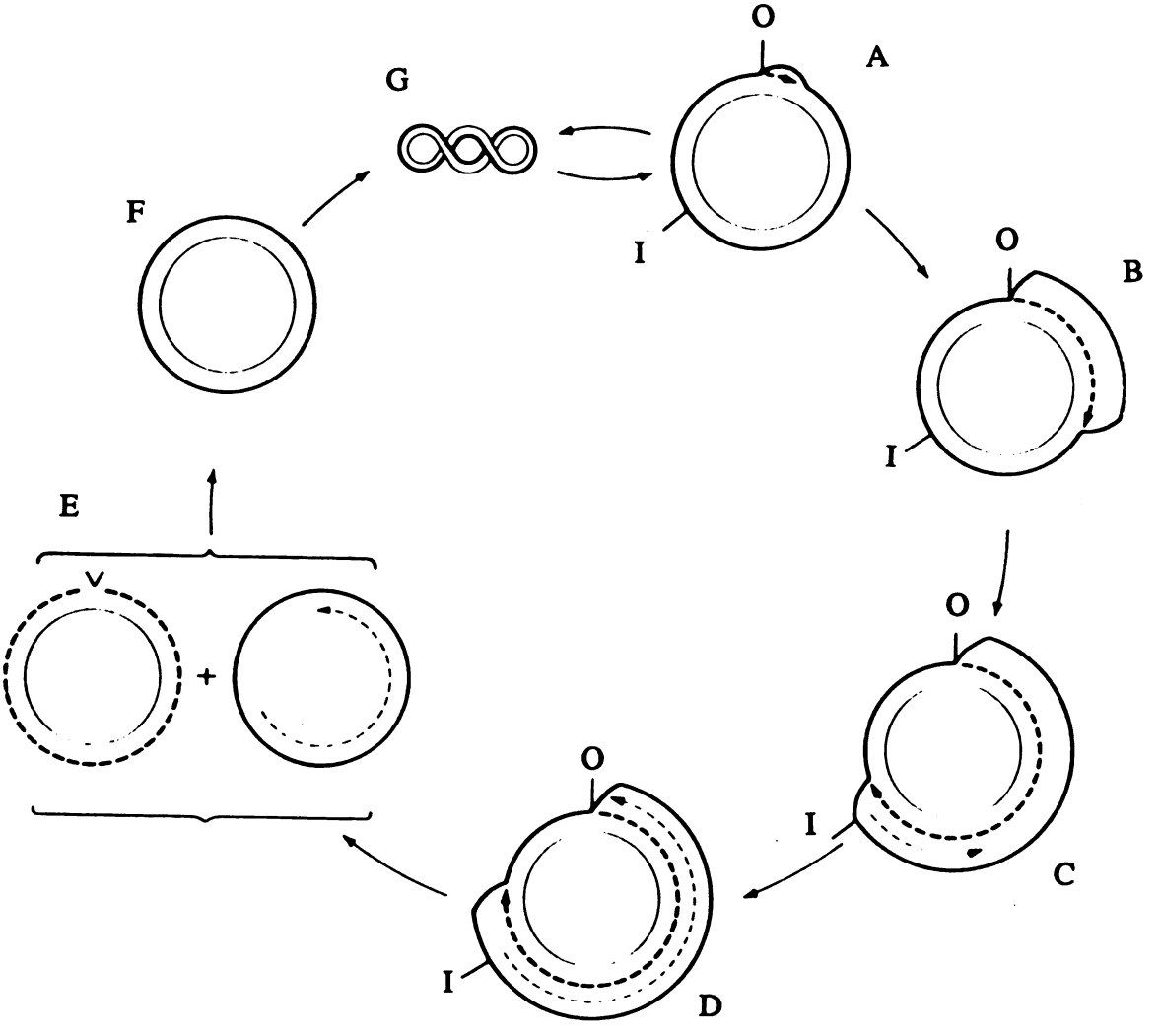

Figure 4

mitochondrial DNA primase, while the RNA/DNA primer to DNA transition occurs near the base of the stem (Wong and Clayton, 1985a, b). Subsequent elongation of the lagging DNA strand appears also to be continuous. Thus, mitochondrial DNA replication may proceed in a fully continuous and processive fashion with no requirement for intricate looping models coordinating synthesis of the two DNA strands. The resultant products of mtDNA replication are two interlocked DNA molecules which are segregated and converted to closed circles into which superhelical turns are introduced (Berk and Clayton, 1974; Bogenhagen and Clayton, 1978).

Drosophila mitochondrial DNA replication appears to be generally similar to that of vertebrate mtDNA. *Drosophila*, like vertebrates, harbor a double-stranded circular genome ranging in size from 15.7 to 19.5 Kb (Fauron and Wolstenholme, 1976). A single non-coding region contains the origin of replication and appears to contain the initiation site for lagging strand DNA replication as well (Goddard and Wolstenholme, 1978, 1980). This non-coding region (A+T region) is composed of 90-96% deoxyadenylate and thymidylate residues (Clary and Wolstenholme, 1985, 1987; Monnerot et al., 1990; Lewis et al., 1994) exhibiting no sequence similarities to its vertebrate mtDNA counterpart (Clary and Wolstenholme, 1985; Lewis et al., 1994). Although the specifics of initiation are unknown, *Drosophila* mtDNA replication originates within the A+T region and proceeds in a unidirectional and asymmetric manner (Figure 5) where leading strand synthesis is up to 99% complete prior to initiation of lagging strand synthesis (Wolstenholme et al., 1979).

Drosophila embryos seem to be a good source of mitochondrial DNA replication proteins, and the study of replication catalyzed by these proteins is valuable as a model for that catalyzed in mammalian systems. In addition, the study of these proteins and processes may enhance comparative analyses between lower eukaryotes, insects and mammalians. Insects are both valuable and pestilent organisms as they carry out many pollinating activities, serve as food for other animals, and conduct valuable scavenger services, but also are responsible for costly crop loss and may disrupt the health of humans

Figure 5. *Model of Drosophila mitochondrial DNA replication.* In (a), the origin of replication, O, and the direction of replication, R, around the molecule are indicated. DNA synthesis from the replication origin is depicted prior to (b) and (c), and after the initiation of lagging strand DNA synthesis (d). Both daughter molecules (e) and (f) are converted to closed circular DNAs. Modified from Wolstenholme and colleagues (1979).

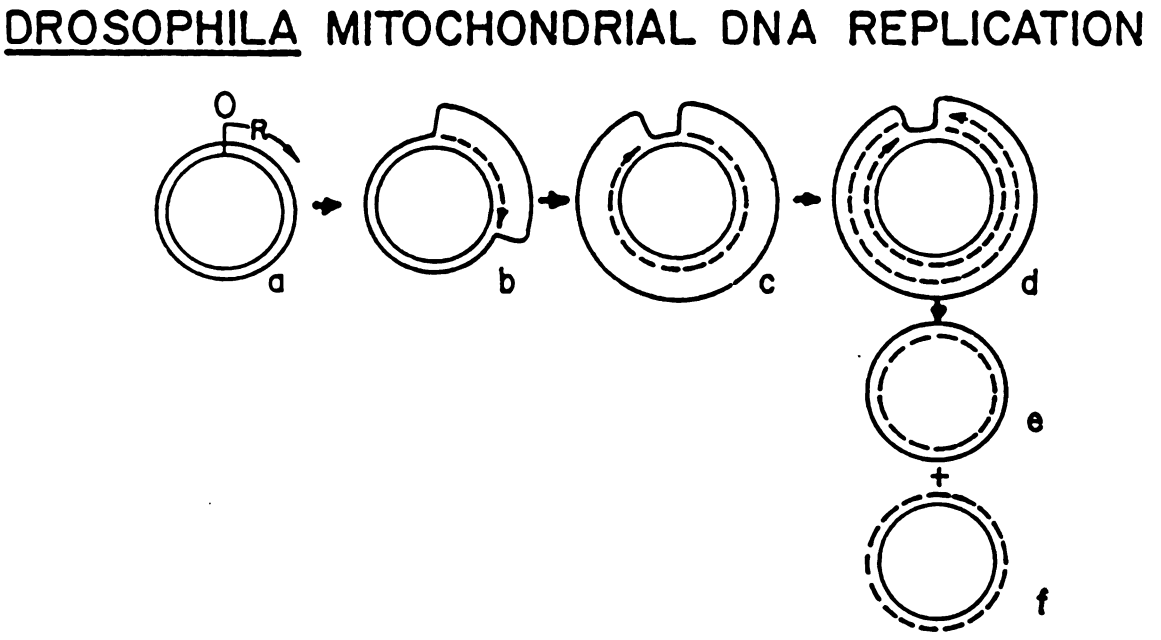

Figure 5

and other mammals. Their ability to breed rapidly and produce a large number of offspring, as well as the ease with which they are raised contributes to the value of *Drosophila* as biochemical and genetic insect models.

Mitochondrial RNA polymerase and DNA primase

Mitochondrial RNA polymerase core is a 140-145 kDa monomer which possesses ribonucleotide polymerizing activity, but requires additional subunits for *in vitro* initiation at promoter sequences (Kelly and Lehman, 1986; Bogenhagen and Insdorf, 1988). Encoded by a nuclear gene which has been cloned from *S. cerevisiae*, core RNA polymerase has been identified and purified from both *S. cerevisiae* and *X. laevis* tissues (Kelly and Lehman, 1986; Bogenhagen and Insdorf, 1988). RNA polymerase holoenzyme, which forms on DNA but not in solution, comprises core and a 43 kDa specificity factor which displays characteristics similar to those of bacterial sigma factors (Jang and Jaehning, 1991). In *X. laevis*, this factor binds an eight nucleotide region which surrounds the transcription initiation site and is required for specific initiation of transcription (Bogenhagen and Insdorf, 1988; Bogenhagen and Romanelli, 1988). RNA polymerase holoenzyme has been implicated in the events leading to initiation of DNA replication even though it cannot incorporate dNMPs, a common feature of DNA primase, and does not synthesize exclusively short RNA chains (Winkley et al., 1985; Kelly and Lehman, 1986).

Mitochondrial DNA primase appears to be involved in the priming of lagging strand DNA synthesis, and has been partially purified from human and rat tissues (Wong and Clayton, 1985b; Ledwith et al., 1986). Products synthesized complementary to the lagging DNA strand initiation site by a protein fraction containing human mtDNA primase and mtDNA polymerase were characterized by the presence of a short (9-12 nucleotide) stretch of RNA covalently linked to greater than 100 nucleotides of DNA (Wong and Clayton, 1985a, b). Similarly, *Xenopus* mtDNA primase catalyzed the formation of 1-12 nucleotide

long RNA primers but also synthesized 25-40 nucleotide stretches of DNA (Ledwith et al., 1986), consistent with the production of mixed RNA/DNA primers characteristic of DNA primase activities.

Mitochondrial DNA topoisomerase

Because mitochondrial DNA is a closed, circular and topologically constrained molecule, its replication likely requires the presence of topoisomerase to relax the positive supercoils introduced into it by DNA unwinding. Further, upon completion of replication, resolution of daughter DNA molecules and their negative supercoiling likely occurs through the action of topoisomerase. Mitochondrial DNA topoisomerase I appears to fill the former role, as it removes positive supercoils (Fairfield et al., 1979; Callen, 1981; Lazarus et al. 1987) and inhibitor studies suggest that it is involved in mtDNA replication and transcription (Fairfield et al., 1985). In addition, its putative association with yeast ABF2 (analogous to human mtTFA) appears to induce a DNA gyrase activity which might be expected to introduce negative supercoils into daughter DNA molecules (Caron et al., 1979; Diffley and Stillman, 1991). In contrast, mitochondrial DNA topoisomerase II appears to play a role in the decatenation of kinetoplast DNA in trypanosome mitochondria as well as the relaxation of negatively supercoiled DNA (Melendy and Ray, 1989).

Mitochondrial DNA helicase

A mitochondrial DNA helicase identified in *S. cerevisiae* is an ATP dependent enzyme which unwinds DNA in the 5'→3' direction (Lahaye et al., 1991) and appears to be involved in mitochondrial DNA repair through recombination (Foury and Kolodynski, 1983). Although genetic analyses indicate that it is not essential to the replication of mtDNA, it may participate in replication events by eliminating local secondary structure (Foury and Lahaye, 1987) similar to *E. coli* DNA helicase II. In fact, yeast mtDNA helicase exhibits homology to this *E. coli* DNA helicase (Foury and Lahaye, 1987) which

plays a role in DNA excision repair and recombination (Kornberg and Baker, 1992). In contrast, a DNA helicase identified in bovine mitochondria exhibits 3'→5' unwinding activity (Hehman and Hauswirth, 1992), a characteristic similar to *E. coli* Rep helicase which is essential in rolling circle DNA replication that some viral DNAs undergo (Kornberg and Baker, 1992). This mechanism of replication is similar to leading strand DNA synthesis in mitochondrial DNA replication.

Replicative DNA polymerase

In general, the mechanistic characteristics of mitochondrial DNA polymerase are expected to be similar to those of its chromosomal counterparts. That is, DNA polymerase should catalyze rapid and highly processive DNA synthesis as well as exhibit a high fidelity of nucleotide selection to ensure the viability of the daughter DNA molecules. However, unlike chromosomal DNA polymerases, the coordination of a dimeric polymerase for concurrent replication of leading and lagging DNA strand is not required. In addition, because neither the leading strand nor the lagging strand DNA polymerase need dissociate until the entire mitochondrial genome is replicated, mitochondrial DNA polymerase may be expected to catalyze only highly processive DNA synthesis, as is characteristic of chromosomal leading strand DNA polymerases.

Animal mitochondrial DNA polymerase, DNA polymerase γ (pol γ) appears to be a heterodimer comprising a catalytic subunit exhibiting 5'→3' DNA polymerase activity and a small subunit of unknown function (Wernette and Kaguni, 1986; Insdorf and Bogenhagen, 1989a; Gray and Wong, 1992). A 3'→5' mismatch specific exonuclease activity is associated with pol γ purified from *Drosophila* (Kaguni and Olson, 1989), *X. laevis* (Insdorf and Bogenhagen, 1989b), and human mitochondria (Gray and Wong, 1992), while sequence analysis of the gene encoding the catalytic subunit from *S. cerevisiae* reveals that it contains a 3'→5' exonuclease motif (Foury, 1989; Ito and Braithwaite, 1990). The subunit structure of γ polymerase appears to be conserved in

Drosophila, chicken, *X. laevis*, and human tissues: a 125-140 kDa catalytic subunit and a 35-54 kDa small subunit (Yamaguchi et al., 1980; Wernette and Kaguni, 1986; Insdorf and Bogenhagen, 1989a; Gray and Wong, 1992). In addition, pol γ identified in porcine liver exhibits a native molecular weight of 140 kDa (Mosbaugh, 1988), while the yeast catalytic subunit is calculated from sequence data to be 143.5 kDa (Foury, 1989).

Processive for 30-90 nucleotides (Wernette et al., 1988), pol γ does not seem well suited for continuous replication of a 15,700 to 19,500 base pair mitochondrial genome (Wolstenholme et al., 1979; Anderson et al., 1981; Bibb et al., 1981). DNA polymerase γ misincorporates 1 nucleotide per 1-5 x 10^5 nucleotides incorporated (Kunkel and Soni, 1988; Wernette et al., 1988; Kunkel and Mosbaugh, 1989), similar to the error rates of pol ε and pol δ in the presence of PCNA (Kunkel et al., 1987; Thomas et al., 1991). Further, the rate of DNA synthesis by γ polymerase of 2 nucleotides per second (Wernette et al., 1988) is similar to the rate of 4.5 nucleotides per second observed *in vivo* (Clayton, 1982). Thus, DNA polymerase γ appears to be enzymatically characteristic of mitochondrial replicative DNA polymerase with regard to its rate and fidelity of DNA synthesis, but is uncharacteristic in its processivity, assuming fully processive replication of mitochondrial DNA. However, even at its moderate processivity, a high concentration of pol γ appears to be sufficient for complete replication of the mitochondrial genome (Wernette et al., 1988)

DNA polymerase accessory factors

Mitochondrial DNA polymerase processivity and primer recognition factors have not yet been identified. Mitochondrial single-stranded DNA binding protein (mtSSB) is a homotetramer (13.3-15.2 kDa monomers) that binds DNA cooperatively covering an 8 to 9 nucleotide region (Van Tuyle and Pavco, 1981; Ghrir et al., 1991; Van Dyck et al., 1992; Hoke et al., 1990). Sequence comparison of mtSSB with *E. coli* SSB reveals 24-30% identity and 43-50% similarity between the two, with the highest homology in the amino terminal two-thirds of the proteins (63-76% identity in some regions; Van Dyck et al.,

1992; Hoke et al., 1990; Ghrir et al., 1991; Mounolou et al., 1988). This homology suggests that subunit interaction and DNA binding domains of the two SSBs are very similar, while the domain involved in SSB interaction with other proteins is not well conserved. This conservation profile is reasonable given the similar function of SSBs to coat single-stranded DNA while interacting with replication proteins which are evolutionarily widely divergent. Mitochondrial DNA polymerase activity is either stimulated up to 10-fold or inhibited up to 20-fold by the presence of mtSSB depending on the purity of the DNA polymerase and the DNA substrate and reaction conditions used (Hoke et al., 1990; Mignotte et al., 1988).

The inability of mitochondrial DNA polymerase to synthesize DNA in a highly processive manner is inconsistent with models of mtDNA replication. As reviewed here, we find that replicative DNA polymerases from *E. coli*, bacteriophage T4, and humans generally display enzymatic characteristics consistent with their roles *in vivo*. However, it is clear that the context in which the rate, processivity, and fidelity of DNA synthesis are measured is critical to their accurate determinations. Therefore, we propose that mitochondrial DNA polymerase catalyzes DNA synthesis with enzymatic characteristics which correlate with models of mitochondrial DNA replication *in vivo*, but may require additional elements to do so.

CHAPTER II

PROCESSIVITY OF MITOCHONDRIAL DNA POLYMERASE FROM *DROSOPHILA* EMBRYOS: EFFECTS OF REACTION CONDITIONS

INTRODUCTION

Replication of the double-stranded circular mitochondrial DNA (mtDNA) genome occurs in the mitochondrial matrix and proceeds in a unidirectional and asymmetric manner (Goddard and Wolstenholme, 1980; Clayton, 1982), suggesting the possibility of continuous synthesis of both DNA strands. Indeed, current models of animal mtDNA replication, derived primarily from biochemical and electron microscopic studies of replication intermediates, favor this possibility. Because DNA polymerase γ (pol γ) is the sole DNA polymerase found in mitochondria (Bolden et al., 1977; Bertazzoni et al., 1977; Hubscher et al., 1977), these models predict that mitochondrial DNA polymerase catalyzes continuous DNA strand synthesis.

Continuous DNA strand synthesis in both pro- and eukaryotic DNA replication generally correlates with processive synthesis (Das and Fujimura, 1979; Fisher et al., 1979; Fay et al., 1981; Hockensmith and Bambara, 1981; Fay et al., 1982; Prelich et al., 1987; Hurwitz et al., 1990). Whereas DNA polymerases responsible for continuous synthesis are highly processive, catalyzing the incorporation of thousands of nucleotides (nt) into the growing DNA chain in a single binding event, those involved in discontinuous synthesis may be processive for only tens or hundreds of nucleotides. While mitochondrial DNA polymerase might be expected to catalyze highly processive DNA synthesis, it was found previously that when assayed under conditions optimized for DNA synthetic rate, *Drosophila* pol γ is only quasi-processive, incorporating 30-90 nt per binding event (Wernett et al., 1988).

The mitochondrial matrix environment is much different from that of the nucleus: the matrix contains a higher protein concentration (calculated as ~50% by weight; Srere, 1980) and higher nucleoside triphosphate levels (Bestwick et al., 1982). In addition, the matrix volume varies up to ~2-fold depending on the metabolic state of the mitochondria (Hackenbrock, 1968), resulting in fluctuating concentrations of metabolites that contribute

to the compartmental ionic strength. These alterations may be important in the regulation of the metabolic pathways within the matrix (Srere, 1980), including mtDNA replication.

Interestingly, the mechanism of DNA synthesis by *E. coli* DNA polymerases I and III and eukaryotic DNA polymerases α and δ was found to vary depending on reaction conditions (Bambara et al., 1978; Kwon-Shin et al., 1987; Hohn and Grosse, 1987). The sensitivity of these replicative enzymes to their assay environments coupled with the unusual mitochondrial environment suggests that mitochondrial DNA polymerase might also be influenced by the composition of the reaction solvent. We have examined the effect of various reagents on the rate and processivity of DNA synthesis by *Drosophila* DNA polymerase γ on a single-stranded DNA template to address the apparent contradiction of continuous DNA synthesis catalyzed by a moderately processive enzyme. The elucidation of *in vitro* reaction conditions that enable mitochondrial DNA polymerase to catalyze highly processive and efficient DNA synthesis concurrently would allow correlation of enzyme function with models of mtDNA replication, as well as suggest a mechanism for its regulation. In contrast, an inability to define such conditions may implicate as yet unidentified DNA polymerase accessory factors in mtDNA replication.

EXPERIMENTAL PROCEDURES

MATERIALS

Nucleotides and nucleic acids. Unlabeled deoxy- and ribonucleoside triphosphates were purchased from P-L Biochemicals; for use at concentrations above 30 μM, ATP, GTP, and ADP solutions were adjusted to pH 7.5 with Tris base (Research Organics). [^{3}H]dTTP was purchased from ICN Biochemicals; [α-^{32}P]dTTP was purchased from New England Nuclear. Calf thymus DNA (highly polymerized Type I) was purchased from Sigma and was activated by partial digestion with DNase I (Boehringer Mannheim) as described by Fansler and Loeb (1974).

Recombinant and wild type M13 viral DNAs (10,650 and 6,407 nt, respectively) were prepared by standard laboratory methods. Synthetic oligodeoxynucleotides (15 nt) complementary to the M13 viral DNAs were synthesized in an Applied Biosystems Model 477 oligonucleotide synthesizer.

Enzymes. Drosophila DNA polymerase γ (Fractions IV, V and VI) was prepared as described by Wernette and Kaguni (1986).

Chemicals. All solutions were prepared in water and stored at room temperature. Polyethylene glycol (8,000 MW, Sigma) was prepared as a 50% stock solution. Polyvinyl alcohol (10,000 MW, Sigma) was prepared as a 17.5% stock solution. Glycerol (Mallinckrodt) was prepared as an 80% stock solution.

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, 30 or 120 mM KCl, 400 μg/ml bovine serum albumin, 20 μM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (1000 cpm/pmol), 10 μM (as nt) of singly-primed M13 DNA, and 0.1 to 0.2 units of Fr VI enzyme. Incubation was at 30°C for 30 minutes. Specific modifications are described in the figure legends. One unit of activity is that amount that catalyzes the incorporation of 1

nanomole of deoxyribonucleoside triphosphate into acid insoluble material in 60 minutes at 30°C using DNase I-activated calf thymus DNA as the substrate. Here, we define standard activity as that exhibited by pol γ in the presence of 120 mM KCl on singly-primed M13 DNA.

Analysis of products of processive DNA synthesis by gel electrophoresis. Reactions were as above except that reaction mixtures contained 30 μM each of dATP, dCTP, dGTP, and 10 μM of [α-^{32}P]dTTP (2 x 10^4 cpm/pmol), 20 μM singly-primed M13 DNA, and 0.02 units of Fr VI enzyme. Incubation was at 30°C for 30 minutes unless otherwise noted. Products to be analyzed by denaturing polyacrylamide gel electrophoresis were made 1% in SDS and 10 mM in EDTA, heated for 4 minutes at 80°C and precipitated with ethanol in the presence of 5 μg of tRNA as carrier. The ethanol precipitates were resuspended in 80% formamide, and 90 mM Tris-borate. Aliquots were denatured for 2 minutes at 100°C and electrophoresed in a 6% polyacrylamide slab gel (13 x 30 x 0.15 cm) containing 7M urea in 90 mM Tris-borate (pH 8.3) and 25 mM EDTA. Alternatively, the ethanol precipitates were resuspended in 0.3 M NaOH and 20 mM EDTA, and aliquots electrophoresed in a 1.5% (agarose) slab gel (13 x 18 x 0.7 cm) containing 30 mM NaCl and 2 mM EDTA in 30 mM NaOH and 2 mM EDTA. Approximately equal amounts of radioactivity (~1000 cpm) were loaded in each lane. In addition, equal sample volumes were loaded on each type of gel to allow direct comparison of product size distribution. Gels were washed in distilled water for 20 minutes, dried under vacuum and exposed at (-)80°C to Kodak X-OMAT AR X-ray film using Du Pont Quanta III intensifying screens. Quantitation of the data was performed by scanning of the 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. In the determination of processivity values, the length of the primer (15 nt) was subtracted from the DNA product strand lengths.

RESULTS

***Drosophila* DNA polymerase γ is highly processive at KCl levels suboptimal for DNA synthesis.** Condensation of the mitochondrial matrix during periods of active respiration likely results in an increase in the concentration of metabolites that contribute to ionic strength (Hackenbrock, 1968). Increasing ionic strength decreases both the activity and processivity of *E. coli* DNA polymerases I and III, calf thymus DNA polymerase α, and T4 phage DNA polymerase (Bambara et al., 1978; Kwon-Shin et al., 1987; Hohn and Grosse, 1987; Newport et al., 1980). Because *Drosophila* mitochondrial DNA polymerase is stimulated by moderate salt (Wernette et al. 1988; Wernette and Kaguni, 1986), we investigated its processivity on singly-primed M13 DNA under conditions of varying monovalent salt concentration. Pol γ activity varied ≈6-fold when assayed over the range of 0-210 mM KCl, with an optimum at ≈120 mM KCl (Fig. 6A). At the same time, processivity varied dramatically (≈600-fold), with the greatest abundance of full length products synthesized in the absence of KCl (Fig. 6B). Reduced polymerase activity under conditions where pol γ is capable of copying a complete 6407 nt template in a single binding event suggests that either primer recognition or enzyme dissociation is rate limiting.

To investigate the effects of other reagents on enzyme activity and processivity, subsequent assays were conducted at 120 mM KCl (moderate salt), where pol γ exhibits its highest (standard) activity yet is only moderately processive (average processive unit (apu) of 45 nt) and at 30 mM KCl (low salt) where it is less active (25% of standard activity), but highly processive (apu of 2500 nt).

pH has little effect on the processivity of *Drosophila* mitochondrial DNA polymerase. The mitochondrial inner membrane is generally impermeable to charged and highly polar molecules, allowing the formation of a pH gradient across the lipid bilayer.

FIGURE 6. *KCl alters the rate and processivity of DNA synthesis by Drosophila pol γ.* **A.** The rate of DNA synthesis was determined as described under "Methods" in the presence of the indicated KCl concentrations. **B.** Product DNA strands were analyzed on denaturing 1.5% agarose (upper panel) and 6% polyacrylamide (lower panel) gels as described under "Methods." Adjacent lanes represent samples obtained after 20 and 40 minutes of incubation at 30°C and containing no KCl (lanes 1 and 2; average processive unit (apu) = 3400 nt), 30 mM KCl (lanes 3 and 4; apu = 2500 nt), 60 mM KCl (lanes 5 and 6; apu = 1600 nt), 90 mM KCl (lanes 7 and 8; apu = 140 nt), 120 mM KCl (lanes 9 and 10; apu = 45 nt), 160 mM KCl (lanes 11 and 12; apu = 20 nt), 210 mM KCl (lanes 13 and 14; apu = 6 nt). Numbers on the left of the photograph indicate the position and size (in nt) of *Hpa* II restriction fragments of M13G*ori*1 replicative form DNA (Kaguni and Ray, 1979) and *Hin*d III restriction fragments of lambda DNA that were electrophoresed in adjacent lanes. In the determination of processivity values, the length of the primer (15 nt) was subtracted from the DNA product strand lengths measured relative to the molecular weight markers shown. Products obtained after 40 minutes of incubation were similar in size and distribution to those obtained after 20 minutes of incubation, indicating that they result from single binding events; subsequent analyses were performed using 30 minute incubations at 30°C.

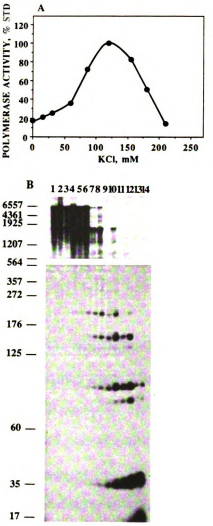
A
POLYMERASE ACTIVITY, % STD
120
100
80
60
40
20
0
0
50
100
150
200
250
KCl, mM
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
6557
4361
1925
1207
564
357
272
176
125
60
35
17

Figure 6

The extent of this gradient varies with matrix pH, as the cytosol seems to be effectively buffered (Moroney et al., 1984). Lowering the pH of the reaction solvent from pH 8.0 to 6.0 increased the processivities of calf thymus DNA polymerases α and δ approximately 30-fold (Sabatino et al., 1988). In contrast, polymerase activity was reduced 5- to 7-fold at pH 6.0 relative to its optimum at pH 7.0. When assayed over the range of pH 6-10, the activity of *Drosophila* pol γ varied ≈3.5-fold at moderate salt and ≈10-fold at low salt, with optimal activity achieved between pH 8.0 to 9.6 and at pH 10, respectively (Fig. 7A). However, processivity varied less than 4-fold regardless of KCl concentration, and the enzyme was most highly processive at pH 8.5 at both moderate and low salt (Fig. 7B). Thus, optimal polymerase activity and processivity were achieved concurrently at 120 mM KCl. However, like pols α and δ, pol γ was most processive at pH suboptimal for activity at 30 mM KCl.

DNA synthetic product length is diminished with increasing $MgCl_2$ concentrations. DNA polymerases require magnesium as a cofactor for nucleotide incorporation in the form of a Mg-dNTP complex. Increasing $MgCl_2$ concentrations decreased the processivity of calf thymus pols α and δ while increasing their activity (Sabatino et al., 1988). Likewise, when assayed from 0.01-64 mM $MgCl_2$, the addition of Mg^{2+} decreased the processivity of pol γ ≈6-fold at moderate salt and ≈700-fold at low salt, at concentrations as low as 1 mM $MgCl_2$ (Fig. 8B). Polymerase activity varies ≈10-fold at 120 mM KCl and is optimal from 0.5-6 mM $MgCl_2$ (Fig. 8A). At 30 mM KCl, pol γ activity varies ≈30-fold and the enzyme exhibits two optima at 0.25 and 20-25 mM $MgCl_2$. Thus, the enzyme is capable of synthesizing long products (>3000 apu) at a high rate (0.2-0.5 mM $MgCl_2$ at 30 mM KCl), yet is also able to make short products (≤ 45 apu) quickly (0.25-7 mM $MgCl_2$ at 120 mM KCl, and 18-27 mM $MgCl_2$ at 30 mM KCl).

FIGURE 7. *pH affects the rate and processivity of DNA synthesis by Drosophila pol γ.* **A.** The rate of DNA synthesis was determined as described under "Methods" in the presence of 50 mM Tris•HCl (pH 6-9.6) or 50 mM CAPS•KOH (pH 9.4-12) and 30 mM (open circles) or 120 mM (closed circles) KCl. **B.** Product DNA strands were analyzed on denaturing 1.5% agarose (upper panel) and 6% polyacrylamide (lower panel) gels. Reactions were performed at 30 mM KCl (lanes 1-3) or 120 mM KCl (lanes 4-6) and pH 7.0 (lanes 1 and 4; apu = 780 nt and 35 nt respectively), pH 8.5 (lanes 2 and 5; apu = 2800 nt and 37 nt), and pH 10.0 (lanes 3 and 6; apu = 1200 nt and 38 nt).

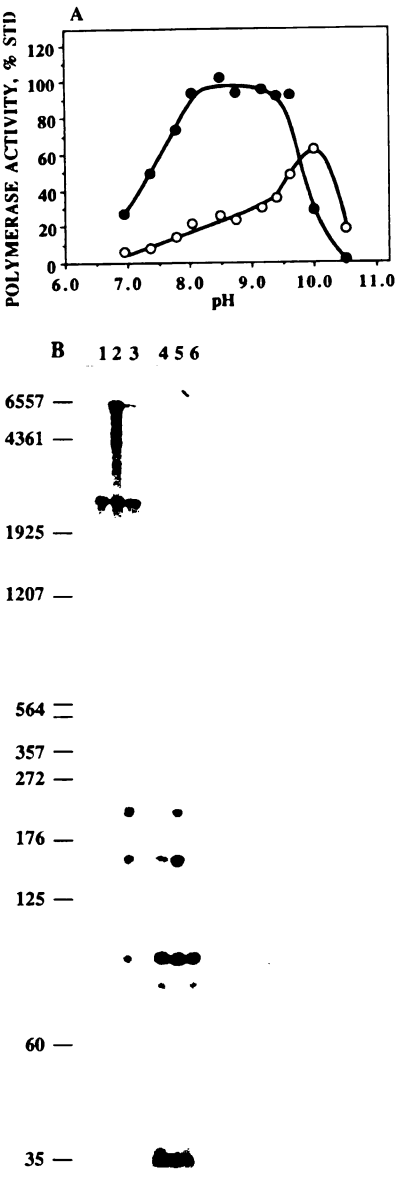
A
POLYMERASE ACTIVITY, % STD
120
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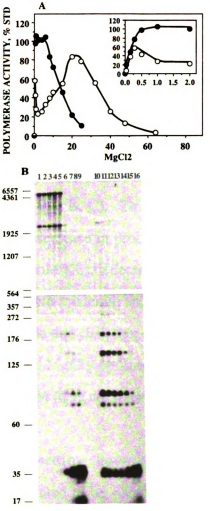
Figure 7

FIGURE 8. *$MgCl_2$ alters the rate and processivity of DNA synthesis by Drosophila pol γ.* **A.** The rate of DNA synthesis was determined as described under "Methods" in the presence of the indicated $MgCl_2$ concentrations and 30 mM (open circles) or 120 mM (closed circles) KCl. **B.** Product DNA strands were analyzed on denaturing 1.5% agarose (upper panel) and 6% polyacrylamide (lower panel) gels. Reactions were performed at 30 mM KCl (lanes 1-9) or 120 mM KCl (lanes 10-16) and 0.15 mM $MgCl_2$ (lanes 1 and 10; apu = 4300 nt and 40 nt respectively), 0.3 mM $MgCl_2$ (lanes 2 and 11; apu = 4300 nt and 40 nt), 0.5 mM $MgCl_2$ (lanes 3 and 12; apu = 3000 nt and 40 nt), 1 mM $MgCl_2$ (lanes 4 and 13; apu = 2900 nt and 25 nt), 4 mM $MgCl_2$ (lanes 5 and 14; apu = 2100 nt and 25 nt), 10 mM $MgCl_2$ (lanes 6 and 15; apu = 25 nt and 15 nt), 20 mM $MgCl_2$ (lanes 7 and 16; apu = 10 nt and 7 nt), 30 mM $MgCl_2$ (lane 8; apu = 6 nt), 40 mM $MgCl_2$ (lane 9; apu = 6 nt).


A
POLYMERASE ACTIVITY, % STD
120
100
80
60
40
20
0
0
20
40
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80
MgCl2
0.0 0.5 1.0 1.5 2.0
B
1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16
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Figure 8

ATP specifically stimulates DNA synthesis by *Drosophila* γ polymerase. Seventy percent of the total cellular ATP is localized within the mitochondrial matrix (Hutson et al., 1989), making it at least 4-fold more abundant than the other nucleoside triphosphates present (Bestwick et al., 1982). ATP hydrolysis is involved in primer recognition by, and increases the processivities of prokaryotic, viral and eukaryotic DNA polymerases (Burgers and Kornberg, 1982b; Cha and Alberts, 1988; Tsurimoto and Stillman, 1990; Burgers, 1991). ATP, but not GTP or ADP, stimulated the activity of *Drosophila* mitochondrial DNA polymerase 4-fold at low salt in the presence of ATP concentrations from 7.5 to 12 mM, such that the activity observed at moderate salt in the absence of ATP was achieved (Fig. 9A, B, C). This specific stimulation by ATP was accompanied by a non-proportional decrease in processivity (Fig. 9D). Notably, while ATP did not stimulate activity at 120 mM KCl, it decreased processivity above 5 mM ATP (Fig. 9D), as did GTP and ADP at both moderate and low KCl concentrations (data not shown). Titration experiments indicate that chelation of Mg^{2+} by ATP is unrelated to the specific stimulation observed: the $MgCl_2$ optimum remains at 4 mM as the ATP concentration is varied from 5 to 12 mM (data not shown).

Effects of macromolecular crowding agents. Protein concentration in the mitochondrial matrix has been calculated to be ≈50% by weight; thus, the behavior of matrix enzymes may be expected to lie between that of enzymes in solution and that of enzymes in ordered complexes (Srere, 1980). The conditions provided by macromolecular crowding agents *in vitro* may mimic those found in the matrix. Crowding agents (dextran and polyethylene glycol (PEG)) enhance binding of *E. coli* DNA polymerase I and T4 DNA polymerase to template-primers, resulting in increased activity, yet having no effect on processivity (Zimmerman and Harrison, 1987; Jarvis et al., 1990). Protein-protein interactions are also enhanced, as indicated by an increased processivity of T4 DNA polymerase in the presence of its accessory proteins (Jarvis et al., 1990). *Drosophila* γ

FIGURE 9. *Effect of ATP, GTP, and ADP on the rate and ATP on the processivity of DNA synthesis by Drosophila pol γ.* The rate of DNA synthesis was determined as described under "Methods" in the presence of the indicated concentrations of ATP (**A**), GTP (**B**), or ADP (**C**) and 30 mM (open circles) or 120 mM (closed circles) KCl. **D.** Product DNA strands were analyzed on denaturing 1.5% agarose (upper panel) and 6% polyacrylamide (lower panel) gels. Reactions were performed at 30 mM KCl (lanes 1-6) or 120 mM KCl (lanes 7-11) and no ATP (lanes 1 and 7; apu = 3000 nt and 95 nt respectively), 2 mM ATP (lanes 2 and 8; apu = 2900 nt and 80 nt), 5 mM ATP (lanes 3 and 9; apu = 3300 nt and 80 nt), 10 mM ATP (lanes 4 and 10; apu = 1800 nt and 50 nt), 15 mM ATP (lanes 5 and 11; apu = 350 nt and 40 nt), 25 mM ATP (lane 6; apu = 80 nt).

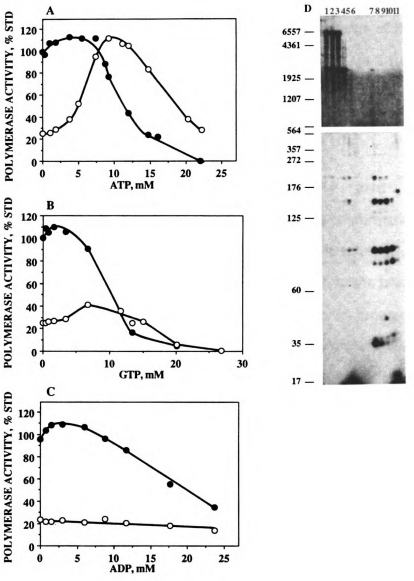
A
POLYMERASE ACTIVITY, % STD
ATP, mM
B
POLYMERASE ACTIVITY, % STD
GTP, mM
C
POLYMERASE ACTIVITY, % STD
ADP, mM
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Figure 9

polymerase exhibited a 7-fold variation in activity upon the addition of PEG in the range of 0%-24%: activity decreased in the range of 4% to 12% at both 120 and 30 mM KCl, and was restored either partially or fully at ≈16% PEG at moderate and low salt, respectively (Fig. 10A). pol γ synthesized longer DNA products in the presence of 8% PEG at both 120 mM and 30 mM KCl (Fig. 10B), where its activity was 2- to 3-fold reduced. In contrast, polyvinyl alcohol has little effect on either the activity or processivity of the mitochondrial DNA polymerase (data not shown).

Glycerol stimulates DNA polymerase activity and processivity concurrently. Glycerol acts to stabilize enzyme activity and conformation (Gekko and Timasheff, 1981), and may enhance the association of proteins in solution. *Drosophila* pol γ activity was stimulated approximately 2-fold when assayed in the presence of ≈20% glycerol at both moderate and low salt (Fig. 11A). In addition, its processivity increased 1.5- to 3.5-fold with the inclusion of ≈15% glycerol (Fig. 11B), demonstrating that pol γ is capable of exhibiting optimal activity and processivity concurrently at each KCl level examined in the presence of glycerol.

Limited template-primer availability does not alter the processivity of *Drosophila* γ polymerase. Protein-protein interactions between DNA polymerase molecules and/or accessory proteins have been demonstrated in both prokaryotic and eukaryotic systems (Kornberg and Baker, 1992). To promote such interactions between pol γ molecules and/or putative sub-stoichiometric accessory proteins, we analyzed enzyme activity under conditions of limiting primer termini. After preincubation of the near-homogeneous enzyme with an M13 DNA template containing 5'-end labeled primers, DNA synthesis was carried out in the presence of a 45-fold excess of unlabeled DNase I-activated calf thymus DNA which serves to trap unbound enzyme molecules, thereby ensuring a single DNA synthetic cycle. The processivity of *Drosophila* γ polymerase was

FIGURE 10. *PEG alters the rate and processivity of DNA synthesis by Drosophila pol γ.* **A.** The rate of DNA synthesis was determined as described under "Methods" in the presence of the indicated PEG concentrations and 30 mM (open circles) or 120 mM (closed circles) KCl. **B.** Product DNA strands were analyzed on denaturing 1.5% agarose (upper panel) and 6% polyacrylamide (lower panel) gels. Reactions were performed at 30 mM KCl (lanes 1-5) or 120 mM KCl (lanes 6-10) and no PEG (lanes 1 and 6; apu = 2500 nt and 45 nt respectively), 4% PEG (lanes 2 and 7; apu = 4000 nt and 50 nt), 8% PEG (lanes 3 and 8; apu = 4100 nt and 330 nt), 14% PEG (lanes 4 and 9; apu = 4000 nt and 100 nt), 18% PEG (lanes 5 and 10; apu = 2400 nt and 40 nt).

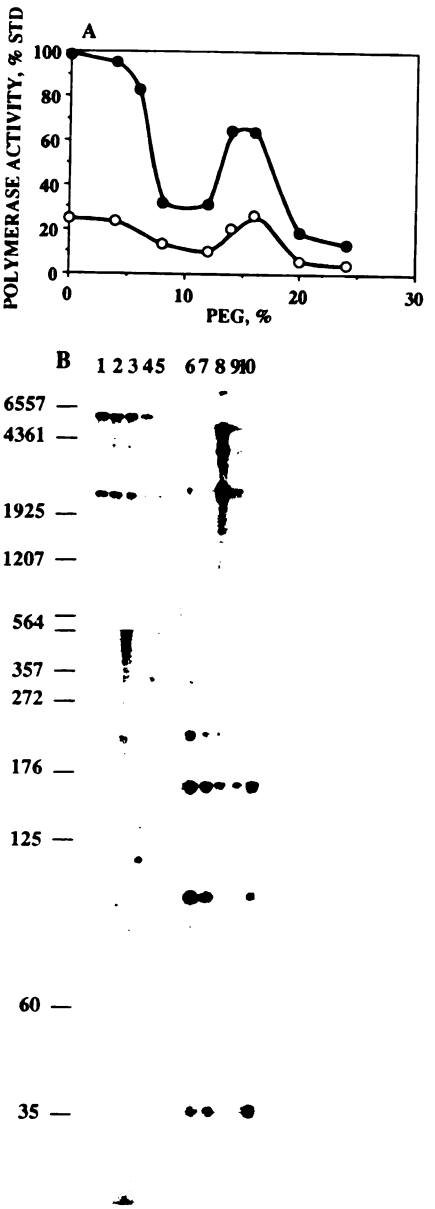

A
POLYMERASE ACTIVITY, % STD
100
80
60
40
20
0
0
10
20
30
PEG, %
B
1 2 3 4 5 6 7 8 9 10
6557
4361
1925
1207
564
357
272
176
125
60
35

Figure 10

FIGURE 11. *Gycerol increases the rate and processivity of DNA synthesis by* *Drosophila pol γ.* **A.** The rate of DNA synthesis was determined as described under "Methods" in the presence of the indicated glycerol concentrations and 30 mM (open circles) or 120 mM (closed circles) KCl. **B.** Product DNA strands were analyzed on denaturing 1.5% agarose (upper panel) and 6% polyacrylamide (lower panel) gels. Reactions were performed at 30 mM KCl (lanes 1-5) or 120 mM KCl (lanes 6-10) and no glycerol (lanes 1 and 6; apu = 2900 nt and 45 nt respectively), 7.5% glycerol (lanes 2 and 7; apu = 3600 nt and 85 nt), 15% glycerol (lanes 3 and 8; apu = 4400 nt and 140 nt), 30 % glycerol (lanes 4 and 9; apu = 4200 nt and 155 nt), 45% glycerol (lanes 5 and 10; apu = 2400 nt and 170 nt).

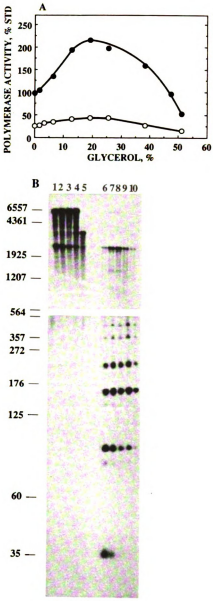

A
POLYMERASE ACTIVITY, % STD
250
200
150
100
50
0
0 10 20 30 40 50 60
GLYCEROL, %
B
1 2 3 4 5 6 7 8 9 10
6557
4361
1925
1207
564
357
272
176
125
60
35

Figure 11

unaffected when the template-primer to enzyme molecule ratio was varied over a 60-fold range, from 0.5 to 30 primer termini per pol γ molecule (Fig. 12).

FIGURE 12. *Varying the pol γ : template-primer molecular ratio has little effect on the processivity of DNA synthesis by Drosophila pol γ.* DNA synthesis reactions were carried out in the presence of a DNA "trap" to ensure processive DNA synthesis. Reaction mixtures were as described under "Methods," except that they contained 2 μM, 8 μM, or 30 μM 5'-end labeled singly-primed M13 DNA and 0.4, 0.2, or 0.1 units of pol γ, respectively. After incubation at 30°C for 5 minutes, prewarmed (30°C) DNase I -activated calf thymus DNA (360 μM) and 30 μM each of dATP, dCTP, dGTP, and dTTP were added simultaneously. Reactions were incubated further at 30°C for 30 minutes, and the product DNA strands isolated and analyzed on denaturing 1.5% agarose (upper panel) and 6% polyacrylamide (lower panel) gels. Reactions were performed at 30 mM KCl (lanes 1-3) or 120 mM KCl (lanes 4-6) at pol γ to template-primer ratios of 2:1 (lanes 1 and 4), 1:4 (lanes 2 and 5), 1:30 (lanes 3 and 6).

Figure 12

DISCUSSION

Replication of the *Drosophila* mitochondrial genome proceeds asynchronously by a mechanism in which up to 98% of the leading DNA strand is copied prior to initiation of lagging DNA strand synthesis (Goddard and Wolstenbolme, 1978; Wolstenholme et al., 1979). Although continuous DNA strand synthesis would be consistent with this mechanism, biochemical characterization of *Drosophila* mitochondrial DNA polymerase does not fully support it; DNA polymerases implicated in continuous DNA strand synthesis are generally highly processive, yet it was previously shown that pol γ is only moderately processive under reaction conditions optimal for DNA synthetic rate (Wernette et al., 1988). By altering various parameters of *in vitro* DNA synthesis, we hoped to increase the processivity of γ polymerase while maintaining a high rate of nucleotide polymerization. We show that mitochondrial DNA polymerase, like other pro- and eukaryotic DNA polymerases, is sensitive to its assay environment, but that changes in reaction conditions yield a highly processive enzyme only under conditions that are suboptimal for DNA synthetic rate. As incorporation of a nucleotide is generally more rapid than dissociation and reassociation of a DNA polymerase at the primer terminus (Kornberg and Baker, 1992), we might expect pol γ to be most active under conditions that limit enzyme cycling, that is, under conditions favoring high processivity. Instead, our results suggest that initiation and/or termination of a processive product are even more rate limiting.

Increasing concentrations of KCl induced a 6-fold increase in the activity, but a 600-fold diminution in the processivity of *Drosophila* pol γ. Ionic strength affects DNA and protein structure as well as molecular interactions, and thus it likely affects the binding of γ polymerase to DNA. Because high processivity correlates with strong enzyme-primer terminus interactions, our data suggests that increasing ionic strength destabilizes them. This destabilization may allow rapid enzyme cycling, resulting in elevated activity if cycling is the rate limiting step. In addition, the stabilization of DNA secondary structure at high ionic strength (Lyons and Kotin, 1964) likely contributes to the multitude of pause sites

observed under these conditions. In contrast, at low ionic strength, the DNA polymerase is tightly bound to the primer terminus and meets fewer impediments on the template DNA, facilitating fully processive DNA synthesis. Here, strong enzyme-DNA associations may inhibit enzyme cycling, resulting in limited overall activity.

Alterations in pH resulted in differential activity optima for *Drosophila* pol γ at low versus moderate salt. However, the enzyme was most processive at pH 8.5 regardless of the KCl level. The different activity optima likely reflect ionic strength effects on shielding of amino acid side groups. At moderate salt, it appears that side groups which are titratable in the range of pH 8.0 to pH 9.6 are sufficiently shielded such that the changes induced lack significant effects on the structure, and therefore the function, of pol γ. On the other hand, at low salt, titration of side groups at more basic pH results in increased DNA polymerase activity. Here, the intrinsic charges of incompatible amino acid side chains may be altered sufficiently to allow their association, stabilizing enzyme structure and stimulating activity.

$MgCl_2$ decreased the processivity of the *Drosophila* mitochondrial DNA polymerase, yet the optimal Mg^{2+} levels for polymerase activity varied at low versus moderate salt. Mg^{2+} is bound by both DNA and DNA polymerases, and is a required cofactor for enzyme activity, as Mg-dNTP complexes are the substrates for nucleotide polymerization. $MgCl_2$ also contributes to the ionic strength of the reaction solvent, thus partially explaining the increase in activity at high $MgCl_2$ levels at low salt, yet the loss of polymerase activity with the addition of $MgCl_2$ at moderate salt. However, this "salt" effect is probably not responsible for the initial peak in DNA polymerase activity observed between 0.25 and 0.5 mM $MgCl_2$ at low salt. Further, although differential binding of Mg^{2+} by the DNA, dNTPs and pol γ may result in a rapid initial increase in the rate of DNA synthesis, the presence of an approximately 20-fold excess of Mg^{2+} molecules over potential binding sites renders this potential explanation unlikely as well. Interestingly, *E. coli* DNA polymerase I possesses at least three types of divalent cation binding sites

exhibiting varying affinities for manganese: the high and intermediate (3-10 μM) affinity sites appear to be stimulatory, while the low affinity sites (800-900 μM) seem to be inhibitory (Slater et al., 1972). Such a binding scenario involving γ polymerase could explain the observed initial peak of polymerase activity. At the same time, diminished processivity may result from the increase in ionic strength due to addition of $MgCl_2$. Alternatively, the decrease may be related to the finding of Griep and McHenry (1988) that the inclusion of Mg^{2+} alters the conformation of the processivity factor of *E. coli* DNA polymerase III, the 37 kDa β subunit: the presence of 10 mM $MgCl_2$ specifically shifts the β subunit from a dimer into a predominantly monomeric form. While the dimeric form is directly implicated in processive DNA synthesis by pol III holoenzyme (Burgers and Kornberg, 1982b; Johanson and McHenry, 1980; Kong et al., 1992), the role of the monomer has not been addressed. *Drosophila* mitochondrial DNA polymerase comprises a 125 kDa polymerase catalytic subunit and a 35 kDa subunit of unknown function (Wernette and Kaguni, 1986). A pol III β subunit-like activity which is affected similarly by Mg^{2+} may be present in the two subunit pol γ.

High levels of ATP, but not GTP or ADP, were able to restore polymerase activity when *Drosophila* pol γ was assayed at low salt. The specificity of this stimulation eliminates the possibility that the ionic strength contribution of added nucleotides alone is responsible. Nucleotide hydrolysis may be involved, as there is some stimulation of pol γ activity by GTP but no effect of ADP. However, we were unable to detect any DNA-dependent ATPase activity in *Drosophila* pol γ (C.M. Wernette, A.J. VonTom, and L.S. Kaguni, unpublished observations). Lack of ATP hydrolysis may be indicative of the lack of a protein factor or factors which work(s) to chaperone the putative processivity factor onto the DNA (Tsurimoto and Stillman, 1990; O'Donnell, 1987; Maki and Kornberg, 1988). Alternatively, ATP bound to pol γ may induce a conformational change that influences polymerase catalytic efficiency. It is clear, although perhaps surprising, that stimulation by ATP is not a result of lowering the effective concentration of Mg^{2+} via

chelation; when the ATP concentration is varied from 5 to 12 mM, the $MgCl_2$ optimum remains at 4 mM (A.J. VonTom, unpublished observation). In addition, GTP would be expected to chelate the Mg^{2+} as well as ATP, yet its effect on enzyme activity is minimal.

The macromolecular crowding agent, polyethylene glycol, stimulated DNA polymerase activity when assayed at low salt, yet inhibited and then partially restored activity when assayed at moderate salt. Enhanced processivity was observed at both KCl levels, but was not coincident with optimal activity. These results may be complicated by the ability of PEG to precipitate protein, DNA, and protein-DNA complexes. However, PEG was shown to enhance the association of *E. coli* DNA polymerase I, its Klenow fragment, and phage T4 DNA polymerase with DNA (Zimmerman and Harrison, 1987), resulting in increased DNA polymerase activity. Stimulation was generally most pronounced at high ionic strength, such that the optimal salt concentration for enzyme activity increased with increasing PEG. Predictably, there was no apparent effect on DNA polymerase processivity since protein-DNA association, but not dissociation should be affected by crowding (Minton, 1983). In contrast, PEG enhanced the assembly of a holoenzyme form of phage T4 DNA polymerase and consequently, its processivity (Jarvis et al., 1990). Our data with pol γ are consistent with either possibility: PEG may enhance binding of γ polymerase to the DNA as a consequence of macromolecular crowding, and/or may promote protein-protein interactions (either polymerase-polymerase or polymerase-accessory factor). Glycerol may also promote these interactions, because its inclusion increased both DNA polymerase activity and processivity, regardless of salt concentration.

The processivity of Drosophila pol γ was unchanged when the enzyme molecule to primer terminus ratio was varied over a 60-fold range, perhaps reflecting an inability to enhance protein-protein interactions by non-chemical means in the near-homogeneous enzyme. Alternatively, some associations which occur may not be detectable because they do not alter the processivity of mitochondrial DNA polymerase.

We have shown here that *Drosophila* γ polymerase is capable of fully processive DNA synthesis on a natural DNA template *in vitro* only under conditions that are suboptimal for DNA polymerase activity. Thus, it is difficult to make a direct correlation of pol γ function with current models of mitochondrial DNA replication or to suggest a mechanism for its regulation. The ATP/ADP ratio and the pH of the mitochondrial matrix have been implicated in the control of oxidative phosphorylation (Brand and Murphy, 1987). Further, the ATP/ADP ratio was shown to be the primary regulator of the tricarboxylate cycle in insect flight muscle (Hansford, 1980). The mechanism of control of DNA replication in the mitochondrion is unknown, but the ability of mitochondrial DNA polymerase to catalyze DNA synthesis under a wide range of experimental conditions suggests that replication can occur in actively respiring, as well as resting organelles. In contrast, only a narrow set of conditions facilitate processive DNA synthesis, possibly restricting continuous DNA synthesis to specific periods of mitochondrial activity. Although the aqueous *in vitro* assay environment may be too unlike that of the mitochondrial matrix to draw accurate conclusions regarding the mechanism of pol γ function, our data suggests that other protein factors involved in mitochondrial DNA replication may function to promote high processivity under conditions optimal for DNA synthetic rate. These protein factors may associate with pol γ or, as suggested by Sabatino et al. (1988), they may exert their effects by altering the microenvironment to which the DNA polymerase is exposed. Furthermore, using prokaryotic, viral, and eukaryotic nuclear systems as precedent, we would predict that mitochondrial DNA polymerase associates with several accessory proteins involved in primer recognition, processivity enhancement, and single-stranded DNA coating. We hope to gain insight into the function of such accessory proteins in mitochondrial DNA replication by reinvestigating the effects of the various reaction parameters examined here, on pol γ in association with such factors, as they are identified.

CHAPTER III

ESCHERICHIA COLI SINGLE-STRANDED DNA BINDING PROTEIN STIMULATES MITOCHONDRIAL DNA POLYMERASE FROM *DROSOPHILA* EMBRYOS

INTRODUCTION

Single-stranded DNA binding protein (SSB) from *E. coli* has numerous roles in the replication of the bacterial genome. In addition to protecting single-stranded DNA from nuclease digestion (Meyer and Laine, 1990), it enhances helix destabilization by DNA helicase by preventing renaturation of unwound duplex (Baker et al., 1987), and helps organize and stabilize replication origins (Baker et al., 1986). Further, SSB enhances the fidelity of DNA polymerase, likely by increasing the rigidity of the template DNA, promoting increased steric hindrance between template and incorrect nucleotides (Kunkel et al., 1979; 1983). Finally, SSB increases the processivity of DNA polymerase by destabilizing the DNA secondary structure which normally causes polymerase pausing and dissociation (LaDuca et al., 1983). In fact, the presence of *E. coli* SSB in sufficient amounts to completely coat all of the ssDNA present in a reaction results in stimulation of *E. coli* DNA polymerase II and III holoenzyme (Sigal et al., 1972; McHenry, 1988), bacteriophage T7 DNA polymerase (Tabor et al., 1987)), herpes simplex virus-1 DNA polymerase (O'Donnell et al., 1987), and human DNA polymerase δ holoenzyme (Kenny et al., 1987). In contrast, SSB has no effect on *E. coli* DNA polymerase I (Molineux et al., 1974) or bacteriophage T4 DNA polymerase (Fay et al., 1982).

The mitochondrial analog to SSB (mtSSB) has been identified and purified from *Saccharomyces cerevisiae* (Jong et al., 1985), *Xenopus laevis* (Mignotte et al., 1985), and rat tissues (Pavco and Van Tuyle, 1985). The gene encoding the *S. cerevisiae* (Van Dyck et al., 1992), and the cDNAs encoding the *X. laevis* (Tiranti et al., 1991), rat (Tiranti et al., 1993) and human (Tiranti et al., 1993) mtSSBs have been cloned and sequenced. Mitochondrial SSB is a 13.3-15.3 kDa monomer with a native molecular weight of 56.2 kDa, implying that it is a tetramer in solution (Pavco and Van Tuyle, 1985; Ghrir et al., 1991; Van Dyck et al., 1992; Tiranti et al., 1993). It binds DNA cooperatively with a

binding site size of 8-9 nucleotides and has an affinity for DNA that is similar to that displayed by *E. coli* SSB (Mignotte, et al., 1985; Hoke et al., 1990).

Mitochondrial SSB is required for mitochondrial DNA (mtDNA) maintenance in yeast, in which it was identified as a suppressor of mitochondrial thermosensitivity associated with a disruption of a mtDNA helicase gene (Van Dyck et al., 1992). Its role in preventing the renaturation of displacement loops in vertebrate mtDNA replication likely aids in enhancing helicase activity (Van Tuyle and Pavco, 1981; Pavco and Van Tuyle, 1985). Further, mtSSB has been shown to coat the displaced ssDNA that is the template for lagging strand synthesis in mtDNA replication (Pavco and Van Tuyle, 1985), and is proposed to aid in the prevention of non-specific lagging DNA strand initiation by mtDNA primase (Wong and Clayton, 1985; Ledwith et al., 1986). The effect of mtSSB on mitochondrial DNA polymerase (pol γ) is unclear. While rat mtSSB increased the activities of both pol γ and *E. coli* DNA polymerase I ≈10-fold on poly(dT)•oligo(dA), a synthetic homo-polymer of low primer density, it had no effect on pol γ on poly(dA)•oligo(dT) for which it exhibits low affinity (Hoke et al., 1990). In contrast, *X. laevis* mtSSB had no effect on DNA synthesis in a mitochondrial lysate, but stimulated partially purified pol γ ≈3-fold on a template with high primer density, poly(rA)•oligo(dT), and ≈1.5-fold on poly(dA)•oligo(dT), while having no effect on either *E. coli* DNA polymerase I or rat DNA polymerase α (Mignotte et al., 1988). However, with the use of singly-primed single-stranded viral DNA as template, mtSSB was completely inhibitory to mitochondrial DNA polymerase activity (Mignotte et al., 1988).

Comparison of the yeast mtSSB amino acid sequence with that of *E. coli* SSB indicated that the two were 24% identical and 50% similar (Van Dyck et al., 1992). This homology was to the amino-terminal two-thirds of *E. coli* SSB which encompasses its DNA binding domain (Meyer and Laine, 1990; Van Dyck et al., 1992). In fact, three amino acids which are known to be involved in DNA binding by *E. coli* SSB are well conserved in the mtSSBs: tryptophan at position 54 and phenylalanine at position 60 are

identical in all of the mtSSBs identified, while tryptophan at position 40 is identical in human, rat, and *Xenopus* SSBs, but not in yeast where an arginine is found instead (Van Dyck et al., 1992; Tiranti et al., 1993). Phenylalanine 60 is the site of DNA crosslinking to *E. coli* SSB (Merrill et al., 1984) and its role in DNA binding, as well as those of tryptophan 40 and 54, was confirmed by site directed mutagenesis (Khamis et al., 1987; Casas-Finet et al., 1987). The amino acid sequence of rat mtSSB is 30% identical and 50% similar overall to *E. coli* SSB, while that of human is 32% identical and 50% similar (Tiranti et al., 1993). Interestingly, human mtSSB is more similar to *E. coli* SSB than to yeast mtSSB (18% identical and 38% similar) even though it is highly homologous to both rat (91% identical) and *Xenopus* (79% identical) mitochondrial SSBs (Tiranti et al., 1993).

Mitochondrial DNA polymerase is capable of highly processive DNA synthesis only under conditions that are suboptimal for DNA synthetic rate. While mitochondrial DNA replication *in vivo* is slow (approximately 4.5 nucleotides per second per DNA strand; Clayton, 1982), DNA polymerase catalyzes DNA synthesis at an even lower rate when carrying out highly processive DNA synthesis *in vitro* (approximately 0.5 nucleotides per second per DNA strand; Wernette et al., 1988 and this report). Because mitochondrial SSB is critical for mtDNA replication *in vivo* and appears to affect the activity of pol γ, we have examined the effect of *E. coli* SSB on mitochondrial DNA polymerase from *D. melanogaster*. Although *Drosophila* mtSSB has not yet been identified, the high amino acid sequence conservation among mtSSBs and with *E. coli* SSB suggests that its effects on γ polymerase may reflect that of mtSSB as well.

EXPERIMENTAL PROCEDURES

MATERIALS

Nucleotides and nucleic acids. Unlabeled deoxy- and ribonucleoside triphosphates were purchased from P-L Biochemicals. [^{3}H]dTTP was purchased from ICN Biochemicals; [α-^{32}P]dTTP and [γ-^{32}P]ATP was purchased from New England Nuclear.

Recombinant and wild type M13 viral DNAs (10,650 and 6,407 nt, respectively) were prepared by standard laboratory methods. Synthetic oligodeoxynucleotides (15 nt) complementary to the M13 viral DNAs were synthesized in an Applied Biosystems Model 477 oligonucleotide synthesizer.

Enzymes. Drosophila DNA polymerase γ (Fraction VI) was prepared as described by Wernette and Kaguni (1986). *Drosophila* SSB (RF-A; DSSB) was generously provided by Dr. I.R. Lehman of Stanford University. *E. coli* SSB and bacteriophage T4 gp32 were from United States Biochemical.

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, 30 or 120 mM KCl, 400 μg/ml bovine serum albumin, 20 μM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (1000 cpm/pmol), 10 μM (as nt) of singly-primed recombinant M13 DNA, and 0.1 to 0.2 units of Fr VI enzyme (3- to 6-fold excess of primer ends over pol γ molecules). Incubation was at 30°C for 30 minutes. Specific modifications are described in the figure legends. One unit of activity is that amount that catalyzes the incorporation of 1 nanomole of deoxyribonucleoside triphosphate into acid insoluble material in 60 minutes at 30°C using DNase I-activated calf thymus DNA as the substrate. Here, we define standard activity as that activity exhibited by pol γ in the presence of 120 mM KCl on singly primed M13 DNA.

Analysis of products of processive DNA synthesis by gel electrophoresis. Reactions were as above except that reaction mixtures contained 30 μM each of dATP, dCTP, dGTP, and 10 μM of [α-^{32}P]dTTP (2 x 10^4 cpm/pmol), 20 μM singly-primed wild type M13 DNA, and 0.02 units of Fr VI enzyme (60-fold excess of primer ends over pol γ molecules). Incubation was at 30°C for 4 or 8 minutes. Products were made 1% in SDS and 10 mM in EDTA, heated for 4 minutes at 80°C, phenol-chloroform extracted, and precipitated with ethanol in the presence of 0.5 μg of tRNA as carrier. The ethanol precipitates were resuspended in 30 mM NaOH and 20 mM EDTA, and electrophoresed in a 1.5% (agarose) slab gel (13 x 18 x 0.7 cm) containing 30 mM NaCl and 2 mM EDTA in 30 mM NaOH and 2 mM EDTA. Approximately equal amounts of radioactivity (~1000 cpm) were loaded in each lane. The gel was washed in distilled water for 20 minutes, dried under vacuum and exposed at (-)80°C to Kodak X-OMAT AR X-ray film using a Du Pont Quanta III intensifying screen.

Time course of pol γ DNA synthesis. Reaction mixtures were as for the DNA polymerase assay, except were 450 μl, contained 0.9 units of Fraction VI enzyme (6-fold excess of primer ends over pol γ molecules), and dNTPs (1000 cpm/pmol or 3000 cpm/pmol) were initially omitted. Incubation was for 5 minutes at 30°C, dNTPs were added, and additional incubation was as indicated in the figure legends. Aliquots (0.05 ml) were stopped by addition to 1 ml of 10% TCA and 10 mM sodium pyrophosphate. For analysis of products by gel electrophoresis, ^{32}P-dTTP was included in place of ^{3}H-dTTP. Aliquots were processed and electrophoresed as for the processivity assay.

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 dATP, dGTP, and dTTP, 20 μM (as nt) of 5'-end labelled singly-primed M13 DNA, and 0.2 units of Fraction VI enzyme (3-fold excess of primer ends over pol γ molecules). Incubation was at 30°C for 5 minutes. Samples were made 1% in SDS and 10 mM in EDTA, heated for 10 minutes at 65°C, phenol-chloroform extracted, and

precipitated with ethanol in the presence of 0.5 μg of tRNA as carrier. The ethanol precipitates were resuspended in 80% formamide and 90 mM Tris borate. Aliquots were denatured for 2 minutes at 100°C and electrophoresed in an 18% polyacrylamide slab gel (13 x 24 x 0.075 cm) containing 7 M urea in 90 mM Tris-borate (pH 8.3) and 25 mM EDTA.

RESULTS

***E. coli* SSB stimulates DNA polymerase γ activity.** *E. coli* SSB was previously shown to have no effect on the activity of partially purified *X. laevis* mitochondrial DNA polymerase (Mignotte et al., 1988). However, when nearly homogeneous *D. melanogaster* γ polymerase was assayed in the presence of SSB and 120 mM KCl, we found that polymerase activity was stimulated approximately 2- to 3-fold (200% to 300% of standard activity; Figure 13). Further, when pol γ was assayed in the presence of SSB and 30 mM KCl, activity was stimulated approximately 25-fold (625% of standard activity), close to a level achieved only on DNase-activated calf thymus DNA optimized for γ polymerase activity. Maximal stimulation was observed when the amount of SSB present was sufficient to coat all of the single-stranded DNA. Because the presence of salt alters the binding characteristics of *E. coli* SSB, the single-stranded DNA is saturated with binding protein at 0.5 μg and 1.0 μg of SSB in the presence of 120 mM and 30 mM KCl, respectively (Lohman and Overman, 1985).

We had found previously that the mechanism of DNA synthesis by pol γ was dependent on the KCl concentration in which it was assayed: At 120 mM KCl, γ polymerase was most active (standard activity) yet only moderately processive (average processive unit of 45 nucleotides), while at 30 mM KCl, γ polymerase was less active (25% of standard) but highly processive (average processive unit of 2500 nucleotides). Therefore, we investigated the effect of KCl concentration on the stimulation of pol γ by *E. coli* SSB. Stimulation of mitochondrial DNA polymerase by SSB was maximal (25-fold) from approximately 20 mM to 90 mM KCl (Figure 14), thus lowering the KCl concentration required to achieve optimal DNA polymerase activity as much as 6-fold. Further, SSB completely inhibited pol γ when the concentration of KCl was greater than 150 mM.

Figure 13. *E. coli SSB stimulates the rate of DNA synthesis by Drosophila pol γ.* The rate of DNA synthesis was determined as described under "Methods" in the presence of the indicated amounts of *E. coli* SSB and 30 mM KCl (open circles) or 120 mM KCl (closed circles).

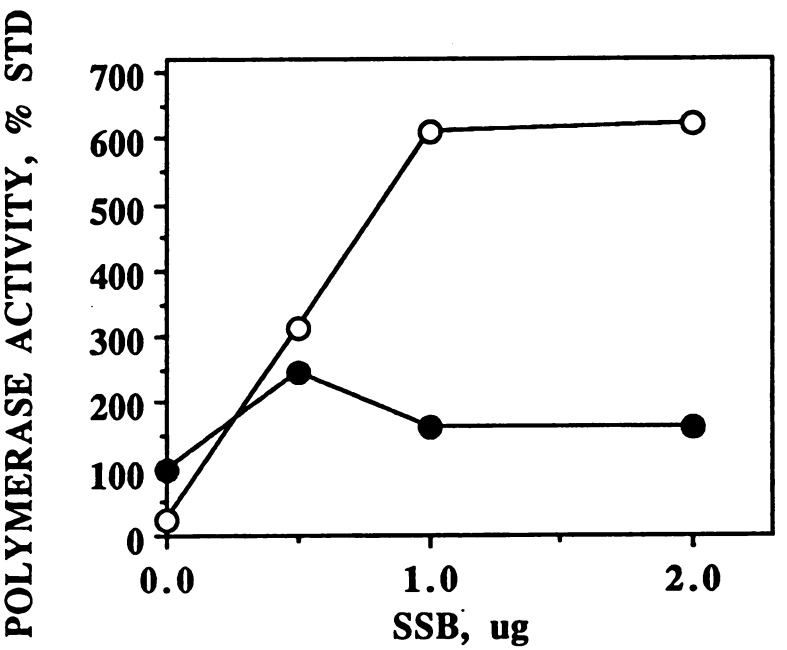

POLYMERASE ACTIVITY, % STD
700
600
500
400
300
200
100
0
0.0
1.0
2.0
SSB, ug

Figure 13

Figure 14. *E. coli SSB lowers the KCl optimum of Drosophila pol γ.* The rate of DNA synthesis was determined as described under "Methods" in the presence of the indicated amounts of KCl and in the absence (closed circles) or presence (open circles) of 1μg of SSB.

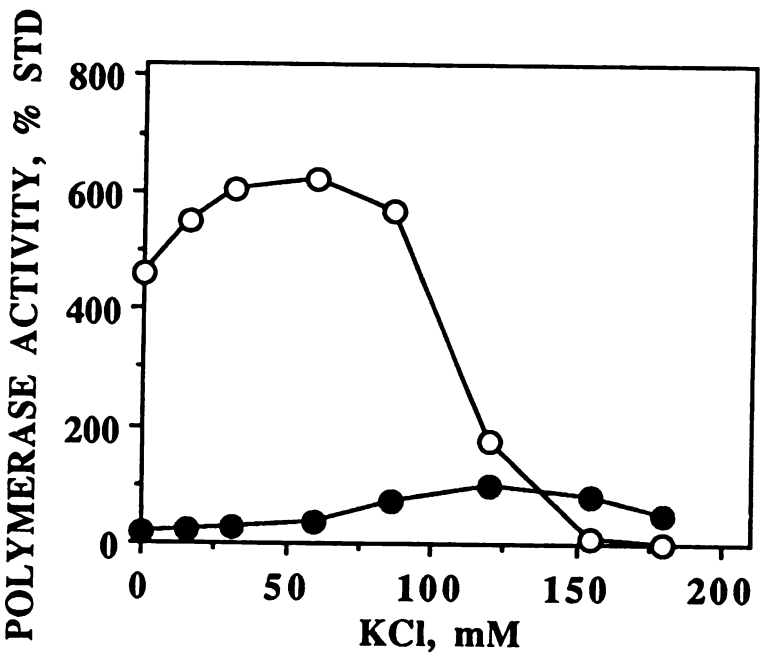

POLYMERASE ACTIVITY, % STD
800
600
400
200
0
0
50
100
150
200
KCl, mM

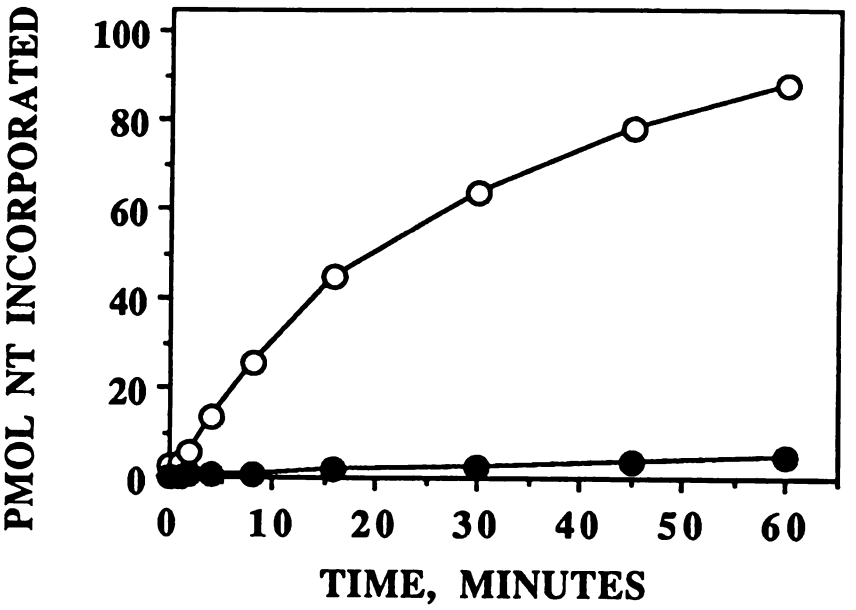
Figure 14

SSB stimulation of DNA polymerase γ activity exhibits a lag time. *E. coli* SSB stimulates bacteriophage T7 DNA polymerase up to 11-fold following two minutes of DNA synthesis, decreasing with time down to 5-fold after 15 minutes of catalysis (Myers and Romano, 1988). To determine the maximal stimulation of pol γ by SSB, we examined the extent of DNA synthesis by pol γ in the absence and presence of SSB over a time course from 1 to 60 minutes in the presence of 30 mM KCl. Incorporation of nucleotides by pol γ was linear up to about 60 minutes of incubation at 30°C (Figure 15A and Kaguni et al., 1988), while incorporation by pol γ in the presence of SSB was linear only from 4 to 16 minutes of incubation. Stimulation of DNA polymerase was 7-fold initially (0.1% of DNA replicated), then over 30-fold from 4 to 16 minutes (2-9% of DNA replicated), but decreased to 17-fold following 60 minutes (18% of DNA replicated) of incubation at 30°C (Figure 15B). In comparison, the 25-fold stimulation of polymerase activity observed in previous experiments was achieved following 30 minutes of incubation (13% of DNA replicated). Thus, a 4 minute lag is required to attain maximal stimulation of pol γ by *E. coli* SSB of 30- to 35-fold in the presence of 30 mM KCl.

SSB has little effect on the processivity of DNA polymerase γ. The stimulation of *Drosophila* mitochondrial DNA polymerase by *E. coli* SSB may be due to its ability to increase the processivity, rate of initiation of DNA synthesis, or intrinsic rate of nucleotide incorporation by γ polymerase. Alternatively, SSB may increase DNA polymerase cycling or stimulate pol γ activity by some combination of all of these parameters. In an initial effort to determine the mechanism by which SSB stimulates pol γ activity, its effect on enzyme processivity was examined at 30 mM KCl (Figure 16). The addition of SSB resulted in a more uniform distribution of DNA synthesis products, likely indicative of the elimination of some DNA polymerase pause sites, and a 2- to 3-fold increase in the amount of full length DNA synthesis products apparent. However, the processivity of mitochondrial DNA polymerase was increased less than 3-fold after 8

Figure 15. *E. coli SSB stimulation of Drosophila pol γ exhibits a lag time.* The rate of DNA synthesis was determined over a time course as described under "Methods" in the presence of 30 mM KCl and in the absence (closed circles) or presence (open circles) of 1 μg of SSB. **A.** Nucleotide incorporation by pol γ was determined following 0, 1, 2, 4, 8, 16, 30, 45, and 60 minutes of incubation. Following 60 minutes and in the absence of SSB, 1% of the DNA available was copied, while in the presence of SSB, 18% of the DNA was copied. **B.** The data from "A" was replotted as the extent of stimulation resultant from the presence of SSB at each time point.

A


PMOL NT INCORPORATED
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME, MINUTES

B

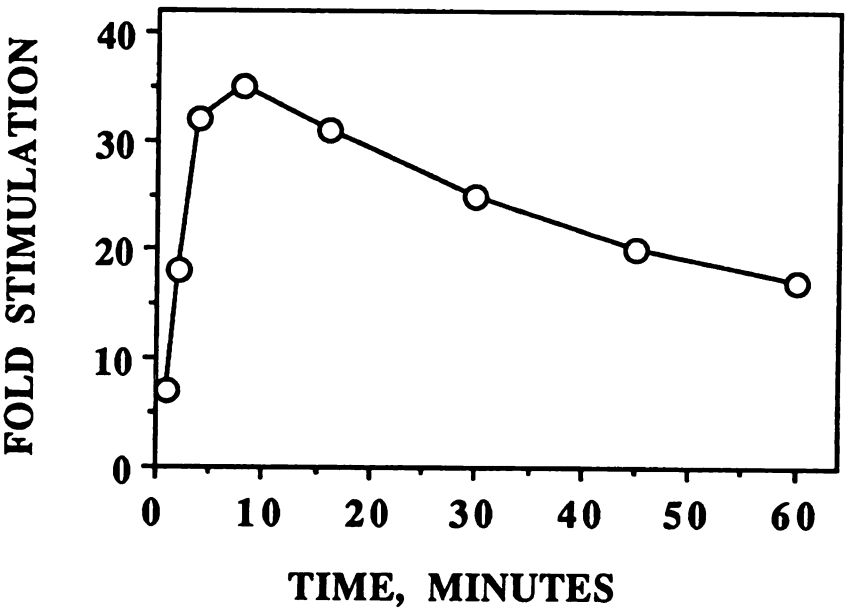

FOLD STIMULATION
40
30
20
10
0
0
10
20
30
40
50
60
TIME, MINUTES

Figure 15

Figure 16. *E. coli SSB increases the processivity of DNA synthesis by Drosophila pol γ.* The processivity of DNA synthesis was determined as described under "Methods" in the presence of 30 mM KCl. Reactions were performed in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of SSB and incubated for 4 (lanes 1 and 3) or 8 (lanes 2 and 4) minutes at 30°C. Numbers at left indicate the position and size (in nt) of *Hin*dIII restriction fragments of λDNA that were electrophoresed in an adjacent lane.

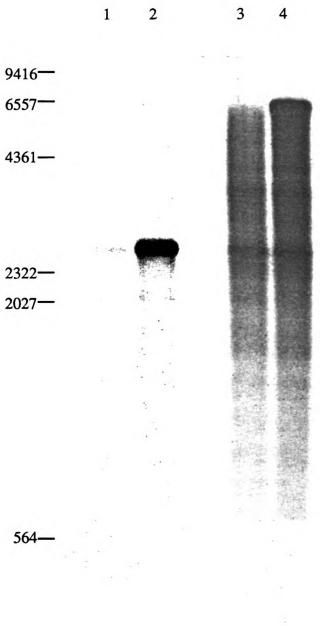


Figure 16

minutes of incubation, indicating that the source of the 35-fold SSB stimulation of polymerase activity may be partially due to an increase in enzyme processivity, but that other mechanisms play a larger role.

SSB increases the rate of initiation of DNA synthesis by γ polymerase. We investigated the effect of SSB on the rate of initiation of DNA synthesis by pol γ by examining the products of limited DNA synthesis from a 5'-end labelled 15 nucleotide primer in the presence of only dATP, dGTP, and TTP. The absence of dCTP causes DNA synthesis to terminate after the incorporation of 8 or 11 nucleotides, the first and second positions where dCTP is required for incorporation. The use of this primer extension assay allows observation of only the initial stages of DNA synthesis: location of and binding to the primer terminus by pol γ and aborted primer extension. Therefore, the aspects of processivity and intrinsic polymerization rate are eliminated.

SSB stimulated primer extension 20- to 25-fold at 30 mM KCl (Figure 17) following 5 minutes of incubation at 30°C. Because the stimulation of polymerase activity was 30- to 35-fold at this time point, these results indicate that 60-80% of pol γ stimulation by SSB is due to an increase in the rate of initiation of DNA synthesis by mitochondrial DNA polymerase. This stimulation may be a result of SSB facilitated primer recognition accomplished by elimination of non-productive binding of DNA polymerase to excess single-stranded DNA or by increasing the affinity of unbound polymerase molecules for DNA. Stimulation of the 3'→5' exonuclease activity of γ polymerase by SSB supports these hypotheses as well (C.L. Farr and L.S. Kaguni, unpublished observations).

SSB has little effect on the intrinsic rate of DNA synthesis by DNA polymerase γ. DNA synthesis in the presence of SSB began to level out after 45 minutes of incubation at 30°C even though only 18% of the available DNA had been replicated (see figure 15A). Because the extent of DNA synthesis is proportional to the

Figure 17. *E. coli SSB increases the initiation of DNA synthesis by Drosophila pol γ.* Primer extension was determined as described under "Methods" in the presence of 30 mM KCl. Reactions were perfomed in the absence (lane 2) or presence (lane 3) of 1 μg of SSB. Lane 1 contains no DNA polymerase or SSB.

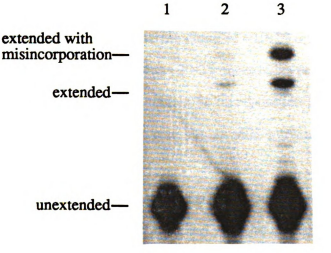


Figure 17

amount of γ polymerase used (data not shown) and pol γ is highly processive at 30 mM KCl, these results suggested a mechanism where each polymerase molecule copied fully a DNA molecule and was slow to cycle to an unused primer terminus. In order to test this hypothesis, we examined the products of DNA synthesis by mitochondrial DNA polymerase in the absence and presence of SSB over the same time course shown in figure 3 (Figure 18).

Full length DNA synthetic products were first observed after 8 minutes of DNA synthesis by pol γ alone and in the presence of SSB. Using these results together with those obtained following 1, 2, and 4 minutes of incubation, we were able to estimate the intrinsic rate of polymerization by γ polymerase alone as ≈10-15 nucleotides per second, while that of pol γ in the presence of SSB was ≈20-25 nucleotides per second. Thus, SSB induced a ≈1.5-fold increase in the rate of nucleotide incorporation by pol γ. In addition, the presence of SSB eliminates a strong pause site at 2600 nucleotides, allowing for a greater accumulation of full length products.

The proportion of DNA polymerase molecules actively synthesizing DNA was calculated by combining the amount of DNA copied with the average length of the DNA products at each time point, and assuming that pol γ was not cycling following processive DNA synthesis. Interestingly, the extent of stimulation due to SSB at each time point is generally proportional to the ratio of DNA polymerase molecules actively synthesizing DNA in the presence and absence of SSB: following one minute of incubation, ≈15% of the pol γ molecules are engaged in DNA synthesis in the presence of SSB, while in its absence only ≈3% (5-fold less) are. DNA polymerase activity is stimulated ≈7-fold at this time point (see figure 15B). Similarly, after 16 minutes, ≈100% and ≈8% (12-fold difference) of polymerase molecules were estimated to be synthesizing DNA in the presence and absence of SSB, respectively, while SSB stimulation of pol γ was 30-fold (see figure 15B). In fact ≈50% of the total stimulation observed at each time point seems to

Figure 18. *Effect of E. coli SSB on a time course of DNA synthesis by Drosophila pol γ.* A time course of DNA synthesis was conducted as described under "Methods" in the presence of 30 mM KCl. Reactions were performed in the absence (lanes 1-8) or presence (lanes 9-16) of 1μg of SSB and were incubated for 1 (lanes 1 and 9), 2 (lanes 2 and 10), 4 (lanes 3 and 11), 8 (lanes 4 and 12), 16 (lanes 5 and 13), 30 (lanes 6 and 14), 45 (lanes 7 and 15), or 60 (lanes 8 and 16) minutes at 30°C. The arrow indicates the position of full length products. Numbers at left indicate the position and size (in nt) of *Hin*dIII restriction fragments of λDNA that were electrophoresed in an adjacent lane.

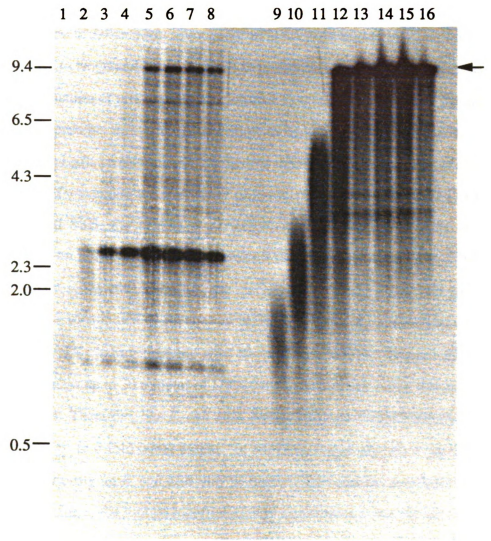


Figure 18

be due to the SSB induced increase in the proportion of actively synthesizing DNA polymerase molecules.

***Drosophila* SSB and bacteriophage T4 gp32 stimulate mitochondrial DNA polymerase.** Stimulation of *Drosophila* mitochondrial DNA polymerase by *E. coli* SSB is dramatic and appears to be caused by an increase in primer recognition and binding, as well as by limited stimulation of processivity and intrinsic rate of DNA synthesis. Single stranded-DNA binding proteins from sources as diverse as bacteria and humans appear to exert their function via specific protein-protein interactions in addition to their ability to coat single-stranded DNA. To examine the specificity of stimulation of mitochondrial DNA polymerase by *E. coli* SSB, the effects of *Drosophila* SSB (RF-A, DSSB) and bacteriophage T4 gp32 on pol γ activity were examined (Figure 19A). Similar to DNA polymerization in the presence of *E. coli* SSB, the reactions containing DSSB and gp32 were linear only from 4-16 minutes of incubation at 30°C. However, stimulation of γ polymerase by these DNA coating proteins was 6- to 7-fold initially (Figure 19B), remained relatively constant up to 30 minutes of DNA synthesis, and was 4-fold following 60 minutes of incubation. Therefore, like *E. coli* SSB, both DSSB and T4 gp32 stimulated DNA synthesis but to a 5- to 6-fold lesser extent. In addition, it appears that a lag time similar to that noted previously for *E. coli* SSB may be required for maximal stimulation by DSSB and T4 gp32, but due to the limited incorporation of nucleotides at the earliest time points, detailed analysis was not attempted.

***E. coli* DNA polymerase III subunits had no effect on Pol γ activity or DNA binding.** The strong homology of mtSSB with *E. coli* SSB and the stimulation of pol γ by SSB suggested that *E. coli* processivity and primer recognition factors may substitute

Figure 19. *Drosophila SSB and bacteriophage T4 gp32 stimulate Drosophila pol γ.* The rate of DNA synthesis was determined over a time course as described under "Methods" in the presence of 30 mM KCl and in the absence (closed circles) or presence of saturating *Drosophila* SSB (1 μg; open circles) or T4 gp32 (1μg; open triangles). **A.** Nucleotide incorporation by pol γ was determined following 0, 1, 2, 4, 8, 16, 30, 45, and 60 minutes of incubation. Following 60 minutes and in the absence of SSB, 1% of the DNA available was copied, while in the presence of *Drosophila* SSB or T4 gp32, 5% of the DNA was copied. **B.** The data from "A" was replotted as the extent of stimulation resultant from the presence of *Drosophila* SSB (open circles) or T4 gp32 (open triangles) at each time point.

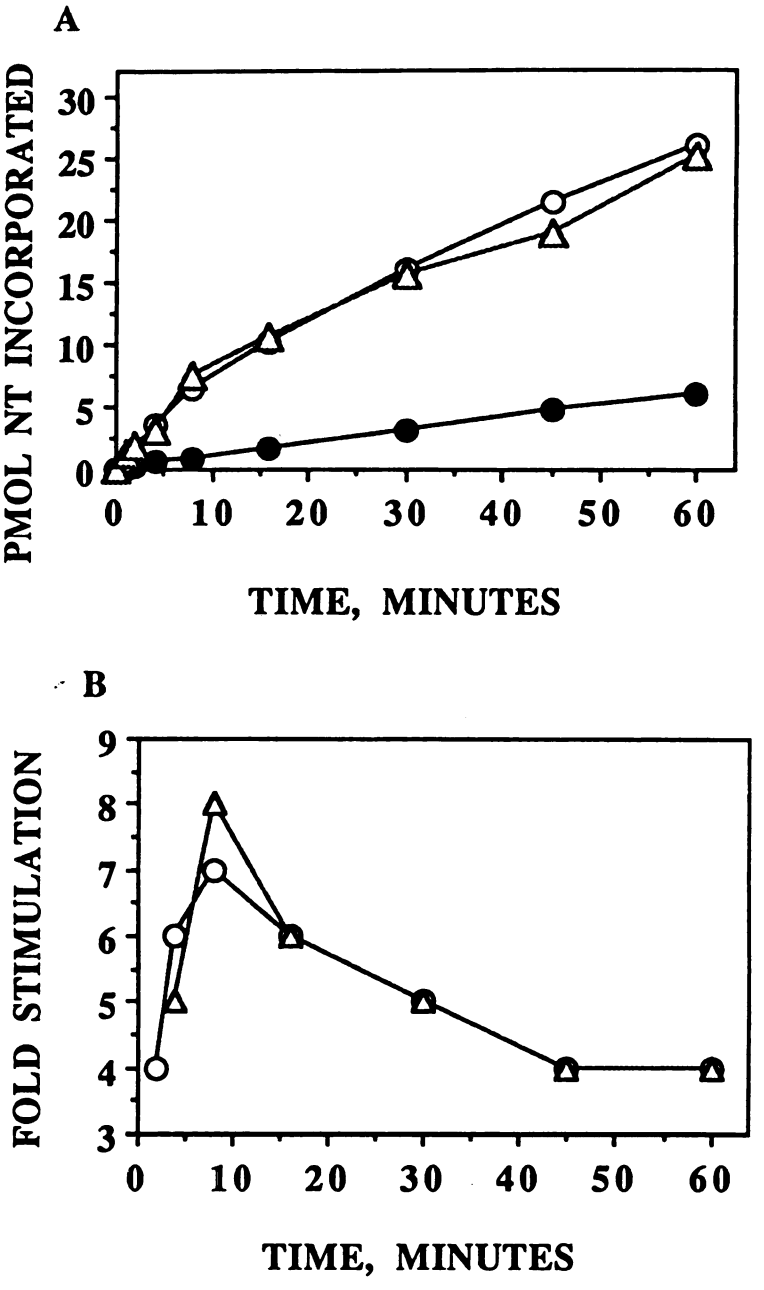

A
PMOL NT INCORPORATED
30
25
20
15
10
5
0
0
10
20
30
40
50
60
TIME, MINUTES
B
FOLD STIMULATION
9
8
7
6
5
4
3
0
10
20
30
40
50
60
TIME, MINUTES

Figure 19

for putative mitochondrial DNA polymerase accessory factors as well. Therefore the subunits of DNA polymerase III responsible for processivity and primer recognition were examined with regard to their effects on mitochondrial DNA polymerase activity and DNA binding. Neither the β subunit (processivity) nor the γ complex (primer recognition) alone or in combination affected pol γ activity at 30 or 120 mM KCl in the absence or presence of SSB (data not shown). In addition, pol γ DNA binding on template-primer DNA was unaffected by either the β subunit or γ complex (data not shown).

DISCUSSION

Efficient DNA replication of bacteriophage, eukaryotic, and viral genomes requires DNA polymerase in addition to its accessory factors and single-stranded DNA binding protein. A ≈15 kDa mitochondrial single-stranded DNA binding protein (mtSSB) homologous (≈25% identical and ≈50% similar) to *E. coli* SSB has been identified in *Xenopus laevis*, *Saccharomyces cerevisiae*, rat and human tissues (Mignotte et al., 1985; Ghrir et al., 1991; Van Dyck et al., 1992; Pavco and Van Tuyle, 1985; Tiranti et al., 1993). This considerable homology, our inability to purify mtSSB from *Drosophila melanogaster*, and the apparent requirement for an SSB-like activity in mitochondrial DNA replication prompted us to examine the effects of *E. coli* SSB on γ polymerase.

E. coli SSB stimulated *Drosophila* pol γ 25-fold in the presence of 1.0 μg SSB and 30 mM KCl, and 3-fold in the presence of 0.5 μg SSB and 120 mM KCl. In the presence of 30 mM KCl, 1.0 μg of SSB is expected to completely coat all of the ssDNA present in the reaction, thus the plateau of stimulation is due to saturated binding of the template DNA. Because maximal stimulation occurs at a ratio of approximately 5000 SSB molecules per DNA polymerase molecule, this result implies that the SSB stimulation of pol γ is caused by its DNA coating capacity and not a stoichiometric interaction with γ polymerase. KCl greatly affects the mode and cooperativity of SSB binding even though it has little effect on the affinity of SSB for DNA: below 10 and above 200 mM NaCl, *E. coli* SSB binds to ssDNA in two modes which have site sizes of 33 and 65 nucleotides per tetramer, respectively. In the range between these salt concentrations SSB tetramers bind in both modes, resulting in apparent binding site sizes between the two limits (Krauss et al., 1981; Ruyechan and Wetmur, 1976; Lohman and Overman, 1985). At 30 mM KCl the apparent site size is approximately 43 nucleotides, while at 120 mM KCl it is 62 nucleotides; therefore, at the higher salt concentration, only 0.5-0.7 μg of SSB is required to saturate the same amount of ssDNA that requires 1.0 μg in the presence of less salt.

Thus, maximal stimulation of DNA polymerase activity occurs in the presence of saturating SSB at both salt concentrations.

Titration of KCl revealed that mitochondrial DNA polymerase activity was greatest from 20 to 90 mM KCl in the presence of SSB, up to 6-fold less KCl than that normally required to achieve maximal polymerase activity in its absence. Further, while 50-80% of optimal DNA polymerase activity is normally supported at 150-180 mM KCl, the addition of SSB completely inhibited it. This shift in KCl optima likely reflects the change of DNA binding mechanisms by both SSB and pol γ with changing salt concentration and the complexity of their interactions with each other and DNA.

Stimulation of several DNA polymerases by *E. coli* SSB has been documented and, in some cases, appears to be due to the ability of SSB to increase primer recognition by either preventing non-productive binding of DNA polymerase molecules or by enhancing the affinity of polymerase for DNA. SSB stimulation of herpes simplex virus-1 DNA polymerase appears to occur by the former mechanism (O'Donnell et al., 1987), while that of bacteriophage T7 DNA polymerase occurs by the latter (Myers and Romano, 1988). The elimination of non-productive DNA polymerase binding may result from a decreased affinity of polymerase for SSB coated ssDNA, causing multiple binding and dissociation events which continue until the primer terminus is bound. Alternatively, the presence of SSB may maintain or enhance DNA binding, and facilitate a sliding or looping mechanism by which DNA polymerase efficiently locates the primer terminus. The ability of SSB to increase the processivity of DNA polymerase by diminishing DNA secondary structure appears responsible, at least in part, for increasing the activities of *E. coli* DNA polymerases II and III, and bacteriophage T7 DNA polymerase (Sherman and Gefter, 1976; LaDuca et al., 1983; Tabor et al., 1987). Alternative putative mechanisms of SSB stimulation of DNA polymerase activity include proposed increases in intrinsic rate of nucleotide incorporation and cycling of DNA polymerase to an unused primer terminus following synthesis of a processive product. Because, by definition, a highly processive

enzyme is one which has a high affinity for a primer terminus, cycling is likely slow during processive DNA replication. Thus, SSB facilitated cycling would increase the apparent rate of DNA replication by providing unbound DNA polymerase molecules for use in the initiation of a new DNA chain.

A time course of DNA synthesis revealed that maximal stimulation (30- to 35-fold) of pol γ by SSB was achieved only after 4 minutes of DNA synthesis at 30°C. While SSB likely binds stably to DNA during its 5 minute preincubation, γ polymerase may require the presence of dNTPs to do so. Alternatively, this lag time may be required to form the putative pol γ-SSB association needed to facilitate an increase in the intrinsic rate of nucleotide incorporation, to allow sufficient DNA synthesis to occur for observation of a stimulation of processivity, or for processive products to be completed so that an increase in cycling due to SSB becomes apparent. The extent of DNA synthesis was increased only 1.3-fold from 30 to 60 minutes of incubation in the presence of SSB even though excess DNA was available and reaction linearity was maintained in its absence. Surprisingly, after 60 minutes of DNA synthesis in the presence of SSB, only 18% of the available DNA was copied, approximately the amount expected if each DNA polymerase molecule completely copied one DNA molecule and did not cycle to an unused primer terminus. Addition of pol γ following this incubation indicated that at least 70% of the DNA in the reaction was viable template for DNA replication (data not shown). Further, the extent of DNA synthesis remained proportional to the amount of DNA polymerase added; that is, the percentage of DNA copied (4-70%) reflected the expected DNA synthesis if each polymerase molecule completely copied only one DNA molecule (data not shown).

The ability of *E. coli* SSB to increase the processivity of *E. coli* DNA polymerases II and III, as well as bacteriophage T7 DNA polymerase (Sherman and Gefter, 1976; LaDuca et al., 1983; Tabor et al., 1987) suggested that perhaps it also increased the processivity of mitochondrial DNA polymerase. The presence of SSB increased the processivity of *Drosophila* pol γ approximately 2- to 3-fold at 30 and 120 mM KCl (C.M.

Wernette and L.S. Kaguni, unpublished observation), and eliminated some polymerase pause sites, likely through removal of DNA secondary structure. While this increase in processivity may account for much of the stimulation of γ polymerase at 120 mM KCl, it is only a minor contributor to SSB stimulation of pol γ at 30 mM KCl.

The ability of mitochondrial DNA polymerase to locate and bind a primer terminus, and catalyze limited incorporation of nucleotides was increased 20- to 25-fold in the presence of SSB following 5 minutes of incubation at 30°C. Because total stimulation of pol γ by SSB is 30- to 35-fold after 5 minutes of DNA synthesis, this result indicates that increased primer recognition is responsible for 60-80% of the total stimulation observed. This data is consistent with mechanisms of stimulation due to increased affinity of DNA polymerase for template-primer, either by prevention of non-productive DNA binding of DNA polymerase or increased affinity of unbound DNA polymerase for DNA, and with increased cycling of DNA polymerase following completion of DNA synthesis.

By examining the products of DNA synthesis over a time course, we were able to estimate that the intrinsic rate of γ polymerase was increased approximately 1.5-fold in the presence of SSB which, together with increased processivity, accounts for 10-15% of the stimulation of mitochondrial DNA polymerase due to SSB at 30 mM KCl. Finally, we were able to estimate the number of DNA polymerase molecules actively polymerizing DNA at each time point by combining the size of DNA synthesis products with the total amount of DNA synthesis achieved. In the presence of SSB, approximately 16 minutes are required for all of the DNA polymerase molecules to be actively engaged in DNA synthesis, while in the absence of SSB, only 5% of the pol γ molecules are incorporating nucleotides after 16 minutes and only 15% following 60 minutes of incubation. In fact, the extent of pol γ stimulation observed at each time point is generally proportional to the difference in the estimated number of polymerase molecules actively synthesizing DNA in the presence and absence of SSB, such that 60-70% of the total stimulation observed throughout the time course can be attributed to this phenomenon. This result supports the

hypothesis that SSB stimulates mitochondrial DNA polymerase by increasing primer recognition, but stimulation is limited by a general lack of enzyme cycling. In addition, this data implies that the lag time required to achieve maximal stimulation of DNA polymerase activity by SSB is due to the time required for primer recognition by pol γ.

We propose that SSB promotes binding of mitochondrial DNA polymerase to the template primer by eliminating non-productive binding of pol γ to single-stranded DNA. Here, SSB either prevents polymerase binding to or facilitates enzyme dissociation from ssDNA until a primer terminus is bound by chance. Because each 10,650 nucleotide long M13 DNA molecule is singly primed, only 0.15% of the total DNA in the reaction is composed of primer annealed to template. Therefore, the time required for all DNA polymerase molecules to locate a primer terminus is long even though there is an approximately 6-fold excess of primer termini over DNA polymerase molecules. Confirmation of this proposed mechanism of stimulation will require comparison of lag times following alteration of preincubation times and conditions, as well as increasing the primer density on the template DNA.

Both *Drosophila* SSB (DSSB, RF-A) and bacteriophage T4 gp32 stimulate mitochondrial DNA polymerase activity 4- to 7-fold over a 60 minute time course at 30°C. Given the proposed mechanism for *E. coli* SSB stimulation of pol γ, it is surprising that all DNA coating proteins do not elicit the same response. While each of these three proteins bind DNA with approximately equal affinity, they may impose different conformations onto single-stranded DNA (Chase and Williams, 1986; Mitsis et al., 1993). Thus, mitochondrial DNA polymerase may bind DSSB and T4 gp32 coated DNA non-productively with high affinity even though *E. coli* SSB appears to prevent this binding. Mitochondrial SSB might be expected to impose a similar conformation onto ssDNA as *E. coli* SSB since it exhibits homology to the DNA binding domain of *E. coli* SSB. While mtSSB is approximately 25% identical and 50% similar to *E. coli* SSB overall, it is 50-67% identical to several regions of the amino terminal two-thirds of *E. coli* SSB which is

involved in DNA binding (Van Dyck et al., 1992; Ghrir et al., 1991; Tiranti et al., 1993; Benedict and Kowalczykowski, 1988). In addition, two amino acids known to be involved in DNA binding by SSB are identical in all mtSSBs identified to date, while an additional amino acid involved in DNA binding is identical in all mtSSBs except yeast mtSSB (Merrill et al., 1984; Casas-Finet et al., 1987; Khamis et al., 1987; Tiranti et al., 1993). Therefore, the effects of mitochondrial SSB on γ polymerase may be expected to be more closely represented by *E. coli* SSB than bacteriophage or eukaryotic SSBs.

The inability of processivity and primer recognition factors from *E. coli* to alter the activity of mitochondrial DNA polymerase is likely due to a lack of stable protein-protein associations. The ability of *E. coli* SSB to stimulate pol γ is likely through its interaction with the DNA and not through a specific pol γ-SSB interaction. However, processivity factors must associate with DNA polymerase to tether it onto the DNA template (Stukenberg et al., 1991). Because DNA polymerase γ and *E. coli* DNA polymerase III lack considerable homology (Ito and Braithwaite, 1991), specific association of the prokaryotic processivity factor with pol γ was probably unlikely.

We have characterized the stimulation of *D. melanogaster* mitochondrial DNA polymerase by *E. coli* SSB and found that 60-80% of this stimulation is due to an increase in the effective enzyme concentration at the primer terminus. We propose that this increase is caused by the ability of SSB to prevent non-productive binding of pol γ to single stranded DNA, resulting in multiple association and dissociation events until the primer terminus is bound by chance. However, it is possible that the effect of SSB is to maintain or increase polymerase DNA binding and transfer these molecules to the primer terminus. Alternatively, SSB may increase the affinity of unbound DNA polymerase molecules to the primer terminus. However, this mechanism is unlikely since it would probably require a specific SSB-polymerase association, which is not consistent with titration experiments indicating that a 5000-fold excess of SSB over DNA polymerase molecules is required for maximal stimulation of pol γ. We found that an additional 10-15% of SSB stimulation of

mitochondrial DNA polymerase is the result of SSB facilitated increases in processivity and rate of DNA synthesis by the DNA polymerase. These increases are likely due to the ability of SSB to remove secondary structure from DNA which act as impediments to DNA synthesis, resulting in decreased enzyme pausing and dissociation (Meyer and Laine, 1990).

Herpes simplex virus-1 (HSV-1) DNA polymerase is stimulated by *E. coli* SSB by a similar mechanism to what we have proposed for mitochondrial DNA polymerase (O'Donnell et al., 1987). Stimulation was suggested to be due to the ability of SSB to eliminate non-productive DNA binding by HSV-1 DNA polymerase, with slow cycling to an unused primer following synthesis of a processive product. In contrast, *E. coli* SSB appeared to increase the affinity of bacteriophage T7 DNA polymerase for DNA at high (30:1) DNA to polymerase ratios (Myers and Romano, 1988).

The asymmetry of mitochondrial DNA replication results in an almost fully single-stranded DNA substrate for lagging strand DNA synthesis *in vivo*. Thus, the singly primed single-stranded viral template DNA used in this study is an excellent model of lagging strand mtDNA synthesis. Mitochondrial DNA polymerase is capable of fully processive (10,650 nucleotides) DNA synthesis *in vitro* only at reaction conditions which are suboptimal for DNA synthetic rate. However, addition of *E. coli* SSB greatly enhances this rate of synthesis while maintaining high processivity. The SSB induced ability of pol γ to efficiently and processively copy long stretches of ssDNA should allow us to more accurately characterize the mechanism of DNA synthesis by mitochondrial DNA polymerase.

Mitochondrial SSB has been identified in several tissues, and is required for mitochondrial DNA replication in yeast (Mignotte et al., 1985; Van Dyck et al., 1992; Pavco and Van Tuyle, 1985; Tiranti et al., 1993;). Although mtSSB exhibits considerable homology to *E. coli* SSB, its effect on mitochondrial DNA polymerase activity is unclear. Single-stranded DNA binding protein isolated from rat mitochondria stimulated its

homologous DNA polymerase approximately 10-fold, while mtSSB from *X. laevis* either completely inhibited or stimulated its homologous polymerase 1.5- to 3-fold depending on the enzyme preparation and reaction conditions utilized (Hoke et al., 1990; Mignotte et al., 1988). We have found that both KCl concentration and enzyme purity affect the stimulation of mitochondrial DNA polymerase by *E. coli* SSB (this report and A.J. VonTom and L.S. Kaguni, data not shown). In the presence of 120 mM KCl, SSB either stimulates pol γ activity 2- to 3-fold or has no effect depending on the purity of g polymerase. Similarly, in the presence of 30 mM KCl, crude pol γ preparations are stimulated 2- to 4-fold by SSB, while more pure fractions are stimulated 20- to 25-fold. Therefore, we suggest that attempts to characterize the effects of mitochondrial SSB on mitochondrial DNA polymerase have been incomplete due to the use of inappropriate reaction conditions and/or DNA polymerase fractions.

Because previous attempts in this laboratory to purify mitochondrial SSB were unsuccessful, we had hoped to use our characterization of *E. coli* SSB stimulation of γ polymerase to identify and purify *D. melanogaster* mtSSB. Although we were able to detect DNA polymerase stimulatory activity (2- to 3-fold) when we examined chromatographic fractions of crude mitochondrial extracts, it was present in most of the fractions and was difficult to follow through several purification steps. Future attempts to purify mitochondrial SSB must rely on a more specific assay.

CHAPTER IV

IDENTIFICATION AND PURIFICATION OF A TEMPLATE-PRIMER DNA BINDING PROTEIN FROM *DROSOPHILA* MITOCHONDRIA

INTRODUCTION

Replication of the mitochondrial genome is unidirectional and asymmetric, and may proceed by continuous DNA synthesis on both DNA strands. In fact, models of mtDNA replication, derived from biochemical and electron microscopic examination of mtDNA replication intermediates, are consistent with this hypothesis (Goddard and Wolstenholme, 1980; Clayton, 1982). Because DNA replication is most rapid when DNA polymerase is processive and does not dissociate from the DNA template during the replication cycle (Young et al., 1992), continuous DNA synthesis is often indicative of processive DNA synthesis. Indeed, in prokaryotic and viral systems, continuous DNA synthesis correlates with processive synthesis (Das and Fujimara, 1979; Fisher et al., 1979; Hockensmith and Bambara, 1981; Fay et al., 1981, 1982; Prelich et al., 1987; Hurwitz et al., 1990). Our results indicate that pol γ is highly processive only under reaction conditions which are suboptimal for DNA synthetic rate, except in the presence of *E. coli* SSB. The ability of γ polymerase to catalyze DNA synthesis with high efficiency and processivity *in vitro* in the presence of SSB suggests that *in vivo* it has the capacity to do so as well.

Mitochondrial SSB is required for mtDNA maintenance in *S. cerevisiae* (Van Dyck et al., 1992) and has been identified and purified from *S. cerevisiae*, *X. laevis*, and rat tissues (Jong et al., 1985, Mignotte et al., 1985; Pavco and Van Tuyle, 1985; Van Dyck et al., 1992). Our attempts to purify SSB from *Drosophila* mitochondria using DNA binding and DNA polymerase stimulation assays have been unsuccessful due, in part, to the presence of nuclease activity in these crude fractions. The homology of mitochondrial SSB to *E. coli* SSB (Van Dyck et al., 1992; Tiranti et al., 1993) and the ability of *E. coli* SSB, but not T4 gp32 or *Drosophila* RF-A, to promote highly efficient DNA synthesis implies that pol γ may utilize mitochondrial DNA polymerase accessory factors similar to those associated with *E. coli* DNA polymerase III. The β subunit of *E. coli* pol III is a processivity factor which increases the activity of core polymerase about 3-fold and its

processivity greater than 50-fold (Fay et al., 1981, 1982; Crute et al., 1983). Similarly, the primer recognition complex associated with pol III (γ complex) increases the activity of core approximately 8-fold, while increasing the processivity 3- to 4-fold (Fay et al., 1982; LaDuca et al., 1983). We hope to utilize these characteristic effects of *E. coli* DNA polymerase accessory factors to identify and purify their putative counterparts in mitochondria.

EXPERIMENTAL PROCEDURES

MATERIALS

Nucleotides and nucleic acids. Unlabeled deoxy- and ribonucleoside triphosphates were purchased from P-L Biochemicals. [^{3}H]dTTP was purchased from ICN Biochemicals; [α-^{32}P]dTTP and [γ-^{32}P]ATP were purchased from New England Nuclear. Calf thymus DNA (highly polymerized Type I) was purchased from Sigma and was activated by partial digestion with DNase I (Boehringer Mannheim) as described by Fansler and Loeb (1974). Recombinant and wild type M13 viral DNAs (10,650 and 6,407 nt, respectively) were prepared by standard laboratory methods. Synthetic DNAs used for DNA binding (see figure 20: LSK-7 (36 nt), JMK-8 (28 nt), or ligations of LSK17 (75 nt) and 18 (20 nt) or LSK 17, 18, and 25 (28 nt)) and synthetic oligodeoxynucleotides (15 nt) complementary to the M13 viral DNAs were synthesized in an Applied Biosystems Model 477 oligonucleotide synthesizer.

Enzymes and antiserum. Drosophila DNA polymerase α and γ and antiserums were prepared as described (Sauer and Lehman, 1982; Kaguni et al., 1983a; Wernette and Kaguni, 1986; Olson et al., 1994). *Drosophila* pol δ was from I.R. Lehman of Stanford University; *Drosophila* topoisomerase I was from Jon Kaguni of this department; *Drosophila* topoisomerase II and antiserum were from Neil Osheroff of Vanderbilt University School of Medicine; human RF-C and antiserum were from Jerard Hurwitz of Sloan-Kettering Institute; yeast mtRNA polymerase and antiserum were from Judith Jaehning of the University of Colorado Health Sciences Center.

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, 30 or 120 mM KCl, 400 μg/ml bovine serum albumin, 20 μM each of dATP, dCTP, dGTP, and [^{3}H]dTTP (1000 cpm/pmol), 10 μM (as nt) of singly-primed recombinant M13 DNA or 250 μg/ml of

FIGURE 20. *Synthetic substrates for DNA binding.* LSK-7, 17, 18, 25, and JMK-8 were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer. Template-primer DNA binding was to LSK-7 or LSK-17/18 which was constructed by ligation of LSK-17 and 18. Double-stranded DNA binding was to LSK-17/18/25 which was constructed by ligation of LSK-25 to LSK-17/18. Single-stranded DNA binding was to JMK-8.

LSK-7

5'-TCTCCATAATTTGACGGCCTG$^{A\ C}_{\ \ \ T}$
3'-CTGCCGGAC$^{\ \ \ C}_{C\ A}$

LSK-17

5'-TCATTCAAATCCTCTCAATCGATATCTTGGCCAGGATCCTAGTGAATTCCGGAAGCTTC$^{T\ T}_{\ \ \ T}$
3'-GGCCTTCGAAG$_{T\ T}$

LSK-18

5'-AATTCACTAGGATCCTGGCC-3'

LSK-17/18

5'-TCATTCAAATCCTCTCAATCGATATCTTGGCCAGGATCCTAGTGAATTCCGGAAGCTTC$^{T\ T}_{\ \ \ T}$
3'-CCGGTCCTAGGATCACTTAAGGCCTTCGAAG$_{T\ T}$

LSK-25

5'-AAGATATCGATTGAGAGGATTTGAATGA-3'

LSK-17/18/25

5'-TCATTCAAAT CCT CTCAATCGATATCTTGGCCAGGATCCTAGTGAATTCCGGAAGCTTC$^{T\ T}_{\ \ \ T}$
3'-AGTAAGTTTAGGAGAGTTAGCTATAGAACCGGTCCTAGGATCACTTAAGGCCTTCGAAG$_{T\ T}$

JMK-8

5'-TCATTCAAATCCTCTCAATCGATATCTT-3'

Figure 20

activated calf thymus DNA, and 0.1 to 0.2 units of Fr VI enzyme. Incubation was at 30°C for 30 minutes. Specific modifications are described in the figure legends. One unit of activity is that amount that catalyzes the incorporation of 1 nanomole of deoxyribonucleoside triphosphate into acid insoluble material in 60 minutes at 30°C using DNase I-activated calf thymus DNA as the substrate. Here, we define standard activity as that exhibited by pol γ in the presence of 120 mM KCl on singly-primed M13 DNA.

Analysis of products of processive DNA synthesis by gel electrophoresis. Reactions were as above except that reaction mixtures contained 30 μM each of dATP, dCTP, dGTP, and 10 μM of [α-^{32}P]dTTP (2 x 10^4 cpm/pmol), 20 μM singly-primed wild type M13 DNA, and 0.02 units of Fr VI enzyme. Incubation was at 30°C for 30 minutes. Products to be analyzed by denaturing polyacrylamide gel electrophoresis were made 1% in SDS and 10 mM in EDTA, heated for 4 minutes at 80°C and precipitated with ethanol in the presence of 5 μg of tRNA as carrier. The ethanol precipitates were resuspended in 80% formamide, and 90 mM Tris-borate. Aliquots were denatured for 2 minutes at 100°C and electrophoresed in a 6% polyacrylamide slab gel (13 x 30 x 0.15 cm) containing 7M urea in 90 mM Tris-borate (pH 8.3) and 25 mM EDTA. Alternatively, the ethanol precipitates were resuspended in 30 mM NaOH and 20 mM EDTA, and aliquots electrophoresed in a 1.5% (agarose) slab gel (13 x 18 x 0.7 cm) containing 30 mM NaCl and 2 mM EDTA in 30 mM NaOH and 2 mM EDTA. Approximately equal amounts of radioactivity (~1000 cpm) were loaded in each lane. In addition, equal sample volumes were loaded on each type of gel to allow direct comparison of product size distribution. Gels were washed in distilled water for 20 minutes, dried under vacuum and exposed at (-)80°C to Kodak X-OMAT AR X-ray film using Du Pont Quanta III intensifying screens. Quantitation of the data was performed by scanning of the 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. In the determination of processivity values, the length of the primer (15 nt) was subtracted from the DNA product strand lengths.

DNA polymerase stimulatory assay. Reaction mixtures were as for the DNA polymerase γ assay, except included 30 mM KCl and either singly-primed or unprimed recombinant M13 DNA and approximately 0.2 ng of 145 kDa polypeptide (0.25 µl of stimulatory fractions).

Construction of synthetic templates for DNA binding. LSK-17 (see figure 20) was 5'-end labelled with [γ^{32}P]-ATP by T4 polynucleotide kinase (USB). Reactions (0.04 ml) contained 1x T4 polynucleotide kinase buffer (USB), 0.75 µM (as primer) LSK-17 (30 pmol ends), 2.25 µM ATP (90 pmol; 8 x 10^5 cpm/pmol), and 7.5 units of T4 polynucleotide kinase. LSK-18 and LSK-25 (see figure 20) were phosphorylated at their 5' ends in reactions (0.06 ml) as for LSK-17, except contained 1.67 µM (as primer) DNA (100 pmol ends) and 1 mM ATP (60,000 pmol). Reactions were incubated for 60 mins. at 37°C, boiled for 5 mins. to inactivate the kinase, and the efficiency of the ^{32}P-LSK-17 kinase reaction was determined. DNAs were annealed by mixing 30 pmol (as primer) of ^{32}P-LSK-17 with 90 pmol (as primer) of phosphorylated LSK-18 for LSK-17/18 or 90 pmol (as primer) each of phosphorylated LSK-18 and LSK-25 for LSK-17/18/25. Reactions (0.3 ml) also contained 10 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 30 mM NaCitrate and were incubated for 30 mins. at 65°C then 30 mins. at 37°C. Reactions were diluted 10-fold, precipitated with ethanol, resuspended in a total volume of 0.4 ml of TE, and the yield of ^{32}P-LSK-17 was determined. The DNA resuspensions were made 10 mM in $MgCl_2$, 1 mM in ATP, and 1 mM in DTT. Ligation was in the presence of 2 units of T4 DNA ligase (Boehringer Mannheim) at 17°C for 3 hours. Reactions were phenol:chloroform extracted 2x, precipitated with ethanol and resuspended in 50 µl of TE. The final DNA concentration was estimated by determining the yield of ^{32}P-LSK-17. DNA binding substrates were electrophoresed in a 10% polyacrylamide gel (13 x 24 x

0.075 cm) containing 6 M urea and 40% formamide in 90 mM Tris-borate (pH 8.3) and 25 mM EDTA to check for their correct construction.

DNA binding assay. Reaction mixtures (0.03 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, 0.04-0.2 pmol (as primer) of the described DNA binding template, and 0.01 pmol of Fr VI DNA polymerase γ or 145 kDa DNA binding protein. Incubation was at 30°C for 10 minutes. Samples were electrophoresed in a 4.5% polyacrylamide (29:1) slab gel (13 x 16 x 0.015 cm) containing 45 mM Tris-borate (pH 8.3) and 12.5 mM EDTA for 1.5 hours at 15 mAmp.

Glycerol gradient sedimentation. Pol γ Fr V was layered on to a preformed 12-30% glycerol gradient containing 50 mM potassium phosphate, pH 7.6, 200 mM $(NH_4)_2SO_4$, 0.015% Triton X-100, 2 mM DTT, 2 mM EDTA, 1mM PMSF, 10 mM sodium metabisulfite, and 2 μg/ml leupeptin, prepared in polyallomer tubes for use in a Beckman SW41 rotor. Centrifugation was at 37,000 rpm for 60 hours at 3°C, after which 60 (185 μl) fractions were collected and assayed for DNA binding and DNA polymerase stimulatory activity. A parallel gradient was calibrated with lactate dehydrogenase, DNA polymerase I, human serum albumin, and carbonic anhydrase.

Gel filtration chromatography. DNA binding fractions were chromatographed at a flow rate of 0.1 column volume per hour on a Sephacryl S-200 column (10 ml) equilibrated with 50 mM potassium phosphate, pH 7.6, 200 mM $(NH_4)_2SO_4$, 0.015% Triton X-100, 20% glycerol, 2 mM DTT, 2 mM EDTA, 1mM PMSF, 10 mM sodium metabisulfite, and 2 μg/ml leupeptin. Fractions (130 μl) were collected and aliquots were assayed for DNA binding and DNA polymerase stimulatory activity. The column was calibrated with DNA polymerase I, yeast alcohol dehydrogenase, rabbit muscle phosphorylase B, bovine liver catalase, and bovine liver glutamate dehydrogenase.

Purification of D. melanogaster DNA Polymerase γ. The procedure for purification of γ polymerase has been described in detail (Wernette and Kaguni, 1986). Briefly,

partially purified mitochondria from *D. melanogaster* (200 g) embryos were extracted in 25 mM HEPES, pH 8.0, 10% glycerol, 0.3 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM sodium metabisulfite, 2 μg/ml leupeptin and 2% sodium cholate. The resulting extract was centrifuged at 96,000 x g for 30 minutes at 3°C. The supernatant was recovered and designated Fraction I. Fraction I was diluted and applied to a phosphocellulose column which was washed with 100 mM potassium phosphate. Elution of pol γ was at 200 mM potassium phosphate during application of a gradient of 150-350 mM potassium phosphate buffers. Active fractions were pooled (Fraction II) and precipitated by the addition of saturated ammonium sulfate to 55% saturation. The precipitate was resuspended (Fr IIb) and loaded onto a single-stranded DNA cellulose column. The column was washed with phosphate buffer containing 100 mM KCl followed by successive elution with buffers containing 250 mM, 600 mM, and 1 M KCl. The γ polymerase eluted at 400 mM KCl and the active fractions were pooled (Fraction III). Solid ammonium sulfate (0.36 g/ml) was added to Fraction III prior to loading this material onto an Octyl-Sepharose column. Elution was achieved with buffer containing 0.3%, 1%, and 2% Triton X-100. The enzyme eluted after application of the buffer containing 1% Triton X-100 (Fraction IV). Fraction IV was applied to a Cibacron-blue agarose column. After washing the column with buffer containing 0.015% Triton X-100 and 50 mM KCl, a step gradient with buffers containing 100 mM, 350 mM, 1 M and 2 M KCl was applied. Pol γ eluted between 400 mM and 500 mM KCl (Fraction V). Fraction V was layered onto a preformed 12-30% glycerol gradient and sedimented by centrifugation at 37,000 rpm for 60 hours at 3°C in a Beckman SW 41 rotor. Active fractions were pooled and designated Fraction VI.

Production of Antiserum. DNA binding fractions containing approximately 30 μg of 145 kDa polypeptide were electrophoresed in an 8% SDS polyacrylamide slab gel (13 x 16 x 0.015 cm). Proteins were stained with Coomassie and water and the region of the gel containing the 145 kDa polypeptide was excised and minced into approximately 1 x 1 x 1.5

mm cubes. Proteins were eluted in 0.1M ammonium bicarbonate (pH 8.2) and 0.1% SDS overnight at room temperature with gentle agitation. The supernatant was removed and the elution procedure repeated. A 30% yield was determined by SDS PAGE and silver staining. The supernatant was dried, resuspended in TE and injected subcutaneously (~5 μg) in the presence of *titermax* adjuvant into a New Zealand white rabbit. Two weeks later, an eluted gel slice was ground finely and injected subcutaneously (~5 μg) in the presence of *titermax* adjuvant. Five weeks following the first injection, the rabbit was bled and the serum tested for reactivity and specificity in immunoblots.

Purification of DBP-145. Purification was as for *D. melanogaster* DNA polymerase γ (Wernette and Kaguni, 1986), except the DNA binding activity eluted at 100 mM potassium phosphate from phosphocellulose.

DBP-145 peptide sequences. DBP-145 was isolated and 20 μg was TCA precipitated and washed with acetone in preparation for sequencing at the protein sequencing facility at Stanford University. DBP-145 was cleaved with endopeptidase Lys c, and the resultant peptides purified on a reverse-phase high performance liquid chromatography system and were sequenced by automated Edman degradation. Peptide sequences are: (Gln Leu Leu Glu Leu Ala Asn Glu Gly Leu Ala), (Arg Asp Glu Pro Asn Asp Arg Ile Ile Ser), (Tyr Glu Glu Asn Phe Thr Phe Val Arg Gln), and (Met Ile Glu Asp Tyr Glu Ala Asp Arg Ala Asp Arg Glu Leu Arg Ser Phe Val Leu Gln). Amino acid sequence analysis was performed by David Lewis using the TFastA program described in the Program Manual for the GCG Package, Version 7, April 1991, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711.

RESULTS

The ability of mitochondrial DNA polymerase to copy efficiently single-stranded DNA is enhanced, then diminished during purification. The extent of DNA synthesis by near-homogeneous *Drosophila* pol γ varies with the template-primer utilized (Wernette et al., 1988). In the two subunit Fraction VI enzyme, DNA polymerase activity is ≈10-fold lower on singly-primed M13 DNA than on DNase-activated calf thymus DNA. To investigate the loss of putative polymerase accessory proteins that are required for efficient DNA synthesis on single-stranded DNA substrates, we compared the pol γ activity ratio on the above DNAs during the course of purification. Whereas the activity ratio increased in the first few steps of purification, likely reflecting the removal of inhibitors of DNA synthesis on M13 DNA, a 4-fold decrease in the ability of pol γ to copy M13 DNA was observed between Fractions V and VI (Fig. 21A). Because there was no change in processivity (Fig. 21B), the data suggest the apparent loss of an accessory factor upon glycerol gradient sedimentation that stimulates DNA synthesis without affecting the mechanism of nucleotide incorporation.

Mitochondrial DNA polymerase stimulatory activity co-sediments with DNA binding activity. Glycerol gradient fractions were assayed for their ability to stimulate DNA polymerase γ activity on singly-primed single-stranded M13 DNA. While DNA polymerase activity was detected in fractions 8-18, stimulatory activity (up to 2.7-fold) was present in fractions 18-40, consistent with its proposed resolution from pol γ during this step of purification (Figure 22A). Determination of the sedimentation coefficient (4.7 S) of the stimulatory activity was accomplished by comparing the position of the peak stimulatory fractions (26-30) with those of known protein standards (data not shown).

Because DNA polymerase accessory factors might associate with either DNA polymerase or DNA in order to increase primer recognition by, or intrinsic rate or cycling

FIGURE 21. *Effect of enzyme purity on the efficiency and processivity of DNA synthesis by Drosophila pol γ on singly-primed M13 DNA.* **A.** The rate of DNA synthesis was determined as described under "Methods" and compared to that on DNase I-activated calf thymus DNA (reaction mixtures contained 380 μM (as nt) DNase I-activated calf thymus DNA and 200 mM KCl). Reactions on M13 DNA were performed at 30 mM KCl (hashed bars) or 120 mM KCl (closed bars). **B.** Product DNA strands were analyzed on denaturing 1.5% agarose (upper panel) and 6% polyacrylamide (lower panel) gels. Reactions were performed at 30 mM KCl (lanes 1-3) or 120 mM KCl (lanes 4-6) and contained pol γ Fraction IV (≈8% of homogeneous; lanes 1 and 4; apu=1800 and 70 nt respectively), Fraction V (≈19% of homogeneous; lanes 2 and 5; apu=3000 and 65 nt), Fraction VI (near homogeneous; lanes 3 and 6; apu=3400 and 50nt). Less pure fractions could not be used due to the nucleases present.

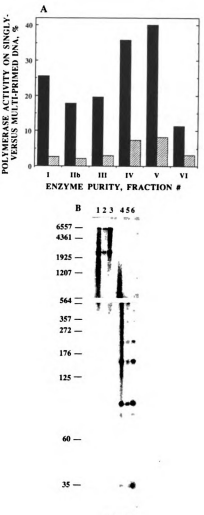

A
POLYMERASE ACTIVITY ON SINGLY-VERSUS MULTI-PRIMED DNA, %
40
30
20
10
0
I
IIb
III
IV
V
VI
ENZYME PURITY, FRACTION #
B
1 2 3 4 5 6
6557
4361
1925
1207
564
357
272
176
125
60
35

Figure 21

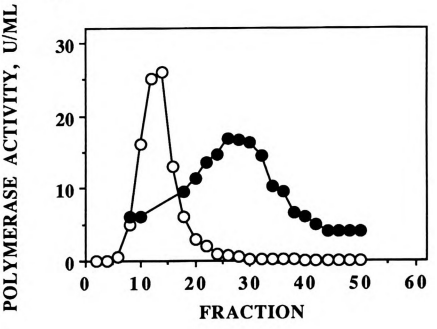
of pol γ, we examined the ability of the stimulatory fractions to bind to template-primer DNA (LSK-7, see figure 20) by a gel electrophoretic mobility shift (gel retardation) analysis. DNA binding was observed in all fractions assayed (18-52): Fractions 18 and 20 contained a DNA binding activity which shifted the template DNA to a position similar to that of purified pol γ (Figure 22B, compare lane P with lanes 18 and 20). Fractions 22-52 contained a DNA binding activity responsible for shifting the template DNA to a more slowly migrating position than pol γ, which generally correlated with DNA polymerase stimulatory activity. Finally, fractions 32-46 contained a third DNA binding activity, likely identical to a mtDNA binding activity previously identified (D.L. Lewis and L.S. Kaguni, unpublished observation), which shifted the template DNA to a much more quickly migrating position than pol γ.

SDS-PAGE analysis of the stimulatory fractions revealed that of approximately fifteen polypeptides present, only about six could be responsible for the DNA binding and DNA polymerase stimulation observed. However, a 145 kDa polypeptide, representing greater than 90% of the total protein present, was evident in fractions 18-52, peaked in fractions 24-30 and appeared to be the most likely source of DNA binding and polymerase stimulatory activities (Figure 22C).

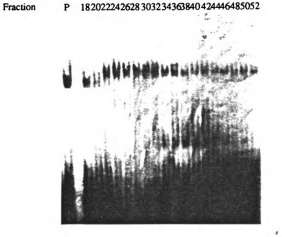
DNA binding and DNA polymerase stimulatory activities are separated by gel filtration chromatography. In order to obtain further purification of protein exhibiting these activities and to determine its native molecular mass, peak fractions were chromatographed on an Sephacryl S-200 gel filtration column, resulting in the separation of the DNA binding and DNA polymerase stimulatory activities (Figure 23). While DNA binding to template-primer DNA was evident in fractions 28-36, DNA polymerase stimulatory activity eluted in fractions 34-46. Comparison of the elution positions of peak fractions with those of known protein standards allowed us to estimate the Stokes radii of the two activities. DNA polymerase stimulatory activity had a Stokes radius of 43 Å,

FIGURE 22. *Co-sedimentation of DNA polymerase stimulatory and DNA binding activities.* **A.** DNA polymerase activity (open circles) was determined on singly-primed M13 DNA in the presence of 120 mM KCl, while DNA polymerase stimulatory activity (closed circles) was determined on singly-primed M13 DNA in the presence of 30 mM KCl. In the absence of stimulatory fractions, pol γ activity was 6.0 u/ml. **B.** DNA binding activity on LSK-7 was determined as described under "Methods". Lane P indicates addition of pol γ. Lanes 18-52 correspond to the glycerol gradient fractions assayed. **C.** Stimulatory and DNA binding fractions were analyzed by SDS-PAGE and silver staining. Lanes 18-52 correspond to the glycerol gradient fractions assayed.

A



B



C

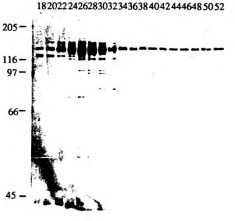


Figure 22

FIGURE 23. *Gel filtration of DNA polymerase stimulatory and DNA binding activities.* DNA binding activity on LSK-7 (open circles) was determined and quantitated as described under "Methods". Bindng activity was plotted as a function of the optical density of each shifted complex (integrated optical density (IOD)). DNA polymerase stimulatory activity (closed circles) was determined on unprimed M13 DNA as described under "Methods".

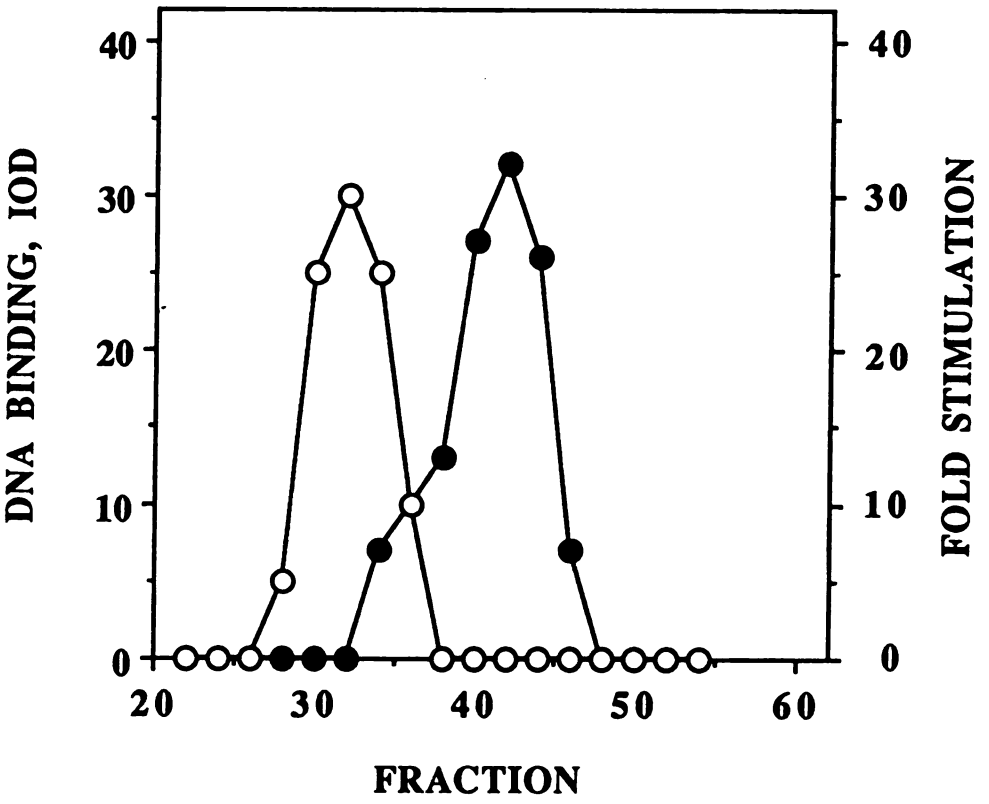


Figure 23

while the position of the DNA binding activity was approximately 56 Å (data not shown). However, the latter value is likely an underestimate due to poor resolution in this region of elution from the column. Combining these data with that from glycerol gradient sedimentation under the same conditions yields a native molecular weight of 112,000 Da for DNA binding activity and 86,000 Da for DNA polymerase stimulatory activity (Siegel and Monty, 1966). Further examination of the DNA polymerase stimulatory fractions revealed that they facilitated the use of unprimed DNA substrates by pol γ and contained a potent nuclease activity which appeared to chromatograph closely with the stimulatory activity (data not shown). Nucleases have been shown to render inert substrates active for DNA polymerase by effectively increasing the available number of primer termini (Lehman, 1967; Kornberg and Baker, 1992). Further, few polypeptides were evident in these fractions and none appeared to correlate to DNA polymerase stimulatory activity (data not shown). The low abundance of this protein and its probable stimulatory activity due to nuclease degradation of template DNA led us to abandon further efforts at characterization. In contrast, SDS-PAGE analysis indicated that the 145 kDa polypeptide, as well as several other minor species appeared to correlate with DNA binding activity (data not shown).

Because the 145 kDa polypeptide was very dilute (≈0.5 ng/μl), extensive characterization of its putative DNA binding activity was not possible. However, we did attempt to determine the specificity of DNA binding to template-primer DNA (LSK-17/18) compared to double-stranded (LSK-17/18/25) and single-stranded DNA (JMK-8) of identical sequence (see figure 20 for substrate description). Although the DNA binding activity appeared to be contaminated with γ polymerase, both the DNA binding activity and pol γ bound template-primer DNA (Figure 24). Similarly, pol γ binding to dsDNA was about equally as efficient as template-primer DNA binding, but dsDNA binding by the DNA binding activity was approximately 3- to 6-fold less efficient than template-primer DNA binding. Surprisingly, neither pol γ nor the DNA binding protein bound ssDNA. These results suggest that, like primer recognition factors from bacteriophage T4 and

FIGURE 24. *Specificity of DNA binding by pol γ and the 145 kDa DNA binding activity.* DNA binding on template-primer (LSK-17/18; lanes 1-3), double-stranded (LSK-17/18/25; lanes 4-10), and single-stranded (JMK-8; lanes 11-17) DNAs was determined as described under "Methods". Reactions contained 0.04 pmol (lanes 1-5, 8), 0.05 pmol (lanes 11-12, 15), 0.08 pmol (lanes 6, 9), 0.10 pmol (13, 16), 0.16 pmol (lanes 7, 10), or 0.2 pmol (lanes 14, 17) DNA (as molecules) and 0.01 pmol of pol γ (lanes 2, 5-7, 12-14) or 145 kDa protein (lanes 3, 8-10, 15-17) or no protein (lanes 1, 4, 12).

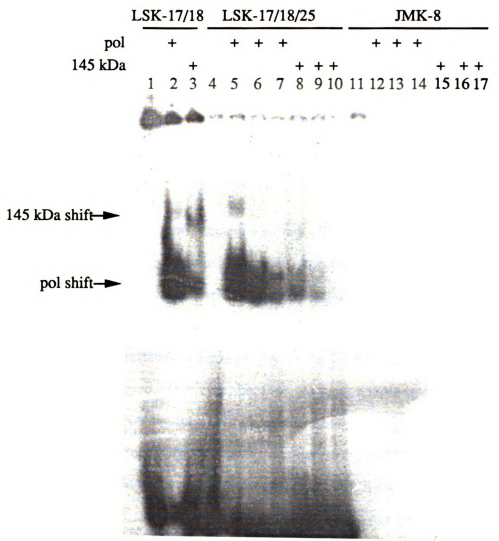

LSK-17/18
LSK-17/18/25
JMK-8
pol
145 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
145 kDa shift
pol shift

Figure 24

humans, the DNA binding activity may be specific for template-primer DNA. Gel retardation and filter binding studies indicate that replication factor-C binds template-primer, but not single-stranded or double-stranded DNA (Lee et al., 1990; Tsurimoto and Stillman, 1990). Further, footprints of RF-C and T4 gp44/62 indicate that they bind to the template-primer junction (Munn and Alberts, 1991a; Tsurimoto and Stillman, 1991). In contrast, the *E. coli* processivity factor, pol III β subunit, does not bind DNA in the absence of its primer recognition complex, but once loaded onto the DNA, binding is stable and non-specific (Stukenberg et al., 1991).

Production of polyclonal antiserum raised against the 145 kDa polypeptide. Further characterization of this putative template-primer specific DNA binding activity required 145 kDa polypeptide of higher purity and in greater abundance. Because the purity and yield of this polypeptide varied in several DNA polymerase γ preparations (data not shown), a purification scheme developed specifically for the DNA binding activity from mitochondrial extracts was desired. However, because we were unable to assay crude mitochondrial fractions for DNA binding activity due to nuclease degradation of the template DNA, we chose to produce polyclonal antiserum against the 145 kDa polypeptide for use in its purification. Due to the impurity of the available DNA binding fractions, the 145 kDa region of an SDS gel was excised and the protein eluted or the gel slice ground finely in preparation for injection. The resultant serum, but not the preimmune serum, recognized the injected material and 2-3 polypeptides of approximately 145 kDa in pol γ fraction III (≈7% pure) in an immunoblot analysis (Figure 25).

Purification of the 145 kDa putative DNA binding protein. In order to confirm the relationship between the DNA binding activity and the 145 kDa polypeptide, estimate the pol γ to DNA binding activity stoichiometry, and to further characterize this activity, we undertook the purification of the 145 kDa polypeptide using an immunoblot assay. Initial

FIGURE 25. *Reactivity and specificity of polyclonal antiserum raised against the 145 kDa polypeptide.* Pol γ fraction III (1.4 μg total protein; lanes 1 and 2) and VI (; 100 ng total protein; lane 3), and glycerol gradient purified 145 kDa polypeptide (200 ng total protein; lane 4) were electrophoresed on an 8% SDS gel, transferred to nitrocellulose and probed with antiserum raised against the 145 kDa polypeptide (lanes 1 and 4), preimmune (lane 2), or antiserum against pol γ (lane 3). Antiserum against pol γ detects the pol γ α-subunit (125 kDa), while antiserum against the 145 kDa polypeptide detects slightly larger polypeptides estimated to be about 145 kDa.

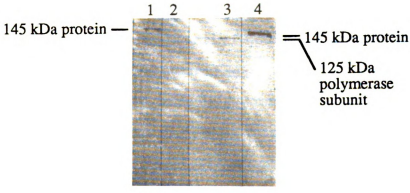

1 2 3 4
145 kDa protein
145 kDa protein
125 kDa
polymerase
subunit

Figure 25

efforts were directed at following the 145 kDa polypeptide through the mitochondrial DNA polymerase purification to determine the level of co-purification of the two proteins. Examination of chromatographic fractions eluted from phosphocellulose, the initial column of the pol γ purification, revealed that less than 10% of the 145 kDa protein was present in the pol γ peak fractions, and that it is approximately 20-fold more abundant than γ polymerase at this stage of the purification. Immunoblots of subsequent pol γ purification steps showed that the 145 kDa protein co-purified almost quantitatively with the DNA polymerase over three columns before resolving from γ polymerase during glycerol gradient sedimentation (data not shown).

Purification of the majority of the 145 kDa protein, which separated from pol γ during chromatography on phosphocellulose, was achieved by following the procedure for mitochondrial DNA polymerase purification (Wernette and Kaguni, 1986): from 200 g of *Drosophila* embryos, approximately 150 μg of the 145 kDa protein (>90% pure) was obtained. In comparison, this purification yields 10 μg of pol γ (Wernette and Kaguni, 1986) and a similar purification yields 500 μg of pol α (Kaguni et al., 1983a). Although DNA binding activity was detectable, nuclease modification of the template DNA was evident even at this stage of the purification (data not shown). Therefore, approximately 25 μg of the 145 kDa protein was chromatographed on a Sephacryl S-200 gel filtration column to yield several polypeptides of approximately 145 kDa, comprising greater than 95% of the protein detectable by SDS-PAGE and silver staining (Figure 26A). Although multiple polypeptides are present, they appear to be due to proteolysis as they are all detected by the antiserum raised against the 145 kDa protein and became more prevalent as the purification proceeded (data not shown). Further, DNA binding analysis revealed that a DNA binding activity which chromatographs closely with the 145 kDa protein is present in these fractions (Figures 26B and C). Thus, the 145 kDa protein is responsible for the observed template-primer specific DNA binding, and has been designated DNA binding protein-145 (DBP-145).

FIGURE 26. *Co-chromatography of DNA binding activity and 145 kDa polypeptide.* **A.** DNA binding fractions were electrophoresed and the protein stained with silver. Lanes 15-41 correspond to the gel filtered fractions assayed. **B.** DNA binding to LSK-7 was determined as described under "Methods". No protein was added to lane 0. Lane L is the load and lanes 15-41 correspond to the gel filtered fractions assayed. **C.** Quantitation of SDS-PAGE and DNA binding analyses was determined as described under "Methods". Protein levels (closed circles) and bindng activity (open circles) were plotted as functions of the optical density of each stained or shifted complex (integrated optical density (IOD)).

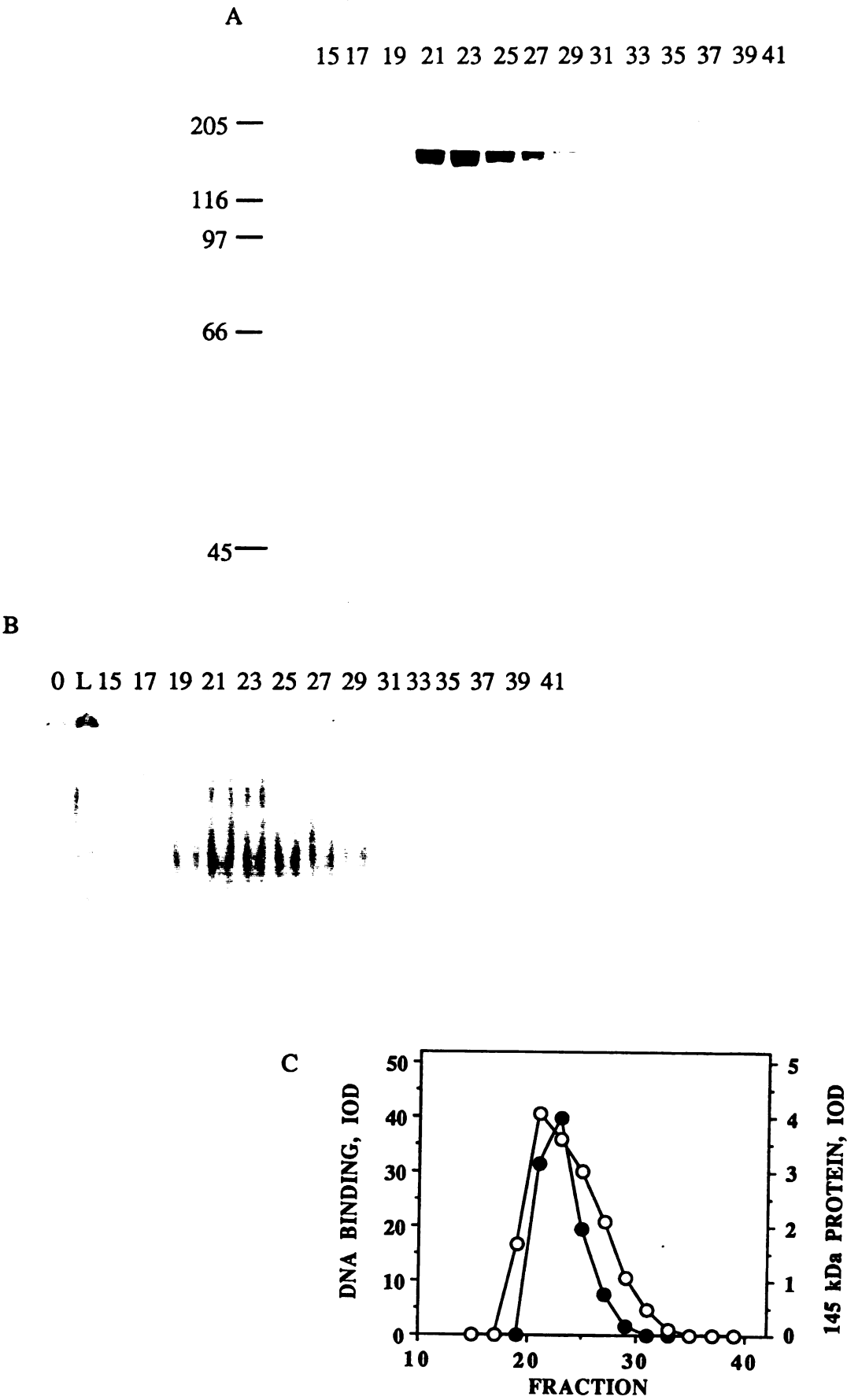

A
15 17 19 21 23 25 27 29 31 33 35 37 39 41
205
116
97
66
45
B
0 L 15 17 19 21 23 25 27 29 31 33 35 37 39 41
C
DNA BINDING, IOD
145 kDa PROTEIN, IOD
FRACTION
50
40
30
20
10
0
5
4
3
2
1
0
10
20
30
40

Figure 26

Physiological role of DNA binding protein-145. Preliminary experiments utilizing glycerol gradient purified DBP-145 indicated that there was no detectable DNA or RNA polymerase, DNA primase, topoisomerase, helicase, ligase, or ATPase activities associated with it (data not shown). In addition, DBP-145 antiserum does not recognize *Drosophila* DNA polymerase α, γ, δ, topoisomerase I or II, human RF-C, or yeast mtRNA polymerase in immunoblot analyses (C.L. Farr, data not shown). Further, antiserum raised against *Drosophila* DNA polymerase α, γ, topoisomerase II, human RF-C, or yeast mt RNA polymerase does not recognize DBP-145 in an immunoblot (C.L. Farr, data not shown).

Elucidation of the physiological role of DBP-145 could likely be attained through a careful survey for all activities known to bind DNA during replication and transcription. However, we chose to obtain amino acid sequence of the protein for several reasons. First, because of our uncertainty of its mitochondrial origin, and because several enzymes involved in cellular DNA metabolism have been identified in *Drosophila*, it would allow us to determine immediately if DBP-145 is one of these proteins. In addition, since we have purified DBP-145 by immunoblot, it is possible that even though DNA binding activity is apparent, it has not retained any other associated activities. Therefore, conventional assays for these putative activities may not be useful. Finally, the generation of PCR primers from protein sequence should allow us to obtain a cDNA clone of DBP-145 and use the derived nucleotide sequence to identify conserved sequence elements which may lead to a suggested role for this protein.

Initial efforts at obtaining internal amino acid sequence have been hampered by the concurrent sequencing of multiple peptides generated by proteolytic cleavage with endo Lys c. However, sequence of four proteolytic fragments, totalling 51 amino acids, has revealed that DBP-145 does not exhibit identity to any sequence available for search by the TFastA program of the GCG Package, Version 7 (D.L. Lewis, personal communication).

DISCUSSION

Our attempts to determine reaction conditions in which mitochondrial DNA polymerase could copy DNA with high efficiency and processivity were unsuccessful: reagents which increased the processivity of the enzyme invariably decreased its activity as well. In contrast, *E. coli* SSB increased the rate while maintaining the high processivity of DNA synthesis by pol γ. In an effort to identify mitochondrial protein factors which might enhance the efficiency of DNA synthesis by γ polymerase, we examined the ability of partially purified fractions of pol γ to copy singly-primed single-stranded DNA. Although its processivity remained unchanged, the rate of DNA synthesis by mitochondrial DNA polymerase was diminished upon glycerol gradient sedimentation, suggesting the loss of a DNA polymerase accessory factor. A putative accessory factor for DNA polymerase α, C1C2, had similar characteristics: it had no effect on the processivity of pol α, but stimulated activity depending on the primer density of the template DNA (Pritchard et al., 1983). In contrast, the primer recognition factors associated with *E. coli* pol III and bacteriophage T4 DNA polymerase increase DNA polymerase activity and processivity (Newport et al., 1980; Huang et al., 1981, Fay et al., 1982; LaDuca et al., 1983), while that associated with human pol δ had no effect on either enzymatic characteristic (Lee and Hurwitz, 1990; Lee et al., 1991).

Further analysis of the glycerol gradient fractions revealed that DNA polymerase stimulatory activity appeared to co-sediment with a DNA binding activity and a 145 kDa polypeptide. Purification of the glycerol gradient fractions by gel filtration resulted in the resolution of the DNA binding and DNA polymerase stimulatory activities. The 145 kDa polypeptide eluted with DNA binding activity, while nuclease activity eluted in the position of DNA polymerase stimulatory activity. The stimulatory effects of nuclease on *in vitro* DNA polymerase activity are well documented: their ability to produce additional primer termini by partially degrading dsDNA or facilitating overlapping of ssDNA renders inert

templates active for DNA polymerase activity (Richardson et al., 1963; Lehman, 1967; Kornberg and Baker, 1992). Thus, our original identification of a factor which stimulated mitochondrial DNA polymerase activity appears to have been misleading. However, further characterization of the DNA binding activity was attempted. Combining the data from gel filtration and sedimentation analyses allowed us to calculate a native molecular weight of 112 kDa for the DNA binding activity, well below the suspected molecular weight of 145 kDa obtained from SDS-PAGE. However, this calculated molecular weight is likely an underestimate due to the poor resolving power in this region of elution from the gel filtration column, as evidenced by the disparate sizes of proteins which eluted with the DNA binding activity (bovine liver catalase (230 kDa) and glutamate dehydrogenase (340 kDa)). Although it may seem surprising that a 145 kDa polypeptide would filter with these larger proteins, it is consistent with the sedimentation results and is indicative of a protein which displays a high degree of asymmetry. Proteins sediment in proportion to their molecular weight and inversely proportional to their frictional coefficient, a function of molecular shape: the further a molecule deviates from a sphere, the higher its frictional coefficient (Cantor and Schimmel, 1980; Zubay, 1988). Therefore, asymmetric molecules sediment slower than spherical molecules and appear to be smaller than they actually are (the DNA binding activity sediments with a spherical marker of 68.5 kDa). In contrast, asymmetric molecules filter faster than spherical molecules and appear larger than they actually are (the DNA binding activity activity filters with spherical markers of 230 kDa and 340 kDa). Calculations of molecular weight obtained by combining these two methods negates these deviations due to asymmetry (Siegel and Monty, 1966). Therefore, because of the suspected underestimate of Stokes radius by gel filtration, the DNA binding activity appears to be an asymmetric protein larger than 112 kDa. Consistent with this hypothesis the frictional coefficient for the DNA binding activity was calculated to be 1.7, whereas "spherical" proteins exhibit frictional coefficients up to only 1.4 (with a sphere having a coefficient of 1.0; Creighton, 1984).

Like primer recognition factors from *E. coli*, bacteriophage T4 and humans, DBP-145 appears to be a template-primer specific DNA binding protein. DNA footprinting studies show that bacteriophage T4 gp44/62 and human RF-C bind the template-primer junction, covering about 20 base pairs and extending 3-4 nucleotides past the junction onto the template strand (Munn and Alberts, 1991a; Tsurimoto and Stillman, 1991). In addition, these factors exhibit a preference for primed template DNA as the cofactor for DNA dependent ATPase activity (Jarvis et al., 1989b; Tsurimoto and Stillman, 1990). Similarly, *E. coli* γ complex appears to bind the template-primer junction and requires primed template as a cofactor for its ATPase activity (Griep and McHenry, 1991; Onrust et al., 1991). The lack of binding by DBP-145 and pol γ to ssDNA appears contradictory to their purification on ssDNA cellulose and to our proposal that SSB eliminates non-productive binding of pol γ to single stranded template DNA. However, these DNAs likely form extensive secondary structure, resulting in regions of dsDNA and apparent template-primer junctions, that serve as binding sites. In fact, putative secondary structure in unprimed M13 DNAs of natural sequence was proposed to be an effector of the *E. coli* γ complex ATPase (Onrust et al., 1991). Thus, our results are consistent with the possibility that DBP-145 is a primer recognition factor similar to those from *E. coli*, bacteriophage T4, and humans. However, we cannot exclude the possibility that DBP-145 is a protein involved in DNA replication or transcription in some role other than as a DNA polymerase accessory factor even though we detected no enzymatic activity associated with DNA binding.

Antiserum raised against the 145 kDa polypeptide recognizes DBP-145 following its resolution from pol γ upon glycerol gradient sedimentation and partially purified DBP-145 present in pol γ Fr III (≈7% pure), but does not recognize *Drosophila* pols α, γ, δ, topoisomerase I, II, human RF-C, or yeast mitochondrial RNA polymerase in immunoblots (C.L. Farr, data not shown). Nor do antibodies raised against *Drosophila* pol α and topoisomerase II, human RF-C, or yeast mitochondrial RNA polymerase

recognize DBP-145. Thus, DBP-145 is neither pol α, γ, or δ nor topoisomerase I or II, but may be *Drosophila* homologs of RF-C or mtRNA polymerase. Further, because the immune serum is specific for DBP-145 in crude fractions, it was an excellent candidate for use in immunoblots to assay for DBP-145 purification even though the resultant protein would potentially be partially or wholly inactive.

Purification of DBP-145 yielded a 145 kDa polypeptide which exhibited template-primer DNA binding activity, and was greater than 95% pure and approximately 15- to 20-fold more abundant than pol γ. DBP-145 may be involved in mtDNA transcription as it appears to be approximately equal in abundance to mtRNA polymerase in protein immunoblots of crude fractions. Alternatively, DBP-145 may be a protein involved in mtDNA replication that is present in mitochondria at greater levels than pol γ is: proteins involved in DNA replication in *E. coli* and bacteriophage T4, including DNA helicase (Venkatesan et al., 1982; Richardson and Nossal, 1989), topoisomerase (Kaguni and Kornberg, 1984; Funnel et al., 1987; Baker and Kornberg, 1988; Baker et al., 1986; Baker et al., 1987), and ligase (Kornberg and Baker, 1992) are up to 500-fold more abundant than replicative DNA polymerase. Although inconsistent with roles as mitochondrial DNA helicase (Lahaye et al., 1991), topoisomerase I (Brun et al., 1981; Lazarus et al., 1987), origin recognition and regulatory proteins (Barat et al., 1985; Mignotte and Barat, 1986; Qureshi and Jacobs, 1993), or transcription factors (Fisher and Clayton, 1985; Diffley and Stillman, 1991), the size of DBP-145 suggests that it may be mitochondrial topoisomerase II (Melendy and Ray, 1989), primase (Wong and Clayton, 1985a; Ledwith et al., 1986), ligase, or RNA polymerase (Kelly and Lehman, 1986; Bogenhagen and Insdorf, 1988) even though we were unable to detect any of these enzymatic activities. However, antiserum against the yeast mtRNA polymerase does not recognize DBP-145 in an immunoblot, but does detect a ≈150 kDa polypeptide in *Drosophila* mitochondrial fractions which co-chromatographs with RNA synthetic activity (C.L. Farr and L.S. Kaguni,

unpublished observations). Thus, it is unlikely that DBP-145 is *Drosophila* mtRNA polymerase.

Amino acid sequence obtained from four proteolytic peptides derived from DBP-145 revealed that it exhibited no identity with any sequence in libraries searched by the GCG TFastA program. This lack of identity does not exclude the possibility that DBP-145 is a component of the *Drosophila* mitochondrial DNA replicational or transcriptional enzymatic machinery since these proteins have not been sequenced. The use of these peptide sequences to produce degenerate oligonucleotides as primers for PCR amplification and sequencing of cDNA encoding DBP-145 should allow us to hypothesize a role of DBP-145 in DNA metabolism.

We have purified a 145 kDa protein which displays template-primer DNA binding specificity but no other detectable enzymatic activities. Although it was originally identified as a putative DNA polymerase accessory factor, it has no effect on the activity or processivity of mitochondrial DNA polymerase. The enzymatic characteristics of pol γ in the presence of 30 mM KCl and *E. coli* SSB are consistent with those predicted by models of *in vivo* mitochondrial DNA replication. Although mitochondrial SSB has been identified and is required *in vivo* for yeast mitochondrial DNA replication, additional DNA polymerase accessory factors need not be necessary. DNA polymerase γ has been identified as a member of the family A DNA polymerases which comprises enzymes that exhibit homology to *E. coli* DNA polymerase I, including bacteriophage T7 DNA polymerase (Ito and Braithwaite, 1991). Bacteriophage T7 DNA polymerase is a heterodimer comprising an 80 kDa catalytic core, exhibiting 5'→3' DNA polymerase and 3'→5' exonuclease activities, and an 11 kDa accessory factor (Adler and Modrich, 1979; Hori et al., 1979a, b). Separation of the two subunits results in diminution of polymerase activity to only 0.5-2% of normal activity (Adler and Modrich, 1979). Similarly, attempts to separate the subunits of pol γ led to loss of enzyme activity (M.W. Olson and L.S. Kaguni, unpublished observations). The accessory factor of T7 DNA polymerase is the

host (*E. coli*) thioredoxin protein and serves to increase DNA polymerase processivity approximately 50-fold to greater than 5000 nucleotides (Huber et al., 1986; Tabor et al., 1987). T7 DNA helicase-primase has been proposed to function as the primer recognition activity for T7 DNA polymerase (Nakai and Richardson, 1986 a, b), although it is not required for processive DNA synthesis. Additional similarities between mitochondrial and T7 DNA replication are evident: the origin of replication of T7 DNA has two RNA polymerase promoters, like the mitochondrial origin, and a 61 base pair A+T region (Saito et al., 1980; Tamanoi et al., 1980; Clayton, 1991). Origin recognition and template melting of both is proposed to occur through transcription by their respective RNA polymerases, and the 3' ends of these transcripts prime leading strand DNA replication (Itoh and Tomizawa, 1980; Chang and Clayton, 1985; Chang et al., 1985; Fisher et al., 1987). Further, yeast mtRNA polymerase appears to be related to T7 RNA polymerase (Masters et al., 1987). Thus the heterodimeric mitochondrial DNA polymerase may, like T7 DNA polymerase, comprise a catalytic core and a processivity factor which does not require an ATP dependent loading mechanism. The cloning and overexpression of mtDNA polymerase should help to address this issue.

CHAPTER V

SCREENING A cDNA EXPRESSION LIBRARY FOR MITOCHONDRIAL DNA POLYMERASE FROM *DROSOPHILA*

INTRODUCTION

Putative accessory proteins to mitochondrial DNA polymerase have proven to be difficult to identify and purify, in part due to the lack of a highly specific functional assay. The generation of such an assay may be dependent on the development of an *in vitro* replication system requiring all of the enzymatic and nucleic acid participants in mitochondrial DNA replication. Our efforts to develop simpler assays to examine activities which contribute to the efficiency of mtDNA replication (e.g. stimulation of DNA polymerase activity, processivity, and primer binding) have been hindered by the modification of DNA substrates due to the presence of nucleases in even partially purified fractions.

The use of DNA polymerase affinity purification to identify γ polymerase accessory factors would alleviate many of the problems introduced by the presence of nucleases in crude fractions. Here, mitochondrial DNA polymerase would be coupled to a chromatographic matrix, to which crude a mitochondrial extract would be applied: polypeptides which exhibited affinity for DNA polymerase would be candidates for polymerase accessory proteins. The identification and purification of DNA polymerase accessory proteins by this method has proven successful in the case of bacteriophage T4 accessory factors (Formosa et al., 1983), but requires a level of DNA polymerase approximately 15,000-fold higher than that obtained from a typical preparation of *D. melanogaster* mitochondrial DNA polymerase performed in our laboratory (Wernette and Kaguni, 1986). Cloning and overproduction of the cDNAs encoding the subunits of DNA polymerase γ will provide the levels of protein necessary to use this strategy.

Given the possible benefits of the cloning and overproduction of DNA polymerase γ we screened a λgt11 cDNA expression library, using antiserum against pol γ as a probe (Young and Davis, 1983). We chose this method due to the immediate availability of polyclonal antiserum and the impetus that this procedure had been used successfully with

the same cDNA library for a protein of similar size, DNA topoisomerase II (Nolan et al., 1986).

EXPERIMENTAL PROCEDURES

MATERIALS

λgt11 cDNA library. The λgtll cDNA library was obtained from the laboratory of Tao-shih Hsieh of Duke University and its preparation has been described (Nolan et al., 1986). Briefly, mRNA was isolated from *Drosophila melanogaster* embryos and cDNA was prepared. *Eco*R1 linkers were added and the cDNAs were inserted into the single *Eco*R1 site of the λgt11 expression vector. The average insert size was 800 base pairs.

Nucleic Acids. Template DNA for Polymerase Chain Reactions (PCR) was prepared from λ phage plate lysates. λ phage were treated with DNase I and RNase A (10 μg/ml each), PEG precipitated, phenol and chloroform extracted, treated with SDS (2%) and proteinase K (200 μg/ml), re-extracted with phenol and chloroform, and ethanol precipitated. Primers complementary to the λgt11 DNA sequence, adjacent to the *Eco*R1 cloning site, for use in PCR were obtained from Dr. Zachary Burton of this department: sense primer 5'-GGTGGCGACGACTCCTGGAGCCCGTCAGTATCGGCG-3', anti-sense primer 5'-TTGACACCAGACCAACTGGTAATGGTAGCGACCGGC-3'.

Enzymes. Sequenase version 2.0 was from United States Biochemical.

METHODS

Screening the λgt11 cDNA library. Screening was carried out as described (Young and Davis, 1983). The λgt11 library was screened with antibody probes as plaques on a lawn of *E. coli* Y1090 ((ATCC no. 37197) = *E. coli* Δ*lacU*169 *proA*$^+$ Δ*lon araD*139 *strA supF*[*trpC*22::Tn*10*] (pMC9) pMC9 = pBR322-*lacIQ*). Infected *E. coli* was plated and grown under lytic conditions until plaques formed, IPTG impregnated nitrocellulose filters were overlaid and plates were further incubated to allow adhesion of the fusion protein. Filters were removed, washed, and probed with polyclonal serum against pol γ and subsequently detected with protein A-alkaline phosphatase. Prior to use in the screening, pol γ antibodies were preadsorbed with filter immobilized total proteins from an *E. coli*

Y1090 lysate, resulting in removal of most anticoliform antibodies and giving significantly lower background in subsequent screening of plaques.

Antibody affinity purification. Plaque purified λgt11 stocks were used to prepare plate lysates. Screening was carried out as above except filters were probed with polyclonal antiserum against pol γ, washed, and incubated for 10 minutes in 100 mM glycine, pH 3 to elute the bound antibodies. BSA and tris base were added to 1% and 50 mM final concentrations, respectively, to neutralize the solution. These antibody solutions were subsequently used in western blots to detect pol γ.

Polymerase chain reaction analysis of insert size. PCR reactions (0.1 ml) contained 50 mM Tris-HCl, 1.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 160 μM dNTPs, 100 ng template DNA, and 1 μg each of primers complimentary to λgt11 DNA adjacent to the *Eco*R1 cloning site. Samples were overlaid with 75 μl light mineral oil and heated at 95°C for 5 mins. Replinase (2 units; DuPont-NEN) was added while the reactions were still hot, and the PCR cycle profile was initiated: 94°C for 1 min. and 72°C for 10 mins. After 25 cycles, the reactions were incubated an additional 7 mins. at 72°C and stored at 4°C. Aliquots of each reaction were made 80% in formamide, 90 mM in Tris-borate (pH 8.3), 0.1% in xylene cyanol, and 0.1% in bromphenol blue, denatured for 2 mins. at 100°C and separated on a 6% polyacrylamide slab gel (13 x 16 x 0.15 cm) containing 7 M urea in 90 mM Tris-borate (pH 8.3) and 25 mM EDTA. DNA was visualized by staining in ethidium bromide (1.5 μg/ml) for 30 min. and washing in water for 20 mins. Sizes of PCR products were determined by comparison to *Hpa*II restriction fragments of M13G*ori*1 replicative form DNA (Kaguni and Ray, 1979) and *Hin*dIII restriction fragments of λ DNA that were electrophoresed in adjacent lanes.

Overproduction and sizing of β-galactosidase-pol γ fusion protein. A crude lysate was prepared as described (Huynh et al., 1984) from a λgt11 recombinant lysogen of λdm-γ71 constructed by Dr. Laurie Kaguni. The proteins in bacterial cell pellets were

separated by SDS-PAGE. Sizing of overexpressed fusion protein was by comparison to molecular weight markers (Sigma) run in an adjacent lane.

Subcloning, DNA Sequencing and alignment with yeast γ polymerase. PCR products of the γ71 insert from λdm-γ71 were subcloned into M13*Gori1* (Kaguni and Ray, 1979) by Dr. Laurie Kaguni and sequenced by Carol Farr using the dideoxy chain termination method of Sanger *et al.* (1977) and Sequenase version 2.0. Sequence analysis was performed by David Lewis using the Program Manual for the GCG Package, Version 7, April 1991, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711.

RESULTS

Screening a λgt11 cDNA library. The low abundance of DNA polymerase γ suggested that its corresponding mRNA levels may be low as well; therefore, the cDNA cloning was initiated by screening 4 x 10^7 plaque forming units (pfu) of a cDNA expression library constructed in lambda phage with polyclonal antiserum raised against pol γ. Primary screening yielded 140 putative positives, 43 of which were carried through subsequent screenings, finally yielding 18 putative pol γ clones.

Verification of putative positive clones. The use of a probe derived from crude polyclonal antiserum introduced the possibility that the phage identified as containing pol γ clones may express polypeptides recognized by antibodies present in the serum other than those specific for γ polymerase. Therefore, verification of the plaque purified clones was performed by affinity purifying antibodies reactive with the putative β-gal : pol γ fusion proteins and subsequently testing the reactivity of these antibodies to pol γ (Fr III, 7% pure) in a protein immunoblot. Antibodies purified from only one (λdm-γ71) of the final 18 putative positives reacted strongly with the α−subunit of mitochondrial DNA polymerase (Figure 27).

Analysis of λdm-γ71. Overproduction of λdm-γ71 fusion protein (data not shown) allowed us to assign this putative cDNA clone a size of ≈300 base pairs (bp), in good agreement with PCR analysis of the insert size of 380 bp. The insert was subcloned into a bacteriophage M13 vector by Dr. Laurie Kaguni and sequenced by Carol Farr. Alignment of the 337 bp insert of λdm-γ71 by David Lewis to the catalytic subunit of yeast mitochondrial DNA polymerase (Foury, 1989) revealed that it exhibited 24% identity and 45% similarity to yeast pol γ (Figure 28A): the putative pol γ clone aligns with sequence outside of the conserved regions common to DNA polymerases (Figure 28B). Shuffling of

Figure 27. *Reactivity of fusion protein affinity-purified antibodies with D. melanogaster DNA polymerase γ.* Antibodies were affinity purified as described in the methods. Pol γ Fraction III (6.4 units, 1.4 ug total protein; lanes 1 and 2) and Fraction VI (8 units, 100 ng total protein; lanes 3 and 4) were denatured, electrophoresed in a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The blot was incubated with unfractionated rabbit antiserum against pol γ (1:1000, lanes 1 and 3) or undiluted affinity purified affinity purified antibodies (lanes 2 and 4) for 3 hours at 25°C and then probed with protein A-alkaline phosphatase.

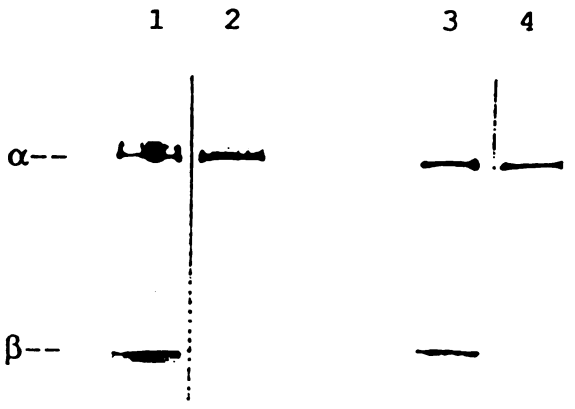


Figure 27

Figure 28. *Amino acid sequence alignment of λdm-γ71 with yeast mitochondrial DNA polymerase catalytic subunit.* **A.** Amino acid sequence was determined in forward and reverse in 3 reading frames and the best alignment shown here. Bars indicate identical matches and dots indicate conservative substitutions. Sequence from λdm-γ71 is numbered 1 through 84 and aligns with yeast pol γ positions 966 through 1078. **B.** The positions of exonuclease (exo) and polymerase (pol) conserved regions are indicated relative to the region of λdm-γ71 alignment.

A.

```
   1 ..............GEVID.......TQPKLVKSIFDLDFDDNDDPLYF 28
                   ||.:|       ...||.|. :|:| . .: :. :
 951 NMDCITPSNKTAIPHGEALDINQLLDKPNSKLGKPSLDIDSKVSQYAYNY 1000
  29 ...IMDEIQKPIAR.......ADELKNNKSDTKNI...........HFGA 57
      :::|..|....        | ::..:|.|...:           .::
1001 REPVFEEYNKSYTPEFLKYFLAMQVESDKRDVNRLEDEYLRECTSKEYAR 1050
  58 SAQNASLNIIHFNDDAQQDNSNQKKKN..................... 84
     .:..||..::.: .|.:.:.... :
1051 DGNTASYSLLDYIKDVEKGKRTKVRIMGSNFLDGTKNAKADQRIRLPVNM 1100
```

B.

Figure 28

the sequence to determine the homology of random alignments revealed similar levels of identity and similarity to the preferred alignment (David Lewis, data not shown).

DISCUSSION

Screening of a λgtll *Drosophila* cDNA expression library with antiserum against pol γ yielded 18 putative DNA polymerase clones from 4 x 10^7 pfu screened. One of these 18 putative clones was confirmed as a *D. melanogaster* γ polymerase clone due to its affinity for antibodies which were specific for the catalytic subunit of mitochondrial DNA polymerase. Further confirmation of this putative pol γ clone was not obtained from its sequencing, as alignment with the yeast γ polymerase catalytic subunit was outside conserved polymerase and exonuclease motifs.

In order to estimate the expected frequency of obtaining a pol γ clone, we compared our results with those obtained from a similar screening of this same library for DNA topoisomerase II (topo II) cDNA. Here, putative topo II clones were obtained at a frequency of 1 in 10^6 pfu screened (Nolan et al., 1986), 10-fold more frequent than for pol γ. Given its role in nuclear chromosomal DNA replication, we can estimate that topo II is at least 50-fold more abundant than γ polymerase (Wernette et al., 1986). Thus, assuming that protein and mRNA levels between topo II and pol γ are approximately proportional, it appears that we have obtained a putative pol γ clone at an appropriate frequency.

Verification of the *D. melanogaster* putative pol γ clones was performed by purifying reactive antibodies and examining their specificity for γ polymerase in an immunoblot. Seventeen of the 18 putative clones exhibited affinity for antibodies which appeared to non-specifically (weakly) interact with either the α-subunit or both the α- and β-subunits of pol γ (data not shown). However, one putative clone, λdm-γ71, exhibited an affinity for antibodies which reacted specifically (strongly) with the catalytic subunit of pol γ. While it is possible that λdm-γ71 expresses a polypeptide which maintains an epitope in common with the α-subunit of pol γ, but is not itself pol γ, it is unlikely that antiserum against pol γ would detect it since it does not recognize either *E. coli* DNA polymerase I or *D. melanogaster* DNA polymerase α (A.K. Kolhoff, M.W. Olson, and

L.S. Kaguni, unpublished observations) which might be expected to have antigenic determinants in common with pol γ.

Mitochondrial DNA polymerase isolated from *D. melanogaster* is a 160 kDa heterodimer comprising 125 kDa (expected cDNA length of 3.3-3.4 Kb) and 35 kDa (expected cDNA length of 0.9-1.0 Kb) subunits. Although it is unknown whether unique mRNAs encode each subunit, it seems likely because bacteriophage and cellular DNA polymerases generally possess separate genes for individual subunits (Wong et al., 1988; McHenry, 1988). Sizing of the confirmed mitochondrial polymerase α subunit clone, λdm-γ71 by PCR and sequencing indicates that it is approximately 0.3 Kb in length: one tenth of the expected size of an α-subunit clone. Sequencing and alignment of λdm-γ71 with yeast mitochondrial DNA polymerase catalytic subunit (analogous to *D. melanogaster* mitochondrial DNA polymerase α-subunit), revealed only 24% identity and 45% similarity. While these levels of identity and similarity do not appear to be significant, alignment is observed outside conserved polymerase and exonuclease regions. Therefore, alignment neither provides confirmatory evidence nor eliminates the possibility that λdm-γ71 is a mitochondrial DNA polymerase clone.

Efforts are currently underway in our laboratory to rescreen the λgt11 *Drosophila* expression library with putative pol γ clone λdm-γ71. In addition, purified γ polymerase will be used to obtain internal amino acid sequence which will also be used either to screen the library or as a secondary screen for those clones which are recognized by λdm-γ71 to finally obtain full length mitochondrial DNA polymerase cDNA clones.

CHAPTER VI

SUMMARY AND PERSPECTIVES

We have examined the effects of reaction conditions, single-stranded DNA binding proteins, and enzyme purity on the mechanism of DNA synthesis by mitochondrial DNA polymerase from *Drosophila* embryos. The alteration of reaction conditions resulted in changes in the rate and processivity of DNA synthesis catalyzed by γ polymerase. Preliminary experiments indicate that the 3'→5' exonuclease activity and fidelity of nucleotide selection by pol γ are also affected by alterations in reaction conditions (M.W. Olson, C.L. Farr, and L.S. Kaguni, unpublished observations). Further characterization of these effects may facilitate our understanding of the coordinate action of the DNA polymerase and exonuclease activities as well as the contribution of the exonuclease activity to the fidelity of pol γ. Although the alteration of reaction conditions facilitated fully processive DNA synthesis by γ polymerase, optimal DNA polymerase activity and processivity were not achieved concurrently: mitochondrial DNA polymerase always exhibited its highest rate of DNA synthesis at moderate salt and its greatest processivity at low salt. These results suggested that protein factors which increase the efficiency of DNA synthesis by pol γ may be involved in mitochondrial DNA replication.

The addition of *E. coli* SSB to a low salt γ polymerase reaction resulted in a dramatic increase in DNA polymerase activity while the high processivity exhibited by pol γ under these conditions was maintained. This increase in activity appears to be due to the ability of SSB to diminish non-productive DNA binding by pol γ, allowing each DNA polymerase molecule to actively engage in DNA synthesis. However, once it completes the replication of a DNA molecule, γ polymerase appears to be slow in cycling to an unused primer terminus, although direct measurement of DNA polymerase cycling is required to confirm this hypothesis. In this experiment, DNA synthesis proceeds in the presence of α^{32}P-TTP on singly-primed single-stranded wild type M13 DNA (6,407 nt). Following 16 minutes of incubation (the time we have estimated for complete loading of the DNA polymerase molecules) an excess of singly-primed single-stranded recombinant M13 DNA (10,650 nt) is added so that any DNA polymerase molecules which cycle will do so onto

the larger DNA. Following further incubation, DNA synthesis products are analyzed by native agarose gel electrophoresis. The amount of 10,650 nt product apparent is indicative of the amount of cycling that has occurred.

We observed a four minute lag time prior to maximal stimulation of pol γ by SSB which seems to be due to an inability of pol γ to locate primer termini efficiently. Altering the conditions of preincubation may aid in determining the cause of this lag time. Possible variations include one where pol γ is incubated in the presence of SSB-coated DNA and dNTPs and DNA synthesis is initiated by the addition of $MgCl_2$. Alternatively, γ polymerase may require the incorporation of dNMPs to remain stably bound to the primer terminus. This hypothesis can be tested by comparing the lag time prior to maximal DNA polymerase stimulation using the above preincubation with one in the presence of SSB-coated DNA, $MgCl_2$ and the dNTP required for initial incorporation. Here, DNA elongation would be initiated by the addition of the remaining three dNTPs. Further confirmation of our hypothesis of inefficient primer location by pol γ may be acquired by altering the primer density of the template-primer DNA: an increase in primer density should decrease the lag time required to achieve maximal pol γ stimulation by SSB.

E. coli DNA polymerase III holoenzyme assembly occurs in two stages: in the rate limiting step, the DNA polymerase accessory factors form an ATP-activated preinitiation complex on a template-primer junction. Subsequently, pol III core locates and tightly binds this preinitiation complex (O'Donnell, 1987). Interestingly, following fully processive DNA synthesis of template DNA, pol III holoenzyme requires 1-2 minutes to cycle to an unused primer terminus (Burgers and Kornberg, 1982b). In contrast, the cycling time can be reduced to as little as 10 seconds if the acceptor template is already endowed with a preinitiation complex (O'Donnell, 1987). These data suggest that DNA polymerase accessory factors may be required for efficient DNA polymerase cycling in mtDNA replication.

Although we were unable to identify *Drosophila* mitochondrial SSB using its putative stimulation of DNA polymerase γ activity, its identification and purification, as well as the characterization of its effect on pol γ activity, are necessary in order to determine the physiological relevance of pol γ stimulation by *E. coli* SSB. In addition, comparative studies using a heterologous mitochondrial SSB may support the conclusions drawn from the results using *E. coli* SSB. The abundance of nuclease activity in crude *Drosophila* mitochondrial fractions precludes the use of assays which require accurate detection of labelled DNAs. However, its characteristic size and chromatographic behavior should aid in the purification of mtSSB by less conventional means. Heat treatment of crude fractions may selectively inactivate nuclease activities over mtSSB activity. DNA binding proteins that are less than 20 kDa in size and bind very tightly to ssDNA cellulose may be good candidates for mtSSB. Further characterization of these putative mtSSBs may reveal a tetrameric structure and cooperative binding to ssDNA which are strong indicators of mtSSB-like proteins. Alternatively, *Drosophila* mtSSB may be recognized by antiserum raised against mtSSBs from other organisms. If so, then this cross-reactivity could be used to purify mtSSB by using immunoblots to assay crude fractions. Similarly, the use of southwestern DNA binding analyses may aid in identification of mitochondrial SSB as well as other mitochondrial DNA binding proteins.

The high degree of sequence conservation between mtSSBs and *E. coli* SSB suggests that *Drosophila* mtSSB cDNA may be identified by using oligonucleotides generated from highly conserved regions of mtSSB amino acid sequence. In fact, the cDNA encoding human mtSSB was identified and cloned using sequence obtained from rat mtSSB (Tiranti et al., 1993). Subsequent overproduction of mtSSB may allow characterization of its activities as well as facilitate our search for additional components of the mitochondrial DNA replicational and transcriptional machinery. The use of T4 single-stranded DNA binding protein (gp32) in protein affinity chromatography resulted in the preferential retention of at least eight proteins involved in DNA replication including the T4

DNA polymerase and primer recognition factor (Formosa et al., 1983). In addition, the eukaryotic single-stranded DNA binding protein, RF-A, interacts with DNA helicase in calf thymus cells (Zhang and Grosse, 1992), with SV40 T-antigen (DNA helicase; Melendy and Stillman, 1993), and with transcriptional activator proteins VP16, GAL4, and p53 (Li and Botchan, 1993; He et al., 1993).

During the course of purification, the ability of *Drosophila* mitochondrial DNA polymerase to copy singly-primed M13 DNA was enhanced then diminished, suggesting the loss of an accessory factor. While an apparent stimulatory factor resolved from pol γ during glycerol gradient sedimentation, we found that it appeared to be associated with a potent nuclease activity. However, a template-primer specific DNA binding activity was also identified and purified to homogeneity. Unfortunately, we were unable to detect any enzymatic activities additional to DNA binding. Amino acid sequence analysis will allow us to construct oligonucleotides for use as primers for PCR, which we can use to obtain a portion of the cDNA encoding the DNA binding protein. Sequencing this DNA may result in the identification of conserved sequence elements, enabling us to develop a hypothesis for the enzymatic role of DBP-145. Confirmation of this role will be obtained by carefully assaying for this activity and characterizing it *in vitro*.

Screening a cDNA expression library with antiserum raised against *Drosophila* mitochondrial DNA polymerase resulted in the identification of a single clone verified by its ability to specifically retain antibodies which recognized the large subunit of pol γ in a immunoblot. Amino acid sequence from pol γ will be obtained and used in conjunction with this putative positive clone to rescreen the cDNA library in order to obtain a full length γ polymerase clone. The overproduction of pol γ will allow us to pursue identification and purification of mitochondrial DNA polymerase accessory factors through the use of DNA polymerase affinity chromatography. In addition, separate overexpression of the two subunits of γ polymerase may allow us to assign a function to the small subunit. If its role

is one of a mitochondrial DNA polymerase accessory factor, our approach for identifying additional putative factors may be altered.

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