Mitochondrial DNA polymerase from Drosophila embryos has been characterized with regard to its mechanism of DNA synthesis under the influence of a variety of compounds in moderate salt (120 mM KCl), where the enzyme is most highly active and only moderately processive, and in low salt (30 mM KCl), where it is less active yet most highly processive. Disparate activity and processivity optima were obtained in low salt in the presence of varying pH or MgCl₂ 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 in either low or moderate salt, the optima were coincident at both salt levels on 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 the highest processivity in low salt. However, while limiting the availability of primer termini had no effect on the mechanism of DNA synthesis, we found that the ability of mitochondrial DNA polymerase to copy singly primed M13 DNA was enhanced then diminished during the course of purification, suggesting loss of an accessory factor.

Replication of the double-stranded circular mitochondrial DNA (mtDNA) 1 genome occurs in the mitochondrial matrix and proceeds in a unidirectional and asymmetric manner (1, 2), 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 (3-5), these models predict that mitochondrial DNA polymerase catalyzes continuous DNA strand synthesis.

Continuous DNA strand synthesis in both prokaryotic and eukaryotic DNA replication generally correlates with processive synthesis (6-12). 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, we 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 (13).

The mitochondrial matrix environment is much different from that of the nucleus; the matrix contains a higher protein concentration (calculated as ~50% by weight; Ref. 14) and higher nucleoside triphosphate levels (15). In addition, the matrix volume varies up to ~2-fold depending on the metabolic state of the mitochondria (16), 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 (14), 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 (17-20). 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 imply that mitochondrial DNA polymerase is not responsible for mtDNA replication. We have therefore here characterized the effects of a variety of compounds on the rate and processivity of DNA synthesis by mitochondrial DNA polymerase y.

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 prepared by dissolving the phosphates in Tris base (Research Organics). [α-32P]dTTP was purchased from ICN Biochemicals; [α-32P]dATP was purchased from DuPont NEN. Calf thymus DNA (highly polymerized Type I) was purchased from Sigma and was activated by partial digestion with DNase I, and the DNA was then purified by exhaustive binding to hydroxyapatite.

**Ref.**

1. The abbreviations used are: mtDNA, mitochondrial DNA; pol γ, DNA polymerase γ; nt, nucleotide(s); apu, average processive unit; pol α, DNA polymerase α; pol δ, DNA polymerase δ; PEG, polyethylene glycol; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
with DNase I (Boehringer Mannheim) as described by Fansler and Loeb (21).

Recombinant and wild type M13 viral DNAs (10,650 and 640 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 471 oligodeoxynucleotide synthesizer.

Enzymes—Drosophila DNA polymerase γ (Fractions IV, V, and VI) was prepared as described by Wernette and Kaguni (22).

Chemicals—All solutions were prepared in water and stored at room temperature. Polyethylene glycol (M, 8000, Sigma) was prepared as a 50% stock solution. Polyvinyl alcohol (M, 10,000, 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 MgCl₂, 10 mM dithiothreitol, 30 or 120 mM KCl, 400 μg/ml bovine serum albumin, 20 μM each of dATP, dCTP, dGTP, and [3H]dTTP (1000 cpm/pmol), 10 μM (as nt) of singly primed M13 DNA, and 0.1–0.2 units of Fraction VI enzyme. Incubation was at 30 °C for 30 min. Specific modifications are described in the figure legends. One unit of activity is that amount that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphate into acid-insoluble material in 60 min at 30 °C using DNA-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 [α-32P] dTTP (2 x 10⁶ cpm/pmol), 20 μM singly primed M13 DNA, and 0.02 units of Fraction VI enzyme. Incubation was at 30 °C for 30 min 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 min 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 min at 100 °C and electrophoresed in a 6% polyacrylamide slab gel (13 x 30 x 0.15 cm) containing 7 M 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. Approximately equal amounts of radioactive (≈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 min, dried under vacuum, and exposed at −80 °C to Kodak X-Omat AR x-ray film using DuPont Quanta III intensifying screens. Quantitation of the data was performed by scanning of the autoradiograms 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 (16). 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 (17–19, 23). Because Drosophila mitochondrial DNA polymerase is stimulated by moderate salt (13, 22), we investigated its processivity on singly primed M13 DNA under conditions of varying monovalent salt concentration. Polymerase γ activity varied ~6-fold when assayed over the range of 0–210 mM KCl, with an optimum at ~120 mM KCl (Fig. 1A). At the same time, processivity varied dramatically (~600-fold), with the greatest abundance of full-length products synthesized in the absence of KCl (Fig. 1B). 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

Fig. 1. 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 min 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 at left indicate the position and size (in nt) of HpaII restriction fragments of M13Gori replicative form DNA (46) and HindIII restriction fragments of λ 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 min of incubation were similar in size and distribution to those obtained after 20 min of incubation, indicating that they result from single binding events; subsequent analyses were performed using 30-min incubations at 30 °C.
Fig. 2. 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 and 35 nt, respectively), pH 8.5 (lanes 2 and 5; apu = 2800 and 37 nt), and pH 10.0 (lanes 3 and 6; apu = 1200 and 38 nt).

highest (standard) activity but 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. The extent of this gradient varies with matrix pH, as the cytosol seems to be effectively buffered (24). 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 (pol α and pol δ, respectively) approximately 30-fold (20). In contrast, polymerase activity was reduced 5-7-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. 2A). 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. 2B). Thus, optimal polymerase activity and processivity were achieved concurrently at 120 mM KCl. However, like pol α and δ, pol γ was most processive at pH suboptimal for activity at 30 mM KCl.

DNA Synthetic Product Length Is Diminished with Increas-
FIG. 4. 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. 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 and 95 nt, respectively), 2 mM ATP (lanes 2 and 8; apu = 2900 and 80 nt), 5 mM ATP (lanes 3 and 9; apu = 3300 and 80 nt), 10 mM ATP (lanes 4 and 10; apu = 1800 and 50 nt), 15 mM ATP (lanes 5 and 11; apu = 350 and 40 nt), 25 mM ATP (lane 6; apu = 80 nt).

**DNA Polymerase γ Processivity**

**ing MgCl₂ Concentrations**—DNA polymerases require magnesium as a cofactor for nucleotide incorporation in the form of a magnesium-dNTP complex. Increasing MgCl₂ concentrations decreased the processivity of calf thymus pol α and δ while increasing their activity (20). Likewise, when assayed using 0.01-64 mM MgCl₂, the addition of Mg²⁺ decreased the processivity of pol γ ~6-fold at moderate salt and ~700-fold at low salt, at concentrations as low as 1 mM MgCl₂ (Fig. 3B). Polymerase activity varied ~10-fold at 120 mM KCl and was optimal from 0.5 to 6 mM MgCl₂ (Fig. 3A). At 30 mM KCl, pol γ activity varied ~30-fold and the enzyme exhibited two optima at 0.25 and 20-25 mM MgCl₂. Thus, the enzyme was capable of synthesizing long products (>3000 apu) at 700-fold at low salt, at concentrations as low as 1 mM MgCl₂ (Fig. 3B).

**ATP Specifically Stimulates DNA Synthesis by Drosophila γ Polymerase**—Seventy percent of the total cellular ATP is localized within the mitochondrial matrix (25), making it at least 4-fold more abundant than the other nucleoside triphosphates present (15). ATP hydrolysis is involved in primer recognition by, and increases the processivities of prokaryotic, viral, and eukaryotic DNA polymerases (26-29). 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. 4, A-C). This specific stimulation by ATP was accompanied by a non-proportional decrease in processivity (Fig. 4D). Notably, while ATP did not stimulate activity at 120 mM KCl, it decreased processivity above 5 mM ATP (Fig. 4D), as did GTP and ADP at both moderate and low KCl concentrations (data not shown). Titration experiments indicated that chelation of Mg²⁺ by ATP was unrelated to the specific stimulation observed; the MgCl₂ optimum remained at 4 mM as the ATP concentration 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 (14). 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-
primes, resulting in increased activity, yet having no effect on processivity (30, 31). Protein-protein interactions are also enhanced, as indicated by an increased processivity of T4 DNA polymerase in the presence of its accessory proteins (31). Drosophila γ 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 4–12% at both 120 and 30 mM KCl and was restored either partially or fully at ~16% PEG at moderate and low salt, respectively (Fig. 5A). pol γ synthesized longer DNA products in the presence of 8% PEG at both 120 and 30 mM KCl (Fig. 5B), where its activity was 2–3-fold reduced. In contrast, polyvinyl alcohol had 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 (32) 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. 6A). In addition, its processivity increased 1.5–3.5-fold with the inclusion of ~15% glycerol (Fig. 6B), demonstrating that pol γ is capable of exhibiting optimal activity and processivity concurrently.
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**Fig. 7.** 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, 8, 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 min, 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 at 30 °C for 5 min, 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 at 30 °C for another 30 min, 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).

at each KCl level examined in the presence of glycerol.

**Limited Template-Primer Availability Does Not Alter the Processivity of DNA Synthesis by *Drosophila* pol γ**—Protein-protein interactions between DNA polymerase molecules and/or accessory proteins have been demonstrated in both prokaryotic and eukaryotic systems (33). 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* pol γ was 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. 7).

**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 (13). In the two-subunit Fraction VI enzyme, DNA polymerase activity was ~10-fold lower on singly primed M13 DNA than on DNase I-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 such as nucleases, a 4-fold decrease in the ability of pol γ to copy M13 DNA was observed between Fractions V and VI (Fig. 8A). Because there was no change in processivity (Fig. 8B), the data suggest the loss of an accessory factor that stimulates DNA synthesis without affecting the mechanism of nucleotide incorporation.
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 (34, 35). 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, but we have shown that pol γ is only moderately processive under reaction conditions optimal for DNA synthetic rate (13). 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 prokaryotic 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 (33), 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 is likely to affect the binding of γ polymerase to DNA. Because high processivity correlates with strong enzyme-primer terminus interactions, our data suggest 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 (36) probably 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.

MgCl₂ decreased the processivity of the *Drosophila* mitochondrial DNA polymerase, yet the optimal Mg²⁺ levels for polymerase activity varied at low versus moderate salt. Mg²⁺ 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. MgCl₂ also contributes to the ionic strength of the reaction solvent, thus partially explaining the increase in activity at high MgCl₂ levels at low salt, yet the loss of polymerase activity with the addition of MgCl₂ 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 MgCl₂ at low salt. Furthermore, although differential binding of Mg²⁺ 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 Mg²⁺ 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 (37). 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 MgCl₂. Alternatively, the decrease may be related to the finding of Griep and McHenry (38) that the inclusion of Mg²⁺ alters the conformation of the processivity factor of *E. coli* DNA polymerase III, the 37-kDa β subunit; the presence of 10 mM MgCl₂ specifically shifts the β subunit from a dimer to a predominantly monomeric form. While the dimeric form is directly implicated in processive DNA synthesis by pol III holoenzyme (26, 39, 40), 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 (22). A pol III β subunit-like activity that is affected similarly by Mg²⁺ may be present in the two subunit pol γ.

High levels of ATP, but not CTP 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. 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 (28, 41, 42). 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 Mg²⁺ via chelation; when the ATP concentration is varied from 5 to 12 mM, the MgCl₂ optimum remains at 4 mM.¹² In addition, GTP would be expected to chelate the Mg²⁺ 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 (30), 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

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¹² C. M. Wernette, A. J. Williams, and L. S. Kaguni, unpublished observations.

¹ª A. J. Williams and L. S. Kaguni, unpublished observation.
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protein-DNA association, but not dissociation, should be affected by capping (43). In contrast, PEG enhanced the assembly of a holoenzyme form of phage T4 DNA polymerase and consequently, its processivity (31). Our data with pol γ is consistent with either possibility; PEG may enhance binding of pol 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 that occur may not be detectable because they do not alter the processivity of mitochondrial DNA polymerase. Because a dissociable accessory factor(s) may be lacking in the near-homogeneous pol γ, the mechanism and efficiency of DNA synthesis by more crude DNA polymerase fractions were examined. We found that while the mechanism of DNA synthesis by pol γ did not change after purification to the homogenous pol γ, the mechanism and efficiency of DNA synthesis by more crude DNA polymerase fractions were decreased 4-fold, suggesting its separation from a dissociable factor present in the less pure fractions. Efforts are currently under way to identify this putative factor.

We have shown here that Drosophila pol 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 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. (20), 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.

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