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Mismatch-specific 3′ → 5′ exonuclease associated with the mitochondrial DNA polymerase from *Drosophila* embryos

(DNA replication fidelity/proofreading/mitochondrial evolution)

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ABSTRACT **The mitochondrial DNA polymerase from *Drosophila* embryos lacks dNTP turnover activity. However, a potent 3′ → 5′ exonuclease activity can be detected by a specific assay in which the exonuclease excises mispaired nucleotides at the 3′ termini of primed synthetic and natural DNA templates. The excision of a mispaired nucleotide occurs at a significantly greater rate than excision of a correctly paired nucleotide and, under conditions of DNA synthesis, hydrolysis of a mispaired terminal nucleotide occurs prior to primer extension. The 3′ → 5′ exonuclease copurifies quantitatively with DNA polymerase γ and cosediments with the nearly homogeneous enzyme under native conditions. These results suggest that the 3′ → 5′ exonuclease provides a proofreading function to enhance the fidelity of DNA synthesis during *Drosophila* mitochondrial DNA replication.**

The high fidelity of chromosomal DNA replication in both prokaryotes and eukaryotes results from a combination of DNA synthesis and postreplicational repair processes (1). Inasmuch as DNA repair is apparently absent in mitochondria (2), accurate replication of the mitochondrial DNA (mtDNA) genome may rely solely on its replication apparatus. The issue of replication fidelity in mitochondria is of particular interest because animal mtDNA evolves at a rate 5- to 10-fold greater than single-copy genomic DNA (3, 4). It is not clear whether an increased mutation rate or an increase in the rate of fixation of mutations is a major factor.

We have shown previously that the nearly homogeneous mtDNA polymerase from *Drosophila melanogaster* embryos is highly accurate in nucleotide polymerization on single-stranded DNA (5). Further, the *Drosophila* γ polymerase does not exhibit a differential affinity for any dNTP nor does it misincorporate ATP (6). This is in contrast to a low-replication fidelity reported for enzymes from HeLa cells and human placenta and fibroblasts (7–9). On the other hand, DNA polymerase γ from chicken embryos has been shown to replicate DNA with high fidelity in an assay system capable of detecting a spectrum of base substitution and frame-shift mutations (10, 11).

Of the four classes of eukaryotic DNA polymerase (α, β, γ, and δ), only δ polymerase has been shown to contain a 3′ → 5′ exonuclease component comparable to the prokaryotic enzymes (12). The 3′ → 5′ exonuclease functions during DNA synthesis to excise misincorporated nucleotides at the 3′ terminus of a nascent DNA chain and contributes as much as a factor of 100 to the overall fidelity of DNA synthesis (13–15). Although high molecular weight forms of α polymerase have been found in association with a 3′ → 5′ exonuclease (16, 17) and the nearly homogeneous replicative enzyme from *Drosophila* embryos contains a cryptic 3′ → 5′ exonuclease (18), DNA polymerases β and γ are generally devoid of such an activity. Recently, however, Kunkel and Soni (19) have shown that γ polymerase from chicken embryos contains an associated proofreading activity. We show here that the nearly homogeneous mitochondrial enzyme from *Drosophila* embryos possesses a potent 3′ → 5′ exonuclease that copurifies quantitatively with the DNA polymerase, and under *in vitro* reaction conditions excises mismatched nucleotides at the 3′ end of a DNA primer prior to nucleotide polymerization.

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MATERIALS AND METHODS

Materials. *Nucleotides and nucleic acids.* [^{3}H]dTTP, [^{3}H]dCTP, and [^{3}H]dATP were purchased from ICN; [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from New England Nuclear. Calf thymus DNA (highly polymerized type I, Sigma) was activated as described by Fansler and Loeb (20). $(dA)_{700}$·p$(dT)_{10}$ was purchased from P-L Biochemicals and contains adenine and thymine in a molar ratio of 20:1.

A recombinant M13 viral DNA [10,650 nucleotides (nt)] was prepared by standard laboratory methods. $(dT)_{15}$ and synthetic oligodeoxynucleotides of 15 nt complementary to the M13 viral DNA but containing a 3′-terminal mismatch (dA·dA or dG·dG) were synthesized in an Applied Biosystems model 477 oligonucleotide synthesizer. $(dA)_n$·$(dT)_{15}$-[^{3}H]dC, -[^{3}H]dA, and -[^{3}H]dT were prepared by the extension of $(dT)_{15}$ by terminal deoxynucleotidyl transferase in the presence of [^{3}H]dCTP, [^{3}H]dATP, or [^{3}H]dTTP, respectively (21), and subsequent hybridization to $(dA)_{700}$ by using a 15:1 molar ratio of adenine to thymine (5).

Enzymes and protein standards. Drosophila DNA polymerase γ (fractions IV–VI) was prepared as described by Wernette and Kaguni (22). *Escherichia coli* DNA polymerase I and its Klenow fragment and phage T4 polynucleotide kinase were purchased from New England Biolabs. Terminal deoxynucleotidyltransferase was purchased from International Biotechnologies. *E. coli* DNA polymerase III holoenzyme (fraction V, ≈50% pure) (23) was the gift of Jon Kaguni of this department.

Methods. *DNA polymerase assay.* DNA polymerase γ was assayed as described by Wernette *et al.* (5). *E. coli* DNA polymerases I and III were assayed in the presence of 20 mM KCl. Incubation was at 30°C for 20 min. One unit of DNA polymerase activity is as defined by Wernette and Kaguni (22).

Deoxynucleotide turnover assay. The turnover assay measures the DNA-dependent conversion of a dNTP to the corresponding monophosphate. Reaction mixtures (0.04 ml) were as described for the DNA polymerase assay on DNase I-activated calf thymus DNA, except that 30 μM [α-^{32}P]dCTP was used (15,000 cpm/pmol for γ polymerase and 5000 cpm/pmol for *E. coli* polymerases I and III); the *E. coli* enzymes were assayed in the presence of 20 mM KCl.

Abbreviations: mtDNA, mitochondrial DNA; nt, nucleotide(s).

Incubation was for 20 min at 30°C. DNA synthesis and deoxynucleoside monophosphate formation were measured as described by Kaguni *et al.* (24).

3′ → 5′ exonuclease assay on synthetic DNA substrates. Reaction mixtures (0.03 ml) contained 50 mM Tris·HCl (pH 8.5), 5 mM $MgCl_2$, 20 mM dithiothreitol, 120 mM KCl, 400 μg of bovine serum albumin per ml, 46 μM $(dA)_n \cdot (dT)_{15}$-[^{3}H]dC (1115 cpm/pmol), -[^{3}H]dA (456 cpm/pmol), or -[^{3}H]dT (735 cpm/pmol), and enzyme. *E. coli* DNA polymerase I was assayed in the presence of 20 mM KCl. Incubation was for 30 min at 30°C. Aliquots (0.012 ml) were spotted in duplicate onto DE-81 filter paper (1 × 1 cm, Whatman). The filters were washed twice for 10 min in 100 ml of 0.3 M ammonium formate (pH 7.6), followed by a 5-min wash in 40 ml of 95% ethanol. After the filters were dried, radioactivity was assayed in a scintillation counter in a toluene-based scintillant.

Preparation of 5′-^{32}P-labeled substrates for product analysis after exonuclease assay. 3′-Terminal mismatched oligonucleotides (15 nt) were 5′-end-labeled in reaction mixtures (0.04 ml) containing 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 15 mM dithiothreitol, 0.5 μM [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq), 85 pmol of oligonucleotide (calculated as nucleotide), and 20 units of T4 polynucleotide kinase. Incubation was for 60 min at 37°C. M13 viral DNA was added to a concentration of 6 mM (in 4-fold molar excess over homologous oligonucleotide), and the DNA mixture was incubated under standard conditions to hybridize the primer to the template.

3′ → 5′ exonuclease assay on natural DNA and product analysis by denaturing gel electrophoresis. The reaction mixtures (0.03 ml) contained 50 mM Tris·HCl (pH 8.5), 5 mM $MgCl_2$, 20 mM dithiothreitol, 110 mM KCl, 400 μg of bovine serum albumin per ml, 4 μM DNA (the exonuclease substrate described above), and DNA polymerase γ fraction V, VI, or VII. Incubation was at 30°C for 20 min unless otherwise indicated. The exonuclease reaction was terminated by incubation for 10 min at 65°C in the presence of 1% $NaDodSO_4$ and 10 mM EDTA. After ethanol precipitation, resuspension, and heat denaturation, the samples were electrophoresed at 600–800 V for 4–6 hr in an 18% polyacrylamide gel (0.75 mm) in the presence of 7 M urea, and the gel was autoradiographed. Product analysis by densitometry was as described by Wernette *et al.* (5).

RESULTS

Drosophila DNA Polymerase γ Does Not Catalyze Detectable dNTP Turnover During DNA Polymerization. The mitochondrial DNA polymerase from embryos of *D. melanogaster* consists of a 125-kDa DNA polymerase catalytic subunit and a 35-kDa subunit of unknown function (22). Because the enzyme replicates DNA with high fidelity (5), we examined the possibility that the γ polymerase contains a 3′ → 5′ exonucleolytic editing function by assay of dNTP turnover. *Drosophila* DNA polymerase γ exhibited no detectable turnover of dCTP even at high polymerase levels, under conditions in which *E. coli* DNA polymerase I and DNA polymerase III yielded substantial amounts of dCMP (Table 1). While the dNTP turnover assay allows direct measurement of exonucleolytic excision during polymerization, it measures removal of both correctly paired and mismatched bases. The data in Table 1 suggest that either the mitochondrial enzyme does not possess a 3′ → 5′ exonuclease activity or, in contrast to *E. coli* DNA polymerases I and III, that this function might be highly mismatch-specific under DNA polymerization conditions. In view of the high fidelity of nucleotide polymerization by DNA polymerase γ, the removal of only mismatched nucleotides during DNA polymerization may not be detectable in the turnover assay.

Table 1. Measurements of dNTP turnover catalyzed by *D. melanogaster* DNA polymerase γ, and by *E. coli* DNA polymerase I and DNA polymerase III holoenzyme

Enzyme	dCMP, pmol		dCTP, % turnover*
	Incorporated	Formed	
DNA polymerase γ	16.9	<0.06	<0.35
	66.7	<0.06	<0.09
	185.8	<0.06	<0.03
DNA polymerase I	17.6	1.74	9.0
	52.1	3.74	6.7
DNA polymerase III	5.8	2.05	26.1
	11.0	3.20	22.5

*% turnover = (dCMP formed/dCMP incorporated + dCMP formed) × 100.

A 3′ → 5′ Exonuclease Activity Associated with _Drosophila_ mtDNA Polymerase Excises 3′-Terminal Mismatched Nucleotides from Synthetic DNA. To determine whether or not the *Drosophila* mtDNA polymerase contains a mismatch-specific 3′ → 5′ exonuclease function, DNA polymerase γ was assayed on the synthetic DNA substrate $(dA)_{700} \cdot p(dT)_{15}$ extended by either a 3′-terminal mismatched nucleotide ([^{3}H]dCMP or [^{3}H]dAMP) or a correctly paired nucleotide ([^{3}H]dTMP). Under nonpolymerization conditions, DNA polymerase γ exhibited hydrolysis of the 3′-terminal nucleotide pairs A·C, A·A, and A·T at rates of 15.0%, 35.8%, and 1.05%, respectively, relative to the rate of nucleotide polymerization on $(dA)_{700} \cdot p(dT)_{15}$ (Table 2). Whereas *E. coli* DNA polymerase I showed a 2.5-fold greater rate of hydrolysis of the mispaired termini, mismatch-specificity for both the A·C and A·A mispairs relative to the A·T pair was 2.5-fold lower than that observed with γ polymerase. At the same time, no hydrolysis of $(dA)_{700} \cdot p(dT)_{15}$ in which the $(dT)_{15}$ was 5′-end-labeled with γ-^{32}P was detected with the mitochondrial enzyme (data not shown). Thus, the data show a 3′ → 5′ exonuclease activity associated with *Drosophila* mtDNA polymerase which has a 14- to 34-fold specificity for A·C and A·A mispairs relative to the correctly paired 3′-terminal nucleotide.

Quantitative Copurification of 3′ → 5′ Exonuclease and DNA Polymerase. To demonstrate the quantitative association of the 3′ → 5′ exonuclease with DNA polymerase γ, cochromatography of the two enzymatic activities was examined at each of three purification steps. DNA polymerase activity obtained upon octyl-Sepharose chromatography (fraction IV) was further chromatographed on Cibacron blue agarose (fraction V) and sedimented in a glycerol gradient as described in our standard purification scheme (22). Finally, the resulting nearly homogeneous enzyme (fraction VI) was

Table 2. 3′ → 5′ exonuclease activity of *Drosophila* DNA polymerase γ

Enzyme	DNA polymerase activity, unit	3′-Terminal nucleotide hydrolyzed, pmol/hr		
		Mismatched		Matched
		[^{3}H]dC	[^{3}H]dA	[^{3}H]dT
DNA polymerase γ				
Preparation 1	0.12	21.3	39.3	0.9
	0.12	17.2	ND	1.2
Preparation 2	0.15	23.2	57.6	2.1
	0.15	18.8	ND	ND
DNA polymerase I	0.12	46.9	102	8.0

DNA polymerase γ (fraction VI) and *E. coli* DNA polymerase I were assayed for DNA polymerase activity and hydrolysis of terminal mismatched nucleotides. $(dA)_{700} \cdot p(dT)_{10}$ was the substrate for nucleotide polymerization, and poly$(dA)_{700} \cdot p(dT)_{15}$-[^{3}H]dC, -[^{3}H]dA, or -[^{3}H]dT were the substrates for exonucleolytic hydrolysis. ND, not determined.

subjected to gel filtration on FPLC (fast protein liquid chromatography) Superose 12 (fraction VII). These enzyme fractions were assayed for DNA polymerase and 3′ → 5′ exonuclease activities in a time course analysis (Fig. 1). In this case, the substrate used for exonuclease assay was a natural DNA to which was annealed a 5′-end-labeled 3′-terminal mismatched primer of 15 nucleotides. Product analysis by denaturing gel electrophoresis and quantitation of the exonuclease assay were as described.

Both DNA polymerase and exonuclease activity were linear for 60 min at 30°C (Fig. 1*A–C*). The ratios of DNA polymerase to 3′ → 5′ exonuclease over the 60-min time course were 14.5, 10.8, and 8.7 for enzyme fractions V–VII, respectively. Thus, the ratio of the two enzyme activities varies only 20% between the nearly homogeneous fraction VI and fraction VII preparations of DNA polymerase γ. The slightly higher DNA polymerase-to-exonuclease ratio in the enzyme for Cibacron blue agarose (fraction V; 20% pure) suggested the presence of inhibitors of the 3′ → 5′ exonuclease, which were removed by subsequent glycerol gradient sedimentation. These were most likely contaminating endonuclease, 5′ → 3′ exonuclease, and/or phosphatase as demonstrated by alternate assays (not shown).

The data show the quantitative association of 3′ → 5′ exonuclease with the *Drosophila* mtDNA polymerase. Further, examination of the products of the exonuclease assay (Fig. 1*D*) clearly shows that the 3′ → 5′ exonuclease is highly mismatch-specific, removing only the 3′-terminal mismatched nucleotide to yield products of 14 nt in length. No detectable hydrolysis of a correctly paired 12-nucleotide primer present as a contaminant in the substrate was observed, nor were other product lengths seen.

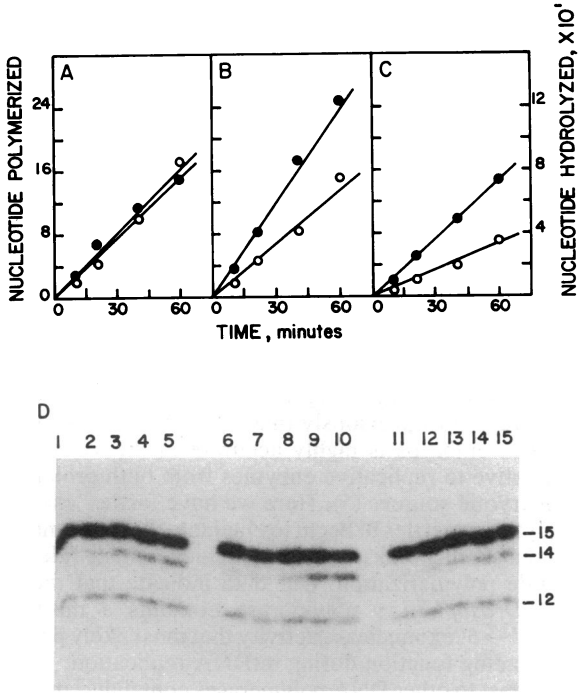

FIG. 1. Copurification of 3′ → 5′ exonuclease and DNA polymerase. (*A–C*) A time course of DNA synthesis and exonucleolytic hydrolysis was performed as described. DNA polymerase activity was assayed on DNase I-activated calf thymus DNA, and 3′ → 5′ exonuclease activity was assayed on phage M13 DNA to which was annealed a 5′-^{32}P-labeled 15-nucleotide primer containing a 3′-terminal mismatched nucleotide (dAMP opposite a template dAMP). Nucleotide polymerized (○) and hydrolyzed (●) are expressed in pmol. (*A*) DNA polymerase γ fraction V. (*B*) DNA polymerase γ fraction VI. (*C*) DNA polymerase γ fraction VII. (*D*) Terminal mismatch excision was analyzed by gel electrophoresis. Products obtained at the time intervals of 0, 10, 20, 40, and 60 min indicated in *A–C* were isolated, denatured, and electrophoresed in a denaturing 18% polyacrylamide gel as described. Lanes: 1–5, fraction V; 6–10, fraction VI; 11–15, fraction VII.

Cosedimentation of 3′ → 5′ Exonuclease and DNA Polymerase. To further substantiate the quantitative association of 3′ → 5′ exonuclease with *Drosophila* DNA polymerase γ, a sedimentation analysis of the fraction V enzyme under native conditions was undertaken. The DNA polymerase and exonuclease activities were precisely coincident (Fig. 2).

Further, the ratio of DNA polymerase to 3′ → 5′ exonuclease across the peak fractions was invariant: the DNA polymerase-to-exonuclease ratios for the indicated fractions between 27 and 35 were 0.91, 0.90, 1.0, 0.94, and 0.82, respectively, where the peak fraction (fraction 31) was assigned an arbitrary value of 1.0. Again, the specificity of hydrolysis of the 3′-terminal mismatched nucleotide from the 15-nucleotide primer is shown (Fig. 2*B*). An insignificant fraction of the correctly paired 12-nucleotide primer (C·G) was hydrolyzed to an 11-mer under conditions where hydrolysis of the mismatched 15-nucleotide primer (A·A) was 54% (fraction 31).

***Drosophila* DNA Polymerase γ Exhibits Exonucleolytic Editing Under Conditions of Nucleotide Polymerization.** The *Drosophila* mitochondrial DNA polymerase specifically hydrolyzes 3′-terminal mismatched nucleotides from synthetic and natural DNA substrates in the absence of DNA synthesis. To determine if exonucleolytic proofreading occurs under conditions of DNA synthesis, a primer extension analysis was performed.

DNA polymerase γ was incubated with an M13 DNA substrate containing a dGMP·dGMP mispaired primer under DNA polymerization conditions in the absence of dCTP, the next nucleotide required for primer extension after exonucleolytic hydrolysis. The time course analysis presented in Fig. 3 (lanes 1–8) shows that no detectable extension of the

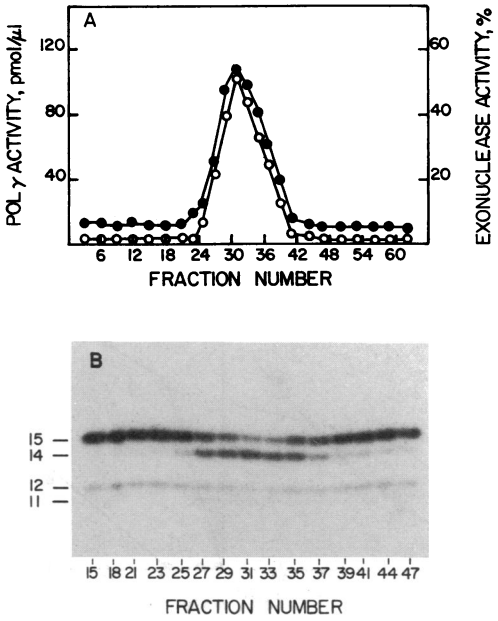

FIG. 2. Cosedimentation of 3′ → 5′ exonuclease and DNA polymerase γ (Pol γ). (*A*) DNA polymerase γ fraction V was sedimented under native conditions in a 12–30% glycerol gradient as described by Wernette and Kaguni (22) and then assayed for DNA polymerase (○) and exonuclease (●) activity. Fractions 1 and 62 represent the bottom and the top of the gradient, respectively. Exonuclease activity is expressed as the percentage of substrate hydrolyzed; the 54% hydrolysis indicated in fraction 31 corresponds to 6.5 pmol of hydrolyzed substrate per μl of enzyme. (*B*) Terminal mismatch excision was analyzed by gel electrophoresis.

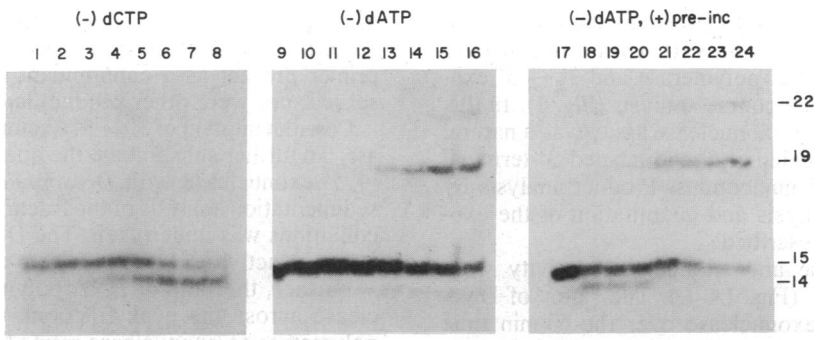

FIG. 3. Proofreading DNA synthesis on phage M13 DNA by DNA polymerase γ. DNA polymerase γ fraction VI was incubated with an M13 exonuclease substrate (4 μM) containing a dGMP·dGMP terminal mismatch under standard DNA polymerase assay conditions with the exceptions indicated below, and terminal mismatch excision was analyzed at the time intervals of 0, 2, 4, 10, 20, 30, and 40 min. Lanes: 1–8, dCTP was omitted from the incubation mixture (lane 1 represents a no enzyme control, and lanes 2–8 correspond to the time intervals indicated above; the product length expected upon primer extension is 23 nucleotides); 9–16, dATP was omitted from the incubation mixture (lane 9 represents a no enzyme control, and lanes 10–16 correspond to the time intervals indicated above; the product length expected upon primer extension is 19 nucleotides); 17–24, a preincubation (pre-inc) for 12 min in the absence of dNTPs was performed prior to DNA synthesis in the absence of dATP (lane 17 represents a no-enzyme control, and lanes 18–24 correspond to the time intervals indicated above).

3′-terminal mismatch occurred. Instead, substantial hydrolysis of the 3′-terminal mispair was observed. Thus, exonucleolytic editing of the mispair is required to permit primer extension by DNA polymerase γ. When dATP was omitted instead of dCTP, proofreading DNA synthesis occurred without significant accumulation of the 14-mer product of the hydrolysis reaction (Fig. 3, lanes 9–16). In fact, the rate of disappearance of the mispaired 15-mer was nearly identical to the rate of appearance of the product 19-mer (the product length expected for primer extension in the absence of dATP; Fig. 4A). The data indicate that exonucleolytic hydrolysis is the rate-limiting step in proofreading DNA synthesis; further, the rate of exonucleolytic hydrolysis is lower during primer extension than that observed in the absence of DNA synthesis (Fig. 4A).

To compare proofreading DNA synthesis to simple primer extension by the mtDNA polymerase, a preincubation in the absence of dNTPs was followed by incubation under DNA polymerization conditions in the absence of dATP. The rate of extension of the correctly paired 14-mer, produced during the preincubation in the absence of DNA synthesis, was dramatically higher than that of the mispaired 15-mer (Fig. 3, lanes 17–24): no significant utilization of the mismatched primer occurred until most of the paired primer was extended to the 19-mer product. Preferential utilization of the paired versus mispaired primers suggests a mechanism in which DNA polymerase γ dissociates more readily from mispaired primers prior to hydrolysis than from paired primers prior to polymerization. In any case, quantitation of the rate of disappearance of the correctly paired (C·G) and mispaired primers (G·G) during the linear phase of each reaction revealed a 4.5-fold greater rate of extension of the former relative to the latter (Fig. 4B), providing further evidence that mismatch hydrolysis is the rate-limiting step in proofreading DNA synthesis.

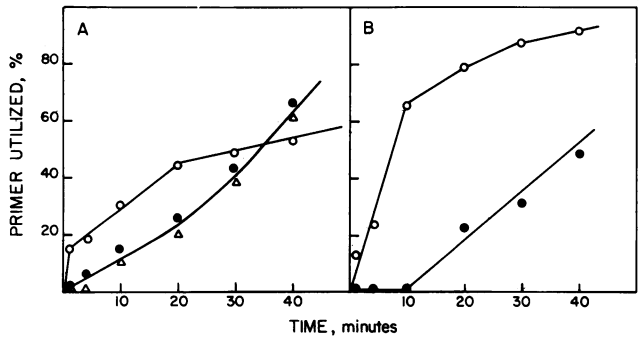

FIG. 4. Time course of utilization of paired versus mispaired primers on M13 DNA. (A) ○, Rate of hydrolysis of the mispaired 15-mer in the absence of dCTP (no primer extension); ●, rate of hydrolysis of the mispaired 15-mer in the absence of dATP (hydrolysis during proofreading); △, rate of primer extension in the absence of dATP (product formation during proofreading). (B) ○, Rate of primer extension of the correctly paired 14-mer in the absence of dATP after preincubation in the absence of all dNTPs; ●, rate of proofreading DNA synthesis in the absence of dATP following preincubation.

DISCUSSION

Replication of chromosomal DNA is by necessity a highly faithful process: the maintenance of genetic integrity is dependent on accurate DNA replication, and infidelity may relate to aging and disease (1). On the other hand, mitochondrial DNA is present in several thousand copies per cell (thereby rendering individual molecules dispensible) and exhibits a significantly higher rate of evolution (3, 4). Nevertheless, mitochondrial genomes from organisms as divergent as *Drosophila* and man have an identical gene content and a highly similar gene organization (25–27).

We have shown previously that DNA polymerase γ from *Drosophila* embryos is highly accurate in the synthesis of DNA relative to replicative enzymes from both prokaryotic and eukaryotic sources (5). Here we have further examined its catalytic properties to begin to elucidate the mechanism by which this mitochondrial enzyme achieves high fidelity in nucleotide polymerization. Our data indicate that the two-subunit *Drosophila* γ polymerase contains a mismatch-specific 3′ → 5′ exonuclease activity that most likely provides a proofreading function during mtDNA replication.

In contrast to other DNA polymerases containing an editing function, *Drosophila* DNA polymerase γ shows no detectable dNTP turnover under optimal conditions for DNA synthesis. Under such assay conditions, *E. coli* DNA polymerases I and III exhibit dNTP turnover values of ≈7.0% (ref. 28; this study) and 23% (ref. 24; this study), respectively. Likewise, turnover values of 80–90% and 34% were reported for eukaryotic δ polymerase (12) and the α subunit of *Drosophila* α polymerase (28), respectively. Because all of these enzymes misincorporate nucleotides at frequencies $< 10^{-5}$ per base pair replicated, most of the dNMP formed either results from hydrolysis of correctly paired nucleotides or from template-dependent hydrolysis of nucleotides that have not actually been incorporated. The latter possibility may result either from conformational or from kinetic proofreading during the base-selection

step prior to nucleotide polymerization (1). Assuming that the optimal salt concentration for polymerization (200 mM KCl) does not inhibit the exonuclease, it appears that *Drosophila* γ polymerase may specifically hydrolyze only misincorporated nucleotides and/or have a more accurate mechanism for nucleotide selection.

Although dNTP turnover was not detected, the *Drosophila* mtDNA polymerase catalyzed substantial hydrolysis of 3′-terminal mispaired nucleotides from synthetic DNA substrates, exhibiting an exonuclease-to-polymerase activity ratio 2.5-fold lower than that of *E. coli* DNA polymerase I. Exonucleolytic excision was in the 3′ → 5′ direction only and was highly specific for the mismatched pairs A·C and A·A relative to the correct A·T pair (14- and 34-fold, respectively). As with the *Drosophila* DNA polymerase α (18), the exonuclease component of DNA polymerase γ shows greater activity in hydrolysis of an A·A mispair relative to an A·C mispair. If uncorrected, an A·A mispair would yield an A ↔ T transversion, the most frequent third-position-codon change in four-codon families of *Drosophila* mitochondrial protein genes (29). Because the exonuclease function of the mtDNA polymerase is particularly effective in hydrolyzing this mispair, it may be more likely that the anomalously high frequency of A ↔ T substitutions in *Drosophila* mtDNA results from a strong selection in favor of fixation of the products of such mutations. It is notable, however, that the specificity and efficiency of exonucleolytic editing and its effect on DNA replication fidelity *in vitro* are dependent on both the particular template and the mispair under examination and on reaction conditions (1, 30).

The specificity of *Drosophila* mtDNA polymerase in hydrolysis of a noncomplementary 3′ terminus is also demonstrated on natural DNA substrates. The product analysis experiments indicate that DNA polymerase γ excises only the mispaired nucleotide (A·A or G·G) to yield products diminished in length by a single nucleotide: there was no significant hydrolysis of either an A·T or a C·G pair at the 14-nucleotide position or of a C·G pair at the 3′ terminus of a contaminating primer of 12 nucleotides. In a simlar analysis, the exonuclease associated with the mitochondrial DNA polymerase from chicken embryos was not highly specific but showed a 2- to 6-fold preference for mispaired termini (19).

Under DNA polymerization conditions, *Drosophila* DNA polymerase γ does not extend a mismatched 3′ terminus: in the absence of the next nucleotide required after mismatch hydrolysis, only the hydrolysis step is observed. The data indicate that exonucleolytic hydrolysis is the rate-limiting step in proofreading DNA synthesis. Further, the rate of the hydrolytic step is slower under polymerization relative to nonpolymerization conditions, suggesting that γ polymerase does not dissociate after the hydrolytic step but utilizes the correctly paired product terminus for subsequent nucleotide polymerization. Finally, γ polymerase extends a complementary 3′-OH terminus at at least a 4-fold greater rate than a mispaired 3′ terminus. Because the rate of exonucleolytic hydrolysis under nonpolymerization conditions is approximately one-third of the rate of DNA polymerization, the slower rate of extension of the mispaired primer most likely reflects the time required for the enzyme to excise the noncomplementary nucleotide. Taken together, the data indicate that *Drosophila* γ polymerase proofreads errors during *in vitro* DNA synthesis. The effect of the exonuclease function on DNA replication fidelity remains to be determined directly.

Finally, we have demonstrated that the mismatch-specific 3′ → 5′ exonuclease quantitatively copurifies with the two-subunit *Drosophila* DNA polymerase γ. The peaks of the two activities were coincident upon glycerol gradient sedimentation of the fraction V enzyme. Further, the DNA polymerase-to-exonuclease activity ratios remained nearly constant upon gel filtration of the nearly homogeneous fraction VI enzyme, as does the 1:1 stoichiometry of the two subunits (22).

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1. Loeb, L. A. & Kunkel, T. A. (1982) *Annu. Rev. Biochem.* **51**, 429–457.
2. Clayton, D. A., Doda, J. N. & Friedberg, E. C. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2777–2781.
3. Dawid, I. B. (1972) *Dev. Biol.* **29**, 139–151.
4. Brown, W. M., George, M., Jr., & Wilson, A. C. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1967–1971.
5. Wernette, C. M., Conway, M. C. & Kaguni, L. S. (1988) *Biochemistry* **27**, 6046–6054.
6. Kaguni, L. S., Wernette, C. M., Conway, M. C. & Yang-Cashman, P. (1988) in *Cancer Cells: Eukaryotic DNA Replication*, eds. Kelly, T. & Stillman, B. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 6, pp. 425–432.
7. Kunkel, T. A. & Loeb, L. A. (1981) *Science* **213**, 765–766.
8. Krauss, S. W. & Linn, S. (1980) *Biochemistry* **19**, 220–228.
9. Krauss, S. W. & Linn, S. (1982) *Biochemistry* **21**, 1002–1009.
10. Kunkel, T. A. (1985) *J. Biol. Chem.* **260**, 12866–12874.
11. Kunkel, T. A. & Alexander, P. S. (1986) *J. Biol. Chem.* **261**, 160–166.
12. Byrnes, J. J., Downey, K. M., Black, V. L. & So, A. G. (1976) *Biochemistry* **15**, 2817–2823.
13. Kunkel, T. A., Schaaper, R. M., Beckman, R. A. & Loeb, L. A. (1981) *J. Biol. Chem.* **256**, 9883–9889.
14. Fersht, A. R., Knill-Jones, J. W. & Tsui, W.-C. (1982) *J. Mol. Biol.* **156**, 37–51.
15. Echols, H., Lu, C. & Burgers, P. M. J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2189–2192.
16. Ottiger, H.-P. & Hübscher, U. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3993–3997.
17. Vishwanatha, J. K., Coughlin, S. A., Wesolowski-Owen, M. & Baril, E. F. (1986) *J. Biol. Chem.* **261**, 6619–6628.
18. Cotterill, S. M., Reyland, M. E., Loeb, L. A. & Lehman, I. R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5635–5639.
19. Kunkel, T. A. & Soni, A. (1988) *J. Biol. Chem.* **263**, 4450–4459.
20. Fansler, B. S. & Loeb, L. A. (1974) *Methods Enzymol.* **29**, 53–70.
21. McHenry, C. S. & Crow, W. (1979) *J. Biol. Chem.* **254**, 1748–1753.
22. Wernette, C. M. & Kaguni, L. S. (1986) *J. Biol. Chem.* **261**, 14764–14770.
23. McHenry, C. S. & Kornberg, A. (1977) *J. Biol. Chem.* **252**, 6478–6484.
24. Kaguni, L. S., DiFrancesco, R. A. & Lehman, I. R. (1984) *J. Biol. Chem.* **259**, 9314–9319.
25. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. & Young, I. G. (1981) *Nature (London)* **290**, 457–465.
26. de Bruijn, M. H. L. (1983) *Nature (London)* **304**, 234–241.
27. Clary, D. O. & Wolstenholme, D. R. (1985) *J. Mol. Evol.* **22**, 252–271.
28. Cotterill, S. M., Reyland, M. E., Loeb, L. A. & Lehman, I. R. (1988) in *Cancer Cells: Eukaryotic DNA Replication*, eds. Kelly, T. & Stillman, B. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 6, pp. 367–371.
29. Wolstenholme, D. R. & Clary, D. O. (1985) *Genetics* **109**, 725–744.
30. Reyland, M. E., Lehman, I. R. & Loeb, L. A. (1988) *J. Biol. Chem.* **263**, 6518–6524.