A cryptic proofreading 3'→5' exonuclease associated with the polymerase subunit of the DNA polymerase-primase from Drosophila melanogaster

(DNA replication fidelity)

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ABSTRACT The DNA polymerase-primase from Drosophila lacks 3'→5' exonuclease activity. However, a potent exonuclease can be detected after separating the 182-kDa polymerase subunit from the other three subunits of the enzyme (73, 60, and 50 kDa) by glycerol gradient sedimentation in the presence of 50% ethylene glycol. The exonuclease activity cosediments with the polymerase subunit, suggesting that the two activities reside in the same polypeptide. The 3'→5' exonuclease excises mismatched bases at the 3' termini of primed synthetic and natural DNA templates. Excision of a mispaired base at the 3' terminus occurs at a 10-fold greater rate than excision of the correctly paired base. When replication fidelity is measured by the bacteriophage φX174 am3 reversion assay, the isolated polymerase subunit is at least 100-fold more accurate than either the intact polymerase-primase or a complex of the 182- and 73-kDa subunits. These results suggest that the 3'→5' exonuclease functions as a proofreading enzyme during Drosophila DNA replication in vitro and very likely in vivo.

Measurement of spontaneous mutation rates suggests that the average frequency of errors during DNA replication is in the range of 10-9 to 10-11 per base pair replicated (1, 2). This high fidelity is thought to be achieved via a multistep process (3, 4), an important component of which in prokaryotes is proofreading at each nucleotide addition step. As a 3'→5' exonuclease activity associated with the DNA polymerase (5). The ratio of polymerase to exonuclease in DNA polymerases isolated from phage T4 mutator strains correlates well with mutagenesis in vivo (6) and with errors during DNA synthesis in vitro (7, 8). Furthermore, inhibition in vitro of the 3'→5' exonuclease of Escherichia coli DNA polymerase I by deoxynucleoside monophosphates can increase error rates by as much as one or two orders of magnitude (3). The contribution of exonucleolytic proofreading to the fidelity of DNA synthesis in eukaryotes is unclear. The DNA polymerases encoded by vaccinia (9), adenovirus (10), and herpes simplex virus (11) possess a 3'→5' exonuclease activity thought to reside in the same polypeptide as the polymerase. A 3'→5' exonuclease has also been described in DNA polymerase preparations from lower eukaryotes, including Ustilago maydis (12) and yeast (13). The exonucleases from Ustilago and yeast can preferentially excise 3'-terminal mismatched nucleotides, suggesting that function in proofreading during DNA synthesis. In contrast, DNA polymerase α purified from higher eukaryotes has no detectable exonuclease activity (14, 15), although a eukaryotic DNA polymerase that does possess 3'→5' exonuclease activity (DNA polymerase δ) has been isolated from rabbit

bonemarrow and calf thymus (16-18). It has been suggested that polymerase δ is a form of polymerase α containing an exonuclease subunit (18). A multiprotein form of the polymerase α with exonuclease activity has been described in mouse myeloma (19), calf thymus (20), and HeLa cells (21).

In this paper we demonstrate that a cryptic 3'→5' exonuclease, which preferentially excises 3'-terminal mismatched nucleotides, is associated with the 182-kDa polymerase subunit of the DNA polymerase-primase from Drosophila melanogaster. The exonuclease is masked in the intact polymerase-primase, appearing only upon dissociation of the 182-kDa subunit from other subunits of the enzyme. The fidelity of the 182-kDa subunit is at least 100-fold greater than that of the intact polymerase-primase, suggesting that the exonuclease is capable of proofreading during DNA replication.

MATERIALS AND METHODS

Materials. Bacterial indicator strains E. coli HF4714 and HF4704, used for growing φX174 am3; E. coli K1, used to prepare spheroplasts; E. coli DNA polymerase I; and preparation of the φX174 am3 single-stranded DNA (ssDNA) templates have been described (22, 23). Unlabeled dNTPs were obtained from P-L Biochemicals, [α-32P]-dTTP and [γ-32P]ATP were purchased from New England Nuclear. Activated calf thymus DNA was prepared as described (24). (dT)3000[H]dC (2100 cpm/pmol of terminal nucleotide) was synthesized using terminal deoxynucleotidyltransferase as described (25) and was hybridized to (dA)400 at 1:20 ratio. (dA)400 and (dT)300 were purchased from P-L Biochemicals. The oligonucleotides (dT)30[H]dA1, (dT)300[H]dC1, and (dT)30[H]dC1 (20 cpm/pmol of terminal nucleotide) were synthesized using terminal transferase and were a gift of F. J. Bollum (Uniformed Services University of the Health Sciences, Bethesda, MD). They were hybridized at a 1:1 ratio with (dA)3000. The 16-base oligonucleotide that contains a 3'-terminal mismatched nucleotide (dCMP opposite position 587 in the φX174 am3 template) was synthesized by the Howard Hughes Chemical Synthesis Facility, University of Washington, Seattle, and phosphorylated using [γ-32P]ATP and T4 polynucleotide kinase (United States Biochemical, Cleveland) (26). It was hybridized to φX174 am3 ssDNA (primer/template ratio 5:1). Unhybridized primers were removed by HPLC using a Bio-Rad G250 Bio-Sil column (7.5 x 300 mm) in 0.1 M KCI/1.0 mM EDTA/20 mM Tris-HCl, pH 7.8, at a flow rate of 1 ml/min. The hybridized template–primer was eluted coincident with the exclusion

Abbreviation: ssDNA, single-stranded DNA.
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volume (5.8 ml), whereas the unhybridized primer was eluted at 11.8 ml. Purification of the *Drosophila* polymerase-primase has been described (27). The polymerase complex containing only the 182-kDa and the 73-kDa subunits was a gift of L. Kaguni (Michigan State University). Ethylene glycol was purchased from Sigma. DE-81 paper was from Whatman. All other chemicals were from commercial sources.

**Dissociation of the Drosophila DNA Polymerase-Primase.** DNA polymerase-primase [fraction IV (27), 30–50 units in 0.05 ml] was layered onto a 0–10% (vol/vol) glycerol gradient containing 50% (vol/vol) ethylene glycol, 20 mM potassium phosphate (pH 7.5), 2 mM dithiothreitol, and 20 mM ammonium sulfate. Centrifugation was performed in a Beckman SW56 rotor at 4°C for 64 hr at 50,000 rpm. Twenty-six gradient fractions (100–150 μl) were collected in siliconized Eppendorf tubes.

**DNA Polymerase Activity.** DNA polymerase activity was assayed in a 50-μl reaction mixture containing 50 mM Tris-HCl (pH 7.5); 10 mM MgCl₂; 2 mM dithiothreitol; 50 μM dATP, dGTP, dCTP, and (α-32P)dTTP (200–600 dpm/pmol); 10 μg of activated calf thymus DNA, and the indicated polymerase. One unit of polymerase catalyzes the incorporation of 1 nmol of labeled nucleotide in 60 min at 30°C.

**Exonuclease Activity.** Exonuclease activity on a synthetic template was measured either as the amount of 3'-labeled polynucleotide rendered acid-soluble after incubation of the polymerase with (dA)₉₀₀₀(dT)₄₆₂[^3H]dc–d, –dT, and –dA or as the amount of radioactivity remaining acid-soluble after incubation with (dA)₉₀₀₀(dT)₄₆₂[^3H]dc–d. Reaction conditions are described in the legends to Table 1 and Fig. 1. Activity on a φX174 template was measured as the removal of the single terminal nucleotide from a 5'-32P-labeled 16-base oligonucleotide containing a 3'-terminal mismatched nucleotide (dCMP opposite the template dAMP at position 587 in the am3 codon) as determined by increased mobility during electrophoresis in a polyacrylamide gel. Reaction conditions are described in the legend to Fig. 2.

**φX174 am3 Fidelity Assay.** The modified φX174 am3 fidelity system using synthetic oligonucleotide primers has been described (28). Reaction conditions are described in the legend to Table 2. Incorporation of labeled nucleotide was calculated by assaying an aliquot for acid-insoluble radioactivity (23). The replicated DNA was transacted and the reversion frequency was determined by the progeny-phage method (29). Background reversion frequency was determined with primed φX174 ssDNA that had not been incubated with polymerase (uncopied DNA).

**Expression of the Newly Synthesized Strand.** Expression of the newly synthesized strand was determined using a heteroduplex molecule consisting of a single-stranded φX174 am3 template primed (at a 10:1 ratio of primer to template) with a 23-base oligonucleotide that spans the amber codon (positions 586–588) and contains the wild type (T-A-G) sequence. Reaction mixtures (60 μl) contained 20 mM Tris-HCl (pH 7.5); 2 mM dithiothreitol; 8 mM MgCl₂; 1 mM dATP; 20 μM dCTP, dGTP, and (α-32P)dTTP (1000–2500 dpm/pmol); 0.2 μg of primed φX174 am3 DNA, and the indicated polymerase.

**RESULTS**

3'→5' Exonuclease Activity Is Associated with the 182-kDa Polymerase Subunit of the Drosophila DNA Polymerase-Primase. The *Drosophila* DNA polymerase-primase consists of a 182-kDa polymerase catalytic subunit, two subunits of 60 and 50 kDa, one or both of which are associated with the primase activity, and a 73-kDa subunit of unknown function (30). The 182-kDa polymerase subunit can be dissociated from the other three subunits by treatment with ethylene glycol followed by ethylene glycol/glycerol gradient sedimentation. As shown in Fig. 1, a potent 3'→5' exonuclease activity is associated with the isolated polymerase subunit. In contrast, only very low levels of exonuclease can be detected in the intact polymerase-primase. The ratio of 3'→5' exonuclease to polymerase in the isolated 182-kDa subunit was up to 200-fold greater than in the intact enzyme (Fig. 1 and Table 1) and 7- to 15-fold greater than in *E. coli* DNA polymerase I (Table 1). Exonuclease activity was observed in the 182-kDa subunit isolated by this procedure from five different preparations of polymerase-primase and was stable during storage at −70°C for at least 6 months.

The lack of exonuclease in the intact enzyme was observed with several different polymerase-primase preparations and is consistent with earlier observations (31). Since more than 40% of the specific activity of the intact polymerase was recovered after gradient centrifugation, the appearance of exonuclease activity in the dissociated 182-kDa subunit was not a result of inactivation of the polymerase by ethylene glycol. Nor was the absence of exonuclease in the intact enzyme the result of a dissociable inhibitor, since addition of a 100-fold greater amount of the polymerase-primase to the 182-kDa subunit had no effect on its exonuclease activity.

**The 3'→5' Exonuclease Associated with the 182-kDa Subunit Can Excise a 3'-Terminal Mismatched Nucleotide from Synthetic DNA.** The 3'→5' exonuclease activity of the intact polymerase-primase, the 182-kDa subunit (isolated from the corresponding polymerase-primase preparation), and *E. coli* DNA polymerase I assayed with (dA)₉₀₀₀(dT)₄₆₂[^3H]dc–d containing at the primer terminus [3H]dCMP or [3H]dAMP, or the correctly matched nucleotide ([^3H]dTMP), are compared in Table 1. The exonuclease activity associated with the 182-kDa subunit

![Fig. 1](image-url)
acting on substrates containing a 3'-terminal mismatched nucleotide was 3- to 10-fold higher than on a template containing the correct T-A base pair (see also Fig. 2). The exonuclease showed a 3-fold greater rate of excision of a mismatched dAMP nucleotide over a mismatched dCMP nucleotide. This preference was not displayed by E. coli DNA polymerase I.

The 3'→5' Exonuclease Can Excise a 3'-Terminal Mismatched Nucleotide from Natural DNA. To determine whether the 3'→5' exonuclease can excise a 3'-terminal mismatched nucleotide from natural DNA templates, fractions obtained following ethylene glycol/glycerol gradient sedimentation of the polymerase-prime was assayed for exonuclease activity on a 5'-32P-labeled synthetic 16-base oligonucleotide containing a 3'-terminal mismatched nucleotide (dCMP opposite dAMP at position 587 in the am3 codon) hybridized to dX174 ssDNA. 3'→5' exonuclease activity can be measured by excision of the 3'-terminal nucleotide, resulting in conversion of the 5'-32P-labeled oligonucleotide to a 15-mer with the correctly paired 3' terminus. Further exonuclease activity should then remove the correctly matched terminal nucleotide. A comparison of the relative levels of the 15-mer and 14-mer should therefore provide an indication of the frequency with which the exonuclease removes mismatched compared to matched nucleotides. As shown in Fig. 2, appearance of the 14-mer was much slower than would be expected if matched nucleotides were removed at the same rate as the mismatched nucleotides. These results are consistent with those observed with the synthetic templates.

Although the levels of exonuclease activity associated with the intact polymerase are very low, it showed the same preference for mismatched over matched bases, suggesting that the exonuclease observed in the intact enzyme is the same as that found in the isolated 182-kDa subunit and may result from a small amount of dissociation of the intact enzyme.

Comigration of Polymerase and Exonuclease During Glycerol Gradient Sedimentation. As shown in Fig. 2, the peak of polymerase activity coincided with the 3'→5' exonuclease peak. Analysis of the peptide composition of these fractions by polycrylamide gel electrophoresis in the presence of NaDodSO4 showed that the only polypeptide corresponding to the profile of the two activities was the 182-kDa polymerase subunit, suggesting that the two activities reside in the same polypeptide (data not shown, but see ref. 30).

The 182-kDa Subunit Is 100-Fold More Accurate in DNA Synthesis Than the Intact Polymerase-Prime. In the dX174 fidelity assay, a primed ssDNA template containing the am3 mutation is copied in vitro with a polymerase. Incorporation of dATP, dCTP, or dGTP in place of the correct nucleotide, dTTP, as shown in Table 2, the reversion frequency of the progeny phage is determined by plating on bacteria that are permissive or nonpermissive for the amber codon (22). As shown in Table 2, the reversion

![Figure 2](image-url)

**Table 1. Exonuclease activity of Drosophila polymerase-prime and the isolated 182-kDa subunit.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>DNA polymerase activity, unit</th>
<th>3'-Terminal nucleotide rendered acid-soluble, pmol</th>
<th>Mismatched</th>
<th>Matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymerase-prime</td>
<td>0.03</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>182-kDa subunit</td>
<td>0.009</td>
<td>14.2</td>
<td>40.0</td>
<td>3.8</td>
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<tr>
<td>Preparation 2</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymerase-prime</td>
<td>0.06</td>
<td>0.9</td>
<td>1.2</td>
<td>ND</td>
</tr>
<tr>
<td>182-kDa subunit</td>
<td>0.009</td>
<td>11.1</td>
<td>34.5</td>
<td>4.5</td>
</tr>
<tr>
<td>E. coli DNA polymerase I</td>
<td>0.20</td>
<td>36.9</td>
<td>31.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

The amount of 3'-terminal nucleotide rendered acid-soluble was determined with paired samples of the intact polymerase-prime and the isolated 182-kDa subunit. Reactions mixtures (60 μl) contained 50 mM Tris-HCl (pH 7.5), 20% ethylene glycol, 10 mM MgCl2, 2 mM dithiothreitol, and the specified template. Concentrations of template were as follows: (dA)2000(dT)46[3H]dC1 and (dA)2000(dT)46[3H]dT1, 2.0 μg/mL. Incubation was for 60 min at 30°C. Reactions were stopped by the addition of 200 μl of 10% trichloroacetic acid and 50 μl of denatured calf thymus DNA (1 ng/mL). After 20 min at 0°C the suspension was centrifuged at 10,000 g for 20 min. The radioactivity of the supernatant (200 μl) was determined by scintillation counting. ND, not determined.

frequency for the isolated 182-kDa subunit was lower by a factor of 100-150 than that for the intact polymerase-primase. This increase in fidelity parallels the 80- to 200-fold increase in 3'→5' exonuclease activity observed upon separation of the 182-kDa subunit from the intact enzyme, suggesting that the exonuclease increases the fidelity of DNA replication by excising mismatched nucleotides at the 3' terminus of the primer. In contrast, when pX174 am3 ssDNA was replicated with a polymerase complex consisting of only the 182-kDa and 73-kDa subunits, the reversion frequency was essentially the same as that seen with the intact polymerase-primase, suggesting that interaction of the catalytic subunit with the 73-kDa subunit may be sufficient to mask the exonuclease activity.

The isolated 182-kDa polymerase subunit is an exceptionally accurate enzyme. The reversion frequency is not more than 2-fold above background even under conditions in which the dNTP pool is biased 50:1 in favor of the incorrect nucleotide. This suggests an approximate error rate of 10−6 to 10−7, which approaches the limits of detection in this system and is comparable to the fidelity obtained with T4 DNA polymerase (32). To demonstrate that the high fidelity we observe is not due to lack of extension of the primer by the polymerase, incorporation of [α-32P]dUTP into the newly synthesized strand was measured. In all cases, the number of nucleotides incorporated was greater than the three required to copy past position 587 in the amber codon. In other experiments, primers labeled at the 5' terminus with [32P]dATP were shown to be extended past the amber codon, as judged by polyacrylamide gel electrophoresis (data not shown). To verify that these extended primers were expressed in the polyphage assay, the percent expression of the newly synthesized strand (penetration) was determined by copying pX174 am3 ssDNA primed with a 23-base nucleotide that spans the amber codon and contains the wild-type sequence. Expression of the primer extended by the 182-kDa subunit was 15%, nearly identical to the value of 16% previously reported for oligonucleotide-primed synthesis of pX174 am3 (28).

**DISCUSSION**

A major distinction between prokaryotic and eukaryotic DNA polymerases has been the presence of a 3'→5' exonuclease activity capable of excising mispaired nucleotides at the 3' primer terminus during DNA replication. In prokaryotes, proofreading by this exonuclease is known to enhance the accuracy of DNA synthesis by up to 100-fold (3, 4). Since the accuracy of DNA replication in eukaryotic cells is likely to be greater than in prokaryotes, it seems reasonable that this activity would be conserved during the course of evolution. However, until now, the presence of such an exonuclease in homogenous preparations of DNA polymerase α, the major replication enzyme in eukaryotes, has escaped detection.

In this paper we demonstrate that the DNA polymerase-primase from Drosophila contains a cryptic 3'→5' exonuclease activity. In agreement with earlier observations (31), we found the intact four-subunit polymerase-primase from Drosophila to be devoid of exonuclease activity. However, a potent 3'→5' exonuclease is revealed when the 182-kDa catalytic subunit is dissociated from other subunits in the complex by sedimentation through a glycerol gradient in the presence of 50% ethylene glycol. Size-determination of the polymerase and exonuclease activities with the 182-kDa subunit suggests that the exonuclease is an integral component of the catalytic subunit. However, the possibility that the exonuclease activity resides in a low molecular weight protein that remains associated with the 182-kDa subunit under these conditions cannot be ruled out. It is worth noting in this regard that cleavage of the 190-kDa catalytic subunit of RNA polymerase from Morris hepatoma yields small peptides with a DNase activity not found in the intact RNA polymerase (33).

There may be several explanations for the cryptic nature of the 3'→5' exonuclease. The appearance of the exonuclease activity upon subunit dissociation may be the result of a conformational change in the catalytic subunit due, in turn, to partial denaturation of the polypeptide by ethylene glycol. An alternative possibility is that one or more of the other subunits masks the activity of the exonuclease. Since a form of the polymerase containing only the 182-kDa and 73-kDa subunits has the same fidelity as the intact enzyme, it is possible that the 73-kDa subunit alone is sufficient to mask the exonuclease activity.

The results in this paper have bearing on the question whether or not DNA polymerase δ is a molecular entity.
distinct from DNA polymerase α. So far, DNA polymerase δ has been identified in only rabbit bone marrow (16) and calf thymus (17, 18). In the latter tissue, it represents as much as 50% of total polymerase activity (18). DNA polymerases α and δ are remarkably similar (17, 18). Of the properties that distinguish these two enzymes, the most striking (and the one used for purification of polymerase δ) is the presence of a 3′→5′ exonuclease (16–18). Other distinguishing properties, such as template specificity (17), differential inhibition by nucleotide analogues (34), and binding to AMP-agarase (18) could be a manifestation of the exonuclease activity. Our finding that the DNA polymerase-prime from *Drosophila* contains a cryptic exonuclease indicates that the presence of a 3′→5′ exonuclease may not be a distinguishing characteristic of polymerase δ.

The 3′→5′ exonuclease associated with the polymerase subunit of *Drosophila* DNA polymerase-prime has all the hallmarks of a proofreading enzyme. Although it is capable of excising both matched and mismatched terminal nucleotides from synthetic and natural DNA, it shows a 10-fold preference for mismatched termini. When assayed using the φX174 fidelity assay, the fidelity of the isolated 182-kDa subunit is at least 100 times greater than that of the intact enzyme. This apparent increase in fidelity is not caused by failure to express the newly synthesized strand or by insufficient DNA synthesis by the isolated 182-kDa subunit, both of which could result in an apparent high fidelity. Since proofreading alone can increase fidelity of DNA synthesis in prokaryotes by 100-fold, the most likely explanation for the high fidelity we observe is that the 3′→5′ exonuclease of *Drosophila* polymerase-prime functions as a proofreading enzyme during DNA replication.

The apparent masking of 3′→5′ exonuclease activity by the 73-kDa subunit of the *Drosophila* polymerase-prime may represent a mechanism whereby this potent exonuclease is regulated *in vivo*. Alternatively, its cryptic nature may be a consequence of the inadequacy of the templates that we have used *in vitro* relative to those that function in DNA replication *in vivo*. Given the high degree of conservation of subunit structure in the DNA polymerase-prime from sources as diverse as yeast and human cells (35), it is likely that a cryptic proofreading 3′→5′ exonuclease may be a common feature of this class of eukaryotic polymerases.

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