Specificity of Proofreading by the 3' → 5' Exonuclease of the DNA Polymerase-Primase of Drosophila melanogaster*

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The DNA polymerase-primase from Drosophila melanogaster contains a cryptic 3' → 5' exonuclease that can be detected after separation of the 182-kDa polymerase subunit from the four-subunit enzyme. To determine the specificity of excision of mispaired nucleotides by the exonuclease, we have utilized primed φX174am3 single-stranded DNA containing a noncomplementary nucleotide at the 3'-primer terminus, opposite deoxyadenosine at position 587 in the amber3 codon of the template strand. In the absence of polymerization, the preference for excision of the mispaired nucleotide from the primer is C > A >> G. Excision under these conditions is inhibited by the addition of deoxyguanosine monophosphate. Under conditions of concomitant DNA synthesis, the preference for excision at this site becomes A = G >> C, and excision is insensitive to deoxyguanosine monophosphate. The high fidelity of DNA synthesis exhibited by the isolated 182-kDa polymerase subunit is not reduced by concentrations of deoxyguanosine monophosphate or adenine monophosphate that inhibit proofreading by prokaryotic DNA polymerases. Thus, the 3' → 5' exonuclease of the Drosophila DNA polymerase-primase participates in exonucleolytic proofreading by excising noncomplementary nucleotides prior to extension of the primer by polymerase action.

The deoxyribose triphosphate analogs N2-(p-butyphenyl)deoxyguanosine triphosphate and N2-(p-butyphenyl)deoxyadenosine triphosphate are potent inhibitors of DNA polymerase α. Like calf thymus DNA polymerase δ, recently determined to have proofreading capability, DNA synthesis by the isolated Drosophila 182-kDa polymerase subunit was not inhibited by the two analogs. In contrast, DNA synthesis by the intact Drosophila polymerase-primase complex was inhibited >95% by these analogs.

DNA replication in vivo is a highly accurate process. Measurements of spontaneous mutation frequencies in animal cells suggest that the average frequency of errors is in the range of 10⁻³ to 10⁻¹ per base pair replicated (1). An important contributor to this high fidelity in prokaryotes and, probably, in eukaryotes is exonucleolytic proofreading of bases incorrectly inserted during DNA synthesis (2). We have recently reported that the 182-kDa polymerase subunit of the DNA polymerase-primase from Drosophila melanogaster contains a potent 3' → 5' exonuclease not detectable in the intact four-subunit enzyme (3). The fidelity of DNA synthesis increases 100-fold upon separation of the 182-kDa polymerase subunit, presumably due to the proofreading action of the 3' → 5' exonuclease.

Although exonucleolytic proofreading is known to enhance the fidelity of DNA synthesis by prokaryotic polymerases up to 100-fold (4), very little is known about the specificity of editing mismatches. Base substitution errors by polymerases capable of proofreading presumably result from errors at both the base insertion and excision steps. Sinha (5) has investigated editing by the 3' → 5' exonuclease of T4 DNA polymerase at two amber codons in φX174 DNA and demonstrated that editing efficiency can vary as much as 400-fold depending on the specific mispair and sequence context. Using oligodeoxynucleotide primers containing a single mismatch, Gillam and Smith (6) have reported that the efficiency of editing by the large fragment of Escherichia coli DNA polymerase I can vary as much as 100-fold depending on the temperature at which deoxyribonucleotide polymerization occurs.

The recent demonstration (7) of proofreading by DNA polymerase δ and our demonstration (3) that the 3' → 5' exonuclease of the Drosophila polymerase-primase enhances the fidelity of DNA synthesis 100-fold suggest that proofreading is an important contributor to the fidelity of DNA synthesis in eukaryotes as well as in prokaryotes. In order to characterize proofreading by the 3' → 5' exonuclease of the Drosophila DNA polymerase-primase, we have investigated the specificity with which misplaced nucleotides are edited. We demonstrate that the efficiency of editing is dependent on the specific mispair and that, in the absence of concomitant polymerization, editing is inhibited by dGMP. Under conditions of DNA synthesis, the efficiency of editing specific mispairs is altered, presumably due to differences in the ability of the polymerase to extend different mismatched termini.

To address the relationship of the isolated Drosophila 182-kDa polymerase subunit to DNA polymerase δ, we have compared the sensitivity of the intact polymerase-primase and the isolated 182-kDa polymerase subunit to the dNTP analogs BuđGTP and BuđATP, inhibitors of DNA polymerase α (8). We find that, whereas these analogs are potent inhibitors of DNA synthesis by the intact polymerase-primase.
mase, the isolated 182-kDa polymerase subunit, like DNA polymerase δ (9, 10), is only minimally inhibited.

EXPERIMENTAL PROCEDURES

Materials

Bacteriophage indicator strains E. coli HF4714 and HF4704, used for growing ϕX174 am3, E. coli K12 (used for preparing spheroplasts), E. coli polymerase I, and preparation of the ϕX174 am3 single-stranded DNA (ssDNA) templates have been described (11, 12). Upanyl acetate was purchased from J. T. Baker Chemical Co. Unlabeled dNTPs were from Pharmacia LKB Biotechnology Inc. dGMP and AMP were from Sigma. Radiolabeled nucleotides were from Du Pont-New England Nuclear. Bu6dGTP and Bu4dATP were a generous gift of G. E. Wright (University of Massachusetts Medical School). The ϕX174 am3 oligodeoxynucleotide primers containing an internal mismatch were synthesized by Operon Technologies Inc. (San Pablo, CA). All other primers for hybridization to ϕX174 ssDNA were synthesized by the Howard Hughes Chemical Synthesis Facility at the University of Washington. They were phosphorylated using [γ-32P]ATP and T4 polynucleotide kinase (Bethesda Research Laboratories) as described (13) and hybridized to ϕX174 am3 ssDNA at a primer/template ratio of 5:1 as previously described (12). Unhybridized primers were removed by high pressure liquid chromatography as described (3). (dA)60 and (dT)6 were purchased from Pharmacia LKB Biotechnology Inc. (dTh)[3H]dC, (2100 cpm/pmol of terminal nucleotide) was synthesized using terminal deoxynucleotide transferase (Pharmacia LKB Biotechnology Inc.) as described (14) and hybridized to (dA)60 at a 1:20 ratio. Hybridization was carried out in H2O at 37 °C for 45 min, followed by cooling to 20 °C for 1 h. Purification of the Drosophila polymerase-primase and dissociation of the complex by sedimentation through glycerol gradients in the presence of 50 °C (8) ethylene glycol were performed as previously described (8, 15). Cult thymus DNA polymerase was purified by immunosaffinity chromatography (16), and calf thymus DNA polymerase 6 II was a gift of R. Szambura (University of Rochester). Ethylene glycol was purchased from Sigma. All other chemicals were from commercial sources.

Methods

DNA Polymerase Activity—DNA polymerase activity was assayed in a 50-μl reaction mixture containing 50 mm Tris-HCl (pH 7.5), 8 mM MgCl2, 2 mM dithiothreitol, 50 μM each dATP, dCTP, and [α-32P]dGTP (600–1000 dpm/pmol), 10 μg of activated calf thymus DNA, and the indicated polymerase (3). One unit of DNA polymerase catalyzes the incorporation of 1 nmol of labeled nucleotide in 60 min at 30 °C.

Terminal Mismatch Excision in the Absence of Nucleotide Polymerization—The autoradiographic assay we described previously (3) was used to measure excision of 3′-terminal nucleotides. Four 16-base oligodeoxynucleotide primers were synthesized, each containing a different 3′-terminal nucleotide. When hybridized to ϕX174 am3 ssDNA, the resulting templates contained, at the 3′-termini, dCMP, dGMP, dAMP, or dTMP opposite the template dAMP at position 587 in the amber3 codon. 3′ → 5′ exonuclease activity was measured as the removal of a single 3′-terminal nucleotide from the 5′-32P-labeled oligonucleotide, as determined by increased mobility during polyacrylamide gel electrophoresis under denaturing conditions. Reactions contained, in 35 μl, 20 mM Tris-HCl (pH 7.5), 17% ethylene glycol, 10 mM MgCl2, 2 mM dithiothreitol, 200 μM each dATP, dCTP, and dGTP, 40 μM [α-32P]dTP (2000–3000 dpm/pmol), 9.2 μg of primed ϕX174 am3 ssDNA, and the indicated polymerase. Incubation was at 36 °C for 60 min, and reactions were terminated by the addition of EDTA to 30 mM. Nucleotide incorporation was measured by assaying an aliquot for acid-insoluble radioactivity. Copied DNA was transfected, and the reversion frequency was determined by the progeny plaque method (18). The background reversion frequency of ϕX174 am3 was determined with primer ϕX174 am3 ssDNA that had not been incubated with polymerase (uncopied DNA).

RESULTS

3′ → 5′ Exonuclease Shows Specificity of Editing 3′-Terminal Mismatches—To investigate the specificity of mispair editing, as illustrated in Fig. 1, we designed a series of ϕX174 am3 ssDNA templates that contain a 3′-terminal mispair (A-A, A-C, or A-G (template-primers)) or the correct base pair (A-T) opposite position 587 in the amber3 codon. Exonucleolytic activity in the absence of polymerization (Fig. 1A) can be measured with a template in which the 5′-terminal nucleotide of the primer strand is labeled with 32P. Removal of the 3′-terminal mismatched nucleotide is detected by increased mobility of the primer during polyacrylamide gel electrophoresis. Using this autoradiographic assay, mispair excision efficiencies of the intact polymerase-primase and the isolated 182-kDa polymerase subunit were determined. As seen in Fig. 2, there was no change in the electrophoretic mobility of any of the oligonucleotide primers after incubation with 0.036 unit of the intact polymerase-primase, indicating that less than 1% of the 3′-terminal A-A, A-C, or A-G (template-primer) mispairs were hydrolyzed. This is in agreement with our previous report (3) that, with an A-C mispair, 3′ → 5′ exonuclease activity was detectable only upon addition of large amounts of the intact polymerase-primase. In contrast, the isolated 182-kDa polymerase subunit excised the terminal nucleotide on all primers tested. Dramatic differences in the efficiency of editing different mispairs were apparent. Visual examination demonstrates that A-A and A-C mispairs were edited efficiently, but that editing of the A (template)-G (primers) mispair was much less even at the highest concentration of the 182-kDa polymerase subunit used. In order to quantify excision efficiency, we analyzed these gels by scanning densitometry. Table I shows the percentage of labeled primers converted from the full-length 16-base oligonucleotide to a 15- or 14-base oligonucleotide. A-A and A-C (template-primer) mispairs were both edited efficiently; excision of a C opposite the template A was nearly twice as efficient as excision of an A opposite the template A. With a homopolymer template, the reverse was observed; the A-A mismatch was excised more rapidly than the A-C mismatch (3). Excision of a G opposite the template A was a rare event, occurring at a 20-fold lower frequency than excision of the mispaired C and at a 10-fold lower frequency than excision of the mispaired A.

Excision of the correctly paired nucleotide (dTMP) was also detected, although at a 2-5-fold lower efficiency than
Fig. 1. Experimental design. The efficiency of specific 3'-terminal mispaired nucleotide excision was determined in the absence or presence of DNA synthesis. Experimental details are described under “Methods.” φX174am3 ssDNA was hybridized to one of four oligonucleotides, each containing a different 3'-terminal nucleotide (X). To determine editing efficiency in the absence of polymerization (A), the 5'-terminal nucleotide of the primer strand is labeled with 32P before hybridization to the φX174am3 ssDNA template. Exonuclease activity is measured as the removal of the 3'-terminal nucleotide, resulting in conversion of the original 16-base oligonucleotide to a 15-base oligonucleotide. Primer length is determined by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. To determine the efficiency of editing during polymerization (B), dNTPs are added to the reaction mixture. Synthesis of the primer past the amber3 codon is verified by determining the extent of incorporation of a labeled dNTP. The partially copied template molecules are then transfected into E. coli spheroplasts which complete replication and produce progeny phage. Progeny phage are plated on E. coli indicator strains which are either permissive or nonpermissive for the amber3 phenotype. DNA synthesis without excision of the mispaired nucleotide results in the formation of heteroduplex molecules that yield revertants upon growth in the nonpermissive E. coli indicator. Excision of the 3'-terminal mismatched nucleotide of the primer strand before extension by the polymerase results in loss of the revertant phenotype.

The addition of nucleoside 5'-monophosphates inhibits the 3' → 5' exonuclease of E. coli polymerase I, presumably by blocking the efficiency of editing during the exonuclease reaction site on the polymerase (19, 20). This inhibition is associated with enhanced misincorporation during polymerization and has been considered a hallmark of proofreading (21, 22). In order to correlate eukaryotic exonuclease proofreading by the 182-kDa polymerase subunit with the more extensively studied prokaryotic DNA polymerases, we wished to determine whether excision by the 3' → 5' exonuclease is altered in the presence of dGMP. As shown in Fig. 3, the addition of 10 mM gGMP inhibited excision of both A-A and A-C mismatches. Densitometric analysis of these gels (Table I) showed that excision of an A-A mispair was reduced 3-fold in the presence of dGMP, whereas excision of an A-C mispair was reduced 6-fold. The decrease in hydrolysis of the 3'-A-A mismatch by addition of dGMP was similar for both the 182-kDa polymerase subunit and the large fragment of E. coli DNA polymerase I (Fig. 3). The loss of labeled primer with E. coli polymerase I is presumably due to the 5' → 3' exonucleolytic activity that removes the 5'-labeled termini.

Specificity of Editing during Deoxynucleotide Polymerization—The efficiency of proofreading during DNA synthesis must reflect the rate constant for both insertion and excision of the noncomplementary nucleotides. The data of Fig. 2 indicate that the rate of excision by the 182-kDa polymerase subunit in the absence of polymerization is dependent upon the nature of the mispair. Furthermore, differences in the thermodynamic stability of various mispairs (23) suggest that the rate constant for extension of a mispaired primer is also likely to vary with the mispair. To determine the efficiency of proofreading during DNA synthesis, we analyzed excision of 3' terminal mismatches from the φX174am3 ssDNA templates previously described using a sensitive infectivity assay based on reversion of the amber3 codon (Fig. 1B). The method for measuring excision of a terminal mispair during polymerization...
Table I
Mismatch excision by the Drosophila polymerase-primase and 182-kDa polymerase-primase subunit in the absence of DNA synthesis

Exposures of the polyacrylamide gels in Figs. 2 and 3 which were in the linear response range of the film were analyzed using a Hoeffer GS-350H scanning densitometer. Values reported are the percent of full-length 16-base oligonucleotide primer converted to a 15- or 14-base oligonucleotide and represent an average of duplicate samples. The amounts of 15- and 14-base oligonucleotides in each 16-base oligonucleotide preparation prior to the reaction were subtracted for each sample and are as follows: 2% 3'-A-A, <1% 3'-A-C, 8% 3'-A-G, and <1% 3'-A-T.

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>3'-Terminal base pair (template-primer)</th>
<th>A-A</th>
<th>A-C</th>
<th>A-G</th>
<th>A-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>182-kDa subunit</td>
<td>units \times 10^{-6}</td>
<td>&lt;1</td>
<td>5</td>
<td>&lt;1</td>
<td>1</td>
</tr>
<tr>
<td>1.8</td>
<td></td>
<td>&gt;5</td>
<td>&lt;1</td>
<td>&gt;1</td>
<td>1</td>
</tr>
<tr>
<td>2.7</td>
<td></td>
<td>26</td>
<td>17</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>3.6</td>
<td></td>
<td>46</td>
<td>6</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>182 kDa subunit + 10 mM dGMP</td>
<td></td>
<td>3</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1.8</td>
<td></td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Polymerase-primase: 3.6

* ND, not determined.

Fig. 3. Inhibition of mismatch excision by dGMP. ϕX174am3 ssDNA templates containing a 3'-terminal A-A or A-C (template-primer) mispair were made as described under “Methods.” Exonuclease reactions and primer analysis were done as described in the legend to Fig. 2. Each reaction contained 0.036 unit of the isolated 182-kDa polymerase subunit or 0.05 unit of E. coli DNA polymerase I (POL I), large fragment. In lanes labeled +dGMP, 10 mM dGMP was included. The original primer length is 16 nucleotides; the 15- and 14-nucleotide primers are exonuclease products.

To determine expression of the mismatched primer strand, we synthesized a series of primers, each containing a different substitution at position 587, followed by eight complementary nucleotides. When hybridized to ϕX174am3 ssDNA, these templates mimic DNA that was extended at the mismatched terminus without editing of the mismatch. (Table II). The expression of these internal mismatches (Table II, third column) was used to correct the observed frequency of revertants (Table II) in order to estimate the efficiency of editing. These analyses with internal mismatches represent mock experiments that permit quantification of the number of revertants one would obtain if 100% of the mismatched nucleotides were expressed and if each primer was elongated by the DNA polymerase. In addition, since transfection of uncopied templates containing terminal mispairs results in extremely efficient removal of the mispaired nucleotide by the 3' → 5' exonucleases in the E. coli host, it is necessary to verify that the primer is indeed extended by DNA synthesis in vitro. By including a radiolabeled nucleotide during synthesis, we verified that the average primer was extended 10 nucleotides beyond the amber3 codon (data not shown).

Table II shows the efficiency of mispair editing during DNA synthesis by the isolated 182-kDa polymerase subunit and the intact polymerase-primase. In the absence of added DNA polymerase, only a small number of wild-type plaques were obtained when an oligonucleotide primer containing a T opposite the A at position 587 was used. This low reversion frequency indicates that the chemically synthesized oligonucleotide was free of contamination by other nucleotides or by degradation products. The higher frequency of revertants observed with oligonucleotides containing terminal A-A, A-C, and A-G mispairs indicates that not all mismatched termini are removed after transfection by the endogenous DNA polymerases in E. coli. With A-A and A-C 3'-mismatched termini, <99.8% of the mismatches were corrected; whereas after transfection, with the A-G mismatch, 97.6% were excised.

The 182-kDa polymerase subunit removed nearly all the mismatched termini prior to polymerization. In fact, the efficiency of mismatch excision by the 182-kDa polymerase subunit is 16–100-fold greater in the presence of polymerization than in its absence. For example, in the absence of synthesis, 46% of the mismatched A-A termini were excised by 0.036 unit of the 182-kDa polymerase subunit (Table I). However, during deoxynucleotide polymerization, <99.8% of the same mismatch was excised with 0.04 unit (Table II). As in the absence of DNA synthesis, there were differences in the efficiency of excision of various mispairs; moreover, the relative efficiencies of editing the various mispairs were altered. During polymerization, A-G and A-A mispairs were edited to a similar extent, whereas A-C mispairs were edited at a 9-fold lower frequency. Less than 1 in 500 A-G and A-A mismatches were left unedited, compared to 1 in 60 A-C mismatches left unedited. Thus, the efficiency of editing mispairs by the 3' → 5' exonuclease during DNA synthesis is A-A = A-G >> A-C at this site.

Surprisingly, significant editing was detected with the intact polymerase-primase during deoxynucleotide polymerization, apparently the result of limited expression of the 3' → 5' exonuclease under these conditions. All mispairs tested were excised by the polymerase-primase, and the order of preference was identical to that observed with the isolated 182-kDa polymerase subunit, i.e. A-A = A-G >> A-C. Editing of A-A and A-G mispairs by the intact enzyme was about 16 times less efficient than editing by the 182-kDa
Proofreading by Drosophila DNA Polymerase-Primase

TABLE II

3'-Terminal mismatch excision during DNA synthesis

Reaction conditions are described under "Methods." Oligonucleotide primers that contain the indicated 3'-terminal noncomplementary base pair (template-primer) at template position 587 were used; 10 mM dGMP was added as indicated. Each reaction contained 0.04 unit of 182-kDa polymerase subunit or 0.05 unit of E. coli DNA polymerase I. The background reversion frequency of an unprimed template is $1 \times 10^{-6}$. Reversion frequencies reported are the average of triplicate experiments. The background reversion frequency of an unprimed template is $1 \times 10^{-6}$.

<table>
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<tr>
<th>DNA polymerase</th>
<th>Hybridized oligonucleotide</th>
<th>Reversion frequency</th>
<th>Corrected reversion frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5'-A-3'</td>
<td>$6.6 \times 10^{-2}$</td>
<td>$2 \times 10^{-5}$</td>
</tr>
<tr>
<td>182-kDa subunit</td>
<td>5'-A-3'</td>
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<td>$16 \times 10^{-5}$</td>
</tr>
<tr>
<td>Polymerase-primase</td>
<td>5'-A-3'</td>
<td>$10.2 \times 10^{-5}$</td>
<td>$34 \times 10^{-4}$</td>
</tr>
<tr>
<td>None</td>
<td>5'-G-3'</td>
<td>$5.6 \times 10^{-2}$</td>
<td>$3 \times 10^{-6}$</td>
</tr>
<tr>
<td>182-kDa subunit</td>
<td>5'-G-3'</td>
<td>$6.1 \times 10^{-2}$</td>
<td>$11 \times 10^{-6}$</td>
</tr>
<tr>
<td>Polymerase-primase</td>
<td>5'-G-3'</td>
<td>$7.8 \times 10^{-2}$</td>
<td>$21 \times 10^{-6}$</td>
</tr>
<tr>
<td>None</td>
<td>5'-A-3'</td>
<td>$5.6 \times 10^{-5}$</td>
<td>$2 \times 10^{-5}$</td>
</tr>
<tr>
<td>182-kDa subunit</td>
<td>5'-A-3'</td>
<td>$6.7 \times 10^{-5}$</td>
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<td>5'-T-3'</td>
<td>$8.3 \times 10^{-5}$</td>
<td>$3 \times 10^{-5}$</td>
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</table>

TABLE III

Effect of dGMP on mismatch excision during DNA synthesis

Reaction conditions are described under "Methods." Oligonucleotide primers that contain the indicated 3'-terminal noncomplementary base pair (template-primer) at template position 587 were used; 10 mM dGMP was added as indicated. Each reaction contained 0.04 unit of 182-kDa polymerase subunit or 0.05 unit of E. coli DNA polymerase I. The background reversion frequency of an unprimed template is $1 \times 10^{-6}$. Reversion frequencies reported are the average of triplicate experiments. The background reversion frequency of an unprimed template is $1 \times 10^{-6}$.

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>dGMP (mM)</th>
<th>Reversion frequency ($\times 10^{-6}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drosophila</td>
<td></td>
<td></td>
</tr>
<tr>
<td>182-kDa polymerase subunit</td>
<td>0</td>
<td>A-A 180 47</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A-C 51 26</td>
</tr>
<tr>
<td>E. coli DNA polymerase I</td>
<td>0</td>
<td>A-G 36 8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>A-G 74 110</td>
</tr>
</tbody>
</table>

polymers were inhibited by nucleoside 5'-monophosphates, presumably by blocking the 3' → 5' exonuclease (19, 20). In contrast, editing by the 182-kDa polymerase subunit during DNA synthesis was not inhibited by dGMP (Table III). This finding may indicate a difference in the mechanism of editing; or more likely, it may result from a higher efficiency of proofreading by the eukaryotic enzyme during polymerization. A similar lack of inhibition of proofreading by nucleoside monophosphates was observed in fidelity studies.

The high accuracy of the 182-kDa polymerase subunit (3) is further indicated by the observation that the reversion frequency is 10-fold lower than that of E. coli DNA polymerase I. The addition of 5 mM AMP or 10 mM dGMP had no effect on the fidelity of DNA synthesis by the isolated 182-kDa polymerase subunit, although it dramatically increased the error frequency of E. coli DNA polymerase I (Table IV).

Inhibition of DNA Synthesis by the dNTP Analogs Bu4dATP and Bu4dGTP—Recent findings suggest the involvement of DNA polymerase δ, which contains a 3' → 5' exonuclease, in cellular DNA replication (24). In order to explore the relationship between polymerase δ and the 182-kDa polymerase subunit of the Drosophila polymerase-primase, we have examined the sensitivity of the intact Drosophila polymerase-primase and the isolated 182-kDa polymerase subunit to the two dNTP analogs, Bu4dATP and Bu4dGTP. These analogs are specific inhibitors of calf thymus DNA polymerase a, but not of polymerase δ (Fig. 4) (8-10). As shown in Fig. 4, Bu4dATP is a potent inhibitor of the intact Drosophila polymerase-primase, but not of the isolated 182-kDa polymerase subunit. At 1 μM Bu4dATP, 5% of the activity of the intact enzyme and 95% of the activity of the isolated 182-kDa polymerase subunit remained. At 100 μM Bu4dGTP, 6% of the activity of the intact polymerase-primase subunit, whereas editing of A-C mispairs was 100 times less efficient.

Effect of Nucleotide 5'-Monophosphates on Fidelity of DNA Synthesis—Exonucleolytic proofreading by prokaryotic polymerases is inhibited by nucleoside 5'-monophosphates, presumably by blocking the 3' → 5' exonuclease (19, 20). In contrast, editing by the 182-kDa polymerase subunit during DNA synthesis was not inhibited by dGMP (Table III). This finding may indicate a difference in the mechanism of editing; or more likely, it may result from a higher efficiency of proofreading by the eukaryotic enzyme during polymerization. A similar lack of inhibition of proofreading by nucleoside monophosphates was observed in fidelity studies.

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Reversion frequency ($\times 10^{-4}$)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
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</tr>
<tr>
<td>dGMP</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td></td>
</tr>
<tr>
<td>182-kDa polymerase subunit</td>
<td>Preparation 1</td>
</tr>
<tr>
<td></td>
<td>Preparation 2</td>
</tr>
<tr>
<td>E. coli DNA polymerase I</td>
<td>22.4 181 118</td>
</tr>
</tbody>
</table>
Proofreading by Drosophila DNA Polymerase-Primase

FIG. 4. DNA polymerase inhibition by Bu\textsubscript{d}ATP. DNA polymerase and the indicated concentration of Bu\textsubscript{d}ATP were added to a DNA polymerase assay as described under "Methods." Amounts of DNA polymerase per reaction were as follows: 0.06 unit of Drosophila 182-kDa polymerase subunit, 0.05 unit of Drosophila polymerase-primase, 0.05 unit of calf thymus DNA polymerase \( \varepsilon \), and 0.024 unit of calf thymus DNA polymerase \( \varepsilon \). Upper, Drosophila 182-kDa polymerase subunit (O) and polymerase-primase (Q); lower, calf thymus DNA polymerase \( \delta \) (A) and polymerase \( \alpha \) (a).

**DISCUSSION**

DNA replication in vivo is of necessity a highly accurate process. Mechanisms that enable eukaryotic cells to achieve this high accuracy are not well-understood. We have previously reported that the DNA polymerase-primase from *D. melanogaster* contains a cryptic 3'→5' exonuclease (3). We demonstrate here that this exonuclease proofreads during DNA synthesis by excising noncomplementary nucleotides prior to primer extension. To address the role of proofreading in preventing base substitution mutations, we have investigated the efficiency of excision of three terminal mispairs in the amber3 codon of \( \phi \)X174. We find that the 3'→5' exonuclease of *Drosophila* edits different mispairs with dramatically different efficiencies and that the relative efficiency of editing the various mispairs is altered when DNA synthesis occurs.

Exonucleolytic proofreading involves a competition between nucleotide polymerization and excision. When an incorrect nucleotide is incorporated, it can be removed by the exonuclease or locked into the nascent DNA chain by incorporation of the next nucleotide. Evidence from other laboratories (5, 6) suggests that the efficiency of editing specific mispairs may vary. Two general types of mechanisms can be invoked to account for discrimination at the level of mispairing. First, excision may be dependent upon the stability of the mispair at the primer terminus. Studies on the stability of the DNA helix in solution suggest that replacement of an A-T duplex base pair lowers the melting temperature in the following order: T·G < T·C, T·T, A·G < A·A or A·C (23). However, interaction of the polymerase with the template-primer, which is thought to exclude water (25), may alter these relative stabilities. Second, excision may be dependent upon the rate of polymerization from a primer containing a terminal mispaired nucleotide. This notion is supported by evidence that proofreading by several polymerases can be overcome by increasing the concentration of the next nucleotide and thus accelerating the forward reaction (20–22).

To discern the relative contribution of each of these mechanisms, a system that allows excision to be uncoupled from polymerization is required. To this end, we have examined the efficiency of excision of three terminal mispairs at the amber3 codon of \( \phi \)X174 in the presence and absence of concomitant DNA synthesis. Under both conditions, we find marked differences in the efficiency of excision of mispairs. In the absence of polymerization, the order of excision of a 3’-terminal nucleotide opposite a template A residue is C > A >> G. These results are in qualitative agreement with those predicted on the basis of melting temperatures (23), suggesting that, under these conditions, base pair stability may be an important determinant of excision specificity. When polymerization occurs, the rate of excision of the 3’-terminal noncomplementary nucleotides increases 16–100-fold. This is in agreement with Maki and Kornberg (26), who recently reported that the activity of the 3’→5’ exonuclease of *E. coli* DNA polymerase III holoenzyme is stimulated 5-fold by concurrent DNA synthesis. Under conditions of DNA synthesis, proofreading by the intact *Drosophila* polymerase-primase can also be detected, albeit at a 100-fold lower efficiency than the isolated 182-kDa polymerase subunit. During DNA synthesis, the efficiency of excising the various mispairs is altered dramatically, and the order of excision becomes A = G >> C. At least three explanations for this change in excision specificity can be proposed. 1) A-C mispairs are poorly proofread by the 3’→5’ exonuclease. Our results on the excision of mispairs in the absence of DNA synthesis suggest this is not the case. 2) The activity of the 3’→5’ exonuclease for different mismatches is dependent upon whether conditions are permissive for excision only or for excision followed by polymerization. Our studies do not address this possibility. 3) Nucleotide addition to an A-C mispaired primer terminus occurs more readily than addition to an A·A or A·G primer terminus. In accord with this concept, we have found that the average number of nucleotides incorporated on the A-C mispaired template-primer is about twice that on the A·G or A·A template-primer and comparable to that seen using the correctly paired A·T (results not shown). This correlation assumes that initiation on a mispaired terminus is rate-limiting and that the 182-kDa polymerase subunit has a low processivity (27).

Most models for the molecular basis of base substitution mutations propose that they arise through unfavored nucleotide tautomers and syn isomers that can base-pair within the constraints of a normal double helix (28, 29). Based on the probability of forming these tautomers and isomers, as well as base-pairing constraints, they predict that transition mutations, resulting from purine-pyrimidine mispairs should predominate. Studies of spontaneous mutations in the lacI gene obtained from *E. coli* lacking a mismatch correction pathway support this prediction (30). Our results suggest that the most probable base pair substitution by both the intact *Drosophila* polymerase-primase and the 182-kDa polymerase subunit at position 587 in the \( \phi \)X174am3 codon is an A·T → G·C transition, the result of a purine-pyrimidine mispair. In addition, studies with *E. coli* polymerase I, a highly accurate polymerase, also demonstrate a preference for A·C mispairs at the \( \phi \)X174am3 locus (31). In contrast, when considering the base substitution frequency at the amber3 locus of several species of polymerase \( \alpha \) that lack a 3’→5’ exonuclease, Kunkel and Alexander (32) have reported that A·A mispairs...
predominate over A-C and A-G mismaps. It is of interest that, in the case of the most accurate eukaryotic DNA polymerase studied by Kunkel and Alexander, polymerase γ, which may contain a 3′ → 5′ exonuclease, A-C mismaps predominate over A-G, and A-A mismaps at the amber3 site (33). This finding raises the interesting possibility that interaction of the fidelity-enhancing component of these enzymes (3′ → 5′ exonuclease in the case of E. coli DNA polymerase I and the Drosophila 182-kDa polymerase subunit) with the polymerase alters the base substitution specificity and that this alteration in specificity may be a marker for identifying proofreading exonucleases.

Whether the specificity of mismatch excision that we describe at the φX174am3 codon is unique to the Drosophila polymerase is not known. Sinha (5) reported that A-G mismaps, but not A-C mismaps, at the amber3 locus are poorly edited by T4 DNA polymerase, using an assay similar to that which we have described. Our experiments are limited to studying the three mismaps at the amber3 site of φX174 which yield viable revertants. In addition, we have not considered the effect of neighboring sequences on the efficiency of excision. Sinha (5) has reported that the absolute (but not the relative) efficiency of editing mismatches at the φX174am3 locus varies with the neighboring sequence. Nonetheless, the specificity of mismatch excision that we describe with the 182-kDa polymerase subunit in the absence of polymerization is in agreement with predicted mismatch stabilities (23). Our data on excision in the presence of DNA synthesis are complex, but suggest that the ability of the polymerase to extend a specific mispaired primer is a major factor that contributes to the overall efficiency of proofreading.

The addition of nucleoside 5′-monophosphates inhibits proofreading by prokaryotic polymerases (19, 20). We find that the addition of 10 mM dGMP inhibits mispair excision in the absence of DNA synthesis, but not in its presence. In addition, dGMP and AMP had no effect on the fidelity of DNA synthesis by the 182-kDa polymerase subunit. We conclude that, during polymerization, these nucleotides are insufficient to inhibit proofreading. It is notable that the ratio of exonuclease to polymerase activity for the isolated 182-kDa polymerase subunit is approximately 10-fold higher than that for E. coli DNA polymerase I (38) and comparable to that reported for the DNA polymerase encoded by the herpes simplex virus (34). The fidelity of DNA synthesis by the herpes polymerase is also insensitive to added monophosphates (34).

We have demonstrated that the 3′ → 5′ exonuclease of the 182-kDa polymerase subunit of the Drosophila polymerase-primase participates in exonucleolytic proofreading during DNA synthesis. Recently, calf thymus DNA polymerase δ has also been reported to proofread (7). These two enzymes are remarkably similar (9, 35). One of the characteristic features used to distinguish DNA polymerase δ from DNA polymerase α is the differential inhibition by the nucleotide analogs BuδdGTP and BuδdATP (8, 9). We have demonstrated that, like calf thymus DNA polymerase-δ, DNA synthesis by the isolated 182-kDa polymerase subunit is insensitive to these inhibitors.

In contrast, DNA synthesis by the intact Drosophila polymerase-primase, like the calf thymus polymerase α, is highly sensitive. Other distinctions remain (36, 37), particularly differential stimulation by cyclin and lack of cross-reactivity of antibodies specific for each of these enzymes. To definitively determine the relationship between these two polymerases, cloning and analysis of their chromosomal location may be required.

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