Articles

Properties of the 3′ to 5′ Exonuclease Associated with Calf DNA Polymerase δ†

Ralph D. Sabatino and Robert A. Bambara*

Departments of Biochemistry, Microbiology, and Immunology and Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received July 29, 1987; Revised Manuscript Received October 13, 1987

ABSTRACT: The 3′ to 5′ exonuclease of calf thymus DNA polymerase δ has properties expected of a proofreading nuclease. It digests either single-stranded DNA or the single-stranded nucleotides of a mismatched primer on a DNA template by a nonprocessive mechanism. The distribution of oligonucleotide products suggests that a significant portion of the enzyme dissociates after the removal of one nucleotide. This mechanism is expected if the substrate in vivo is an incorrect nucleotide added by the polymerase. Digestion of single-stranded DNA does not proceed to completion, producing final products six to seven nucleotides long. Digestion of a long mismatched terminus accelerates when the mismatched region is reduced to less than six nucleotides. At the point of complementation, the digestion rate is greatly reduced. These results suggest that short mismatched regions are a preferred substrate. The use of a mismatched primer-template analogue, lacking the template single strand, greatly lowers digestion efficiency at the single-stranded 3′-terminus, suggesting that the template strand is important for substrate recognition. When oligonucleotides were examined for effectiveness as exonuclease inhibitors, $(dG)_8$ was found to be the most potent inhibitor of single-stranded DNA digestion. $(dG)_8$ was less effective at inhibiting digestion of mismatched primer termini, again suggesting that this DNA is a preferred substrate. Overall, these results indicate that the exonuclease of DNA polymerase δ efficiently removes short mismatched DNA, a structure formed from misincorporation during DNA synthesis.

Four types of eucaryotic DNA polymerases have been identified: α, β, γ, and δ. DNA polymerase α has been classified as the enzyme responsible for nuclear chromosomal replication by several criteria [see review by Fry and Loeb (1986)]. These include the observation that both DNA replication and purified DNA polymerase α are inhibited by similar concentrations of the drug aphidicolin. Also, rapidly proliferating tissue, such as regenerating liver, contains high levels of DNA polymerase α whereas nonproliferating tissue (e.g., brain) has low levels of DNA polymerase α. Similar observations, however, have recently been made for DNA polymerase δ, equivalently supporting it as an essential enzyme for nuclear chromosomal replication. DNA polymerase δ has been shown to be identically sensitive as DNA polymerase α to aphidicolin (Crute et al., 1986; Wahl et al., 1986; Lee et al., 1984; Byrnes, 1984) and to parallel the levels of DNA polymerase α during maturation and terminal differentiation of the heart (Zhang & Lee, 1987). Also, Miller et al. (1987) have suggested that DNA polymerase δ plays a significant role in DNA replication.

Except for several reports, suggesting that DNA polymerase α may have an associated 3′ to 5′ exonuclease (Chen et al., 1979; Ottiger & Hubscher, 1984; Skarnes et al., 1986; Cotterill et al., 1987), DNA polymerase δ appears to be the only eucaryotic enzyme to have a tightly associated 3′ to 5′ exonuclease (Wahl et al., 1986; Lee & Toomey, 1987; Byrnes, 1984). This 3′ to 5′ exonuclease has been shown to hydrolyze 5′-monophosphate deoxynucleotides from the 3′-termini of single-stranded DNA molecules and the 3′-termini of mismatched double-stranded DNA molecules (Lee & Toomey, 1987; Crute et al., 1986; Lee et al., 1984; Byrnes, 1984). These results led investigators to speculate that DNA polymerase δ has a "proofreading" capability similar to those of procaryotic DNA polymerases (Kornberg, 1980). Recently, we have addressed this question using an *M13LacZα* nonsense codon reversion assay (Kunkel et al., 1987). We have been able to demonstrate that indeed DNA polymerase δ does proofread and that proofreading contributes to the fidelity of DNA polymerase δ.

This prompted us to examine the enzymatic characteristics of the 3′ to 5′ associated exonuclease of DNA polymerase δ. We have found the exonuclease to have substrate specificities and a nonprocessive mechanism consistent with its role in proofreading.

Materials and Methods

$Poly(dA)_{4000-5000}$, $poly(dT)_{400}$, $oligo(dT)_{16}$, and terminal deoxynucleotidyltransferase were purchased from Midland Certified Reagent Co. (Midland, TX). $(dG)_4$, $(dG)_6$, $(dG)_8$, $(dG)_{10}$, $(dT)_{10}$, $(dC)_{10}$, $(dA)_{10}$, and $(dA)_{16}$ were from Pharmacia–P-L Biochemicals, Inc. (Milwaukee, WI). Venom phosphodiesterase was from Worthington Diagnostic System, Inc. (Freehold, NJ), and T4 polynucleotide kinase was from New England Biolabs, Inc. (Beverly, MA). [γ-^{32}P]ATP (3000 Ci/mmol), [^{3}H]deoxythymidine 5′-triphosphate (80 Ci/mmol), [^{3}H]deoxyguanosine 5′-triphosphate (10.4 Ci/mmol), [^{3}H]-deoxycytosine 5′-triphosphate (31 Ci/mmol), and NENSORB columns were purchased from New England Nuclear Corp. (Boston, MA). Sephadex G-50 was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). (Diethylaminoethyl)nitrocellulose (DEAE-nitrocellulose)[1] filters (NA45, 24

†Supported by National Institutes of Health Grant GM24441, Cancer Center Core Grant 5-P30-CA-11198-15, and a postdoctoral fellowship grant from the United Cancer Council of Rochester to R.D.S.

*Address correspondence to this author at the Department of Biochemistry, Box 607, University of Rochester Medical Center, 601 Elmwood Ave., Rochester, NY 14642.

0006-2960/88/0427-2266$01.50/0

mm in diameter, pore size 0.45 μm) and Elutrap (Electroseparation chamber) were purchased from Schleicher & Schuell (Keene, NH). Electrophoresis-grade acrylamide, *N,N'*-methylenebis(acrylamide), and *N,N,N',N'*-tetramethylethylenediamine were purchased from Bethesda Research Laboratories Life Technologies, Inc. (Gaithersburg, MD). Ammonium persulfate was from Bio-Rad Laboratories (Richmond, CA). Thin-layer chromatography plates containing a fluorescent indicator were purchased from Eastman Kodak (Rochester, NY). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

DNA Polymerase δ. DNA polymerase δ was purified by a modification of the method of Crute et al. (1986). Postmicrosomal supernatant solution was prepared from calf thymus glands (200 g) and 1 L of 50 mM imidazole, pH 6.5, 200 mM NaCl, 10% glycerol (v/v), 20% Me_2SO (v/v), 1.0 mM DTT, 2.0 mM ATP, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 μg of leupeptin/mL, 0.5 μg of pepstatin A/mL, and 0.5 mM PMSF (buffer I). The solution was applied directly to a column of Bio-Rex-70 (75 mL, 5 × 3.5 cm) equilibrated with buffer I and eluted with a 13 column volume linear gradient from buffer I to 50 mM imidazole, pH 6.5, 450 mM NaCl, 30% glycerol (v/v), 1.0 mM DTT, 2.0 mM ATP, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 μg of leupeptin/mL, 0.5 μg of pepstatin A/mL, and 0.5 mM PMSF (buffer IA). Five-milliliter fractions were collected at a flow rate of 1 column volume/h. The peak of DNA polymerase δII was collected and dialyzed against 20 volumes of 50 mM imidazole, pH 6.5, 20% glycerol (v/v), 4% Me_2SO (v/v), 20 mM NaCl, 1.0 mM DTT, 1.0 mM EDTA, 1.0 mM EGTA, 0.5 μg of leupeptin/mL, 0.5 μg of pepstatin A/mL, and 0.5 mM PMSF (buffer II) with two changes over a 6-h time period. The dialyzed sample was then applied to a column of DEAE-Sephadex-A25 (60 mL, 5 × 3 cm) equilibrated with buffer II. After being washed with 1 column volume of buffer II, the DEAE-Sephadex-A25 column was eluted with 17 column volume linear gradient from buffer II to buffer II without Me_2SO but containing 300 mM NaCl. Five-milliliter fractions were collected at a flow rate of 1 column volume/h. Fraction III DNA polymerase δII was collected and dialyzed against 15 volumes of 50 mM Tris-HCl, pH 7.5, 20% glycerol (v/v), 100 mM NaCl, 1.0 mM DTT, 1.0 M EDTA, 1.0 mM EGTA, 0.5 μg of leupeptin/mL, 0.5 μg of pepstatin A/mL, and 0.5 mM PMSF (buffer III) with two changes over a 6-h time period. Fraction III DNA polymerase δII was further purified by chromatography on a phosphocellulose column (Whatman P-11, 1 mL, 0.9 × 1.6 cm) equilibrated with buffer III. DNA polymerase δ was eluted by using a 20 column volume linear gradient from buffer III to buffer III containing 500 mM NaCl. One-milliliter fractions were collected at a flow rate of 1.3 column volumes/h. The resulting DNA polymerase was dialyzed overnight against 200 volumes of buffer III containing 20 mM NaCl and 5.0 mM DTT and stored frozen in liquid N_2.

The DNA polymerase δII purified for the present study is equivalent, by previously used criteria, to DNA polymerase δII purified by our original scheme. Both polymerase and exonuclease activities copurify through DEAE-Sephadex-A25 and phosphocellulose chromatography. Phosphocellulose chromatography was utilized in order to remove contaminating endonuclease (Kunkel et al., 1987) and to increase the specific activity 10-fold with a higher yield when compared to sucrose gradients used in the original scheme. Aphidicolin and BuPdGTP drug sensitivities are the same as reported earlier. Also, the molecular weight is similar, and the specific activity is comparable to that used in our initial studies.

Enzymatic Assays. DNA polymerase assays (25 μL) contained 20 mM Tris-HCl, pH 7.5, 5% glycerol, 10 mM $MgCl_2$, 5.0 mM β-mercaptoethanol, 250 μg of BSA/mL, 2 mM ATP, 25 μM 4.0 Ci/mmol [^{3}H]dTTP, and 32 μM poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$ (20:1). Reactions were incubated at 37 °C for 30 min followed by the addition of 30 μL of 100 μM EDTA. The radiolabeled DNA was then collected by sedimentation through centrifuge columns of Sephadex G-50 (Penefsky, 1977). One unit of DNA polymerase incorporates into DNA 1 nmol of nucleotide/h at 37 °C.

Conditions for the exonuclease assays were the same as those for polymerase assays except that poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$, [^{3}H]dTTP, and ATP were omitted, the appropriate exonuclease substrate was added, and the incubation time was increased to 60 min. When necessary, DNA was collected on DEAE-nitrocellulose disks according to LaDuca et al. (1986).

We routinely perform our assays at pH 7.5, since the optimum for polymerization is pH 7.0 and the optimum for exonuclease is pH 8.0. At pH 7.5, the exonuclease retains approximately 80% of the optimum activity, while the polymerase retains 50% of the optimum activity.

Exonuclease Substrates. The 3'-terminal end of poly(dT)$_{400}$ was labeled at 37 °C in a reaction mixture containing (25 μL) 0.1 M potassium cacodylate, pH 7.2, 40 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM $CoCl_2$, 2 mM β-mercaptoethanol, 9.4 mM poly(dT)$_{400}$, 20 units of terminal deoxynucleotidyltransferase, and either 300 μM 10 Ci/mmol [^{3}H]dTTP or 300 μM 10.4 Ci/mmol [^{3}H]dGTP. The reactions were terminated by the addition of 30 μL of 100 mM EDTA. The DNA was then purified by centrifuge columns containing Sephadex G-50 (Penefsky, 1977). The eluates containing terminally labeled poly(dT)$_{400}$([^{3}H]dTMP)$_2$, poly(dT)$_{400}$([^{3}H]dGMP)$_2$, or poly(dT)$_{400}$([^{3}H]dGMP)$_4$ were collected.[2]

Oligo(dT)$_{16}$ was purified by electrophoresis on 20% polyacrylamide gels containing 7 M urea, 100 mM Tris–borate, pH 8.3, and 2 mM EDTA. After 5 h of electrophoresis at 1500 V, oligonucleotides were localized by placing the gel over a thin-layer chromatographic plate containing a fluorescent indicator and exposing the gel to shortwave ultraviolet light. The oligomers were cut out of the gel and eluted overnight using an Elutrap electrophoresis chamber containing 100 mM Tris–borate, pH 8.3, and 2 mM EDTA. The oligomers were then desalted by using an NENSORB column as described by the supplier.

Gel-purified oligo(dT)$_{16}$ was 3' terminally labeled under the above conditions in a reaction containing 500 μM 0.2 Ci/mmol [^{3}H]dCTP, 364 μM gel-purified oligo(dT)$_{16}$, and 25 units of terminal deoxynucleotidyltransferase. The reaction products oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$ or oligo(dT)$_{16}$([^{3}H]dCMP)$_{3-5}$ were purified on a NENSORB column as described.

Oligo(dT)$_{16}$ was also 3' terminally labeled with [^{3}H]dTMP or [^{3}H]dGMP as above with the exception that the oligo(dT)$_{16}$ reaction concentration was 470 μM and the resultant oligo(dT)$_{16}$([^{3}H]dTMP)$_1$ or oligo(dT)$_{16}$([^{3}H]dGMP)$_1$ was purified on a NENSORB column as described.[2]

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; Me_2SO, dimethyl sulfoxide; DEAE, diethylaminoethyl; BuPdGTP, N^2-(*p-n*-butylphenyl)-2'-deoxyguanosine 5'-triphosphate.

[2] The number of terminal nucleotides added per 3'-hydroxyl to either poly(dT)$_{400}$ or oligo(dT)$_{16}$ was calculated from the DNA concentration and the specific activity of the nucleotide added.

Processivity of the Associated Exonuclease of DNA Polymerase δ. To measure the processivity of the associated exonuclease of DNA polymease δ, oligo(dT)$_{16}$, (dC)$_{10}$, (dA)$_{10}$, and (dG)$_{10}$ were 5′-end labeled using 3000 Ci/mmol [γ-^{32}P]ATP and T4 polynucleotide kinase in an exchange reaction according to Maniatis et al. (1982). DNA polymerase δ (0.3 unit) was added to reactions (25 μL) containing either 10 μM [^{32}P]oligo(dT)$_{16}$, (dC)$_{10}$, (dA)$_{10}$, or (dG)$_{10}$ under exonuclease assay conditions (see above). At time intervals of 0, 15, 30, 45, 60, 75, 90, 105, 120, and 135 min, 10% of the initial DNA was removed, and deionized formamide and EDTA were added to final concentrations of 50% and 25 mM, respectively. After an aliquot was removed, DNA polymerase δ (0.3 unit) was added at each time point. Samples were boiled for 5 min and then subjected to electrophoresis on 20% polyacrylamide gels as described. After electrophoresis, polyacrylamide gels were wrapped with Saran wrap, overlayed with Kodak X-Omat RP film, and exposed.

A homologous series of oligonucleotides was made by digesting [^{32}P]oligo(dT)$_{16}$ (0.3 mM) with 1×10^{-4} unit of the snake venom phosphodiesterase under exonuclease assay conditions (25 μL). At 0, 5, 15, and 25 min, 5 μL was removed and the reaction terminated with formamide and EDTA as above. The samples from each time point were combined and used as size standards on 20% polyacrylamide gels.

Processivity of the associated exonuclease of DNA polymerase δ was also measured on a double-stranded DNA molecule, poly(dA)$_{4000-5000}$·[^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$ (see above for details). Reactions (15 μL) containing 0.63 μM (as 3′-hydroxyl) [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$ annealed to poly(dA)$_{4000-5000}$ (40:1) were treated with DNA polymerase δ (0.5 unit) under exonuclease conditions. At 30-min intervals, 20% of the initial DNA was removed and terminated (as above) over a 2-h period. At each time interval, 0.5 unit of DNA polymerase δ was added. As controls, one reaction contained 4 mM AMP to inhibit 3′ to 5′ exonuclease, another utilized single-stranded [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$, another utilized double-stranded poly(dA)$_{4000-5000}$·[^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{3-5}$, and another contained 25 μM dTTP to support DNA synthesis.

Results

Processivity of DNA Polymerase δ Associated Exonuclease on Single-Stranded DNA Substrates. Processivity of DNA polymerase δ associated 3′ to 5′ exonuclease was measured on ^{32}P-end-labeled single-stranded DNA oligomers. The reactions were sampled at time intervals to observe the formation of intermediate digestion products and to observe the limiting size of single-stranded DNA that the exonuclease could digest. If digestion occurred by a highly processive mechanism, a progressive loss of the initial oligomer would be followed by the production of the limiting digestion products. In this case, essentially no intermediate products would be observed. If, however, the mechanism were nonprocessive, i.e., the exonuclease dissociated after the removal of each nucleotide, a distribution of intermediate products would be observed. The production of the limiting digestion products would then follow the digestion of these intermediate products. As shown in Figure 1, the digestion of [^{32}P]oligo(dT)$_{16}$ produces intermediate products that begin to appear (lane 2) at early reaction times and have a distribution between 11 and 16 nucleotides in length. As the reaction progresses, the size distribution becomes shorter (i.e., lanes 3–7) and eventually approaches the limiting size of single-stranded DNA that can be digested by DNA polymerase δ exonuclease (lanes 8–10). As shown, the minimum length of single-stranded DNA that can be digested by the 3′ to 5′ exonuclease is oligo(dT)$_{6-7}$. Essentially identical results were observed for other oligomers, (dC)$_{10}$ and (dA)$_{10}$ (not shown). The exonuclease digestion reaction thus occurs by a nonprocessive mode, and the minimum size for the reaction is not dependent upon nucleotide composition.

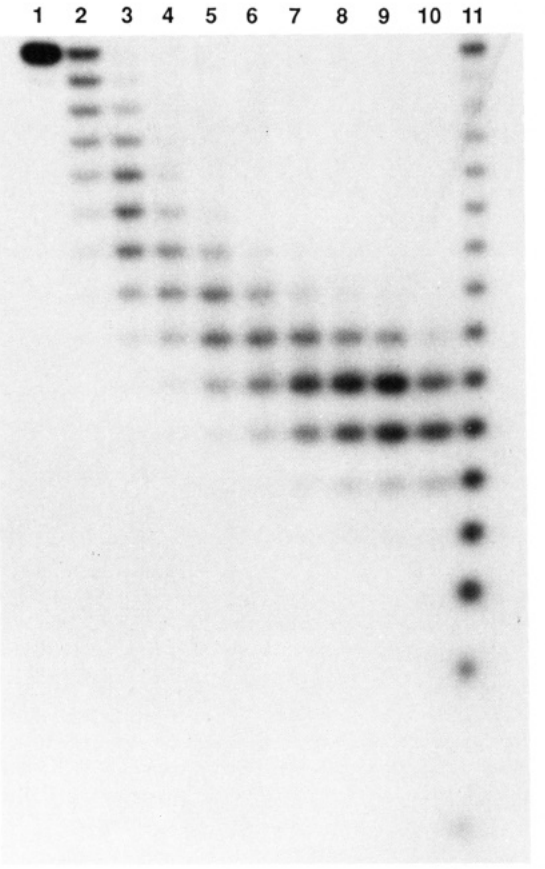

FIGURE 1: Processivity of DNA polymerase δ 3′ to 5′ exonuclease on single-stranded DNA. [^{32}P]Oligo(dT)$_{16}$ (10 μM) was digested with the 3′ to 5′ exonuclease of DNA polymerase δ (0.3 unit). Lane 1 indicates zero time of the digestion. Lanes 2–11 are successive 15-min time intervals of the reaction. At each time interval, 10% of the initial DNA was removed, and 0.3 unit of DNA polymerase δ was added. After the addition of formamide and EDTA, samples were electrophoresed on a 20% polyacrylamide gel. Lane 11 shows the products produced by the partial digestion of [^{32}P]oligo(dT)$_{16}$ with snake venom phosphodiesterase (1×10^{-4} unit), a nonprocessive 3′ to 5′ exonuclease.

Processivity and Reaction Rate of Exonucleolytic Activity at a Mismatched Primer Terminus. To measure the characteristics of DNA polymerase δ exonuclease on a mismatched primer terminus, [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$ was constructed (see Materials and Methods) and annealed to poly(dA)$_{4000-5000}$. Digestion of this substrate by the 3′ to 5′ exonuclease (Figure 2, lanes 7–11) demonstrates that hydrolysis of terminally mismatched mononucleotides occurs nonprocessively until the exonuclease approaches the region at which the DNA molecule becomes double stranded. At that point, the hydrolysis rate is greatly reduced, suggesting that double-stranded 3′-terminal nucleotides are poor substrates for DNA polymerase δ exonuclease. There is, however, a significant rate of removal of correctly base-paired nucleotides. This may be the result of digesting the 16 nucleotide long region during transient melting of the complementary base pairing. Another experiment that supports this conclusion is shown in Figure 3. Under DNA synthesis conditions (dTTP added to reactions), removal of the mismatched region is followed by the extension of the complementary 3′-terminus. As can be seen, oligomers shorter than 16 are not observed

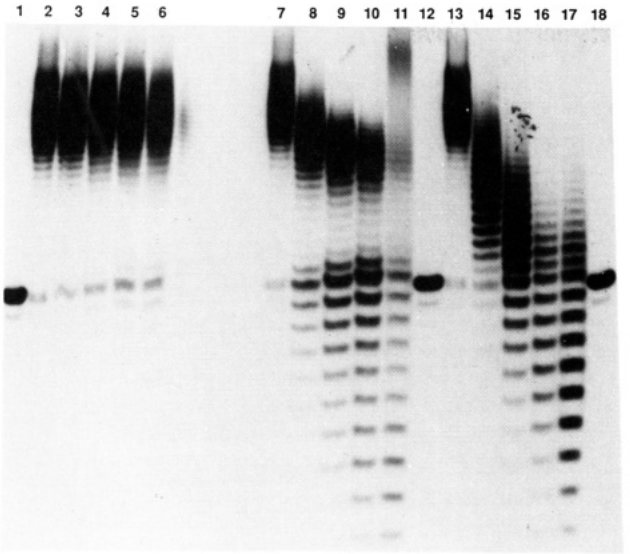

FIGURE 2: Digestion of single-stranded mismatched DNA by the 3′ to 5′ exonuclease of DNA polymerase δ. [^{32}P]Oligo(dT)$_{16}$([^{3}H]-dCMP)$_{14-28}$ was constructed as described under Materials and Methods and digested with the 3′ to 5′ exonuclease of DNA polymerase δ (0.5 units) under various conditions. Lanes 2–6 show the digestion of 0.63 μM (in 3′-hydroxyl ends) [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$·poly-(dA)$_{4000-5000}$ (40:1) in the presence of 4 mM AMP. Lanes 7–11 show the digestion of the same substrate in the absence of AMP. Lanes 13–17 show the digestion of the single-stranded [^{32}P]oligo(dT)$_{16}$-([^{3}H]dCMP)$_{14-28}$ (0.63 μM in 3′-hydroxyl ends). For each digestion, the left lane represents zero time. Subsequent lanes moving to the right represent successive 30-min time points. At each 30-min time point, 20% of the initial DNA was removed, and 0.5 unit of DNA polymerase δ was added. After the reactions were terminated with formamide and EDTA, samples were analyzed by 20% polyacrylamide gel electrophoresis. Lanes 1, 12, and 18 show the marker [^{32}P]oligo(dT)$_{16}$.

after mismatch removal (lanes 3 and 4).

It is noteworthy to mention that in the course of the digestion, the population of 1–6 nucleotide long mismatched reaction intermediates (Figures 2 and 3) is very low compared to longer intermediates. This suggests that there is either an acceleration of mismatch removal as the mismatch becomes shorter or an increase in the processivity. To distinguish between these two mechanisms, a shorter substrate, [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{3-5}$, was constructed and annealed to poly(dA)$_{4000-5000}$. Degradation, by the 3′ to 5′ exonuclease, indicates that this shorter mismatch is also degraded by a nonprocessive mechanism (not shown). Thus, we concluded that the low level of digestion intermediates with short mismatches (1–6 nucleotides) resulted from an increase in the rate of nucleotide hydrolysis.

The single-stranded [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$ was also digested as a control to determine whether the digestion kinetics of the mismatched substrate reflected the sequence heterogeneity of the primer. As can be seen (Figure 2, lanes 13–17), hydrolysis of single-stranded [^{32}P]oligo(dT)$_{16}$([^{3}H]-dCMP)$_{14-28}$ appears to occur at a uniform rate. This demonstrates that the observations made during digestion of the mismatched double-stranded DNA resulted from the double- and single-stranded character of the mismatched primer and not from nucleotide specificity of the exonuclease during digestion of the primer.

Finally, it is shown in Figure 2, lanes 2–6, that 5′-AMP, the product of 3′ to 5′ exonuclease hydrolysis (Wahl et al., 1986; Byrnes et al., 1976), inhibits the exonucleolytic degradation of [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$·poly(dA)$_{4000-5000}$, demonstrating that our observations are a direct result of 3′ to 5′ exonuclease digestion.

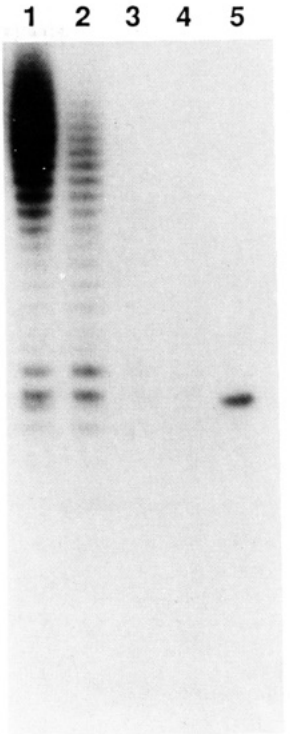

FIGURE 3: Digestion of single-stranded mismatched DNA by DNA polymerase δ 3′ to 5′ exonuclease under conditions allowing DNA synthesis. In the presence of 25 μM dTTP, 0.63 μM (in 3′-hydroxyl ends) [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$·poly(dA)$_{4000-5000}$ (40:1) was digested by the 3′ to 5′ exonuclease. The reaction was sampled at 0, 30, 60, and 90 min (lanes 1–4, respectively), 25% of the initial DNA was removed, and 0.5 unit of DNA polymerase δ was added. The reactions were terminated with formamide and EDTA and analyzed by 20% polyacrylamide gel electrophoresis. Synthesis results in a large diffuse area of radioactivity in lanes 2–4 not shown in Figure 3. Lane 5 shows the marker [^{32}P]oligo(dT)$_{16}$.

Exonuclease Activity on Oligo(dA)$_{16}$·Oligo(dT)$_{16}$([^{3}H]-dCMP)$_{14-28}$. We then attempted to determine whether the single-stranded poly(dA) template adjacent to the mismatched primer tested above was required for efficient digestion of a long single-stranded mismatch. For this experiment, digestion of [^{32}P]oligo(dT)$_{16}$([^{3}H]dCMP)$_{14-28}$ was titrated with oligo-(dA)$_{16}$ which can form a double strand with the (dT)$_{16}$ but does not contain a template single strand. The results (not shown) demonstrate that at an approximate (dA)$_{16}$:(dT)$_{16}$ ratio of 1:1, the exonuclease is unable to digest this DNA molecule. To eliminate the possibility that oligo(dA)$_{16}$ inhibits digestion of dC oligomers, oligo(dC)$_{10}$[^{3}H]dCMP (10 μM) was titrated, up to a 10-fold excess, with oligo(dA)$_{16}$. Under these conditions, oligo(dA)$_{16}$ only slightly inhibited the digestion of oligo(dC)$_{10}$[^{3}H]dCMP. Evidently, oligo(dA)$_{16}$·(dT)$_{16}$([^{3}H]-dCMP)$_{14-28}$ is an inactive analogue of the exonuclease substrate.

Inhibition of Polymerase and Exonuclease Activity by Oligonucleotides. The capacity of various oligonucleotides to differentially inhibit polymerase and exonuclease activities of DNA polymerase δ was assessed (Table I). When the homologous series (dG)$_4$, (dG)$_6$, and (dG)$_8$ was examined, it was found that (dG)$_4$ could inhibit digestion of (dT)$_{16}$[^{3}H]dTMP. This indicates that although the length of (dG)$_4$ is below the digestion limit, it is still capable of interfering with interaction of an effective substrate with the exonuclease active site. Although each member of the series was capable of inhibition, the maximum potency was not achieved until (dG)$_8$ was utilized as a digestion inhibitor. It should also be noted that this effect is specific for dG oligomers, since (dT)$_{16}$ and (dC)$_{10}$

Table I: Capacities of Oligonucleotides To Inhibit Differentially DNA Polymerase δ and the Associated 3′ to 5′ Exonuclease[a]

inhibitor	inhibn of DNA synthesis on poly(dA)·oligo-(dT)	inhibn of δ-exonuclease on poly(dT)[^{3}H]-dTMP	inhibn of δ-exonuclease on oligo(dT)$_{16}$[^{3}H]-dTMP
$(dG)_4$	0 (100 μM)[a]		10 (20 μM)
			40 (100 μM)
$(dG)_6$	0 (100 μM)		50 (20 μM)
			80 (100 μM)
$(dG)_8$	88 (20 μM)	0 (93 μM)	21 (2 μM)
	98 (100 μM)		100 (19 μM)
$(dT)_{10}$		0 (117 μM)	
$(dT)_{16}$		0 (153 μM)	11 (100 μM)
$(dT)_{400}$		60 (50 μM)	95 (2.5 μM)
		83 (100 μM)	
$(dC)_{10}$	0 (70 μM)	2 (93 μM)	4 (74 μM)
$(dA)_{12-18}$	0 (47 μM)		

[a] DNA synthetic activity on poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$ and 3′ to 5′ exonuclease activity on either long single-stranded DNA [poly(dT)$_{400}$-[^{3}H]dTMP] at 10 μM or short single-stranded DNA [oligo(dT)$_{16}$-[^{3}H]dTMP] at 10 μM were titrated with the various oligonucleotides indicated above. The values given are the percent inhibition by each oligonucleotide and represent a point on the titration curve at the indicated concentration.

inhibited only slightly even at high concentrations.

Comparing the digestion of $(dT)_{16}$[^{3}H]dTMP with that of poly(dT)$_{400}$[^{3}H]dTMP shows that none of the oligomers tested was capable of inhibiting digestion of the longer dT polymer. Since similar nucleotide concentrations of short- and long-labeled substrates were used, this phenomenon is not the result of a concentration difference between experiments. Instead, the exonuclease must have a binding preference for longer substrates. This conclusion is supported by the result showing that 100 μM $(dT)_{400}$ inhibits the digestion of the $(dT)_{400}$-[^{3}H]dTMP to 83%, while very low concentrations of $(dT)_{400}$ (2.5 μM) completely inhibit the digestion of $(dT)_{16}$[^{3}H]dTMP.

Among the oligonucleotides tested, only $(dG)_8$ inhibited DNA synthesis. In fact, $(dG)_8$ had the unique capacity to inhibit strongly both DNA synthesis and exonuclease activity on short oligomers, while having no inhibitory effect on digestion of $(dT)_{400}$ (Table I and Figure 4). This result suggests that exonuclease activity, at least for digestion of long polymers, may not require that the polymerase active site (or binding site) be functional. To support this conclusion, digestion of $(dT)_{16}$[^{3}H]dGMP·poly(dA)$_{4000-5000}$ was titrated with $(dG)_8$. As can be seen in Figure 4, digestion of the mismatched nucleotide is inhibited less than digestion of the free primer, suggesting that binding interactions between the enzyme and the dA polymer may interfere with $(dG)_8$ inhibition.

Salt Sensitivities of DNA Polymerase δ and the Associated 3′ to 5′ Exonuclease. DNA polymerase δ synthetic activity on poly(dA)$_{4000-5000}$·oligo(dT)$_{16}$ and the 3′ to 5′ exonuclease activity on poly(dT)$_{400}$([^{3}H]dTMP)$_2$, poly(dT)$_{400}$([^{3}H]-dTMP)$_2$·poly(dA)$_{4000-5000}$, and poly(dT)$_{400}$([^{3}H]dGMP)$_2$·poly(dA)$_{4000-5000}$ were titrated with KCl (Figure 5). Results

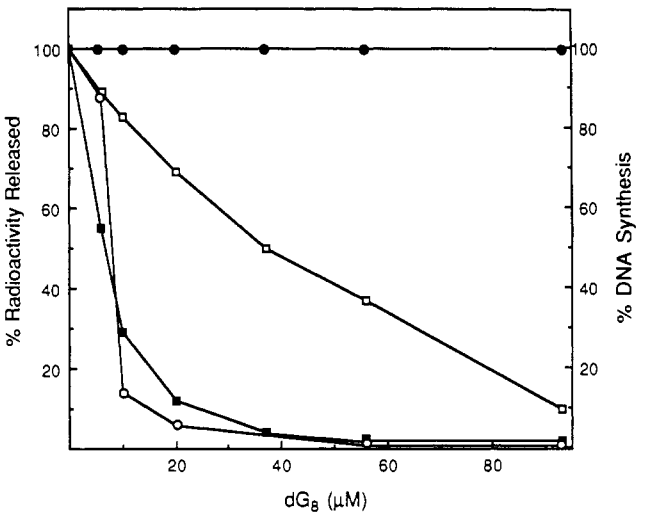

FIGURE 4: Inhibition of DNA polymerase δ and the associated 3′ to 5′ exonuclease by $(dG)_8$. DNA synthetic activity of DNA polymerase δ (0.10 unit) was determined in the presence of various amounts of $(dG)_8$ as indicated (open circles). Inhibition of the 3′ to 5′ exonuclease was also assessed by digesting 25 nM (in 3′-hydroxyl ends) poly-(dT)$_{400}$([^{3}H]dTMP)$_2$ (closed circles), 0.63 μM (in 3′-hydroxyl ends) (dT)$_{16}$[^{3}H]dGMP (closed squares), and 0.63 μM (in 3′-hydroxyl ends) (dT)$_{16}$[^{3}H]dGMP·poly(dA)$_{4000-5000}$ (80:1) (open squares) using DNA polymerase δ (0.8 unit) at the indicated amount of $(dG)_8$.

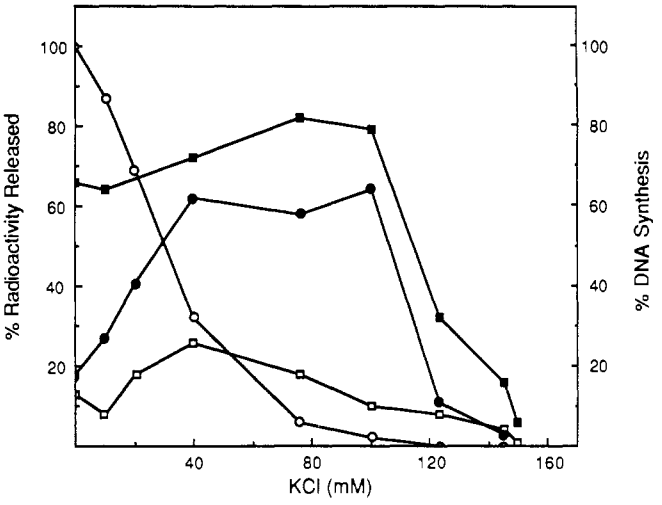

FIGURE 5: Salt sensitivities of DNA polymerase δ and the associated 3′ to 5′ exonuclease. DNA synthetic activity using 0.05 unit of DNA polymerase δ was determined in the presence of increasing amounts of KCl (open circles). The 3′ to 5′ exonuclease activity using 0.3 unit of DNA polymerase δ was determined on 25 nM (in 3′-hydroxyl ends) poly(dT)$_{400}$([^{3}H]dTMP)$_2$ (closed squares), 25 nM (in 3′-hydroxyl ends) poly(dT)$_{400}$([^{3}H]dTMP)$_2$·poly(dA)$_{4000-5000}$ (2:1) (open squares), and 25 nM (in 3′-hydroxyl ends) poly(dT)$_{400}$([^{3}H]dGMP)$_2$·poly(dA)$_{4000-5000}$ (2:1) (closed circles).

indicate that DNA polymerase δ synthetic activity is highly sensitive to salt. The rate of DNA synthesis is inhibited approximately 50% by addition of 30 mM KCl to the standard reaction. On the other hand, DNA polymerase δ exonuclease

Table II: Differential Inhibition of DNA Polymerase δ and the Associated 3′ to 5′ Exonuclease by Mononucleotides and Aphidicolin[a]

inhibn	inhibn of δ-exonuclease on poly(dT)([^{3}H]dGMP)$_4$	inhibn of δ-exonuclease on poly(dA)·poly-(dT)([^{3}H]dGMP)$_4$	inhibn of DNA synthesis on poly(dA)·oligo(dT)
20 mM AMP	20[b]	15	
20 mM GMP	80	85	
aphidicolin[c] (10 μg/mL)	6	5	78

[a] 3′ to 5′ exonuclease activity on poly(dT)$_{400}$([^{3}H]dGMP)$_4$ and on this substrate annealed to poly(dA)$_{4000-5000}$ (at a 1:4 ratio in nucleotide) was titrated with 5′-AMP and 5′-GMP. [b] The value for the inhibition of the exonuclease represents the percent inhibition at the indicated concentration. [c] The effect of aphidicolin on the 3′ to 5′ exonuclease on these substrates and on DNA synthesis was measured at 10 μg of aphidicolin/mL.

activity is very resistant to salt on both single-stranded and mismatched double-stranded DNA substrates. Inhibition to a level of 50% requires addition of more than 100 mM KCl. Exonuclease activity on complementary poly(dT)$_{400}$([^{3}H]-dTMP)$_2$·poly(dA)$_{4000-5000}$ is relatively low and is least affected by salt.

Inhibition of Polymerase and Exonuclease Activity by Mononucleotides and Aphidicolin. Results in Table II demonstrate that although AMP and GMP inhibit exonuclease activity, GMP is severalfold more effective. Inhibition of exonuclease activity on single-stranded poly(dT)$_{400}$([^{3}H]dG)$_4$, or on this substrate annealed to poly(dA) to form a mismatched primer terminus, was nearly equivalent.

Aphidicolin at 10 μg/mL inhibited DNA synthesis but had little effect on the exonuclease.

Discussion

Our results show that the 3′ to 5′ exonuclease of calf DNA polymerase δ has characteristics expected of a nuclease that functions to remove mismatched nucleotides incorrectly incorporated during DNA synthesis. The nonprocessive behavior of DNA polymerase δ exonuclease observed is similar to that of other proofreading exonucleases associated with *Escherichia coli* DNA polymerase I and T4 DNA polymerase (Thomas & Olivera, 1978). Crute et al. (1986) suggested that the 3′ to 5′ exonuclease of DNA polymerase δ has low processivity. We have now demonstrated, however, that the exonuclease is completely nonprocessive. The distribution of the oligonucleotide products suggests that a significant portion of the enzyme dissociates after the removal of one nucleotide. Such a mode of digestion should be preferred for a nuclease designed to remove a single mismatched nucleotide followed by the release of the 3′-terminus to the polymerase active site for further synthesis.

Although the 3′ to 5′ exonuclease is capable of efficient removal of single-mismatched nucleotides, it is incapable of completely digesting single-stranded DNA to mononucleotides. Digestion is limited on single-stranded DNA molecules to final products six to seven nucleotides in length. This result strongly suggests that the mismatched terminus is the natural substrate for this 3′ to 5′ exonuclease. During mismatched digestion, enzyme–DNA contacts are presumably made with either the double- and/or the single-stranded regions of the primer template that allow the enzyme to completely digest the entire mismatched region.

The exonuclease substrate specificity for misincorporated nucleotides was further suggested by several additional results. Exonuclease activity on a primer-template with a mismatch 14–28 nucleotides long seemed to accelerate as the mismatch length was reduced to 1–6, a structure resembling that likely to form from misincorporation during DNA synthesis. Also, when (dG)$_8$ was used in inhibition studies, mismatch removal occurred while digestion of single-stranded DNA was inhibited, suggesting that extra favorable enzyme–DNA contact points occur at the 3′-terminal mismatch. Digestion of [^{32}P]oligo-(dA)$_{16}$·oligo(dT)([^{3}H]dCMP)$_{14-28}$, a structure similar to a mismatch that is lacking the adjacent single-stranded template, also demonstrated that the increased rate of digestion, observed when a mismatched primer is annealed to poly(dA), does not occur. These results strongly suggest that DNA–protein binding sites, beyond those that occur with single-stranded DNA, are required for the removal of nucleotides misincorporated during DNA synthesis.

Our results do not suggest, however, that the polymerase and exonuclease activities are interdependent. Titration of these activities with salt demonstrates that the polymerase activity can be almost completely inhibited with little effect on the exonuclease. These salt sensitivities are similar to those observed for calf DNA polymerase δ purified by Lee et al. (1984).

The polymerase is also highly sensitive to aphidicolin (10 μg/mL). However, at this drug concentration, exonuclease digestion on either single-stranded DNA or mismatched DNA is not inhibited. The exonuclease resistance to aphidicolin on single-stranded DNA is similar to that reported for DNA polymerase δ from calf (Lee et al., 1984) and rabbit bone marrow (Byrnes, 1984). Resistance on mismatched DNA, however, contrasts that reported by Lee et al. (1984) and Byrnes (1984). The apparent contradiction may result from use of different substrates, i.e., poly(dT)$_{400}$([^{3}H]dGMP)$_4$·poly(dA)$_{4000-5000}$ in our work, compared to alternating dA·[^{3}H]dT used in previous work.

Current results are all consistent with our previous report demonstrating that the 3′ to 5′ exonuclease of DNA polymerase δ functions as a proofreading exonuclease (Kunkel et al., 1987). We have found the exonuclease digests single-stranded mismatches nonprocessively, evidently requires multiple DNA contact points at the mismatched 3′-terminus, and has substrate specificities expected for a proofreading exonuclease.

Acknowledgments

We thank David Berube for contributing to early stages of this project and Michelle Dziejman for expert technical assistance.

References

Byrnes, J. J. (1984) *Mol. Cell. Biochem. 62*, 13–24.

Byrnes, J. J., Downey, K. M., Black, V. L., & So, A. G. (1976) *Biochemistry 15*, 2817–2823.

Chen, Y.-C., Bohn, E. W., Planck, S. R., & Wilson, S. H. (1979) *J. Biol. Chem. 254*, 11678–11687.

Cotterill, S. M., Reyland, M. E., Loeb, L. A., & Lehman, I. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5635–5639.

Crute, J. J., Wahl, A. F., & Bambara, R. A. (1986) *Biochemistry 25*, 26–36.

Fry, M., & Loeb, L. A. (1986) in *Animal Cell DNA Polymerases*, CRC, Boca Raton, FL.

Hammond, R. A., Byrnes, J. J., & Miller, M. R. (1987) *Biochemistry 26*, 6817–6824.

Harwell, L. W., Lord, E. M., & Bambara, R. A. (1986) *Biochemistry 25*, 7821–7827.

Kornberg, A. (1980) in *DNA Replication*, pp 201–229, W. H. Freeman, San Francisco, CA.

Kunkel, T. A., Sabatino, R. D., & Bambara, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4865–4869.

LaDuca, R. J., Crute, J. J., McHenry, C. S., & Bambara, R. A. (1986) *J. Biol. Chem. 261*, 7550–7557.

Lee, M. Y. W. T., & Toomey, N. L. (1987) *Biochemistry 26*, 1076–1085.

Lee, M. Y. W. T., Tan, C., Downey, K. M., & So, A. G. (1984) *Biochemistry 23*, 1906–1913.

Ottiger, H. P., & Hubscher, U. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3993–3997.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Skarnes, W., Bonin, P., & Baril, E. (1986) *J. Biol. Chem. 261*, 6629–6636.

Thomas, K. R., & Olivera, B. M. (1978) *J. Biol. Chem. 253*, 424–429.

Wahl, A. F., Crute, J. J., Sabatino, R. D., Bodner, J. B., Marracinno, R. L., Harwell, L. W., Lord, E. M., & Bambara, R. A. (1986) *Biochemistry 25*, 7821–7827.

Zhang, S. J., & Lee, M. Y. W. T. (1987) *Arch. Biochem. Biophys. 252*, 24–31.