Characterization of the 5′ to 3′ exonuclease associated with *Thermus aquaticus* DNA polymerase

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ABSTRACT

***Thermus aquaticus* DNA polymerase was shown to contain an associated 5′ to 3′ exonuclease activity. Both polymerase and exonuclease activities cosedimented with a molecular weight of 72,000 during sucrose gradient centrifugation. Using a novel *in situ* activity gel procedure to simultaneously detect these two activities, we observed both DNA polymerase and exonuclease in a single band following either nondenaturing or denaturing polyacrylamide gel electrophoresis: therefore, DNA polymerase and exonuclease activities reside in the same polypeptide. As determined by SDS-polyacrylamide gel electrophoresis this enzyme has an apparent molecular weight of 92,000. The exonuclease requires a divalent cation ($MgCl_2$ or $MnCl_2$), has a pH optimum of 9.0 and excises primarily deoxyribonucleoside 5′-monophosphate from double-stranded DNA. Neither heat denatured DNA nor the free oligonucleotide (24-mer) were efficient substrates for exonuclease activity. The rate of hydrolysis of a 5′-phosphorylated oligonucleotide (24-mer) annealed to M13mp2 DNA was about twofold faster than the same substrate containing a 5′-hydroxylated residue. Hydrolysis of a 5′-terminal residue from a nick was preferred threefold over the same 5′-end of duplex DNA. The 5′ to 3′ exonuclease activity appeared to function coordinately with the DNA polymerase to facilitate a nick translational DNA synthesis reaction.**

INTRODUCTION

Thermus aquaticus (*Taq*) DNA polymerase was originally purified and characterized as a relatively thermostable enzyme (1,2). The enzyme preparation was reported to be free of detectable phosphomonoesterase, phosphodiesterase, and single-stranded exonuclease activities (1). In addition, *Taq* DNA polymerase was shown to lack an associated 3′ to 5′ exonuclease activity capable of excising a 3′-terminally mismatched base (3). Recently the *Taq* DNA polymerase gene was isolated, sequenced, and expressed in *Escherichia coli* (4). From the predicted 832-amino acid sequence and Western blot analysis, *Taq* DNA polymerase appears to have a polypeptide molecular weight of ~94,000 (4). Significant amino acid sequence similarity exists between *Taq* DNA polymerase and *E. coli* DNA polymerase I. The C-terminal region of *Taq* DNA polymerase resembles the *E. coli* DNA polymerase I domain known to contain DNA polymerase activity (4,5,6); whereas, the N-terminal region (residues 1−300) corresponds to the *E. coli* DNA polymerase I domain containing the 5′ to 3′ exonuclease activity (4,7). In contrast, no corresponding similarity to the 3′ to 5′ exonuclease domain of *E. coli* DNA polymerase I has been noted.

Taq DNA polymerase has proven to be a suitable enzyme for *in vitro* amplification of DNA fragments via the polymerase chain reaction (PCR) and also for DNA sequencing (8,9,10). The increased use of *Taq* DNA polymerase for various molecular biology applications warrants further characterization of this DNA polymerase. In this paper, we (i) identify a 5′ to 3′ exonuclease within the *Taq* DNA polymerase polypeptide; (ii) characterize the catalytic activity of this exonuclease; and (iii) examine the ability of the polymerase/exonuclease to conduct nick translational DNA synthesis.

MATERIALS AND METHODS

Reagents and Chemicals

Prestained molecular weight protein markers (SDS-7B), unlabeled 2′-deoxyribonucleoside 5′-triphosphates, dGMP and ATP were obtained from Sigma. Pharmacia was the source of 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP); whereas [γ-^{32}P]ATP, [α-^{32}P]dGTP, and [^{3}H]dTTP were from New England Nuclear.

E. coli DNA polymerase I large fragment (LF) and *Eco*R1 were purchased from New England Biolabs. T4 polynucleotide kinase and *Taq* DNA polymerase were from Bethesda Research Laboratories and calf intestinal alkaline phosphatase was from Sigma.

M13mp2 and M13mp2 (-C106) DNA were isolated as described by Kunkel et al. (11). Oligonucleotides GGCG-ATTAAGTTGGG (15-mer), TAACGCCAGGGTTTTCCCA (19-mer), GTGCTGCAAGGCGATTAAGTTGGT (24-merT) and GTGCTGCAAGGCGATTAAGTTGGG (24-merG) were

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prepared using an Applied Biosystems Model 380A Synthesizer by the Gene Research Center (Oregon State University).

Preparation of DNA Substrates

Deblocked/deprotected oligodeoxyribonucleotides were purified (12) and phosphorylated at the 5'-end in reactions (75 μl) containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.33 μM [γ-^{32}P]ATP (6000 Ci/mmol), 10.5 pmol oligonucleotide and 30 units of T4 polynucleotide kinase. After incubation at 37°C for 60 min, reactions were terminated by the addition of 9 μl of 0.1 M EDTA and by heating at 70°C for 5 min. Analysis on a 12% polyacrylamide DNA sequencing gel indicated that labeled oligonucleotides were ≥95% pure and had a specific activity of $3.0-3.5\times10^6$ cpm/pmol of 5'-ends. Double-stranded DNA substrates were formed by annealing oligonucleotide to M13 DNA (0.2 pmol of 5'-ends/μg DNA at 63 μg/ml) by heating to 70°C followed by slow cooling to 25°C as previously described (12).

Oligonucleotides to be labeled at the 3'-end were 5'-phosphorylated as above except [γ-^{32}P]ATP was replaced with 1 mM ATP and EDTA was omitted from the termination reaction. M13mp2 DNA (53 μg in 24 μl) was added and hybridization was performed as above. Labeling reactions (106 μl) contained 24-merT/M13 hybrid DNA, 21 pmol [α-^{32}P]dGTP (3,000 Ci/mmol), and 5 units of *E. coli* DNA polymerase I (LF). After DNA synthesis at 37°C for 40 min, reactions were terminated by adding 10.6 μl of 0.1 M EDTA and by heating at 70°C for 5 min. Then the mixture was adjusted to 150 mM NaCl and 15 mM sodium citrate and rehybridized as above (63 μg/ml M13 DNA). A 5'-phosphorylated oligonucleotide with a matched 3'-terminus was similarly labeled except 13.3 pmol of 19-mer, hybridized to 47.6 μg M13mp2 DNA, was incubated with 26.7 pmol [α^{32}P]dGTP on ice for 5 min. Labeled oligonucleotides were determined to be ≥95% pure with a specific activity of $1.8-3.1\times10^6$ cpm/pmol of 3'-ends.

Enzyme Assays

Standard DNA polymerase reaction mixtures (100 μl) contained 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 50 mM NaCl, 40 μg bovine serum albumin (BSA), 15% (w/v) glycerol, 100 μM each dATP, dGTP, dCTP, and [^{3}H]dTTP (56 cpm/pmol), 10 μg activated calf thymus DNA and 0.003−0.1 units of *Taq* DNA polymerase. When necessary, polymerase was diluted in 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 100 μg/ml BSA and 15% (w/v) glycerol. Reactions were incubated at 70°C for 60 min and stopped on ice by addition of 200 μl of 1 mg/ml of BSA in 0.1 M sodium pyrophosphate. The DNA was precipitated with 1 ml of 10% (saturation) trichloroacetic acid and collected on Schleicher and Schuell #30 glass filters as previously described (13). Acid-insoluble radioactivity was measured using a Beckman LS-6800 liquid scintillation spectrometer with 0.4% 2,5-bis-2-(5-tertbutyl-benzoxazolyl)-thiophene in toluene as the scintillator. One unit of *Taq* DNA polymerase activity catalyzes incorporation of 10 nmol total dNMP into DNA in 30 minutes at 70°C. One unit of *E. coli* DNA polymerase I (LF) was defined similarly except at 37°C.

Standard exonuclease reaction mixtures (25 μl) contained 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 50 mM NaCl, 12.6 μg/ml ^{32}P-labeled oligonucleotide annealed to M13mp2 DNA and 0.1−0.3 units of *Taq* DNA polymerase. Following incubation at 50°C for 60 min, reactions were transferred to ice and terminated by the addition (25 μl) of a mixture containing 95% deionized formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. Samples (3 μl) were analyzed by electrophoresis using a 12% polyacrylamide (29:1), 8.3 M urea DNA sequencing gel at 1000 V (45 mA/gel) described by Kunkel and Mosbaugh (14). Autoradiography was performed at room temperature using Kodak XAR-5 film and radioactive bands were quantified after excision from the gel using a liquid scintillation spectrometer.

Sucrose Gradient Centrifugation

The native molecular weight of *Taq* DNA polymerase was determined by centrifugation through a linear 6−20% sucrose gradient containing 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, 500 mM NaCl, 10 mM NaN_3, and 8% (w/v) glycerol. Samples (200 μl) containing i) *Taq* DNA polymerase and 10 mg/ml BSA or ii) 10 mg/ml BSA and 4.3 units of calf intestinal alkaline phosphatase were layered onto gradients. Centrifugation was performed in a Beckman SW50.1 rotor at 48,000 rpm for 24 h at 4°C. Fractions (7 drops) were collected from the bottom of the gradient. The location of BSA (Mr = 68,000; S = 4.7) was determined using the Bio-Rad protein assay, originally described by Bradford (15) and alkaline phosphatase (Mr = 100,000; S = 6.2) was detected as previously described (16). Molecular weights were determined by the method of Martin and Ames (17).

In Situ Activity Polyacrylamide Gels

Detection of *Taq* DNA polymerase and exonuclease activities *in situ* following nondenaturing polyacrylamide gel electrophoresis was performed by a modification of the procedure described by Longley and Mosbaugh (18). In brief, the resolving gel contained 10% acrylamide, 0.37% N,N'-methylene-*bis*-acrylamide, 375 mM Tris-HCl (pH 8.8), 50 μg/ml bovine fibrinogen, 2 mM EDTA, 0.95 μg/ml each of 5'-end ^{32}P-labeled 15-mer/M13 DNA and 3'-end ^{32}P-labeled 24-merG/M13 DNA, 0.07% ammonium persulfate and 0.03% TEMED. Stacking gels were composed of 2.5% acrylamide, 0.625% N,N'-methylene-*bis*-acrylamide, 62.5 mM Tris-HCl (pH 6.8), 20% sucrose, 2 mM EDTA, 5 μg/ml riboflavin, and 0.1% TEMED. Samples of *Taq* DNA polymerase were adjusted to final concentrations of 30% (w/v) glycerol and 0.008% bromophenol blue and 26 μl loaded into a well.

In situ detection of *Taq* DNA polymerase using SDS-polyacrylamide activity gels was performed essentially as described by Longley and Mosbaugh (12,18). The enzyme was resolved through a 10% polyacrylamide resolving gel (acrylamide:N,N'-methylene-*bis*-acrylamide, ratio 37.5:1) containing the components described above, except that 0.1% SDS was included. The stacking gel was composed of 4.5% acrylamide, 0.2% N,N'-methylene-*bis*-acrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% SDS and 2 mM EDTA. Samples (23 μl) were prepared by heating at 37°C for 3 min in 54 mM Tris-HCl (pH 6.8), 1.7 mM EDTA, 120 mM 2-mercaptoethanol, 6.25% (w/v) glycerol, 0.8% SDS and 0.03% bromophenol blue.

Electrophoresis on both nondenaturing and denaturing activity gels was performed at 4°C using an analytical gel format as described previously (18). For denaturing activity gels only, the SDS was extracted from the resolving gel at 25°C and the enzyme renatured at 4°C as described (18). Following electrophoresis (or enzyme renaturation for SDS-polyacrylamide gels) *in situ* DNA polymerase/exonuclease reactions were initiated by

immersing resolving gel slices (~0.2×5 cm) in reaction buffer (5 ml) containing 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 50 mM NaCl, 400 μg/ml BSA, 15% (w/v) glycerol and 100 μM ddTTP. After incubation at 50°C for 15 or 30 min, the reactions were stopped, placed on ice, and adjusted to 10 mM EDTA. Following incubation at 4°C for 30 min, ^{32}P-labeled DNA reaction products within the gel were analyzed by electrophoresis through a second dimensional DNA sequencing gel (12,18). Briefly, the analytical activity gel slices were rotated 90° and cast horizontally within a 20% polyacrylamide/urea DNA sequencing gel. Electrophoresis was conducted, the gel was dried, and autoradiography was performed using Kodak XAR-5 film.

RESULTS

Detection of 5′ to 3′ Exonuclease Activity

Taq DNA polymerase was examined for associated 5′ to 3′ exonuclease activity following sedimentation in a sucrose gradient containing 0.5 M NaCl. Using a 3′-end ^{32}P-labeled oligonucleotide (24-mer) hybridized to M13mp2 DNA as a substrate, both DNA polymerase and exonuclease activities were detected as a single peak with a molecular weight of 72,000 ± 4,000 (Figure 1). From this denaturing DNA sequencing gel analysis, two shorter products (22- and 23-mers) were observed

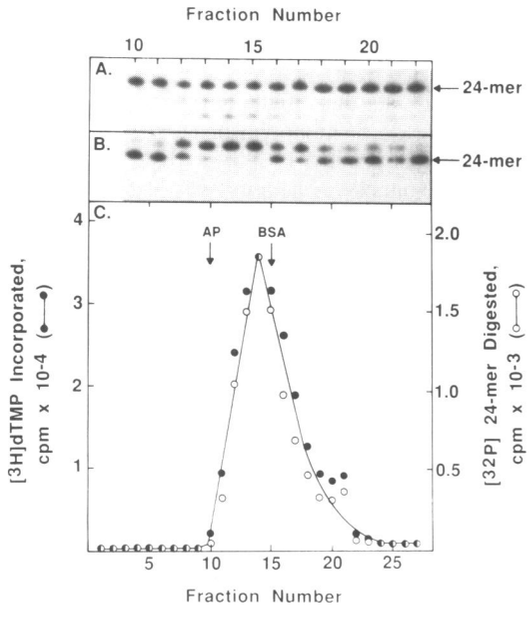

Figure 1. Sucrose gradient sedimentation of *Taq* DNA polymerase. A sample (200 μl) containing *Taq* DNA polymerase (20 units) and BSA (2 mg) was layered onto a linear 6–20% sucrose gradient and centrifugation carried out as described under Materials and Methods. Fractions (185 μl) were collected and dialyzed into buffer containing 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, and 50 mM NaCl. (A) Standard exonuclease reactions containing 12.6 μg/ml 3′-end ^{32}P-labeled 24-merG/M13mp2 DNA and 12.5 μl of each fraction were incubated at 50°C for 120 min. (B) DNA polymerase activity was measured in identical reactions containing 100 μM ddTTP and 7.5-fold dilutions of gradient fractions. Both reactions were terminated on ice, samples (3 μl) analyzed by electrophoresis and ^{32}P-labeled DNA detected by autoradiography. (C) Standard DNA polymerase reactions were carried out on activated calf thymus DNA using 10 μl of each undialyzed fraction as described under Materials and Methods. After incubation at 70°C for 60 min, acid insoluble [^{3}H]dTMP incorporation was measured using a liquid scintillation counter. The vertical arrows indicate the location of the internal protein marker (BSA) and the external marker alkaline phosphatase (AP). Sedimentation was from right to left.

which indicated the exonuclease acted in the 5′ to 3′ direction (Figure 1A). Furthermore, no 3′ to 5′ exonuclease activity was observed. DNA polymerase activity was detected in identical reactions containing 100 μM ddTTP. These reactions detected DNA polymerase activity at 50°C by the incorporation of ddTMP onto the ^{32}P-labeled DNA primer (24-mer) generating a product 25 nucleotides in length (Figure 1B). To verify the thermostability of this activity, we also monitored DNA polymerase activity at 70°C for the incorporation of [^{3}H]dTMP into activated DNA (Figure 1C). Again a single peak of activity coincident with the exonuclease was observed. Taken together, these results suggest that *Taq* DNA polymerase exists in association with the 5′ to 3′ exonuclease activity.

Taq DNA Polymerase and 5′ to 3′ Exonuclease are Contained in the Same Polypeptide

To determine if the DNA polymerase and 5′ to 3′ exonuclease activities reside in the same or separate polypeptides we utilized a novel *in situ* activity gel technique (12,18). A sample of *Taq* DNA polymerase was first resolved through a nondenaturing polyacrylamide gel containing two defined ^{32}P-labeled oligonucleotides, both annealed to M13mp2 DNA. One oligonucleotide (15-mer) was ^{32}P-labeled at the 5′-end and the other oligonucleotide (24-mer) was ^{32}P-labeled at the 3′-end. After electrophoresis, the *Taq* DNA polymerase catalyzed *in situ* reactions (polymerase and exonuclease) in the presence of ddTTP and the resulting [^{32}P]DNA products were resolved from the protein gel by a second dimension of electrophoresis through a denaturing DNA sequencing gel. Detection of elongated or degraded [^{32}P]oligonucleotide chains was used to locate the polymerase and exonuclease activities, respectively. As observed in Figure 2A, incorporation of ddTMP onto one DNA primer (15-mer) produced the expected 16-mer product by polymerization (Rf = 0.15). At the same location in the protein polyacrylamide gel, 5′ to 3′ exonucleolytic activity was detected by the appearance of products smaller than the 24-mer. Thus, both activities comigrated during electrophoresis. To ascertain whether these activities existed in the same polypeptide, the *in situ* activity gel analysis was repeated under the polypeptide-denaturing conditions of a SDS-polyacrylamide gel. Following electrophoresis, the SDS was extracted from the gel and the enzyme was allowed to renature in the presence of the same two [^{32}P]DNA substrates. As before, *in situ* reactions were performed in the presence of ddTTP and polymerization or degradation products were located by autoradiography (Figure 2B). Both *Taq* DNA polymerase and 5′ to 3′ exonuclease migrated in the same band with a molecular weight of 92,000 ± 6,000. From these results we concluded that both activities reside in the same polypeptide.

Characterization of 5′ to 3′ Exonuclease Activity

Reaction Requirements

The exonuclease activity has an absolute requirement for a divalent cation which can be satisfied by either $MgCl_2$ or $MnCl_2$. Optimal activity was observed at 4–8 mM $MgCl_2$ or 3 mM $MnCl_2$; however, $MnCl_2$ was the preferred divalent cation. Reactions carried out in 3 mM $MgCl_2$ resulted in 3% of the exonuclease activity detected with the same concentration of $MnCl_2$.

pH Optimum

Exonuclease activity was detected over a broad pH range (6.5–9.5) with maximal activity observed with 50 mM Tris-HCl

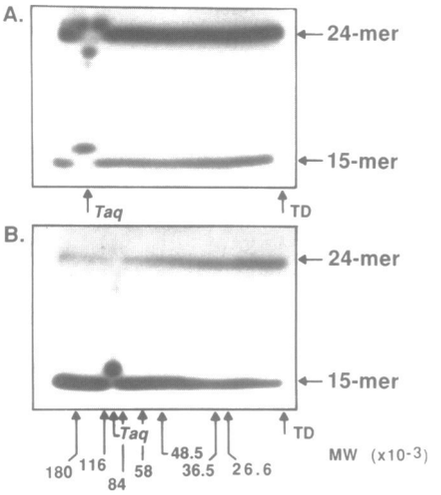

Figure 2. *In situ* detection of *Taq* DNA polymerase and exonuclease following polyacrylamide gel electrophoresis. (A) *Taq* DNA polymerase (0.04 units) was loaded (26 μl) onto a 10% polyacrylamide gel. Electrophoresis, *in situ* enzyme reactions and [^{32}P]DNA product analysis using a second dimensional DNA sequencing gel were as described under Materials and Methods. The protein gel was sliced vertically into segments (~ 0.2×5 cm) and *in situ* reaction buffer (5 ml) was added. After 15 min at 50°C, the *in situ* reaction was stopped by adding 500 μl of 0.1 M EDTA on ice, the buffer replaced with 20 mM Tris-HCl (pH 9.0), 10 mM EDTA, 50 mM NaCl, 400 μg/ml BSA, and 15% (w/v) glycerol and the gel slice agitated at 4°C for 30 min. The gel slice was reoriented (rotation of 90° from the first dimension of electrophoresis) and cast within a 20% polyacrylamide/8.3 M urea DNA sequencing gel which allowed size analysis of ^{32}P-labeled DNA products after a second dimension of electrophoresis. Following migration at 1200 V until the bromophenol blue dye moved 18−20 cm, the gel was dried and autoradiography performed at room temperature for 24 hr. (B) A sample (23 μl) of *Taq* DNA polymerase (0.51 units) in denaturation buffer was loaded onto a 10% polyacrylamide gel containing 0.1% SDS. Electrophoresis, SDS extraction, enzyme renaturation, *in situ* enzyme reactions (30 min at 50°C), and [^{32}P]DNA product analysis by DNA sequencing gel electrophoresis were as described under Materials and Methods. The horizontal arrows locate the ^{32}P-labeled 15- and 24-mer DNA substrates. Polypeptide molecular weight was determined using the prestained proteins (vertical arrows): α_2-macroglobulin (180,000), β-galactosidase (116,000), fructose-6-phosphate kinase (84,000), pyruvate kinase (58,000), fumarase (48,500), lactic dehydrogenase (36,500) and triosephosphate isomerase (26,600) which were resolved in an adjacent lane. Vertical arrows also locate the DNA polymerase/exonuclease activity (*Taq*) and the tracking dye (TD).

buffer at pH 9.0. At pH 8.5 and 9.5 the exonuclease activity was reduced to 89% and 96% maximal activity. Near maximal activity was also observed with 50 mM Hepes-KOH (pH 8.5) and CAPS-KOH (pH 9.0−9.5) buffers. However, MES-KOH (pH 6) resulted in 25% maximal activity.

Substrate Specificity

To analyze the substrate specificity and characterize the exonuclease reaction products, standard reactions were carried out using a 5′-end ^{32}P-labeled oligonucleotide (24-mer) which in some experiments was annealed to M13mp2 (-C106) DNA as substrate. These reactions were performed at either 50°C or 70°C, and reactions products were analyzed by polyethylenimine (PEI) thin layer chromatography. Double-stranded DNA was clearly the preferred substrate as the rate of hydrolysis on the free oligonucleotide at 50°C and 70°C was >100-fold reduced (Figure 3). Although exonuclease activity was detected at 70°C using the 24-merT/M13mp2(-C106) DNA substrate, the rate was about 45% of that observed at 50°C. The result at 70°C illustrates the thermostability of the exonuclease. The reduction in activity is most likely due to a significant fraction of the oligonucleotide

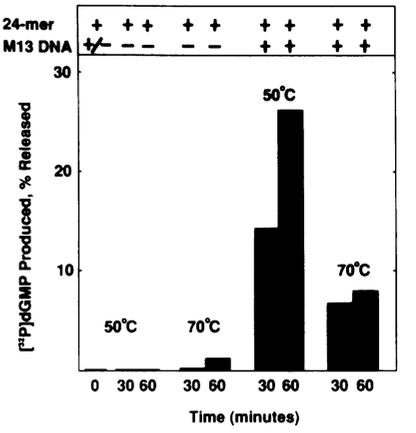

Figure 3. Exonuclease activity of *Taq* DNA polymerase on single- and double-strand DNA substrates. Standard exonuclease reaction mixtures (25 μl) were prepared contained 12.6 μg/ml 5′-end ^{32}P-labeled 24-merT/M13mp2 (-C106) DNA and 0.2 units of *Taq* DNA polymerase. Incubation was for either 30 or 60 min at 50°C or 70°C as indicated above. Reactions were terminated on ice with the addition of 3 μl of 10.7 mM EDTA. Samples (5 μl) were spotted along with 5 μl of 10 mM dGMP on a PEI thin layer chromatographic sheet. Ascending chromatography was performed using 1.2 M LiCl as solvent. After the solvent migrated 17 cm, the chromatogram was examined under a Mineralight Model UVGL-25 (254 nm) to locate the internal dGMP marker. Each lane was sliced into 1 cm segments and incubated with 500 μl of H_2O for 30 min before 5 ml of Ready Protein$^+$ (Beckman) was added and the radioactivity measured in each slice. Total release of the 5′-terminal nucleotide would equal 66 fmol of [^{32}P]dGMP per reaction.

being denatured at this high temperature. In all cases, deoxyribonucleoside 5′-monophosphate ([^{32}P]dGMP) was the primary hydrolysis product (>90%) (data not shown).

The rate of exonuclease activity was also determined using 3′-end ^{32}P-labeled oligonucleotides constructed with either a 5′-phosphoryl or a 5′-hydroxyl terminus. When these oligonucleotides were annealed to M13mp2 DNA, exonuclease activity was about 2-fold greater on the 5′-phosphorylated oligonucleotide compared to the same substrate containing a 5′-terminal hydroxyl group (Figure 4). As expected with the 5′-phosphorylated DNA substrate, the 23-mer and then the 22-mer oligonucleotide products were generated with time, suggesting sequential removal of mononucleotides from the 24-mer. Curiously, it appeared that the 22-mer was produced almost exclusively in reactions involving the 5′-hydroxylated DNA substrate. Whether this resulted from the release of a dinucleotide or from an altered degree of exonuclease processivity remains to be determined.

The rate of exonuclease activity on the 5′-end of a nick in DNA was determined using the DNA substrates schematically illustrated in Figure 5A. Hydrolysis rates were measured on the 20-mer/M13mp2 DNA in the presence and absence of a second oligonucleotide (24-mer) annealed immediately upstream. We observed that the 5′ to 3′ exonuclease acted threefold faster at the 5′-end in the nicked substrate (Figure 5B). Comparison of competing rates of hydrolysis of the 20-mer and 24-mer when both were present confirmed the preference for the 5′-end at the nick (Figure 5B, lanes 8−14). Clearly the majority of the 20-mer (3′-terminal ^{32}P-label) was degraded to a 19-mer and some smaller products, whereas the 24-mer (5′-terminal ^{32}P-label) almost completely escaped degradation. Only a small amount of [^{32}P]dGMP was detected at the bottom of the gel. Although the exact amount of [^{32}P]dGMP was difficult to quantitate, the

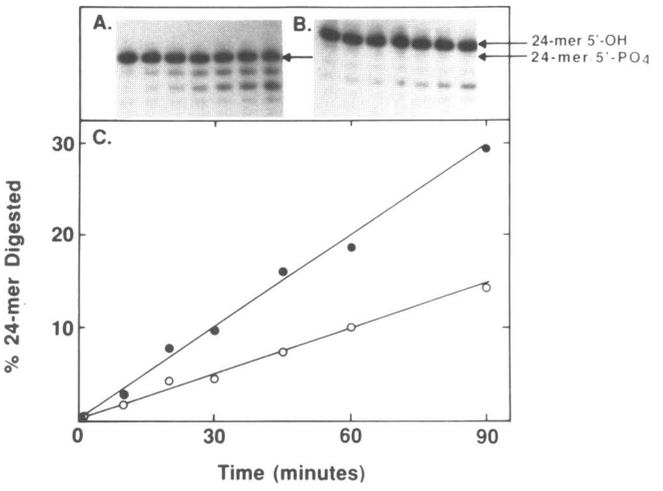

Figure 4. Exonuclease activity of *Taq* DNA polymerase on DNA substrates containing a 5′-terminal phosphoryl or hydroxyl group. Standard exonuclease reaction mixtures (80 μl) were prepared each containing 1 unit of *Taq* DNA polymerase and 12.6 μg/ml 3′-end ^{32}P-labeled 24-merG/M13mp2 DNA containing either (A) 5′-phosphoryl, or (B) 5′-hydroxyl termini. After incubation at 50°C for 0, 10, 20, 30, 45, 60, and 90 min (lanes 2–7 or 9–14, respectively), 10 μl aliquots were removed into 10 μl of formamide/dye mix. Lanes 1 and 8 contain substrates from control reactions incubated without DNA polymerase. Samples (3 μl) were analyzed by electrophoresis using a DNA sequencing gel and autoradiography was performed as described under **Materials and Methods**. The arrows indicate the location of the two DNA substrates (24-mer/PO_4 and 24-mer/OH). Total digestion of the 24-mer would result in 26 fmol of [^{32}P]DNA products per aliquot. (C) A time course of exonuclease activity on 5′-phosphoryl (●–●) and 5′-hydroxyl (○–○) termini is shown.

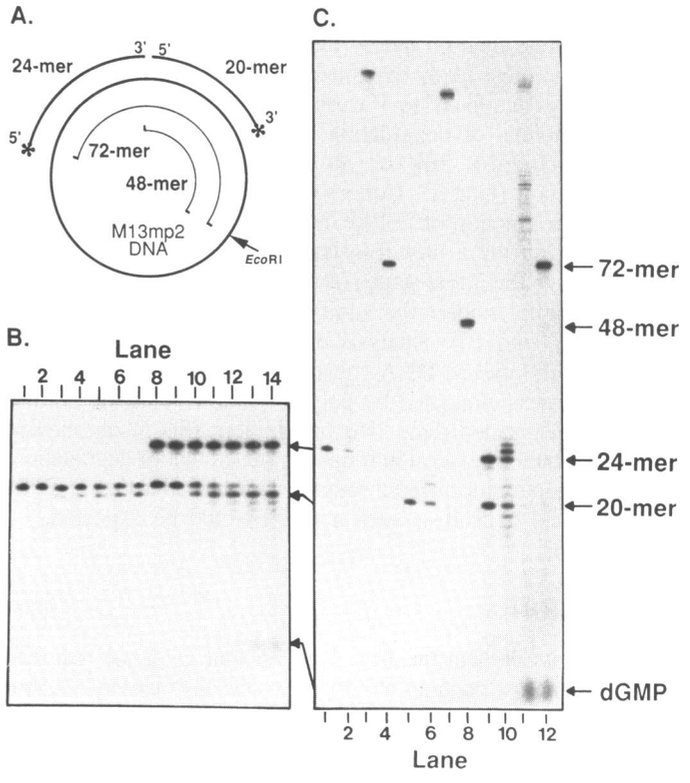

Figure 5. *Taq* DNA polymerase and exonuclease activity on a nicked DNA substrate. (A) A 5′-end ^{32}P-labeled 24-merG (oligonucleotide I) and/or a 3′-end ^{32}P-labeled 20-mer (oligonucleotide II) were singly or dually hybridized (0.28 pmol each oligonucleotide/μg M13 DNA) upstream from the unique *Eco*R1 restriction endonuclease site of M13mp2 DNA. The singly primed molecules were designated substrates I and II and the dually primed hybrid containing the juxtaposed oligonucleotides formed the nicked substrate III. (B) Exonuclease reaction mixtures (70 μl) contained 50 mM Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 50 mM NaCl, 12.6 μg/ml [^{32}P]DNA substrate II or III and 0.46 units of *Taq* DNA polymerase. Samples (10 μl) of each reaction were removed after 0, 10, 20, 30, 45 and 60 min of incubation at 50°C into 10 μl of formamide/dye mix. The DNA products (3 μl) were analyzed by DNA sequencing gel electrophoresis as described under **Materials and Methods**. Lanes 2–7 represent increasing times of incubation for substrate II and lanes 9–14 represent the same for substrate III. Lanes 1 and 8 contain substrates II and III, respectively, from control reactions incubated at 50°C for 60 min without DNA polymerase. (C) DNA polymerase reaction components were as in (B) except 100 μM of each dNTP was added. After incubation at 50°C for 10 min, reactions were divided into two 10-μl aliquots. To the first was added 7.6 units of *Eco*R1 in 5 μl of 150 mM Tris-HCl (pH 7.0), 50 mM NaCl, 100 μg/ml BSA; the second aliquot received the same buffer without *Eco*R1. After 10 min additional incubation at 37°C, the reaction was terminated by adding 15 μl of formamide/dye mix and samples (3 μl) were analyzed by DNA sequencing gel electrophoresis as in (B). Reaction products for substrate I (lanes 1–4), substrate II (lanes 5–8) and substrate III (lanes 9–12) are shown. Lanes 1, 5 and 9 were incubated without *Taq* DNA polymerase while lanes 2, 6 and 10 were incubated with polymerase and the reactions were immediately stopped. Lanes 3, 4, 7, 8, 11 and 12 were incubated with polymerase for 10 min, and those polymerized products treated with *Eco*R1 are shown in lanes 4, 8 and 12.

observed rate of dGMP production from the 24-mer/M13mp2 DNA was approximately the same whether the 20-mer was present or absent (data not shown).

Polymerase and Exonuclease Activity on a Nicked DNA Substrate

DNA synthesis reactions containing the substrates described in Figure 5A were carried out to determine if *Taq* DNA polymerase acts in concert with the 5′ to 3′ exonuclease to perform nick translation. Under the conditions described in Figure 5C, the polymerase extended both the 20-mer and 24-mer primers when individually hybridized to M13mp2 DNA (lanes 3 and 7). In both cases, the primers were extended past the *Eco*R1 restriction endonuclease site located 48 and 72 nucleotides downstream from the 5′-ends of the 20-mer and 24-mer, respectively (lanes 4 and 8). Upon longer autoradiographic exposure, hydrolysis of [^{32}P]dGMP was observed from the 5′-end of the 24-mer (lanes 3 and 4) but not from the internalized [^{32}P]dGMP residue of the 20-mer (lanes 7 and 8). Thus, during chain elongation on singly primed substrates, degradation at the 5′-end was limited to only a few nucleotides, certainly <20 nucleotides. This observation was substantiated since *Eco*R1 digestion products of the extended 20-mer primers generated oligonucleotides a few nucleotides shorter than the expected 48-mer (lane 8). When both the 20-mer and 24-mer primers were annealed to the same M13 DNA molecule, forming the nicked DNA substrate, significant chain elongation occurred from both primers (lane 11). After *Eco*R1 digestion, we observed that all the extended DNA molecules were cleaved which resulted in a single band that migrated as a 72-mer (lane 12). A 48-mer band was not observed. Thus, we conclude that polymerization occurred by nick translation. If the downstream primer (20-mer) had been displaced prior to extension, a 20-mer would have been recovered from these reactions (lane 11 and 12). Alternatively, if the 20-mer has been extended at the 3′-end and displaced from the 5′-end by polymerization on the 24-mer primer, *Eco*R1 digestion would not have efficiently cleaved the displaced single-stranded DNA, and ^{32}P-labeled DNA would have remained in high molecular weight forms. Since neither of these results were observed it is unlikely that strand displacement occurred. Further evidence for nick translation stems from the observation that considerably more

[^{32}P]dGMP was produced during polymerization on the nicked DNA substrate then on the 24-mer primed M13 DNA (lane 3 vs. 11). The most likely explanation for additional [^{32}P]dGMP production is that the 5′ to 3′ exonuclease degraded the 20-mer. Similar amounts of degradation did not occur without DNA synthesis (Figure 5B) or in the absence of upstream polymerization (lane 7). Collectively these results suggest the increased production of [^{32}P]dGMP was due to degradation of the 20-mer during a nick translation reaction.

To observe the initial stages of the reactions, we immediately removed samples after the addition of *Taq* DNA polymerase (lanes 2, 6, and 10). Analysis of products from the reaction containing the nicked DNA substrate appeared to indicate that the 24-mer was elongated by polymerization while the 20-mer was being degraded (lane 10). In addition, this result showed that the amount of extension roughly equaled that of degradation. If nick translation occurred through a coordinated action of DNA synthesis and hydrolysis such a result would be expected.

DISCUSSION

These results present the first experimental evidence that *Taq* DNA polymerase contains a 5′ to 3′ exonuclease activity resident in the same molecular weight polypeptide. This observation is not surprising since the N-terminal region of the enzyme shows significant similarity to the 5′ to 3′ exonuclease domain of the *E. coli* DNA polymerase I gene (4). Furthermore, Joyce et al. (19) have characterized four *E. coli* DNA polymerase I point mutants (*pol*A107, *pol*A214, *pol*A480ex, and *pol*A4113) with known amino acid changes which are defective in 5′ to 3′ exonuclease activity. In all cases the corresponding critical amino acid required for exonuclease activity was conserved in *Taq* DNA polymerase (4). Our results also suggest that *Taq* DNA polymerase does not contain a 3′ to 5′ exonuclease activity. This conclusion is based on the observation that no 3′ to 5′ degradation of 5′-end ^{32}P-labeled oligonucleotide was observed during *in situ* or *in vitro* detection of *Taq* DNA polymerase/exonuclease activity. This lack of 3′ to 5′ exonuclease activity is in agreement with the previous study by Tindall and Kunkel (3).

As determined by *in situ* detection of *Taq* DNA polymerase and exonuclease activities following SDS-polyacrylamide gel electrophoresis, both activities reside in the same polypeptide (Mr = 92,000). This result is in good agreement with the predicted molecular weight of ~94,000 deduced from the *Taq* DNA polymerase gene (4). Both values are significantly larger than the native molecular weight we observed (Mr 72,000) and that (Mr 62,000–68,000) reported by Trela and coworkers (1) for the enzyme purified from *Thermus aquaticus*. The lower apparent molecular weight observed during sedimentation analysis could suggest that the native polymerase exists in a nonglobular conformation.

The 5′ to 3′ exonuclease activity exhibits the following properties which imply it functions in conjunction with the DNA polymerase in nick translational DNA synthesis: (1) the exonuclease strongly preferred double-stranded over single-stranded DNA as substrate; (2) exonuclease activity was stimulated on a nicked DNA substrate; (3) in the absence of polymerization, hydrolysis at the nick appeared to be restricted to only 1–2 nucleotides; (4) during polymerization from a nick, concomitant degradation of the downstream DNA produced deoxyribonucleoside monophosphates; and (5) during DNA synthesis dNMPs were preferentially released from the downstream oligonucleotide compared to the upstream oligonucleotide primer. Whether nick translation occurred via precise coordination of extension and hydrolysis reactions or by the transient generation of short single-stranded overhangs as observed for nick translation by *E. coli* DNA polymerase I (20) requires more detailed mechanistic studies. Nevertheless, awareness of the 5′ to 3′ exonuclease activity associated with *Taq* DNA polymerase should be stressed in the design and interpretion of experiments using this enzyme.

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