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Exonuclease IX of *Escherichia coli*

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ABSTRACT

The bacteria *Escherichia coli* contains several exonucleases acting on both double- and single-stranded DNA and in both a 5′→3′ and 3′→5′ direction. These enzymes are involved in replicative, repair and recombination functions. We have identified a new exonuclease found in *E.coli*, termed exonuclease IX, that acts preferentially on single-stranded DNA as a 3′→5′ exonuclease and also functions as a 3′-phosphodiesterase on DNA containing 3′-incised apurinic/apyrimidinic (AP) sites to remove the product *trans*-4-hydroxy-2-pentenal 5-phosphate. The enzyme showed essentially no activity as a deoxyribophosphodiesterase acting on 5′-incised AP sites. The activity was isolated as a glutathione S-transferase fusion protein from a sequence of the *E.coli* genome that was 60% identical to a 260 bp region of the small fragment of the DNA polymerase I gene. The protein has a molecular weight of 28 kDa and is free of AP endonuclease and phosphatase activities. Exonuclease IX is expressed in *E.coli*, as demonstrated by reverse transcription–PCR, and it may function in the DNA base excision repair and other pathways.

INTRODUCTION

Several enzymes have been identified in *Escherichia coli* containing exonuclease activity that function during DNA synthesis, repair and recombination (1,2). These exonucleases can act in either the 5′→3′ direction, removing mono- or oligonucleotides as part of DNA excision repair, or in the 3′→5′ direction, removing a single mismatched nucleotide during DNA strand extension (2–4). The most widely studied enzyme containing exonuclease activity in *E.coli*, and the first to be discovered, is DNA polymerase I. Three primary activities have been identified for this enzyme; it acts as a polymerase and contains both 5′→3′ and 3′→5′ exonuclease activity (3). Proteolytic analysis of DNA polymerase I has revealed the presence of a large and a small fragment (5). The larger C-terminal fragment, referred to as the Klenow fragment, contains the polymerase and 3′→5′ exonuclease activities. The 5′→3′ exonuclease activity is encoded within the smaller N-terminal fragment (5).

Recently, through a homology search of the *E.coli* genome, a previously unrecognized open reading frame encoding a protein of 251 amino acid residues was identified (6). The protein sequence encoded by this putative gene was found to be 60% identical to a 260 bp region of the small fragment of DNA polymerase I. This putative gene is positioned downstream of an efficient translation initiation sequence, indicating a likelihood that the gene would be expressed (6).

In this study, we have expressed the putative exonuclease gene in *E.coli* and isolated a glutathione S-transferase (GST) fusion product of the gene. The protein was purified and GST was removed by proteolytic treatment with factor Xa. The purified enzyme was found to be a 3′→5′ exonuclease acting preferentially on single-stranded DNA. The protein also contained a 3′-phosphodiesterase activity that was able to remove the 3′ unsaturated sugar–phosphate product (*trans*-4-hydroxy-2-pentenal 5-phosphate) at a 3′-incised AP site. The enzyme, termed exonuclease IX (ExoIX) and expressed by the *xni* gene, may play an important role in base excision repair and other replicative, repair and recombination pathways.

MATERIALS AND METHODS

Enzymes and reagents

Amplitaq DNA polymerase was purchased from Perkin-Elmer. The large fragment (Klenow) of DNA polymerase I, RNase-free DNase I and factor Xa were purchased from Boehringer Mannheim. M13mp18 single-stranded DNA, M13 24mer sequencing primer (–47) and *E.coli* uracil-DNA glycosylase were purchased from US Biochemical. T4 polynucleotide kinase was purchased from New England Biolabs. Endonuclease III was a gift from Dr Richard Cunningham (SUNY, Albany, NY) and endonuclease IV was prepared as described previously (7).

Overexpression and purification of the *E.coli* ExoIX protein

The 755 bp gene coding *E.coli* ExoIX protein was amplified by PCR using *E.coli* AB1157 genomic DNA as template and the oligonucleotides (A) 5′-GGGGAATTCCGTGGCTGTTCATTTGCTT-3′ and (B) 5′-CCCCTCGAGCTTACCGTACCAACCGCAA-3′ containing *Eco*RI and *Xho*I restriction sites at the 5′- and 3′-ends respectively, for subsequent cloning into the pGEX (Pharmacia) vector system. The amplified fragment was purified following agarose gel electrophoresis and was subcloned into pGEM-T (Promega). The resulting plasmid was transformed into *E.coli* JM109 competent cells. The *lacZ* marker of the pGEM-T vector was used for blue/white screening of colonies on LB plates containing 100 µg/ml ampicilin, X-gal and IPTG. Plasmid DNA from white colonies was isolated and digested with *Eco*RI and *Xho*I. Following agarose gel electrophoresis, the

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*Eco*RI–*Xho*I fragment containing the gene was ligated into pGEX-5X-3 (Pharmacia) replacing its *Eco*RI–*Xho*I fragment. The hybrid plasmid was transformed into BL-21(DE 3) competent cells. An isolated colony was then inoculated into LB medium containing 100 µg/ml ampicilin (LB-amp) and grown overnight at 37°C. The saturated culture was diluted 1:50 in 300 ml LB-amp medium and grown with shaking at 37°C until A_{600} reached 0.5, then IPTG was added to a final concentration 0.1 mM and the cell culture was incubated for an additional 3 h at room temperature. Cells were pelleted by centrifugation at 3000 *g* at 4°C for 10 min and were resuspended in 8 ml ice-cold phosphate-buffered saline. Cells were lysed by sonication in 4 × 10 s bursts, then Triton X-100 was added to a final concentration of 1%. The cell debris was pelleted by centrifugation at 12 000 *g* at 4°C for 30 min. Expression of the GST–ExoIX fusion protein was determined by reaction with 1-chloro-2,4-dinitrobenzene. GST–ExoIX was isolated from the crude cell extract (8 ml) by addition of 140 µl of a 50% slurry of glutathione–Sepharose 4B and was incubated at room temperature for 30 min. After centrifugation at 3000 *g* the pellet was washed three times with 1 ml phosphate-buffered saline. ExoIX was cleaved from GST–ExoIX by adding 20 µg factor Xa in 400 µl factor Xa buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$) followed by incubation overnight with gentle shaking at room temperature. The suspension was centrifuged and the supernatant was stored at 4°C. Enzyme purity was determined by SDS–PAGE. Protein concentration was determined by the method of Bradford (8).

Gel filtration chromatography

For gel filtration, either GST–ExoIX (140 µg) or ExoIX (50 µg) was loaded onto a Superdex 75 HR 10/30 FPLC column (Pharmacia) previously equilibrated with 50 mM Tris–HCl, pH 7.6, 1 mM DTT, 1 mM Na_2EDTA, 250 mM NaCl, 5% glycerol. Protein was eluted from the column at a flow rate of 0.5 ml/min and fractions were collected every 0.5 min.

Reverse transcription–PCR

Total RNA was extracted from *E.coli* strain AB1157 using the RNeasy Mini Kit (Qiagen) and trace DNA was removed by incubation with DNase I (RNase-free). RT–PCR was carried out in a 50 µl reaction containing 20 ng RNA, 1 µM oligonucleotides A and B described above, AMV/Tfl buffer supplied by the manufacturer (Promega), 1.5 mM $MgSO_4$, 200 µM dATP, dTTP, dCTP and dGTP, 5 U AMV reverse transcriptase and 5 U Tfl DNA polymerase. Following an initial incubation at 48°C for 45 min and a subsequent incubation at 94°C for 2 min, 40 cycles of PCR were performed at 94°C for 1 min, 50°C for 2 min and 68°C for 2 min. Following the PCR reaction, the DNA was incubated for 7 min at 68°C. The DNA was analyzed by agarose gel electrophoresis.

DNA substrates for exonuclease activity

A 7.2 kb plasmid, pGAD-GL (9), was linearized by digestion with *Bam*HI. Following dephosphorylation, a 5′-end-labeled substrate was prepared with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) and T4 polynucleotide kinase and a 3′-end-labeled substrate was prepared with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) and the Klenow fragment of DNA polymerase I. Single-stranded DNA substrates were prepared by heat denaturation at 100°C for 10 min of the 5′- and 3′-end-labeled double-stranded DNA substrates, followed by immediate chilling on ice. Exonuclease activity was assayed in a 100 µl reaction containing 50 mM HEPES–KOH, pH 7.8, 1 mM Na_2EDTA, 5 mM DTT, 10 mM $MgCl_2$, 10 pmol DNA substrate and 70 ng ExoIX protein. After incubation for 30 min at 37°C, the DNA was precipitated with 5% trichloroacetic acid and was centrifuged. Release of ^{32}P-labeled nucleotides was determined by liquid scintillation counting. AP endonuclease activity was determined using a depurinated plasmid substrate as described previously (10).

M13 double-stranded DNA containing labeled incised AP sites

A M13 DNA substrate containing ^{33}P-labeled AP sites was prepared essentially as described previously (11,12). [^{33}P]dUMP-containing M13 DNA was treated with uracil-DNA glycosylase and subsequently with either *E.coli* endonuclease IV to create a substrate containing 5′-incised AP sites or with *E.coli* endonuclease III to create a substrate containing 3′-incised AP sites as described previously (13,14).

DNA dRpase assays

DNA dRpase activity was assayed in a reaction measuring either the release of 2-deoxyribose 5-phosphate from a M13mp18 DNA substrate containing 5′-incised AP sites or *trans*-4-hydroxy-2-pentenal 5-phosphate from a M13mp18 DNA substrate containing 3′-incised AP sites. A typical reaction (100 µl) contained 220 fmol M13mp18 DNA substrate containing incised AP sites, 70 ng ExoIX enzyme, 50 mM HEPES–KOH, pH 7.4, 5 mM DTT, 0.1 mM Na_2EDTA. Some reactions were supplemented with 10 mM $MgCl_2$. Release of sugar–phosphate products was determined either by precipitation with trichloroacetic acid in the presence of Norit charcoal or by HPLC anion exchange chromatography, as described previously (13,14).

RESULTS

Purification of exonuclease IX

The 755 bp *xni* gene (6), originally designated *exo*, was expressed in *E.coli* as a GST fusion protein (GST–ExoIX). The protein was isolated following chromatography on glutathione–Sepharose and GST was cleaved from the fusion protein by treatment with factor Xa. Purification of the protein is shown in Figure 1; the purity of GST–ExoIX was estimated to be >95% (lanes 5 and 6). All of the subsequent experiments utilized the cleaved form of the protein (lane 5), as it was found that this preparation was 3- to 4-fold more active for exonuclease activity than the fusion protein (data not shown). Although the preparation also contained the factor Xa protease (<5%), factor Xa alone did not contain any detectable exonuclease or dRpase activity.

ExoIX is a 3′→5′ single-stranded DNA exonuclease

Given the high degree of sequence homology of the *xni* gene to the region of the DNA polymerase I gene containing 5′→3′ exonuclease activity, it was assumed that ExoIX would act in a similar manner. DNA exonuclease activity was measured in either a 5′- or 3′-^{32}P-end-labeled DNA substrate, double- or single-stranded. All of the reactions required the presence of Mg^{2+} cations. A time–course study of the release of nucleotides

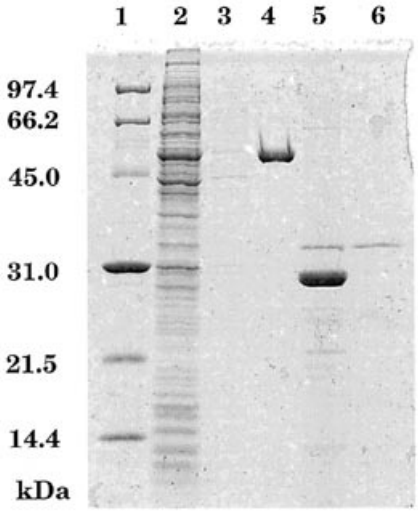

Figure 1. Purification of the fusion protein GST–ExoIX. GST–ExoIX was overexpressed in *E.coli* and proteins were visualized on a 12% SDS–polyacrylamide gel stained with Coomassie blue. Lane 1, molecular weight markers (lyoszyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase b); lane 2, *E.coli* BL21 crude cell extract transformed with pGEX-5X-3(gst-exoIX); lane 3, fraction eluted from glutathione–Sepharose following the third wash with phosphate-buffered saline; lane 4, fraction eluted from glutathione–Sepharose with 10 mM reduced glutathione (GST–ExoIX protein); lane 5, fraction eluted from glutathione–Sepharose following proteolytic cleavage with factor Xa; lane 6, factor Xa alone.

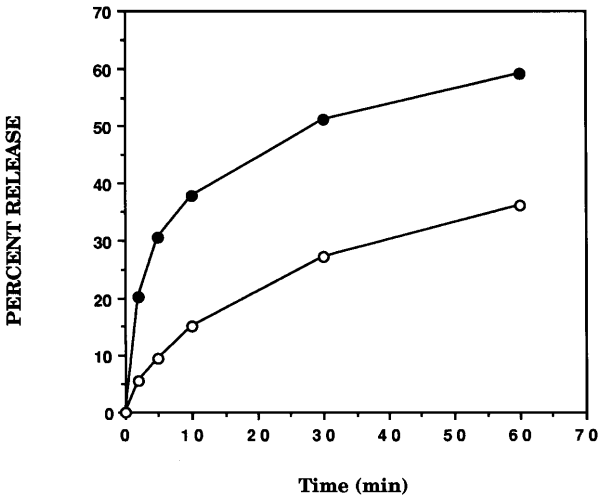

Figure 2. Time–course for the release of 3′-deoxynucleoside monophosphates from 3′-^{32}P-end-labeled plasmid substrates. The release of 3′-[^{32}P]dCMP was determined by precipitation with trichloroacetic acid. Reactions contained double-stranded (○) or single-stranded DNA (●).

from a 3′-end-labeled plasmid substrate is shown in Figure 2. The rate of nucleotide release was ~3-fold higher for the single-stranded DNA substrate as compared with the double-stranded DNA substrate. A time–course study of the release of nucleotides from a 5′-end-labeled plasmid substrate is shown in Figure 3. Both double- and single-stranded 5′-end-labeled DNA were poor substrates for the enzyme. Release of the end-labeled nucleotide (5′-[^{32}P]dGMP or 3′-[^{32}P]dCMP) was confirmed by anion exchange HPLC (data not shown).

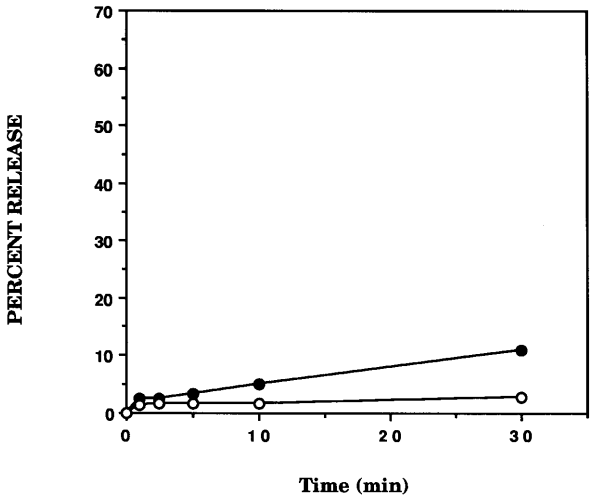

Figure 3. Time–course for the release of 5′-deoxynucleoside monophosphates from 5′-^{32}P-end-labeled plasmid substrates. The release of 5′-[^{32}P]dGMP was determined by precipitation with trichloroacetic acid. Reactions contained double-stranded (○) or single-stranded DNA (●).

To determine that the 3′→5′ exonuclease activity associated with ExoIX was not due to another *E.coli* exonuclease, both the GST–ExoIX fusion protein and ExoIX (following cleavage of GST–ExoIX with the factor Xa protease) were separated on a Superdex 75 gel filtration column. The 3′→5′ single-stranded DNA exonuclease activity contained in each fraction was determined. As seen in Figure 4, the 3′→5′ exonuclease activity associated with GST–ExoIX eluted as a single peak with a corresponding molecular weight of ~70 kDa, slightly higher than the expected molecular weight of 64 kDa. However, when ExoIX was purified following cleavage of the GST protein with factor Xa, the 3′→5′ exonuclease activity associated with this protein eluted as a single peak with a molecular weight of ~28 kDa, as expected. We conclude that these preparations of ExoIX are free from other known *E.coli* exonucleases.

DNA dRpase activities associated with exonuclease IX

Several *E.coli* exonucleases also have associated activities that remove sugar–phosphate moieties at AP sites that have been incised with either an AP endonuclease or AP lyase (13,15,16). To test whether ExoIX had associated dRpase activities, an M13 DNA substrate was employed that contained either 2-deoxyribose 5-phosphate groups at 5′-termini produced by cleavage with endonuclease IV or *trans*-4-hydroxy-2-pentenal 5-phosphate groups at 3′-termini produced by cleavage with the AP lyase endonuclease III. As seen in the time–course in Figure 5, ExoIX efficiently removed the *trans*-4-hydroxy-2-pentenal 5-phosphate product; this activity required the presence of Mg^{2+} cations. The enzyme was not efficient in removing 2-deoxyribose 5-phosphate from the substrate containing 5′-incised AP sites (<5% release in 30 min) in the presence or absence of Mg^{2+} cations. Removal of *trans*-4-hydroxy-2-pentenal 5-phosphate from the DNA containing 3′-incised AP sites was confirmed by anion exchange HPLC, as seen in Figure 6; no detectable DNA phosphatase activity was found associated with the enzyme. The apparent K_m for the release of *trans*-4-hydroxy-2-pentenal 5-phosphate, as determined by Lineweaver–Burk analysis, is shown in Figure 7 and

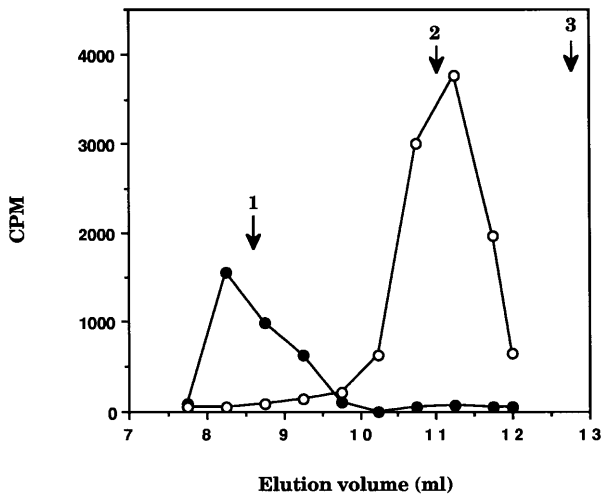

Figure 4. Superdex 75 gel filtration of either GST–ExoIX (●) or ExoIX protein (○). The arrows indicate the elution positions of the molecular weight markers bovine serum albumin, 66 kDa (1), carbonic anhydrase, 31 kDa (2) and cytochrome c, 12.4 kDa (3). The release of 3′-[^{32}P]dCMP from the 3′-end-labeled single-stranded plasmid substrate was determined by precipitation with trichloroacetic acid.

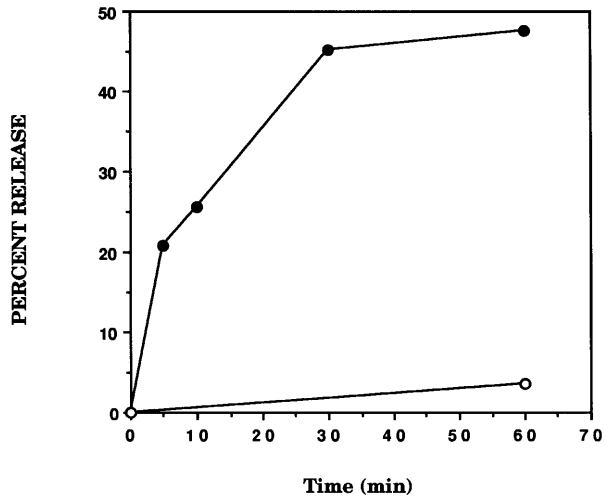

Figure 5. Time–course for the release of *trans*-4-hydroxy-2-pentenal 5-phosphate from a M13mp18 double-stranded DNA substrate containing 3′-incised AP sites. Reactions incorporated M13mp18 DNA containing 2 nM ^{32}P-labeled sugar–phosphate end groups. The release of *trans*-4-hydroxy-2-pentenal 5-phosphate was determined by precipitation with trichloroacetic acid in the presence of Norit charcoal. Reactions contained no enzyme (○) or 70 ng ExoIX (●).

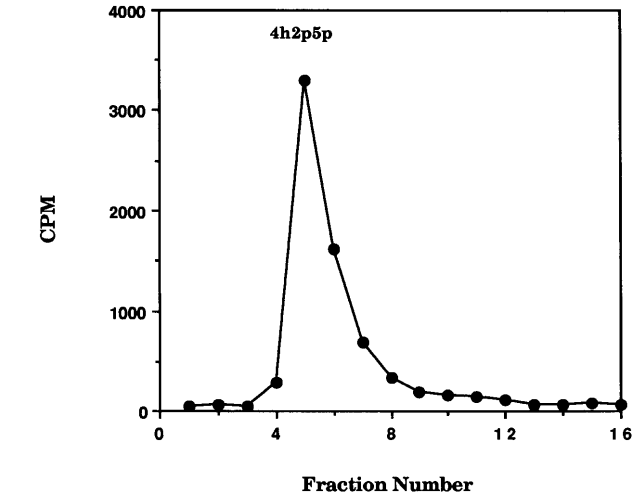

Figure 6. Enzymatic release of *trans*-4-hydroxy-2-pentenal 5-phosphate from a M13mp18 double-stranded DNA substrate containing 3′-incised AP sites. The reaction products were resolved on a MPLC AX HPLC column. The sugar–phosphate product *trans*-4-hydroxy-2-pentenal 5-phosphate (4h2p5p) released by ExoIX elutes between fractions 6 and 7 (3.5 min) under these conditions; inorganic phosphate (Pi) elutes between fractions 10 and 11 (5.5 min) (13,17).

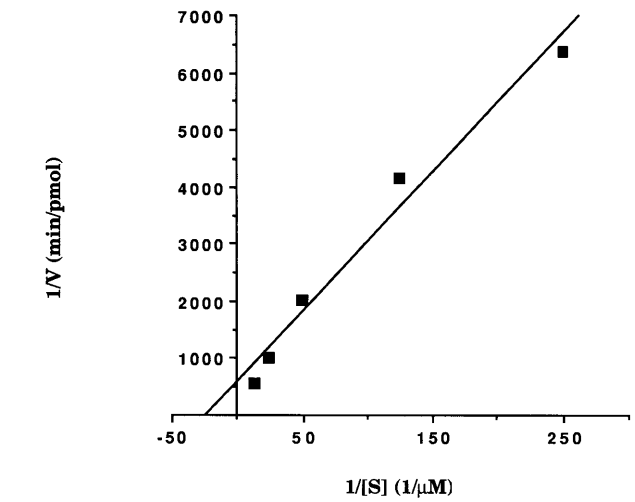

Figure 7. Lineweaver–Burk plot for the determination of K_m for the release of *trans*-4-hydroxy-2-pentenal 5-phosphate from a M13mp18 double-stranded DNA substrate containing 3′-incised AP sites. Substrate range, 0.004–0.08 μM; K_m = 0.04 μM.

was found to have a value of 0.04 μM, using a limiting amount of enzyme (0.7 nM). The enzyme was also found to be free of AP endonuclease activity using a DNA plasmid containing AP sites produced by depurination (data not shown).

ExoIX is expressed in *E.coli* cells

The exonuclease protein was expressed from a 762 bp gene found in *E.coli* genomic DNA. To demonstrate that the *xni* gene is actually expressed in bacteria, reverse transcription–PCR (RT–PCR) reactions were performed with mRNA isolated from *E.coli* strain AB1157. As seen in Figure 8, a DNA product of corresponding size was produced following RT–PCR using DNA primers complimentary to the 5′- and 3′-ends of the *xni* gene (lane 4). No product was produced when the reverse trancriptase step was omitted from the reaction (lane 5). As seen in lane 2, a DNA product was produced corresponding to the size (1401 bp) of the exonuclease I gene product when DNA primers complimentary to the 5′- and 3′-ends of the *xon* gene were used (12). This DNA was expressed less than that for ExoIX; *E.coli* exonuclease I has been shown to be expressed at a low level in bacterial cells (18). We have also found that the *E.coli* single-stranded DNA binding protein gene *ssb* is also well expressed following RT–PCR (data not shown). We conclude that the *xni* gene is expressed in *E.coli* cells.

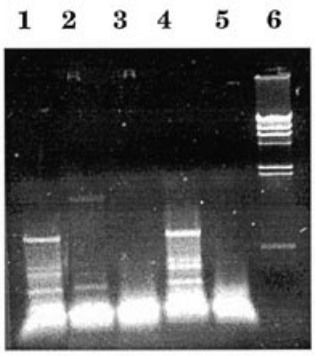

Figure 8. RT–PCR of DNA products using *E.coli* AB1157 mRNA as template. Products were resolved on a 0.8% agarose gel and stained with ethidium bromide. Lane 1, PCR of the *xni* gene using genomic DNA as template; lane 2, RT–PCR of the *xon* gene; lane 3, RT–PCR of the *xon* gene with omission of the reverse transcriptase step; lane 4, RT–PCR of the *xni* gene; lane 5, RT–PCR of the *xni* gene with omission of the reverse transcriptase step; lane 6, λ DNA digested with *Hin*dIII (23 130, 9416, 6557, 4361, 2322, 2027 and 564 bp).

DISCUSSION

We have characterized the biochemical functions of a previously unrecognized exonuclease found in *E.coli*. The enzyme is the product of a gene which codes for a protein of 251 amino acids and shows a high degree of similarity with the N-terminal region of *E.coli* DNA polymerase I and other bacterial DNA polymerases that have intrinsic 5′→3′ exonuclease activity (6). However, the product of this gene, ExoIX, was found to act poorly as a 5′→3′ exonuclease, but acted preferentially as a 3′→5′ exonuclease on single-stranded DNA. The enzyme also had a 3′-phosphodiesterase activity capable of removing the unsaturated sugar–phosphate product at a 3′-terminus created by cleavage of an AP site with an AP lyase such as endonuclease III of *E.coli*.

Other enzymes in *E.coli* shown to have 3′-phosphodiesterase activity acting on 3′ sugar–phosphate termini include exonuclease I (13), exonuclease III and endonuclease IV (19,20). Endonuclease IV and exonuclease III have associated AP endonuclease activities; no such activity was found for ExoIX. In a study examining multiple (at least five) DNA repair activities for 3′-deoxyribose fragments, an unknown activity with an apparent molecular weight of 28 kDa was observed (20). We believe that ExoIX may correspond to this previously unidentified activity.

AP lyases such as endonuclease III remove oxidatively damaged bases in DNA (16,21,22), cleave the AP site and leave an unsaturated sugar–phosphate (*trans*-4-hydroxy-2-pentenal 5-phosphate) at the 3′-terminus. This sugar–phosphate group is a block to DNA polymerases. The ability of ExoIX to remove these groups suggests that ExoIX may function as part of a base excision repair pathway, possibly as a back-up for the other enzymes. ExoIX also appears to have an activity that can remove 3′-phosphoglycolate end groups from DNA (manuscript in preparation), suggesting that this enzyme is involved in the repair of oxidatively damaged DNA. The enzymes exonuclease I (23), exonuclease III and endonuclease IV (19) have been demonstrated to remove 3′-phosphoglycolate end groups.

We have named the activity described in this report exonuclease IX and have named the gene *xni*, according to a convention originally suggested by Weiss (24). The previous named gene product in this series is exonuclease VIII, the product of the *recE* gene (25,26). Not all of the exonucleases identified in *E.coli* have adopted this nomenclature; for example, the RecJ protein (27), which is a 5′→3′ exonuclease.

What are other biological roles for ExoIX? Other 3′→5′ exonucleases, such as exonuclease I, have been shown to be involved in recombination (28), mismatch repair (29) and base excision repair (13). Whether ExoIX functions in these pathways remains to be determined. Certainly, creation of *xni* mutants will give insights into the possible role of the enzyme in several of these pathways.

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