Previous characterization of *Escherichia coli* endonuclease IV has shown that the enzyme specifically cleaves the DNA backbone at apurinic/apyrimidinic sites and removes 3’ DNA blocking groups. By contrast, and unlike the major apurinic/apyrimidinic endonuclease exonuclease III, negligible exonuclease activity has been associated with endonuclease IV. Here we report that endonuclease IV does possess an intrinsic 3’-5’ exonuclease activity. The activity was detected in purified preparations of the endonuclease IV protein from *E. coli* and from the distantly related thermophile *Thermotoga maritima*; it co-eluted with both enzymes under different chromatographic conditions. Induction of either endonuclease IV in an *E. coli* overexpression system resulted in induction of the exonuclease activity, and the *E. coli* exonuclease activity had similar heat stability to the endonuclease IV AP endonuclease activity. Characterization of the exonuclease activity showed that its progression on substrate is sensitive to ionic strength, metal ions, EDTA, and reducing conditions. Substrates with 3’ recessed ends were preferred substrates for the 3’-5’ exonuclease activity. Comparison of the relative apurinic/apyrimidinic endonuclease and exonuclease activity of endonuclease IV shows that the relative exonuclease activity is high and is likely to be significant in *vivo*.

Apurinic and apyrimidinic (AP)1 sites threaten genetic stability because they block replication and are mutagenic (1, 2). They arise in DNA through the spontaneous loss of normal or damaged bases or through the release of modified or mismatched bases from DNA by DNA glycosylases (3, 4). The first general step of base excision repair following base loss is the removal of the DNA backbone at apurinic/apyrimidinic sites and AP endonuclease activity. The first enzyme family is typified by exonuclease (Exo) III from *Escherichia coli* (7, 8) and the homologous APE-1 enzyme in humans (9), which are major AP endonucleases in these organisms. The second conserved AP endonuclease family is typified by *E. coli* endonuclease (Endo) IV (10, 11) and includes the APN-1 protein from *Saccharomyces cerevisiae* (12) and *Schizosaccharomyces pombe* (13) and the CeAPN1 gene from the nematode *Caenorhabditis elegans* (14). In *E. coli*, Endo IV expression is induced by superoxide anion generators (15), but in *S. cerevisiae*, APN-1 is the predominant constitutive AP endonuclease.

Genetic studies indicate that Exo III and Endo IV have overlapping but distinctive repair specificities in *vivo*. In *E. coli* Exo III is encoded by the *xth* gene (16) and is a constitutive enzyme accounting for 80–90% of the total AP activity in the cell. Endo IV is encoded by the *nfo* gene (17) and accounts for 5–10% of the total cellular AP activity (8). Exo III is a divalent metal ion-dependent enzyme and is inactivated by metal chelating agents (18). In contrast, the AP activity of *E. coli* Endo IV is resistant to inactivation by EDTA in normal assay conditions (11). Exo III and Endo IV also have a 3’-phosphatase and a 3’-repair phosphodiesterase in common. These activities are responsible for removing a multitude of blocking groups, including 3’-phosphoglycolate and 3’-phosphate, that are present at single-stranded breaks in DNA induced by oxidative agents (8, 18). Endo IV is the only known enzyme that is active against damaged nucleotides with bases in the α configuration (19). Exo III has a 3’-5’ exonuclease activity, the functional significance of which is unknown (20). Even though *E. coli* Endo IV is a minor AP endonuclease, its expression can be induced more than 20-fold by superoxide-generating agents, such as paraquat (15), which thus enhances the capability of the cell for repairing oxidative DNA damage or damage that is refractory to enzymatic processing by Exo III. *nfo*-like endonucleases have also been reported to nick DNA on the 5’ side of various oxidatively damaged bases (21).

The Endo IV active site contains a trinuclear zinc center that is ligated by conserved protein side chains that cluster at the center of a deep, crescent shaped groove (22). Biochemical experiments have suggested a role for manganese (9), although the crystal structure of the Endo IV complexed with DNA indicates that manganese is not needed for activity (22). Two of the zinc atoms are partially buried in the enzyme, whereas the third atom is relatively accessible. The high resolution structures suggest that the geometry of the Endo IV trinuclear zinc cluster is exquisite tuned for cleaving phosphodiester bonds, with all three zinc ions participating in catalysis (8, 22). To date, no significant exonuclease activity has been associated with Endo IV (10, 11). Here we report detection and characterization of a significant Endo IV 3’-5’ exonuclease activity.
Exxonucleolytic activity associated with E. coli and T. maritima Endo IV proteins. A. SDS-PAGE (15%) analysis of MonoQ and MonoS purified fractions of E. coli and T. maritima Endo IV proteins used for the exonuclease assay. 1. Following electrophoresis the proteins were visualized by staining with Coomassie Brilliant Blue. Lane 1, protein standard marker; lane 2, 3.5 µg of E. coli Endo IV; lane 3, 1.7 µg of T. maritima Endo IV. B. The 5’ end-labeled substrate subDS-18R (500 fmol) was incubated with the purified E. coli or T. maritima Endo IV proteins. Lane 1, subDS-18R, no enzyme added; lanes 2 and 3, subDS-18R incubated with 100 ng of E. coli Endo IV or 100 ng of T. maritima Endo IV, respectively. n, nucleotides.

**Characterization of AP Endonuclease IV Exonuclease Activity**

**EXPERIMENTAL PROCEDURES**

### Materials

- [γ-32P]ATP (3000 Ci/mmole) was obtained from Amer sham Biosciences.
- E. coli Endo IV was obtained from Epicentre Technologies and Fermentas and generously supplied by Bruce Demple (Harvard University, Boston, MA).

### Overexpression of the E. coli and Thermotoga maritima Endo IV Genes

- The E. coli Endo IV gene (Ec-Endo IV) was amplified by PCR from E. coli K12 genomic DNA using the following forward and reverse primers: 5′-ATGAAATACATTGGAGCGCA-3′ and 5′-GGCTACCCCGT-GCTTGACATTC-3′, designed from the published sequence accession number M22591 (16). The T. maritima Endo IV gene (Tm-Endo IV) was amplified from T. maritima genomic DNA using the primers 5′-ATGATAAAATAGGAGCTCACA-3′ and 5′-ATCGACCTTATAACCGATT-T-3′ designed from sequence data obtained from the Institute for Genomic Research (www.tigr.org). The reverse primers used for the amplification of both genes were designed to exclude the native stop codon. Amplification of the genes was performed by PCR (95°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 35 cycles followed by 72°C for 10 min) in a reaction volume of 100 µl containing 300 nM of each primer, 200 µM of each deoxynucleoside triphosphate, 500 ng of T. maritima or E. coli genomic DNA, and 2.6 units of expand high fidelity DNA polymerase (Roche Molecular Biochemicals) in the supplied buffer. The amplified products of the anticipated sizes, 855 bp for the Ec-Endo IV proteins.

### Purification of E. coli and Thermotoga maritima Endonuclease IV—E. coli and T. maritima Endo IV proteins were expressed in E. coli top10 cells according to the manufacturer’s instructions (Invitrogen).

- **Purification of E. coli and T. maritima Endo IV Proteins—**E. coli TOP10 cells harboring the pBAD-TOPO-Ec-Endo IV or Tm-Endo IV constructs were grown overnight at 37°C in 10 ml of LB containing 50 µg/ml ampicillin (LB-amp). This overnight culture was used to inoculate a 1-liter LB-amp culture, which was grown to an A600 of 0.5, at which point arabinose was added to a final concentration of 0.2% w/v.

### Enzymatic Assays

- The assays were performed using 500 fmol of the end-labeled substrate in a total volume of 25 µl containing 1 unit of T. maritima or E. coli Endo IV. Fractions of 0.5 µl were collected, and samples were analyzed on a 15% SDS-polyacrylamide gel electrophoresis and visualized by staining with Coomassie Brilliant Blue. The crude extract was clarified by centrifugation at 3000 × g for 20 min at 4°C. Following this, they were resuspended in 20 ml of buffer A (50 mM phosphate buffer, pH 7.5, 500 mM NaCl, 20 mM imidazole) and lysed by sonication (four 10-s bursts at a medium setting). The crude extract was clarified by centrifugation at 12,000 × g for 30 min at 4°C. Batch application of the supernatant to 1 ml of Pre-Bond resin (Invitrogen), pre-equilibrated with buffer A, was performed at 4°C for 1 h with gentle agitation. Unbound proteins were collected in the flow through, which was followed by two subsequent 10-ml washes with buffer A and then buffer B (50 mM phosphate buffer, pH 7.5, 50 mM NaCl, 35 mM imidazole). The Ec-Endo IV and Tm-Endo IV His-tagged proteins were collected overnight at 37°C in 10 ml of LB containing 50 µg/ml ampicillin (LB-amp). This overnight culture was used to inoculate a 1-liter LB-amp culture, which was grown to an A600 of 0.5, at which point arabinose was added to a final concentration of 0.2% w/v.

### Overexpression and Purification of E. coli and T. maritima Endonuclease IV—E. coli and T. maritima Endo IV genes were amplified from genomic DNA using primers designed from the published sequence and cloned into the pBAD-TOPO expression vector under the control of the E. coli arabinose-inducible pBAD promoter. The primers were designed so that the EcoRI HindIII digestion site was maintained. Following growth of E. coli strains harboring the pBAD-TOPO-Ec-Endo IV and pBAD-TOPO-Tm-Endo IV, the cells were allowed to grow for a further 6 h at 37°C (Tm-Endo IV) for 15 min. Prior to optimization, all of the assays were carried out in 10 mM Tris/HCl, pH 8.3, 10 mM KCl. For determination of optimal pH, the assays were performed in buffers (25 mM) of varying pH: sodium acetate buffer, pH 5, MES/NaOH with pH 5.5, 6.0, or 6.5, Tris/HCl with pH 7.0, 7.5, 8.0, or 8.5, and glycine/NaOH with pH 9.0, 9.5, 10.0, or 10.5. Following pH optimization, all of the subsequent reactions were performed in 25 mM glycine/NaOH, pH 9.0. The reactions were stopped by transferring the tubes to ice and adding an equal volume of loading solution (98% formamide, 10% DMSO, 0.5% xylene cyanol, and 0.5% bromphenol blue). The reaction products were analyzed by denaturing gel electrophoresis (20% polyacrylamide, 7 M urea) gels and quantified by phosphorimaging analysis using ImageQuant Software (Molecular Dynamics, Inc.).
IV fusion proteins were purified by immobilized metal affinity chromatography. Pooled samples bearing the eluted proteins of the expected sizes were further purified using ion exchange chromatography, anion exchange (MonoQ) for the *E. coli* protein, and cation exchange MonoS for the (*T. maritima*) protein. Analyses of the purified fractions by SDS-PAGE and Coomassie Blue staining showed that the Ec-Endo IV and the Tm-Endo IV fusion proteins were >95% pure (Fig. 1A).

**Evaluation of the Exonuclease Activity**—Exonuclease activity in the MonoQ and MonoS Ec-Endo IV and Tm-Endo IV fractions was initially evaluated by incubation of the enzymes with a double-stranded 5’ end-labeled 3’ recessed 18-nt substrate (subDS-18R) at 37 and 60 °C, respectively. The buffer used in this initial assay was different from that used previously in reports investigating Endo IV activity (11). In particular, EDTA and DTT were absent, and the salt concentration was low. Analysis of samples incubated with either Endo IV revealed multiple bands smaller in size than the labeled 18-nt fragment and indicating the presence of exonuclease activity. The band sizes decreased approximately one base at a time, consistent with the presence of a 3’–5’ exonuclease activity (Fig. 1B). The fact that significant exonuclease activity was present in the MonoQ-purified Ec-Endo IV and the MonoS-purified Tm-Endo IV showed that the activity was co-purified with the two Endo IV proteins under very different ion exchange conditions and indicated that the activity might be intrinsic to the Endo IV enzymes. In agreement with this, the exonuclease activity associated with the purified Ec-Endo IV sample has a heat stability (stable after incubation at 65 °C for 5 min) similar to that described for the AP endonuclease activity of the Ec-Endo IV (data not shown) (23). The possibility that the exonuclease activity was an arabinose-inducible *E. coli* gene was investigated. *E. coli* harboring the empty inducible pBAD-TOPO expression vector or the vector bearing the Ec-Endo IV or Tm-Endo IV genes were grown under identical conditions and induced with arabinose. Crude cell extracts were preincubated at 65 °C for 7 min and assayed for exonuclease activity. A low level of background activity was detected in cells harboring the empty vector, but no significant difference in activity was observed between extracts from noninduced and induced cells (Fig. 2A). Extracts from noninduced cells harboring the Endo IV genes had levels of exonuclease activity similar to that observed in the control extracts. By contrast, significant exonuclease activity was present in extracts from the induced cells harboring either Endo IV gene, confirming that the activity is associated with the Endo IV proteins (Fig. 2A).

The natural substrate for the Endo IV enzymes is an AP site within a double-stranded DNA context. To determine whether the exonuclease activity was active at cleaved AP sites, a 5’ end-labeled double-stranded 29-nt bearing a central synthetic (tetrahydrofuranyl) AP site at position 19 (subDS-19AP) was incubated with heat-treated extracts prepared from noninduced and induced cells harboring the plasmids, pBAD-TOPO, pBAD-TOPO-Ec-Endo IV, or pBAD-TOPO-Tm-Endo IV. Cleav-

**FIG. 2.** Induction of exonuclease activity in *E. coli* harboring the Endo IV gene under an inducible promoter. Crude cell extracts were prepared from *E. coli* harboring the pBAD-TOPO expression vector (control) or the vector bearing the Ec-Endo IV or Tm-Endo IV genes after induction for 6 h with 0.2% arabinose. Following heat treatment at 65 °C for 7 min, the extracts (1 μl) were incubated with the 5’ end-labeled subDS-18R (500 fmol) (A) or the 5’ end-labeled AP containing substrate subDS-19AP (500 fmol) (B). Lanes 1, no extract added; lanes 2 and 3, noninduced and induced control extract; lanes 4–7, noninduced and induced extracts from cells harboring the vector bearing the Ec-Endo IV or Tm-Endo IV genes, respectively. n, nucleotides.

**FIG. 3.** Exonuclease activities of Endo IV proteins from different sources. The 5’ end-labeled substrates, subDS-19AP (500 fmol; lanes 1–6) and subSS-19AP (500 fmol; lanes 7–12) were incubated with different preparations of Endo IV proteins. Lanes 1 and 7, no enzyme added; lanes 2 and 8, 100 ng of the Ec-Endo IV MonoQ fraction; lanes 3 and 9, 2 units of Ec-Endo IV (Epican; lanes 5 and 11, 40 ng of Ec-Endo IV (Demple); lanes 6 and 12, 100 ng of the Tm-Endo IV MonoS fraction. n, nucleotides.

**TABLE I**

| Product size | No. EIV | subDS-19AP | subSS-19AP |
|--------------|---------|------------|------------|
|              | EcEIV   | Epi        | MonoQ      | EcEIV   | Epi        | MonoQ      |
| 29           | 100     | 26.78      | 36.30      | 15.09   | 44.08      | 35.80      |
| 18           | 33.42   | 30.01      | 25.79      | 17.61   | 32.35      | 24.99      |
| 17           | 17.57   | 25.33      | 20.82      | 21.52   | 24.99      | 24.99      |
| 16           | 5.29    | 5.40       | 9.01       | 6.18    | 4.64       | 4.64       |
| 15           | 12.17   | 2.42       | 15.92      | 6.86    | 1.92       | 1.92       |
| 14           | 0.82    | 0.19       | 2.04       | 0.81    | 0.30       | 0.30       |
| 12           | 2.53    | 0.35       | 5.98       | 1.76    | 0.64       | 0.64       |
| 11           | 0.63    | 2.24       | 0.52       | 0.52    | 0.64       | 0.64       |
| 10           | 0.33    | 1.64       | 0.33       | 0.33    | 1.64       | 1.64       |
| 9            | 0.46    | 1.33       | 0.28       | 0.28    | 1.33       | 1.33       |
| 8            | 0.14    | 0.05       | 0.05       | 0.05    | 0.05       | 0.05       |
Characterization of AP Endonuclease IV Exonuclease Activity

**Fig. 4.** Effect of pH and NaCl on the exonuclease activity of E. coli Endo IV protein. The 5' end-labeled substrate subDS-19AP (500 fmol) was incubated with the MonoQ fraction of Ec-Endo IV under various pH or NaCl conditions. A, lanes 1–12, substrate was incubated with 600 ng of Ec-Endo IV at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, and 10.5, respectively. B, substrate was incubated with 100 ng of Ec-Endo IV protein and varying NaCl concentrations. Lane 1, no Endo IV added; lanes 2–14, NaCl added to reaction mix to a concentration of 0.0, 0.05, 0.1, 0.15, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90, and 1 M, respectively. n, nucleotides.

**Fig. 5.** Effect of metal ions on the E. coli Endo IV exonuclease activity. The 5' end-labeled substrate subDS-19AP (500 fmol) was incubated with 100 ng of the MonoQ fraction of Ec-Endo IV with various metal ions (2 mM) or EDTA (2 mM). Lane 1, no Endo IV added; lane 2, Endo IV added; lane 3, with MgCl2; lane 4, with MnCl2; lane 5, with ZnCl2; lane 6, with MgSO4; lane 7, with MnSO4; lane 8, with ZnSO4; lane 9, EDTA added. n, nucleotides.

**Fig. 6.** Effect of DTT in different buffers on the exonuclease activity of E. coli Endo IV. The 5' end-labeled substrate subDS-19AP (500 fmol) was incubated with 100 ng of the MonoQ fraction of Ec-Endo IV in different reaction buffers with various additions to each buffer. Lanes 1–3, 50 mM glycine/NaOH reaction buffer, pH 9.0, with no enzyme added, enzyme added, and enzyme plus 1 mM DTT added, respectively; lanes 4–7, 10 mM Tris/HCl reaction buffer, pH 9.0, with no enzyme added, enzyme added, enzyme plus 1 mM DTT, and enzyme plus 100 mM DTT added, respectively; lanes 8–11, 10 mM Tris/HCl reaction buffer, pH 7.6, with no enzyme added, enzyme added, enzyme plus 1 mM DTT, and enzyme plus 100 mM DTT added, respectively; lanes 12–15, 25 mM Hepes/KOH reaction buffer, pH 7.6, with no enzyme added, enzyme added, enzyme plus 1 mM DTT, and enzyme plus 100 mM DTT added, respectively. n, nucleotides.

**Fig. 7.** Inhibition of the exonuclease activity of E. coli Endo IV protein. The 5' end-labeled substrate subDS-19AP (500 fmol) was incubated with 100 ng of the MonoQ fraction of Ec-Endo IV with various added reagents. The standard reaction contains 25 mM glycine/NaOH, pH 9.0, using 100 ng of Endo IV and 500 fmol of subDS-19AP. Lane 1, no enzyme; lane 2, with enzyme; lane 3, 1 mM EDTA added; lane 4, 1 mM DTT added; lane 5, 100 mM KCl added; lane 6, 1 mM EDTA and 1 mM DTT added; lane 7, 1 mM EDTA, 1 mM DTT, and 100 mM KCl added. n, nucleotides.
Characterization of AP Endonuclease IV Exonuclease Activity

The Endo IV exonuclease activity was very sensitive to the salt concentration, with little activity observed at NaCl concentrations of 75% cleavage occurring in the presence of NaOH reaction buffer, pH 9.0 (Fig. 4A). At this pH, there was complete cleavage of the AP site in the substrate. In the absence of exonuclease activity, the 18-nt PCP should account for 100% of the product. At pH 9.0, the 18-nt PCP only accounted for 100% of the cleaved product. The smallest digestion products were detectable at this pH, showing that the enzyme progressed furthest on the substrate at this pH. Both the exonuclease activity and the extent of progression on the substrate decreased as pH was increased above 9.0 (Fig. 4A).

Prior to pH optimization, all of the exonuclease assays were performed in a reaction mixture containing 10 mM Tris/HCl, pH 8.3, 10 mM KCl. Following pH optimization, all of the subsequent assays were performed using 25 mM glycine-NaOH buffer, pH 9.0.

The Endo IV exonuclease activity was very sensitive to the salt concentration, with little activity observed at NaCl concentrations of >150 mM (Fig. 4B). Similar results were obtained for KCl (data not shown). In contrast, the AP endonuclease activity of Endo IV was active at low and high salt concentrations with >95% substrate cleavage occurring in the absence of added NaCl and ~75% cleavage occurring in the presence of 1 mM NaCl (Fig. 4B).

The effect of magnesium, the metal ions associated with the enzyme (9), and EDTA on the exonuclease activity was analyzed using both substrates (Fig. 5). 100 ng of the Ec-Endo IV cleaved ~65% of the AP sites in the subDS-19AP substrate, whereas in the presence of MgCl₂, MnCl₂, ZnCl₂, MgSO₄, MnSO₄, and ZnSO₄, cleavage was ~95, 67, 93, 90, 90, and 93%, respectively. It was difficult to estimate exonuclease activity using this substrate because the level of AP site cleavage varied. However, it was clear that the exonuclease activity was most progressive in the presence of zinc (Fig. 5). Progression of the enzyme on the substrate was also high in the presence of MnSO₄ but not in the presence of MnCl₂. Although the presence of MgCl₂ or MgSO₄ appeared to stimulate AP activity, the progression of the exonuclease was lower than that for ZnCl₂, ZnSO₄, or MnSO₄. Interestingly in the presence of EDTA, the highest level of digestion of the 18-nt PCP was observed, but progression was lowest. Overall, the metal ion effects were reproducible; however, significant variation was observed between experiments. The reason for this is unclear.

The effect of the reducing agent DTT on the exonuclease activity of the Endo IV was investigated (Fig. 6). Addition of DTT to the reaction mixture reduced the AP activity of the enzyme by 2–20% depending on the buffer used and the concentration of agent. Addition of 1 mM DTT to the standard reaction containing subDS-19AP and Endo IV in the glycine/NaOH reaction buffer, pH 9.0, did not appear to significantly alter the level of 18-nt PCP generated. However, in the presence of DTT, the number and intensity of fragments smaller than 17 nt were much lower than in the absence of DTT. This indicates that the progression of the exonuclease on the substrate was impaired by the DTT. The addition of the same amount of DTT to the standard reaction in Tris/HCl buffer, pH 9.0, had less effect on the progress of the exonuclease activity. The inhibitory effect was much more pronounced in 100 mM DTT. Using a Tris/HCl buffer, pH 7.6, or a Hepes/KOH buffer, pH 7.6, no inhibitory effect on exonuclease activity was observed in the presence of 1 mM DTT, but impairment progression of the exonuclease was high at a 100 mM concentration (Fig. 6).

Inhibition of the Exonuclease Activity—Ec-Endo IV has previously been characterized extensively. However, a significant exonuclease activity associated with the enzyme has not been reported. In the majority of characterizations, EDTA, DTT, and KCl have been included in reaction buffers (10, 11). The effect of these agents, singularly and combined, on the exonuclease activity of the Endo IV was investigated (Fig. 7). The addition of EDTA (1 mM), DTT (1 mM), or KCl (100 mM) to the standard reaction inhibited the exonuclease activity significantly but not completely. The addition of all three agents to the reaction mixture inhibited the activity to the extent that only the 18-nt PCP of the AP endonuclease activity was detected.

Determination of the Relative Activity of the Exonuclease Activity of Ec-Endo IV on Different Substrates—To determine the relative activity of the exonuclease activity of Ec-Endo IV on different substrates, 100 ng of the enzyme was incubated with 20 and 18-nt duplex oligonucleotides with 3’ recessed ends, a 30-nt duplex substrate with blunt ends, and a 20-nt duplex substrate with a 3’ overhang (Fig. 8). Comparison of the extent of digestion of the substrates showed that ~20% of the labeled 20-nt oligonucleotide of subDS-20R was digested by one nucleotide or more, and more than 45% of the 18-nt oligonucleotide of subDS-18R was digested. By contrast, the activity on the labeled 30-nt oligonucleotide of the substrate subDS-30B, was significantly less at ~12%, and the enzyme had little or no activity on 3’ overhangs (Fig. 8). Digestion of the 18-nt PCP of subDS-19AP was greatest, with ~72% of the 18-nt product digested by one or more nucleotides.

Comparison of the Relative AP Endonuclease and Exonuclease Activity of Endo IV—The relative AP endonuclease and exonuclease activities of Ec-Endo IV were compared (Fig 9 and Table II) using both the single- and double-stranded AP substrates, subSS-19AP and subDS-19AP, respectively. The level of exonuclease activity on the single-stranded substrate was

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**Fig. 8. Exonuclease activity of E. coli Endo IV on different DNA substrates.** The MonoQ-purified fraction of Ec-Endo IV was incubated with 500 fmol of each of the 5’ end-labeled DNA substrates. Lanes 1 and 2, subDS-20R (3’ recessed) without and with enzyme respectively; lanes 3 and 4, subDS-18R (3’ recessed) without and with enzyme, respectively; lanes 5 and 6, subDS-30B (blunt ended) without and with enzyme, respectively; lanes 7 and 8, subDS-20O (3’ overhang) without and with enzyme respectively; lanes 9 and 10, subDS-19AP without and with enzyme, respectively. n, nucleotides.

**Fig. 9. Comparison of the AP endonuclease and exonuclease activity of E. coli Endo IV.** 500 fmol of the 5’ end-labeled single and double-stranded substrates, subSS-19AP (lanes 1-11) and subDS-19AP (lanes 12-22) were incubated with increasing amounts of the MonoQ fraction of Ec-Endo IV. Lanes 1 and 12, no Endo IV added; lanes 2 and 13, 0.2 ng; lanes 3 and 14, 0.4 ng; lanes 4 and 15, 0.8 ng; lanes 5 and 16, 1.6 ng; lanes 6 and 17, 3.2 ng; lanes 7 and 18, 6.4 ng; lanes 8 and 19, 12.8 ng; lanes 9 and 20, 25.6 ng; lanes 10 and 21, 51.2 ng; lanes 11 and 22, 100 ng. n, nucleotides.
low. For this substrate, 78.6% of substrate remained uncleaved after incubation with 100 ng of Endo IV under the conditions used. Of the 21.4% of cleaved product, the single-stranded 18-nt PCP accounted for ~17.2%, and a 17-nt product accounted for ~4%. This shows that the 18-nt PCP generated was subsequently digested further by one nucleotide to a level of ~20%. Additional smaller products were not observed. Thus, exonuclease activity on the single-stranded substrate is relatively low. By comparison, the level of exonuclease activity on the double-stranded substrate was significantly higher. Only 40% of substrate remained uncleaved after incubation with 100 ng of Endo IV. Of the 60% of cleaved product, the 18-nt PCP accounted for ~28%, a 17-nt product accounted for ~23%, a 16-nt product accounted for 4%, and additional smaller products were visible.

DISCUSSION

Previous characterization of Endo IV has shown that the enzyme specifically cleaves the DNA backbone at AP sites and also removes 3'-DNA blocking groups such as 3'-phosphates, 3'-phosphoglycolates, and 3'-α,β-unaturated aldehydes that arise from oxidative base damage and the action of the combined glycosylase/lyase enzymes (1, 2, 4). By contrast, and unlike the major AP endonuclease Exo III, negligible exonuclease activity has been associated with Endo IV. The results reported here show that Endo IV does possess an intrinsic 3'-5' exonuclease activity; the activity was detected in purified preparations of the Endo IV protein from E. coli and from the distantly related thermophile T. maritima; it co-eluted with both enzymes under different chromatographic conditions; induction of either Endo IV in an E. coli overexpression system resulted in induction of the exonuclease activity; and the E. coli exonuclease activity had similar heat stability to the Endo IV AP endonuclease activity.

Considering that Ec-Endo IV is a highly characterized and widely explored enzyme, we sought to identify the reason that this activity was not detected previously. Characterization of the exonuclease activity was carried out using a variety of substrates under a variety of conditions. The Endo IV exonuclease was active over a broad pH range with labeled fragments lower than 5 nt detectable at the optimum pH, indicating that the exonuclease progresses extensively on the substrate and is active on substrates with small duplexed regions. The Endo IV exonuclease activity was very sensitive to the ionic strength of the reaction mixture. As the salt concentration increased, the portion of substrate digested to smaller fragments decreased. At 0 mM added NaCl, the 18-nt PCP was extensively digested into several smaller fragments. By 100 mM NaCl, it was predominantly digested by one nucleotide; by 150 mM, significant inhibition was observed; and by 200 mM NaCl, the exonuclease activity was almost completely inhibited. By contrast, the AP activity appeared relatively insensitive to the NaCl concentration. The progression of the exonuclease on the substrate was enhanced by the presence of excess zinc, indicating that zinc may be important for processivity of the Endo IV on its substrate in addition to its known role in phosphodiester bond cleavage (22). By contrast, magnesium and manganese appeared to inhibit progression of the exonuclease activity on the substrate (although MnSO₄ appeared to have no effect). EDTA inhibited progression of the exonuclease activity and also had an inhibitory effect on the AP activity. The addition of the reducing agent DTT inhibited the progression of the enzyme on the substrate. This was an unexpected finding and suggests that the exonuclease activity of the enzyme may be more active when the cell is under oxidative stress. The Endo IV exonuclease activity may be processive or distributive, and further experiments are needed to clarify this issue.

Typical Endo IV assays in previous reports contained 100 mM KCl, 1 mM DTT, and 1 mM EDTA in the reaction mixture (10, 11). The effect of cumulative addition of the agents to the reaction mixture on the Endo IV exonuclease activity was assessed. Addition of any of the agents reduced progress of the enzyme on the substrate, and addition of all three agents completely inhibited the exonuclease activity. Because previous characterizations of the Endo IV enzyme have predominantly used buffers including all three agents, this may explain why the exonuclease activity was not observed previously.

The results shown indicate that the Endo IV exonuclease activity works very well within an AP site cleavage context and has a strong preference for substrates with either 3' recessed ends or an incised AP site (Fig. 8) and raises the question as to what the extent of exonuclease activity in vivo is at such sites. Reconstitution of the E. coli base excision-repair pathway using crude or purified enzymes, including Endo IV showed no detectable resynthesis of DNA 5' of AP sites (5). However, the conditions used are likely to have been refractory to Endo IV exonuclease activity. Comparison of the relative AP endonuclease and exonuclease activity of Endo IV indicates that the exonuclease activity is high and that exonuclease action is likely to follow AP incision and have a functional significance in vivo. Although the function of the 3'-5' exonuclease activity of Exo III is unknown and has been considered redundant, the discovery of substantial exonuclease activity associated with Endo IV argues in favor of a significant functional importance for this activity in vivo.
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