Partial Purification of Pde1 from *Saccharomyces cerevisiae*: Enzymatic Redundancy for the Repair of 3′-Terminal DNA Lesions and Abasic Sites in Yeast

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ABSTRACT: Earlier work indicates that the major DNA repair phosphodiesterase (PDE) in yeast cells is the well-characterized Apn1 protein. Apn1 demonstrates both Mg^{2+}-independent PDE activity and Mg^{2+}-independent class II apurinic/apyrimidinic (AP) endonuclease activity and represents greater than 90% of the activity detected in crude extracts from wild-type yeast cells. Apn1 is related to *Echerichia coli* endonuclease IV, both in its enzymatic properties and its amino acid sequence. In this work, we report the partial purification of a novel yeast protein, Pde1, present in Apn1-deficient cells. Pde1 is purified by sequential BioRex-70, PBE118, and MonoS chromatography steps using a sensitive and highly specific 3′-phosphoglycolate-terminated oligonucleotide-based assay as a measure of PDE activity. Mg^{2+}-stimulated PDE and Mg^{2+}-stimulated class II AP endonuclease copurify during this procedure. These results indicate that yeast, like many other organisms studied to date, has enzymatic redundancy for the repair of 3′-blocking groups and abasic sites.

Abasic sites, DNA strand breaks, altered miscoding base moieties, and many other lesions accumulate in DNA continuously (Friedberg et al., 1995). Cells harbor extensive and elaborate DNA repair systems designed to prevent genome instability by repairing DNA lesions that would otherwise accumulate or be fixed by the cellular replicative machinery. The abasic site, which is among the most common lesion, is estimated to accumulate spontaneously in the genome at a rate of 10^4 lesions $cell^{-1}$ day^{-1} (Lindahl & Nyberg, 1972). Repair of abasic sites is facilitated by apurinic/apyrimidinic (AP)[1] endonucleases and AP lyases, enzymes that recognize this form of DNA damage and cleave the phosphodiester backbone, thus preparing for polymerase-catalyzed replacement of the missing base. The hydrolytic AP endonucleases, known as class II enzymes, produce 3′-hydroxyl-terminated DNA ends after cleavage of AP sites (Friedberg et al., 1995; Linn et al., 1981), and these enzymes, which include *Escherichia coli* exonuclease III and endonuclease IV, also repair biologically important oxidative lesions that often occur at DNA strand breaks. Mutant yeast or *E. coli* cells deficient in the repair of abasic sites demonstrate an increase in their spontaneous mutation rate and large increases in lethality after exposure to agents that induce DNA damage (Kunz et al., 1994; Ramotar et al., 1991; Robson & Hickson, 1991; Cunningham et al.,1986).

At least five AP endonucleases and AP lyase activities are known in *E. coli* (Friedberg et al., 1995). The class II AP endonucleases, endonuclease IV and exonuclease III, are each members of a conserved family of proteins that have been extensively studied (Popoff et al., 1990; Sander et al., 1991a; Demple et al., 1991). Exonuclease III, encoded by the *xth* gene, is a constitutive and abundant enzyme, representing 80−90% of the total measurable AP endonuclease activity in a crude extract. Endonuclease IV, encoded by the *nfo* gene, is an inducible activity: it represents either 5% or up to 50% of the activity in uninduced or induced cells, respectively (Chan & Weiss, 1987). Exonuclease III is a Mg^{2+}-dependent multifunctional enzyme that functions as a 3′-exonuclease specific for dsDNA, a class II AP endonuclease, a 3′-phosphatase, and a 3′-phosphodiesterase (PDE). The AP endonuclease activity of exonuclease III is essential for repair of abasic sites as indicated by the fact that a *dut* (dUTPase) *xth* double mutant cell has a conditional lethal phenotype that can be rescued by a third mutation in the *ung* gene (uracil−DNA *N*-glycosylase) (Taylor & Weiss, 1982). *xth* mutants are hypersensitive to hydrogen peroxide (Demple et al., 1983), and *xth nfo* double mutants, deficient in both exonuclease III and endonuclease IV, show large increases in lethality in comparison to either the *nfo* or the *xth* single mutants after exposure to DNA damaging agents (Cunningham et al., 1986).

Nine proteins related to *E. coli* exonuclease III have been identified from *Streptococcus*, *Pseudomonas*, *Dictyostelium*, *Drosophila*, *Arabidopsis*, rat, mouse, bovine, and human (Puyet et al., 1989; MacGregor et al., 1996; Freeland et al., 1996; Sander et al., 1991a; Babiychuk et al., 1994; Wilson et al., 1994; Seki et al., 1991; Robson et al., 1991; Demple et al., 1991; Robson & Hickson, 1991). The *E. coli* and human homologues demonstrate 27.5% amino acid identity. All members of the family share a homologous carboxy-terminal region approximately 250 amino acids in length and a divergent amino-terminal region of variable length. Each of these proteins is an efficient AP endonuclease. Only exonuclease III, exonuclease A, mouse APEX, and *Drosophila* Rrp1 demonstrate readily detectable 3′-exonuclease activity on undamaged DNA (Rogers & Weiss, 1980; Puyet

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[1] Abbreviations: AP, apurinic/apyrimidinic; PDE, 3′-phosphodiesterase; DTT, dithiothreitol; BSA, bovine serum albumin; dsDNA, double-strand DNA; ssDNA, single-strand DNA.

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et al., 1989; Seki et al., 1991; Sander et al., 1991b). No protein from *Saccharomyces cerevisiae* with structural homology to exonuclease III has yet been identified. Three proteins related to *E. coli* endonuclease IV, from *S. cerevisiae*, *Schizosaccharomyces pombe* (unpublished results), and *Caenorhabditis elegans* (Masson et al., 1996), have been identified. Each of these proteins shows approximately 45% identity to endonuclease IV.

To date, AP endonuclease activities belonging to one or the other, but not both, of these families of DNA repair enzymes have been found in eukaryotic cells. In yeast, the major AP endonuclease is an endonuclease IV related protein, Apn1 (Ramotar et al., 1991; Popoff et al., 1990). Apn1-deficient yeast demonstrate 5% residual AP endonuclease activity and a 4-fold increase the in spontaneous mutation rate (Ramotar et al., 1991). Apn1-deficient cells are sensitive to DNA damage including MMS and hydrogen peroxide, but not bleomycin or radiation (Ramotar et al., 1991). The following experiments were carried out to search for additional DNA repair functions present in *S. cerevisiae*.

MATERIALS AND METHODS

DNA and Enzymes. T4 polynucleotide kinase was from New England Biolabs. Uracil−DNA Glycosylase was from USB/Amersham. Oligonucleotides were from Oligo's, Etc. Inc. In some cases, oligonucleotides were gel-purified prior to 5′-end labeling. Radiolabeled nucleotide was from USB/Amersham. Bleomycin was from Sigma. Apn1 was purified as described in Johnson and Demple (1988).

The DNA sequences of the oligonucleotides used in AP endonuclease and PDE assays are as follows: BL1, ^{32}P-CCCCAAAAAAAAAAAAGCAAAAAAAAAATTA; BL2, TAATTTTTTTTTTGCTTTTTTTTTTTTGGGGGGGG; 1U, 5′-^{32}P-CCCCCTCTCTCTCTCTUCTCTCTCT-CTCTTTTTTTTT-3′; 2T, 5′-AAAAAGAGAGAGAGAG-TAGAGAGAGAGAGGGGGTTTT-3′

Preparation of DNA Substrates for AP Endonuclease and PDE Assays. (A) AP Endonuclease Substrate. A 37 base oligonucleotide containing a unique uracil residue was 5′-^{32}P-end-labeled. The specific activity of the labeled oligonucleotide was determined by running an aliquot of the kinase reaction on a 16% polyacrylamide gel and quantitating the amount of incorporated radioactivity [(5−10) × 10^4 cpm/ng]. The oligonucleotide was incubated at 30 °C overnight in 25 μL of 10 mM Tris-HCl, pH 8.0, 50 mM EDTA containing 3 units of uracil−DNA glycosylase. For the AP endonuclease substrate, the reaction was terminated by addition of 8 μL of formamide gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF).

(B) PDE Substrate [See Sander and Huang (1995)]. A 31 base oligonucleotide containing a unique GpC dinucleotide was 5′-^{32}P-end-labeled and specific activity determined as described above. The oligonucleotide was precipitated by 6 volumes of ethanol and resuspended in 50 mM $NaHPO_4$, pH 7.2, 50 mM NaCl and annealed with an equimolar amount of the complementary oligonucleotide by heating at 70 °C and slow-cooling to room temperature. The sample was chilled on ice, and additions were made to the following final conditions: 0.4 mM DTT, 100 μM bleomycin, 100 μM iron(II) ammonium sulfate. Incubation proceeded on ice for 10 min. The sample was concentrated using a Microcon-3 device, and 0.5 volume of formamide loading buffer was added. The substrates were purified on 12% (AP endonuclease) or 16% (PDE) denaturing polyacrylamide gels and eluted using an Elutrap (Schleicher & Schuell). The recovery of oligonucleotide was determined, and a 2.5-fold molar excess of unlabeled complementary 35 base oligonucleotide was added. Annealing was carried out at 70 °C for 1 min in 100 mM Tris-HCl, pH 8.0, 20 mM NaCl, containing 50 μg/mL BSA followed by slow-cooling to room temperature. (Some unlabeled complementary oligonucleotide remains present in the substrate as ssDNA.) The annealing efficiency was determined by comparing the electrophoretic mobility of the ssDNA oligonucleotide and the annealed dsDNA substrate on a nondenaturing 18% polyacrylamide gel, or in some cases by carrying out a Klenow polymerase fill-in reaction, followed by sequencing gel analysis.

Nuclease Assays. AP endonuclease and PDE reactions were carried out in buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 50 μg/mL BSA, and 4−9 nM dsDNA oligonucleotide substrate in a final volume of 10 μL. Incubations were carried out at 30 °C for 10−30 min, as indicated, and stopped by the addition of proteinase K to 0.1 mg/mL and EDTA to 25 mM. Proteinase K digestion was carried out at 42 °C for 15 min. Samples were prepared for gel electrophoresis by addition of 5 μL of formamide gel loading buffer. Reaction products were analyzed on 16% denaturing polyacrylamide gels. The reaction products were quantitated using a Molecular Dynamics Phosphorimager. The detection limit of this assay is estimated to be ≈1% of input substrate.

Partial Purification of Pde1. A 200 mL inoculum of DRY377 (MATa, his3Δ-200, ura3−52, leu2Δ, apn1Δ::HIS3) was grown in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 30 °C, shaking in an orbital shaker at 250 rpm until stationary phase. The cells were subcultured into 20 L of YPD medium and allowed to grow for 16 h. Cells were centrifuged at 500*g* for 5 min, and the cell pellet was washed once with cold sterile-distilled water. This yielded 100 mL of cell pellet which was stored at −80 °C. The pellet was thawed on ice for 1 h followed by the addition of 100 mL of cold extraction buffer (50 mM Tris-HCl, pH 7.5, 30 mM KCl, 10% glycerol, 1 μg/mL each of aprotinin, leupeptin, chymostatin, and TPCK, and 1 mM each of PMSF and benzamidine). Crude extract was prepared by using a bead beater (Biospec Products, Bartlesville, OK) equipped with a 30 mL chamber and a surrounding ice-cooling chamber. Zirconium beads (0.5 mm) were added to the 30 mL chamber to the 15 mL mark, and the chamber was filled with the resuspended cell pellet so no air space remains in the chamber. The cells were lysed by spinning for 20 s followed by cooling in the ice-filled surrounding chamber. This was repeated 12 times. The total extract was sedimented at 32000*g* for 20 min. The crude extract was aliquoted and stored at −80 °C. The yield of protein was approximately 50 mg/mL.

A 15−30 mL sample of cell extract containing approximately 1 g of protein was adjusted to 0.5 M NaCl with 0.1 volume of 5 M NaCl. Polymin P was added to 0.1%, and the sample was stirred for 20 min and sedimented for 20 min at 16000*g*. The supernatant was removed. Chilled, pulverized, solid ammonium sulfate was added (0.5 g/mL) gradually with stirring. When the salt was solubilized, the

sample was stirred for 20 min, and centrifuged for 20 min at 16000*g* in an SS34 Sorval rotor. The pellet was resuspended in buffer S2 (10% glycerol, 50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.2 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl chloride, and 0.25 μg/mL each of the peptides pepstatin A, leupeptin, chymostatin, antipain, and aprotinin) containing 25 mM NaCl. The sample was dialyzed vs 100 volumes of buffer S2 containing 25 mM NaCl, for 2 × 2.0 h (FII). The conductivity of the sample was measured and adjusted to 50 mM monovalent salt equivalent, as necessary. This sample was applied to a 10 mL BioRex-70 column equilibrated with buffer S2 containing 0.05 M NaCl at a flow rate of 1.0 mL/min. The column was washed with 30 mL of buffer S2 containing 0.1 M NaCl and eluted with a 160 mL gradient from 0.1 M NaCl to 0.7 M NaCl. The highest PDE activity was found in the 0.1 M NaCl wash fraction (fraction III; approximately 3−5 mg of protein).

The active pool from BioRex-70 chromatography was applied to a 5 mL PBE118 (Pharmacia Biotech) column equilibrated with buffer S2 containing 0.1 M NaCl at a flow rate of 0.5 mL/min. The column was washed with 10 mL of buffer S2 containing 0.1 M NaCl and eluted with a 40 mL gradient from 0.1 M NaCl to 0.6 M NaCl followed by step elution at 1 M NaCl. The highest PDE activity eluted at about 0.4 M NaCl (fraction IV; approximately 0.5 mg of protein). Active fractions were pooled and concentrated to less than 1 mL by Centricon-10.

Active material from several preparations of fraction IV Pde1 was pooled for MonoS (Pharmacia Biotech) chromatography. The sample was diluted with buffer S2 containing no NaCl, resulting in a final NaCl concentration of 0.1 M. The sample was applied to a 1 mL MonoS column equilibrated with buffer S2 containing 0.1 M NaCl at a flow rate of 0.75 mL/min. The column was washed with 10 mL of buffer S2 containing 0.1 M NaCl and eluted with a 10 mL gradient from 0.1 M NaCl to 0.75 M NaCl followed by step elution at 1 M NaCl. The highest PDE activity eluted at about 0.5 M NaCl (fraction V).

Other Methods. Piperidine cleavage products were prepared in a volume of 50 μL containing 1 M piperidine and 5 μg of tRNA, and heated for 30 min at 90 °C. The cleaved sample was chilled on ice, evaporated to dryness in a Savant speedvac, and resuspended in formamide gel loading buffer.

RESULTS

In general, class II AP endonucleases demonstrate the capacity to remove 3′-blocking groups such as phosphoglycolate and phosphate. To detect putative repair enzymes in yeast extracts, a sensitive PDE assay was employed to test for the capacity to remove a 3′-terminal DNA lesion (Sander & Huang, 1995). The PDE assay measures removal of a unique 3′-terminal phosphoglycolate from a ^{32}P-labeled oligonucleotide substrate. The 3′-phosphoglycolate substrate (BL1−17PG; Figure 1) is generated by bleomycin-dependent cleavage of a 31mer containing a unique GpC dinucleotide, and utilizes the sequence dependence of DNA cleavage by bleomycin. The substrate design and preparation are shown schematically in Figure 1. Enzymatic removal of the phosphoglycolate results in a 3′-hydroxyl terminated 17 base oligonucleotide product (BL1−17). The reaction product and substrate have distinct electrophoretic mobilities and can be

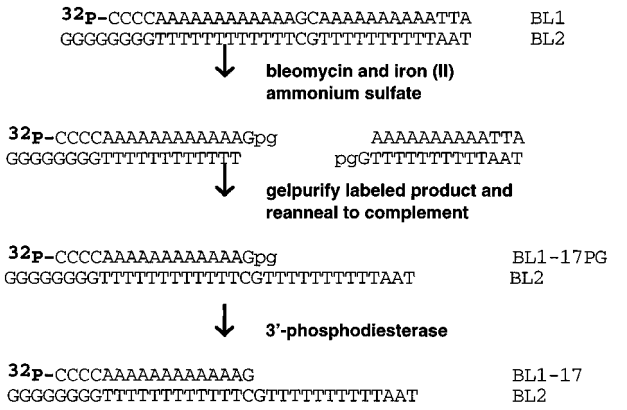

FIGURE 1: Substrate design for PDE activity assay. Preparation of the oligonucleotide substrate is shown schematically (see Materials and Methods for a detailed protocol). Bleomycin cleavage occurs at the GpC dinucleotide, which results in a 3′-phosphoglycolate-terminated DNA end. PG indicates phosphoglycolate.

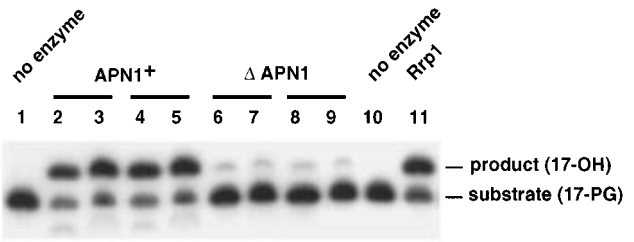

FIGURE 2: PDE assay of crude yeast extracts. Two Apn1$^+$ strains (FY86, lanes 2−3; DRY214, lanes 4−5) and two Δ apn1 strains (DRY377, lanes 6−7; DRY387, lanes 8−9) were assayed in the presence of 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 8.0, 0.2 mM Na_3-EDTA, 50 μg/mL BSA with the addition of 20 mM NaCl (lanes 1−9) or 150 mM NaCl (lanes 10−11). Additions of enzyme were as follows (μg): 0.0, 3.5, 0.9, 5.0, 1.2, 6.5, 1.6, 5.7, 1.4, 0.0, 0.01 in lanes 1−11, respectively. Assays were incubated at 30 °C for 10 min.

quantitated following DNA sequencing gel analysis. This assay is sensitive and specific (Sander & Huang, 1995), and the substrate can be readily prepared in the amounts required for protein isolation and characterization.

Figure 2 shows the total PDE activity detected in yeast cells by this assay method. Crude extract of wild-type yeast cells is highly active (lanes 2−5) and produces a major product species that comigrates with a 3′-hydroxylterminated marker (lane 11). Comparing Apn1-deficient cell extracts to the wild-type extract indicates that the major yeast PDE activity is Apn1. However, weak PDE activity is detected in the mutant cell extract (lanes 6−9). Because of the relative abundance of these two activities, characterization and partial purification of this second yeast PDE activity were carried out using extracts of Apn1-deficient cells.

A flow chart diagram of the steps used to obtain partially purified Pde1 is shown in Figure 3. Nucleic acid was removed from the total cell extract by precipitation with poly(ethylenimine), followed by protein concentration by ammonium sulfate precipitation. Partial purification of the PDE activity was achieved using three sequential ion exchange chromatography steps (Figure 3); BioRex-70, PBE118, and MonoS. Details of the PBE118 and MonoS chromatography steps are shown in Figures 4 and 5.

Comparison of the activity in the crude extract (FI; Figure 2) and the BioRex-70 eluate (FIII; Figure 4 inset: column load) indicates a large enhancement of activity in the initial purification steps. This enhancement may be due in part to

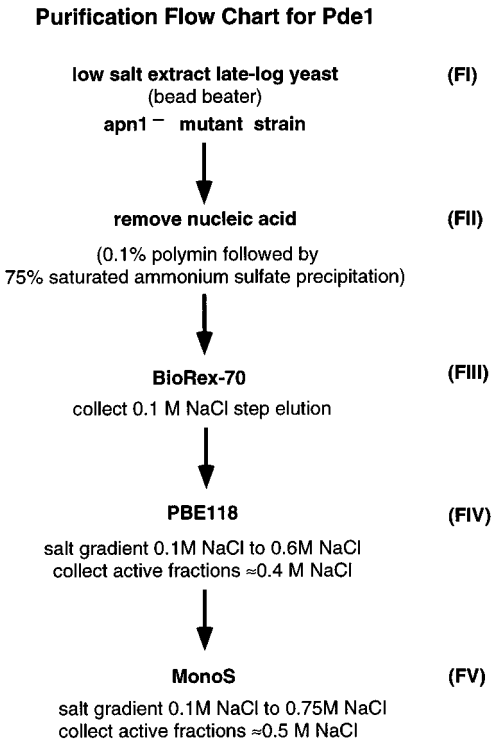

FIGURE 3: Flow chart for partial purification of Pde1.

Table 1: Characteristics of Pde1 PDE and AP Endonuclease Activities

conditions	enzyme (ng)	fmol prod	relative activity
(A) Phosphodiesterase Assay			
complete	Pde1 (31)	27.6	100
	Pde1 (15)	4.0	
$-Mg^{2+}$	Pde1 (31)		0
$-Mg^{2+} + 1$ mM Ca^{2+}	Pde1 (31)		1.6
+50 mM NaCl	Pde1 (31)		75.7
+100 mM NaCl	Pde1 (31)		14.1
heat Δ 65 °C, 30 min	Pde1 (31)		6.3
proteinase K	Pde1 (31)		8.6
complete	Rrp1 (5)	80.2	
complete	Apn1 (4)	72.6	(100)
$-Mg^{2+}$	Apn1 (4)	63.6	(88)
(B) AP Endonuclease Assay			
complete	Pde1 (62)	9.7	100
	Pde1 (31)	6.8	
$-Mg^{2+}$	Pde1 (62)	1.1	11
$-Mg^{2+} + 1$ mM Ca^{2+}	Pde1 (62)		36
+100 mM NaCl	Pde1 (62)		64
heat Δ 65 °C, 30 min	Pde1 (62)		5
complete	Apn1 (4)	9.9	(100)
$-Mg^{2+}$	Apn1 (4)	9.3	(94)

the removal of endogenous nucleic acid. During chromatography on the zwitterionic PBE118 resin, a single strong peak of activity is detected in the second half of the NaCl gradient elution (Figure 4). PDE activity binds with relatively high affinity and elutes close to 0.4 M NaCl, well separated from the main peak of A_{280} absorbance (at 0.15 M NaCl). Pde1 is thereby enriched during this step, since a good separation from other protein species is achieved. The first two column steps, BioRex-70 and PBE118, remove the majority of the 3′-exonuclease and class I AP endonuclease present in the crude extract (see below and data not shown). The active fractions from PBE118 chromatography were pooled (FIV) and used for further characterization (Table 1; Figures 5 and 6).

FIV Pde1 from several preparations were pooled together for further purification by MonoS chromatography (Figure 5). A sample of 1.2 mg of total protein was applied to a 1 mL MonoS column. A single peak of PDE activity elutes from the salt gradient during MonoS chromatography at about 0.5 M NaCl (Figure 5, solid bars). Analysis of the fractions for AP endonuclease also reveals a single peak of activity (Figure 5, patterned bars), and these two activities demonstrate very similar elution profiles. This suggests that one enzyme is likely to carry out both DNA repair reactions (see below). Protein analysis of the peak fractions from the MonoS column reveals the presence of six major and several minor bands (data not shown). The yield of protein at this stage was less than 0.1 mg of total protein. The very low total protein yield at the latter purification steps has hindered further purification of Pde1.

In the absence of exogenous Mg^{2+}, only a large excess of Pde1 reveals a low level of residual PDE activity (Figure 6A). In the linear range of Pde1 titration, no Mg^{2+}-independent PDE is observed (Table 1). This behavior is in contrast to the behavior of Apn1, which demonstrates Mg^{2+}-independent PDE activity (Table 1). In addition, 1 mM $CaCl_2$ will not support the phosphoglycolate removal reaction by Pde1, indicating that the divalent cation requirement is somewhat specific. Pde1 is moderately inhibited by NaCl, with 76% and 14% activity remaining in reactions that contain 50 and 100 mM NaCl, respectively. Pde1 is inactivated by heat (65 °C, 30 min) or treatment with proteinase K, consistent with the active molecule being a protein molecule. The activity level observed with FIV Pde1 is at least 250-fold higher than the level in the crude extract, and approximately 70-fold lower than that of homogeneous endonuclease IV from *E. coli* (data not shown).

Figure 6B demonstrates the presence of highly specific class II AP endonuclease associated with partially purified Pde1. In the presence of a 37mer oligonucleotide with a unique abasic site at position 17, Pde1 efficiently produces a specific endonucleolytic cleavage at the abasic site. The reaction product comigrates with the product generated by *Drosophila* Rrp1, another class II AP endonuclease, and migrates slightly slower than the piperidine cleavage product which is terminated with 3′-phosphate. This AP endonuclease activity is also dependent on exogenous $MgCl_2$. The ratio of AP endonuclease to PDE activity observed with FIV Pde1 is very close to 1:1 (Figure 6). The similar characteristics of the PDE and AP endonuclease functions, including Mg^{2+} dependence, similar specific activity, and coelution during MonoS chromatography, support the argument that both activities are intrinsic to a single enzyme. Further, the copurification of 3′-PDE and class II AP endonuclease is commonly seen for other well-characterized DNA repair proteins (i.e., exonuclease III, Rrp1, hAPE), and thus the results are consistent with the expected characteristics of a functional base excision repair enzyme.

The 5′-^{32}P-labeled oligonucleotide substrates used in the enzyme assays shown in Figures 2, 4, and 6 are sensitive to electrophoretic mobility shifts caused by numerous enzymes of DNA metabolism that would be detected during product analysis. Figure 6 demonstrates that partially purified Pde1 lacks significant contamination by 5′-exonuclease, 5′-phosphatase, nonspecific dsDNA endonuclease, and class I AP endonuclease. This conclusion is supported by other experiments carried out with higher enzyme input (data not shown).

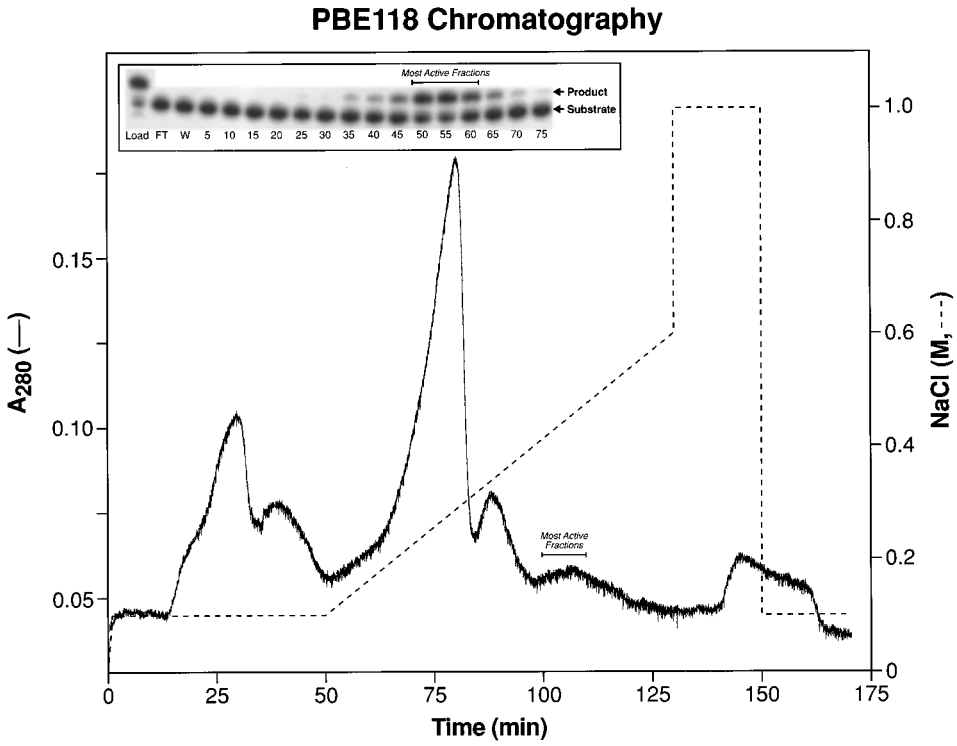

FIGURE 4: PBE118 chromatography. 4.0 mg of FIII Pde1 in a volume of 15 mL of buffer S2 containing 100 mM NaCl was applied to a 5 mL PBE118 column. A_{280} absorbance was monitored (solid line) as the column was washed and eluted with a gradient from 0.1 M to 0.6 M NaCl. 500 μL fractions were collected through the salt gradient elution (dashed line). Most active fractions elute at approximately 0.4 M NaCl. Inset: Standard PDE assays were carried out using 1 μL of selected fractions incubated for 20 min at 30 °C. Substrate and product bands are indicated. Load indicates material applied to column. FT indicates material not bound to the column. The number of the fraction used in each assay is indicated.

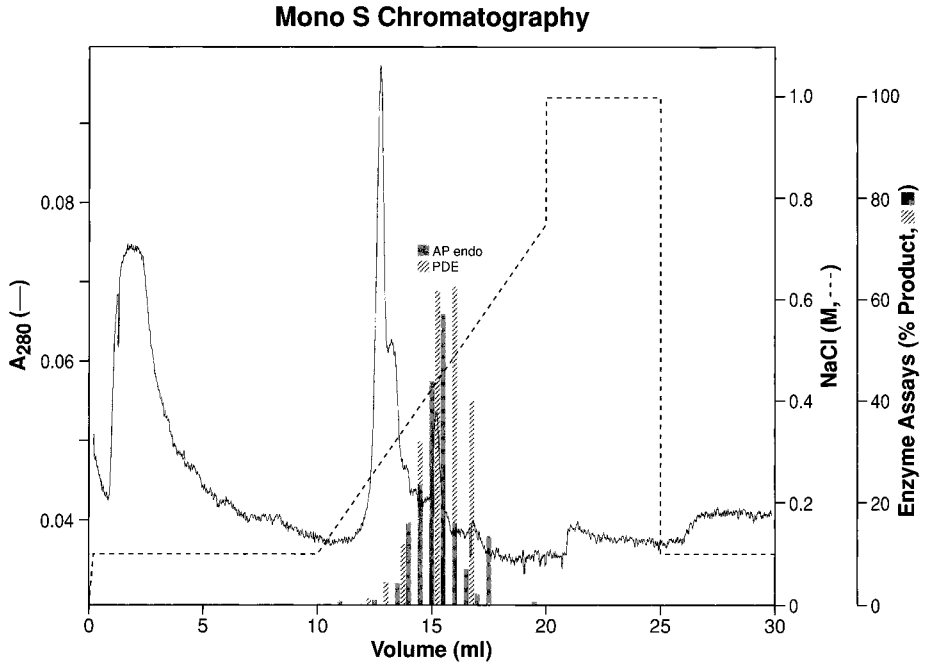

FIGURE 5: MonoS chromatography. 1.2 mg of FIV Pde1 in a volume of 4 mL of buffer S2 containing 100 mM NaCl was applied to a 1 mL MonoS column. A_{280} absorbance was monitored (solid line) as the column was washed and eluted with a gradient from 0.1 M to 0.75 M NaCl. 250 μL fractions, collected through the salt gradient elution (dashed line), were assayed for AP endonuclease (solid bars) and PDE activity (patterned bars). Standard assays were carried out using 1 μL of selected fractions incubated for 20 min at 30 °C.

In contrast, while 3′-exonuclease activity is not evident in reactions with low enzyme input, some 3′-exonuclease is associated with Pde1 at high concentrations (Figure 7, lanes 2, 4). In addition, assays for 3′-phosphatase activity indicate that a Mg^{2+}-dependent 3′-phosphatase also copurifies with Pde1. Additional work will determine if these enzyme functions are intrinsic to a single enzyme polypeptide or multimer.

The substrate specificity of Pde1 was further defined by comparing Pde1 activity with a ssDNA and dsDNA substrate containing an abasic site. Cleavage of an abasic site requires duplex DNA surrounding the abasic site and is not observed

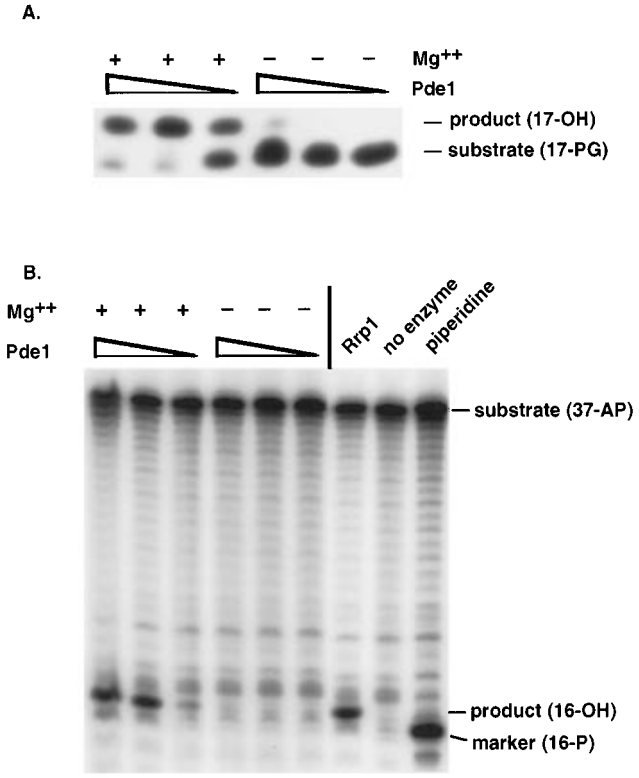

FIGURE 6: Phosphodiesterase and AP endonuclease assays of partially purified Pde1. FIV Pde1 (see purification flow chart) was used in all assays. Mobilities and size in nucleotides of substrate and product are indicated. (A) Enzyme additions to phosphodiesterase assay were as follows: 500, 125, 32, 500, 125, and 32 ng of Pde1 in lanes 1−6, respectively. Reactions were incubated at 30 °C for 20 min. PG indicates 3′-terminal phosphoglycolate. (B) The labeled 37mer oligonucleotide contains a single abasic site at postition 17. Enzyme additions to the AP endonuclease assay were as follows: 125, 50, 5, 125, 50, and 5 ng of Pde1 in lanes 1−6, respectively; 0.2 ng of Rrp1, lane 7; no enzyme, lane 8. Piperidine-treated substrate is shown in lane 9. Reactions were incubated at 30 °C for 15 min. P indicates 3′-terminal phosphate.

with a ssDNA abasic oligonucleotide (compare Figure 7, lanes 2 and 4). The degradation pattern visible in Figure 7, lane 2, is attributable to the weak nonspecific 3′-exonuclease associated with FIV Pde1.

DISCUSSION

The distribution of AP endonuclease activities found in extracts from various species demonstrates significant differences in the number of enzymes found and their characteristics. In extracts from *E. coli*, the pattern of expression of class II AP endonucleases includes exonuclease III, an abundant constitutive enzyme, and endonuclease IV, a much less abundant but inducible activity; this pattern has not been documented in extracts from cells of other species. However, functional redundancy of DNA repair nucleases has been noted in extracts from several cell types, including human (Chen et al., 1991) and mouse (Wakabayashi et al., 1996). In this work, we demonstrate the presence of a low-abundance repair nuclease from yeast cells, Pde1, which is redundant in enzymatic function to the well-characterized abundant and constitutive yeast enzyme, Apn1. By corollary to evidence that strongly supports important biological roles for Apn1 and many similar DNA repair enzymes, it is likely that Pde1 contributes to genomic stability in yeast.

FIGURE 7: Pde1 requires a dsDNA substrate. AP endonuclease assay was carried out for 20 min at 30 °C. The labeled 37mer oligonucleotide contains a single abasic site at postition 17. The labeled substrate was annealed to a complementary oligonucleotide (lanes 3 and 4) or mock-annealed in the absence of complement (lanes 1 and 2). 125 ng of FIV Pde1 was added to the assays in lanes 2 and 4. Piperidine-treated substrate is shown in lane 5.

Pde1 is a Mg^{2+}-dependent class II AP endonuclease and 3′-phosphodiesterase present in low amounts in Apn1-deficient cells. After approximately 500-fold purification, Pde1 demonstrates highly specific interactions with DNA damage and low levels of nonspecific nuclease activity on undamaged DNA. However, a weak exonuclease active on 3′-DNA ends, which decreases in specific activity during the purification, remains associated with FIV Pde1. While enzymatically similar to Apn1, Pde1 is clearly distinct from that enzyme: first, it is present in cells lacking a functional Apn1 gene; and second, unlike Apn1, it demonstrates strong dependence on exogenous Mg^{2+} for activity. While several yeast nucleases have been characterized, including those involved in base excision repair or nucleotide excision repair (Eide et al., 1996; Habraken et al., 1995; Sung et al., 1993; Chow & Resnick, 1987; Dake et al., 1988), no other report of a yeast nuclease with the enzymatic properties of Pde1 has been made.

The exonuclease III family of proteins includes members from nine species including *E. coli*, *Streptococcus*, *Pseudomonas*, *Dictyostelium*, *Arabidopsis*, *Drosophila*, mouse, rat, and human (Puyet et al., 1989; MacGregor et al., 1996; Freeland et al., 1996; Sander et al., 1991a; Babiychuk et al., 1994; Wilson et al., 1994; Seki et al., 1991; Robson et al., 1991; Demple et al., 1991; Robson & Hickson, 1991); thus, it is unusual that, to date, a yeast protein related to exonuclease III has not been reported. While Pde1 is enzymatically similar to exonuclease III, experiments have not established any structural or physical relationship between Pde1 and either exonuclease III or endonuclease IV. Antigenic similarity was tested by probing partially purified Pde1 with polyclonal antibodies to exonuclease III, APN1, and Rrp1, or anti-peptide antibodies to Apn1 and Rrp1 (data not shown), revealing no specific cross-reactivity. Further, no genes other than Apn1 are identified by searches of the

genome of *S. cerevisiae* for genes containing conserved motifs from known AP endonuclease protein sequences. Thus, it is unclear if the amino acid sequence of Pde1 is homologous to that of other similar DNA repair enzymes, and it remains possible that Pde1 is a novel enzyme whose homologues in other species have not yet been characterized.

The amount of detectable Pde1 was compared for both normal and petite cultures of DRY377 (lacking Apn1), and in mitochondrial and nuclear extracts of DRY377. Similar activity levels were observed for all four extracts (data not shown). These experiments suggest that Pde1 is not localized exclusively to either the mitochondrion or the nucleus, and that Pde1 is encoded by a nuclear gene. The amount of 3′-phosphodiesterase was also tested in crude extracts of wild-type and Apn1-deficient cells following exposure to various levels of bleomycin, hydrogen peroxide, or MMS: no change in the activity level was observed (data not shown), suggesting that Pde1 may not be inducible by DNA damage.

The biochemical properties of Pde1 suggest that it may play a role in maintaining genomic stability, since it has a high specificity for recognition of biologically important DNA lesions. As mentioned above, by precedent, it was expected that additional 3′-PDE enzymes would be present in yeast. In addition, previous work also suggests that an Apn1-independent pathway may exist for repair of 3′-blocking groups, since Apn1-deficient yeast cells are resistant to treatment with bleomycin. Future experiments will address how Pde1 may contribute to DNA repair in yeast.

In conclusion, this report identifies a low-abundance yeast DNA repair enzyme, Pde1, with specificity for repair of abasic sites and oxidative DNA damage at 3′-DNA termini. Enzymatically identified as a class II AP endonuclease, Pde1 is biochemically similar to the abundant yeast protein Apn1. The DNA repair specificity of Pde1 suggests that it is likely to help maintain genomic stability in yeast; understanding the role of Pde1 will contribute to a complete analysis of DNA repair mechanisms in yeast.

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REFERENCES

Babiychuk, E., Kushnir, S., van Montagu, M., & Inze, D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3299.

Chan, E., & Weiss, B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3189.

Chen, D. S., Herman, T., & Demple, B. (1991) *Nucleic Acids Res. 19*, 5907.

Chow, T. Y., & Resnick, M. A. (1987) *J. Biol. Chem. 262*, 17659.

Cunningham, R. P., Saporito, S. M., Spitzer, S. G., & Weiss, B. (1986) *J. Bacteriol. 168*, 1120.

Dake, E., Hofmann, T. J., McIntire, S., Hudson, A., & Zassenhaus, H. P. (1988) *J. Biol. Chem. 263*, 7691.

Demple, B., Halbrook, J., & Linn, S. (1983) *J. Bacteriol. 153*, 1079.

Demple, B., Herman, T., & Chen, D. S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11450.

Eide, L., Bjoras, M., Pirovano, M., Alseth, I., Berdal, K. G., & Seeberg, E. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10735.

Freeland, T. M., Guyer, R. B., Ling, A. Z., & Deering, R. A. (1996) *Nucleic Acids Res. 24*, 1950.

Friedberg, E. C., Walker, G. C., & Siede, W. (1995) in *DNA Repair and Mutagenesis*, ASM Press, Washington, D.C.

Habraken, Y., Sung, P., Prakash, L., & Prakash, S. (1995) *J. Biol. Chem. 270*, 30194.

Johnson, A. W., & Demple, B. (1988) *J. Biol. Chem. 263*, 18009.

Kunz, B. A., Henson, E. S., Roche, H., Ramotar, D., Nunoshiba, T., & Demple, B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8165.

Lindahl, T., & Nyberg, B. (1972) *Biochemistry 11*, 3610.

Linn, S., Demple, B., Mosbaugh, D. W., Warner, H. R., & Deutsch, W. A. (1981) in *Chromosome damage and repair* (Seeberg, E., & Kleppe, K., Eds.) pp 97−112, Plenum Press, New York.

MacGregor, C. H., Arora, S. K., Hager, P. W., Dail, M. B., & Phibbs, P. V., Jr. (1996) *J. Bacteriol. 178*, 5627.

Masson, J.-Y., Tremblay, S., & Ramotar, D. (1996) *Gene* (in press).

Popoff, S. C., Spira, A. I., Johnson, A. W., & Demple, B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4193.

Puyet, A., Greenberg, B., & Lacks, S. A. (1989) *J. Bacteriol. 171*, 2278.

Ramotar, D., Popoff, S. C., Gralla, E. B., & Demple, B. (1991) *Mol. Cell Biol. 11*, 4537.

Robson, C. N., & Hickson, I. D. (1991) *Nucleic Acids Res. 19*, 5519.

Robson, C. N., Milne, A. M., Pappin, J. C., & Hickson, I. D. (1991) *Nucleic Acids Res. 19*, 1087.

Rogers, S. G., & Weiss, B. (1980) *Methods Enzymol. 65*, 201.

Sander, M., & Huang, S.-M. (1995) *Biochemistry 34*, 1267.

Sander, M., Lowenhaupt, K., Lane, W. S., & Rich, A. (1991a) *Nucleic Acids Res. 19*, 4523.

Sander, M., Lowenhaupt, K., & Rich, A. (1991b) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6780.

Seki, S., Ikeda, S., Watanabe, S., Hatsushika, M., Tsutsui, K., Akiyama, K., & Zhang, B. (1991) *Biochim. Biophys. Acta 1079*, 57.

Sung, P., Reynolds, P., Prakash, L., & Prakash, S. (1993) *J. Biol. Chem. 268*, 26391.

Taylor, A. F., & Weiss, B. (1982) *J. Bacteriol. 151*, 351.

Wakabayashi, H., Tsuji, T., & Seki, S. (1996) *Acta Med. Okayama 50*, 131.

Wilson, T. M., Carney, J. P., & Kelley, M. R. (1994) *Nucleic Acids Res. 22*, 530.

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