Analysis of class II (hydrolytic) and class I (β-lyase) apurinic/apyrimidinic endonucleases with a synthetic DNA substrate

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ABSTRACT

We have developed simple and sensitive assays that distinguish the main classes of apurinic/apyrimidinic (AP) endonucleases: Class I enzymes that cleave on the 3′ side of AP sites by β-elimination, and Class II enzymes that cleave by hydrolysis on the 5′ side. The distinction of the two types depends on the use of a synthetic DNA polymer that contains AP sites with 5′-[^{32}P]phosphate residues. Using this approach, we now show directly that *Escherichia coli* endonuclease IV and human AP endonuclease are Class II enzymes, as inferred previously on the basis of indirect assays. The assay method does not exhibit significant interference by nonspecific nucleases or primary amines, which allows the ready determination of different AP endonuclease activities in crude cell extracts. In this way, we show that virtually all of the Class II AP endonuclease activity in *E. coli* can be accounted for by two enzymes: exonuclease III and endonuclease IV. In the yeast *Saccharomyces cerevisiae*, the Class II AP endonuclease activity is totally dependent on a single enzyme, the Apn1 protein, but there are probably multiple Class I enzymes. The versatility and ease of our approach should be useful for characterizing this important class of DNA repair enzymes in diverse systems.

INTRODUCTION

Apurinic/apyrimidinic (AP)[1] sites in DNA are formed by spontaneous hydrolysis (1), by exposure to ionizing radiation (2), and as products of N-glycosylases acting on modified bases in DNA (3). Unrepaired AP sites block DNA synthesis and act as mutagenic targets *in vivo* (4). Two major classes of enzymes are known that cleave DNA at AP sites (Fig. 1). Class I AP endonucleases, otherwise known as AP lyases, catalyze β-elimination reactions at AP sites, leaving 3′-(4-hydroxy-5-phospho-2-pentenal) residues (5–7); these structures are also referred to as 3′-(2,3-didehydro-2,3-dideoxyribose) termini (3′-ddR5P)). All known Class I AP lyases have associated DNA glycosylase activity for modified bases (8,9). Class II AP endonucleases, in contrast, do not have associated glycosylase activity, and cleave hydrolytically 5′ to the AP site, producing nucleotide-3′-hydroxyl and 5′-deoxyribose-5-phosphate (5′-dR5P) residues on opposite sides of the nick (10–12, see Fig. 1). Quantitatively, Class II AP endonucleases are the predominant AP endonucleases in most organisms (3).

Class II AP endonucleases also remove various blocking groups, including ddR5P, from 3′-termini of DNA to produce active primer-termini for repair synthesis by DNA polymerase I (13–16). Thus, it has been proposed that repair of AP sites requires a Class II AP endonuclease at some point, either to precede the action of a 5′-deoxyribophosphodiesterase (dRPase) that removes 5′-terminal dR5P (17), or to unblock the 3′-ends left by Class I AP lyase (11). *Escherichia coli* and *Saccharomyces cerevisiae* mutants deficient in Class II AP endonuclease activity are hypersensitive to a variety of cytotoxic agents that produce AP sites (18, footnote 2). However, no such phenotypic sensitivity has been demonstrated for mutants deficient in Class I AP lyase activities (19,20).

A widely used assay for AP endonucleases involves nicking of heat/acid-depurinated, supercoiled plasmid or viral DNA (for example, see ref. 11). This type of assay does not distinguish between Class I and Class II enzymes. Because of the physiological importance of AP endonucleases, it would be useful to have methods specific for the two types of enzymes. In this paper, we describe assays that distinguish between Class I and Class II AP endonucleases. In addition to specificity, these methods have the advantages of simplicity, speed and high sensitivity.

MATERIALS AND METHODS

Enzymes and chemicals

E. coli endonuclease IV was purified essentially as described (14) with the following modifications: Fraction IV (14) was eluted from the single-stranded DNA agarose column using a linear

[1] The abbreviations are: AP, apurinic/apyrimidinic; dR5P, deoxyribose-5-phosphate; ddR5P, 2,3-didehydro-2,3-dideoxyribose-5-phosphate; dRPase, 5′-deoxyribophosphodiesterase; EDTA, ethylenediaminetetraacetic acid.
[2] S. Popoff and B.D., unpublished data
[3] D. Chen, T. Herman, and B.D., in preparation
[4] S. Popoff, A. Johnson, and B.D., unpublished data

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gradient from 50 to 350 mM NaCl, and this fraction (after passage through DEAE-cellulose; ref. 14) was concentrated in a Centriprep apparatus (Amicon) instead of by dialysis against polyethlylene glycol. *E. coli* uracil glycosylase was extensively purified from wild-type *E. coli* by sequential chromatography of a Polymin P-treated extract on phosphocellulose (eluted with 0.3 M NaCl), single-stranded DNA cellulose (eluted with 0.18–0.20 M NaCl), and Ultrogel AcA54. The enzyme was assayed by monitoring the release of [^{3}H]uracil from a synthetic poly[d(A-T)] DNA substrate containing [α-^{32}P, *uracil*-^{3}H]dUMP residues (15). One unit of uracil glycosylase releases 1 pmol uracil per minute at 37°C. Endonuclease III of *E. coli* (21) was a generous gift from Dr. Richard P. Cunningham (State University of New York, Albany). T4 endonuclease V (22), yeast AP endonuclease (15) and human T-lymphoblast AP endonuclease[3] were provided by Drs. Arlen Johnson, Sonya Popoff, and Davis Chen, respectively. Exonuclease III and lambda exonuclease (New England Biolabs), micrococcal nuclease (Pharmacia), and DNase I and spermidine (Sigma) were obtained from commercial sources. Units for nonspecific nucleases are defined by the supplier.

Preparation of crude extracts of *E. coli*

Strain AB1157 was a stock in this laboratory. Strains BW9109 (Δ(*xth-pnc*)), BW527 (*nfo*::*kan*), and BW528 (Δ(*xth-pnc*) *nfo*::*kan*) were kindly supplied by Dr. Bernard Weiss (University of Michigan). Extracts were prepared by French-press treatment of mid-log phase cells grown in LB broth (14). Extracts of *S. cerevisiae* strains DBY747 (*APN1*) and SCP762 (*apn1*::*URA3*) were prepared by Dr. Sonya Popoff as described (23). Protein concentrations were determined by the method of Bradford (24) using bovine serum albumin as the standard.

Assay for AP endonuclease activity

Poly[d(A-T)] containing [α-^{32}P, *uracil*-^{3}H]dUMP at a frequency of one dUMP per ~1000 nucleotides was synthesized as previously described (15), except that [α-^{32}P]dUTP was prepared from labelled dCTP by deamination of dCTP in 3 M $NaNO_2$, 1 M acetic acid for 4 h at ambient temperature followed by neutralization with 10% triethylamine. The polymer was stored frozen at −20°C. The [5′-^{32}P]AP sites were introduced into the polymer on the day of use. The dUMP-containing polymer (40 pmol of dUMP residues) was incubated in a final volume of 80 μl containing 35 mM Hepes·KOH, pH 7.6, 50 mM NaCl, 2 mM EDTA, 7 units of uracil glycosylase for 30 min at 37°C, then for 3 min at 65°C to inactivate the enzyme. The reaction was monitored by following the release of TCA-soluble [^{3}H]uracil.

The AP polymer at a concentration of 40 nM AP sites was incubated in 25 μl of the appropriate enzyme buffer (detailed below) and the indicated enzyme or reagent at 37°C for 10 min. In cases where a second enzyme was to be added, the first enzyme was inactivated by incubation at 65°C for 3 min. For post-treatment with alkali, 25 μl of 0.4 M NaOH, 20 mM EDTA were added, and the reaction mixture was incubated at 65°C for 45 min. For post-treatment with endonuclease IV, 0.5 unit of endonuclease IV was added, followed by incubation at 37°C for 45 min. The reactions were stopped by chilling on ice, followed by treatment with TCA and acid-washed Norit (25). Radioactivity in the Norit supernatent was quantitated by liquid scintillation counting. Descending paper chromatography of the Norit-nonadsorbed fraction was performed as described (15). One unit of AP endonuclease activity releases 1 pmol of radioactive material per minute at 37°C.

The following buffers were used for the enzyme reactions. Endonuclease IV, endonuclease III, and T4 endonuclease V reactions contained 50 mM Hepes·KOH pH 7.6, 50 mM KCl, 0.05 mg/ml bovine serum albumin, 1 mM dithiothreitol, 1 mM EDTA. Spermidine treatment was performed in the same buffer except at 65°C. For exonuclease III, 5–10 mM $MgCl_2$ was substituted for the EDTA. For yeast AP endonuclease, both EDTA and dithiothreitol were omitted and 200 mM KCl was used. Transition metals were not required in reactions with this enzyme, which had been purified in the absence of EDTA and reducing agents[4]. For the human AP endonuclease, reactions were performed in 50 mM Hepes·KOH pH 7.6, 100 mM KCl, 0.05 mg/ml bovine serum albumin, 10 mM $MgCl_2$. Other enzymes were used according to the supplier's specifications.

RESULTS

Strategy for Class I- and Class II-specific assays

The rationale for the assays is shown in Fig. 1 and is based on a method developed by Weiss (25) to determine the incision position of exonuclease III. Cleavage of an AP site by a Class II AP endonuclease leaves 5′[^{32}P]dR5P-DNA. The label can be

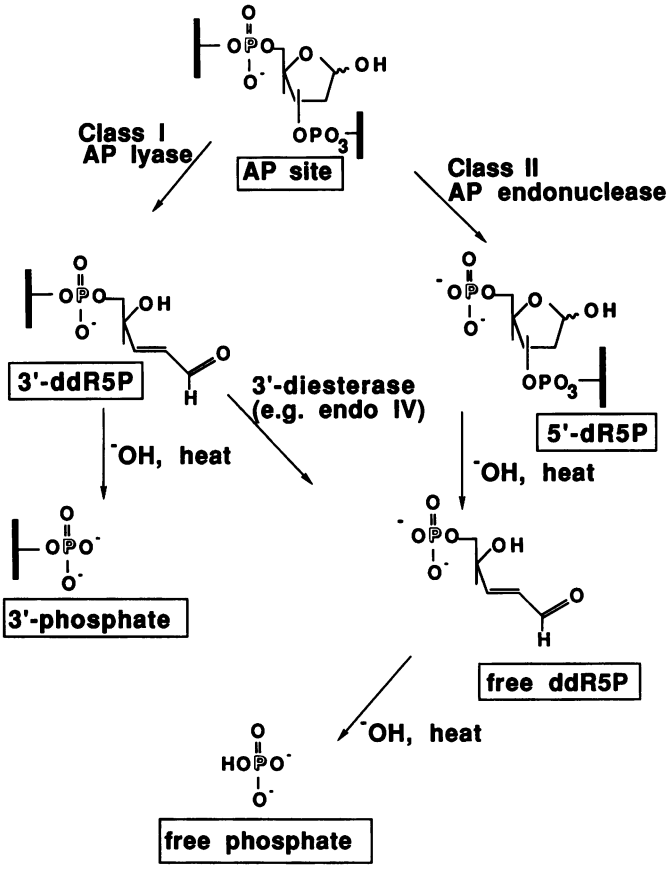

Figure 1: Principle of Class I and Class II AP endonuclease assays. The AP site is drawn attached to DNA at both ends (thick vertical bars). The ^{32}P-labelled phosphate (outlined and shadowed) is on the 5′-side of the AP site. Cleavage of AP sites by Class II AP endonucleases follows the right-hand pathway, the action of Class I AP lyases the left-hand pathway. The products expected from secondary reactions are shown. Endo: endonuclease.

released by alkali-catalyzed β-elimination, yielding free [^{32}P]4-hydroxy-5-phospho-2-pentenal (ddR5P) and a small amount of [^{32}P]phosphate from subsequent δ-elimination (see below). In contrast, enzymatic cleavage of AP sites by a Class I AP lyase leaves the radiolabel attached to the 3'-end of DNA, where it is not released by subsequent alkali treatment. However, treatment of this 3'-labelled DNA by a 3'-diesterase such as endonuclease IV releases the radiolabel from DNA (14). This difference allows Class I and Class II enzymes to be distinguished from each other by treatment of their reaction products with endonuclease IV or alkali, respectively.

Site of action of endonuclease IV

E. coli endonuclease IV was believed to cleave AP sites via a Class II (hydrolytic) mechanism, based on the observation that the enzyme generates nicks in DNA that are active primers for DNA polymerase I (11,14). The experiments presented here confirm this conclusion. Radioactive, Norit-nonadsorbable product was efficiently released from the synthetic AP substrate by the sequential action of endonuclease IV and alkali. The kinetics of endonuclease IV action in this reaction were approximately linear (Fig. 2A). The two-stage reaction also exhibited linearity over a wide range of endonuclease IV concentrations (Fig. 2B). The specific activity of purified endonuclease IV on this substrate was comparable to the specific activity of the enzyme on partially depurinated plasmid DNA and on a DNA-3'-phosphoglycolaldehyde substrate (14).

Specificity of the Class II AP endonuclease assay

The utility of the site-specifically labeled polymer was tested with respect to different classes of AP endonucleases from both prokaryotic and eukaryotic sources, and for interference by nonspecific enzymes and chemicals. *E. coli* exonuclease III cleaves on the 5' side of AP sites, leaving 3'-hydroxyl and 5'-phosphoryl ends (10). Indeed, the release of labelled material from the products of exonuclease III action depended almost entirely on subsequent treatment with alkali (Fig. 3A). In contrast, AP lyases such as *E. coli* endonuclease III (21) and T4 endonuclease V (22) did not release significant Norit-nonadsorbable radioactivity when followed by alkali, but only upon incubation with a 3'-diesterase such as endonuclease IV (Figure 3A). Thus, AP lyases (Class I enzymes) can be distinguished from hydrolytic (Class II) AP endonucleases by their dependence on a particular post-treatment to release the label. This specificity is in contrast to the usual AP nicking or DNA degradation assays (11,18), which do not distinguish between the different types of incisions. A small amount of label was reproducibly released in $MgCl_2$-containing enzyme blank reactions, and the products were identical to those observed with exonuclease III or endonuclease IV followed by alkali (data not shown).

Two nonspecific endonucleases, DNase I and micrococcal nuclease, were tested for interference in the Class II assay. These enzymes released insignificant amounts of Norit-nonadsorbable product when followed by alkaline treatment, although they efficiently generated TCA-soluble material (Fig. 3B). This result shows that these enzymes were active on this substrate without hydrolyzing the phosphodiesters 5' to the AP sites. Lambda exonuclease, a nonspecific exonuclease, did not release significant amounts of TCA-soluble material from the AP DNA substrate (data not shown), which indicates that this enzyme may be blocked by AP sites. Spermidine, a β-elimination catalyst that mimics the action of a class I AP lyase, allowed the release of significant amounts of labelled product by endonuclease IV, but not by alkali (Fig. 3B). Higher concentrations of spermidine led to reduced amounts of Class I-type release (data not shown), perhaps by interfering with the action of endonuclease IV or by forming precipitates with DNA.

The major AP endonucleases from *Saccharomyces cerevisiae* (15,16) and from a human T-lymphoblast cell line[3] have been purified to physical homogeneity in our laboratory. Both of these enzymes led efficiently to the release of label from the AP

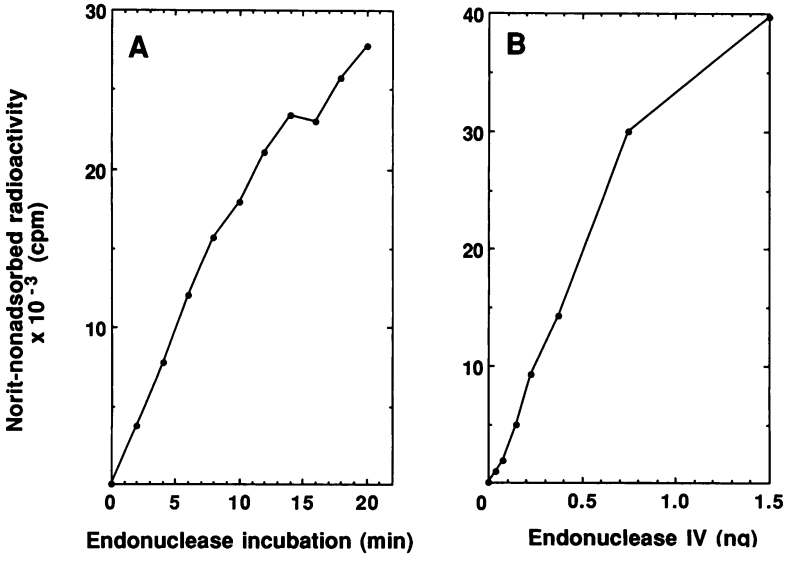

Figure 2: Endonuclease IV action on the AP polymer: Dependence on time and enzyme concentration. The specific radioactivity of the substrate was 176 cpm per fmol of AP sites. The radioactivity released was corrected for Norit-nonadsorbable counts without enzyme addition. *A*: Endonuclease IV (2 ng) was added to AP polymer in 350 μl reaction buffer at 37°C. Aliquots (25 μl) were removed to ice at the indicated times and treated with alkali as described in the text. *B*: The indicated amounts of endonuclease IV were added to the AP polymer in 25 μl reaction buffer and incubated at 37°C for 10 min. The samples were then treated with alkali as described in Methods.

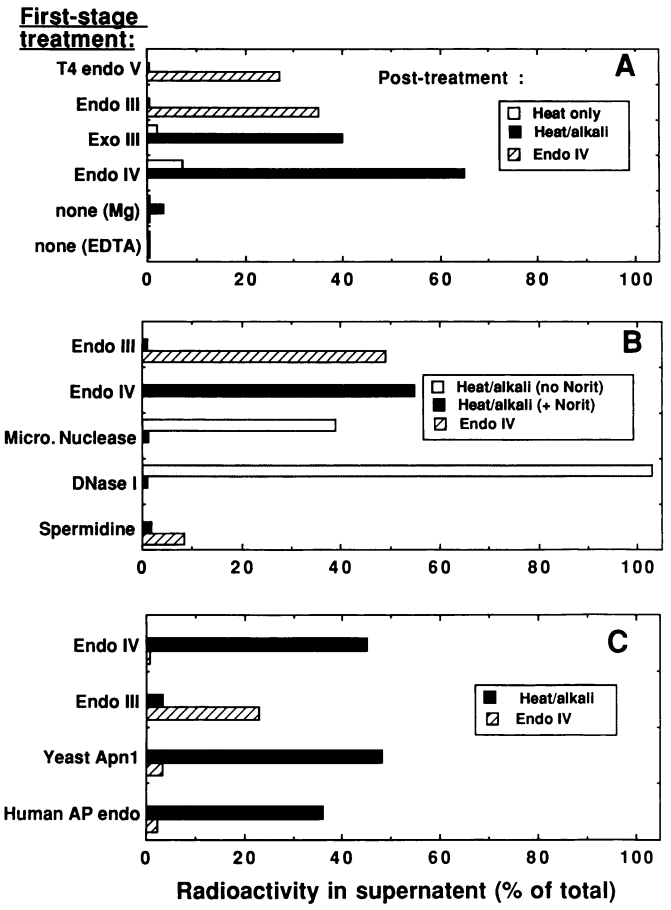

Figure 3: Dependence of the release of Norit-nonadsorbable products on enzyme and chemical treatments. AP polymer (1 pmol of AP sites) in 25 μl reaction buffer was treated as indicated and then incubated 10 min at 37°C (enzymes) or 65°C (spermidine). The reactions were then treated as indicated. Enzyme units are defined in Materials and Methods. In cases where no bar appears, that combination was not tested. The specific radioactivity in all cases was 140–170 cpm per fmol of AP sites. The detection limit in all cases was ≤ 3% release of label (≤ 1000 cpm for reactions without Mg^{++}, ≤ 5000 cpm for reactions with Mg^{++}). Endo: endonuclease. Exo: exonuclease. *A: Class I AP lyases vs. Class II AP endonucleases.* The rows 'none ($MgCl_2$)' and 'none (EDTA)' are enzyme blanks with buffer that included $MgCl_2$ or EDTA, respectively. The following amounts of enzyme were used: T4 endo V, 0.1 unit; endo III, 0.2 unit; exo III, 0.07 unit; endo IV, 1.0 unit. The radioactivity released was corrected for Norit-nonadsorbed counts obtained with untreated substrate. The short bars in the 'none (EDTA)' rows represent the detection limit of the experiment. *B: AP-specific vs. nonspecific enzymes and chemicals.* The following amounts of enzyme were used: Endo III, 0.2 unit; endo IV, 0.5 unit; micrococcal nuclease, 0.08 unit; DNase I, 5.5 units. Spermidine treatment was in 10 mM spermidine in endonuclease IV buffer for 10 min at 65°C. The amount of label released was corrected for Norit-nonadsorbed counts obtained with no first-stage enzyme treatment. The black bar in the 'Endo III' row represents the detection limit in this experiment. Micro: micrococcal. *C: Prokaryotic vs. eukaryotic AP endonucleases.* The following amounts of enzyme were used: Endo IV, 0.25 unit; endo III, 0.35 unit; yeast Apn1 protein, 0.15 unit; human AP endonuclease, 0.06 unit. The amount of radioactivity released was corrected for Norit-nonadsorbed counts obtained with no first-stage enzyme treatment. The striped bar in the 'Human AP endo' row represents the detection limit for this enzyme, which was slightly higher than the detection limit for endonuclease IV. The detection limit for yeast Apn1 was similar to the detection limit for the human enzyme.

substrate upon subsequent treatment with alkali but not endonuclease IV (Fig. 3C) and are therefore Class II AP endonucleases. Thus, the assay approach is generally useful for the identification and measurement of AP endonucleases from a variety of organisms.

Table 1: Class II AP endonuclease activity in *E. coli* mutants

Strain	Class II AP endonuclease activity (units/mg)	
	in 5 mM $MgCl_2$	in 1 mM EDTA
xth$^+$ *nfo*$^+$	46	4.9
Δ*xth* *nfo*$^+$	4.1	3.6
xth$^+$ *nfo*::*kan*	43	0.8
Δ*xth* *nfo*::*kan*	1.2	<0.2

The data were obtained from the initial portions of the curves in Fig. 4A,B.

Class II AP endonucleases in crude extracts

Exonuclease III is responsible for ~90% of the AP endonuclease activity in *E. coli* crude extracts detected using DNA degradation assays (18,26). In the presence of EDTA, this enzyme is inactive, but at least three other AP endonucleases are active: endonuclease IV (27), endonuclease III (28), and Fpg protein (9). Of these enzymes, only endonuclease IV is a hydrolytic AP endonuclease. This distribution is reflected accurately in the activities detected in the Class II assay under different conditions and with extracts of different mutant *E. coli* strains (Fig. 4A,B). Exonuclease III (encoded by *xth*) was the predominant Class II AP endonuclease in the presence of Mg^{++} (Fig. 4A), but was almost undetectable when Mg^{++} was replaced with EDTA (Fig. 4B). In contrast, endonuclease IV (the *nfo* gene product) made only a small contribution when exonuclease III was active (Fig. 4A), but accounted for most of the activity detected in the presence of EDTA (Fig. 4B). The small amount of EDTA-resistant endonuclease activity in *nfo*::*kan* extracts (Fig. 4B) was due to residual activity of exonuclease III in the presence of EDTA, as this activity was absent from extracts of cells deficient in both enzymes (Fig. 4B). These experiments demonstrate that the Class II AP endonuclease assay can be applied quantitatively to activities in crude bacterial extracts to compare enzyme levels under different conditions. The enzyme levels in different strains, with and without Mg^{++} in the assay, are summarized in Table 1.

The Apn1 protein from *S. cerevisiae*, which is homologous to endonuclease IV (23), is the only Class II AP endonuclease detectable in yeast crude extracts (23). This result is confirmed in Fig. 4C, which demonstrates the utility of this assay in extracts of a eukaryotic cell.

Linearity and utility of the Class I assay

When endonuclease III was present in a first-stage reaction, the subsequent release of labeled product by endonuclease IV was linear with respect to the amount of endonuclease III used (Fig. 5). In crude extracts of *E. coli*, however, substantial amounts of labelled product were released even with no post-treatment (data not shown). This result could indicate sequential action of Class I AP lyase followed by endonuclease IV, or endonuclease IV followed by a dRPase (17). The amount of label released by crude extracts of *E. coli* in the absence of any post-treatment was unaffected by EDTA, but was dependent on the presence of Class II AP endonuclease activities (data not shown). Thus, the reaction in *E. coli* crude extracts is probably due to the sequential action of AP lyase followed by hydrolytic AP endonuclease, because the known dRPase of *E. coli* requires $MgCl_2$ for its activity (17). This is consistent with the high level of total Class I AP lyase activity estimated to be present in *E. coli* (9).

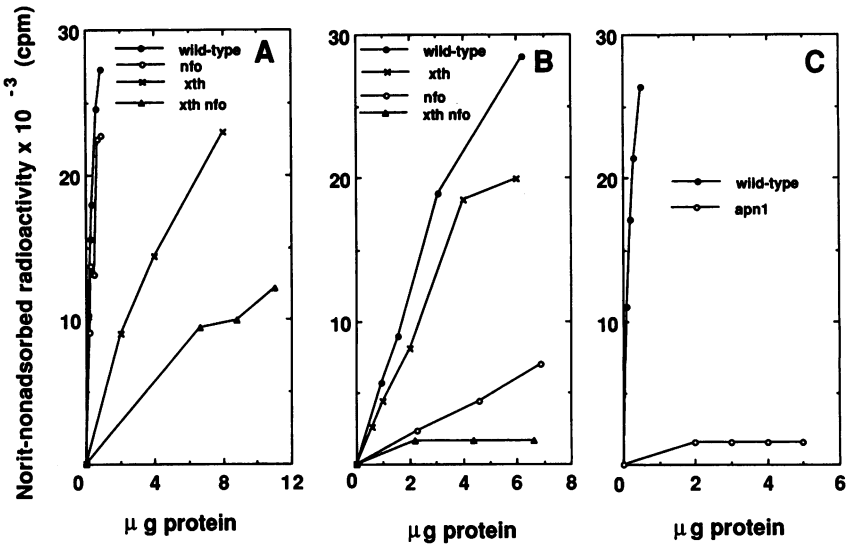

Figure 4: Class II Activities in Crude Extracts. Crude extracts of *E. coli* and *S. cerevisiae* were assayed for Class II AP endonuclease activity as described in the text. *A*, *E. coli* crude extracts assayed in the presence of 5 mM $MgCl_2$. *B*, *E. coli* crude extracts assayed in the presence of 1 mM EDTA. *C*, yeast crude extracts assayed as described in Methods. The relevant mutant genes in the individual strains are indicated in the insets. The amount of Norit non-adsorbed radioactivity released was corrected for the amount of label released with no extract addition. Specific activity was 105−130 cpm per fmol of AP sites in all cases.

Since yeast mutants deficient in Class II AP endonuclease are available (23), the amount of Class I AP lyase activity in yeast crude extracts could be determined. Extracts of SCP762 (*apn1::URA3*) released no detectable label in the absence of any post-treatment (data not shown), but post-treatment with endonuclease IV efficiently liberated significant amounts of radioactivity. The specific activity of Class I activity in a yeast *apn1::URA3* extract was 3.5 units/mg. This level is apparently much higher than that of yeast 'redoxyendonuclease' (an enzyme with enzymatic properties similar to endonuclease III) in crude extracts (estimated to be 0.03 units/mg, ref. 29), which indicates that there are multiple Class I AP lyases in yeast.

Identification of products released from the AP site

Chromatography of the alkali-released, Norit-nonadsorbed material obtained from the action of exonuclease III or endonuclease IV on the AP polymer showed three major products (Fig. 6A,B). The largest peak of radioactivity co-migrated with dR5P marker. This may be authentic dR5P (formed by hydration of ddR5P) or the β-elimination product itself, which separates poorly from dR5P on paper or thin-layer chromatography (16,22). A second peak of radioactivity co-migrated with phosphate, indicating that a substantial amount of δ-elimination had occured. The identity of the third peak is unknown, but it may correspond to 2-oxocyclopent-1-enyl phosphate, a known product of alkali degradation at AP sites in DNA (30). The AP polymer was also treated sequentially with endonuclease III and endonuclease IV, and the products were chromatographed. Only a single discrete species, which co-migrated with dR5P, was observed (Fig. 6C).

DISCUSSION

We have developed a convenient and sensitive assay approach that distinguishes between AP endonucleases that cleave by hydrolysis (Class II) or by β-elimination (Class I). Endonuclease IV exhibits a linear response in this assay with respect to both time and enzyme concentration, and the sensitivity is similar to

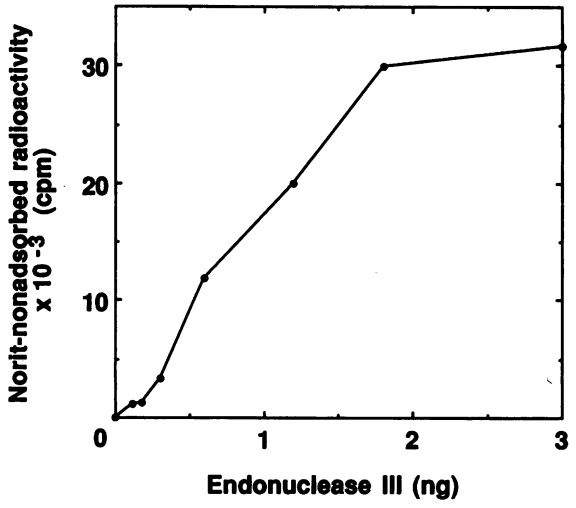

Figure 5: Endonuclease III action on the AP polymer. The indicated amounts of purified endonuclease III (21) were added to 40 nM AP polymer in 25 μl reaction buffer and the mixture was incubated at 37°C for 10 min. The samples were then treated with endonuclease IV as described in the text. The data were corrected for the amount of Norit-nonadsorbable radioactivity obtained from an enzyme blank. The specific radioactivity was 89 cpm per fmol of AP sites.

that of the 3′-phosphoglycolaldehyde diesterase assay for this enzyme (14) with a detection limit of approximately 0.001 unit (i.e. 1 fmol AP sites cleaved per minute). Nonspecific deoxyribonucleases and β-elimination catalysts do not interfere significantly with this assay, allowing the quantitation of Class II activities in crude extracts. The Class I assay is linear with a purified enzyme (endonuclease III), but may be difficult to apply to crude extracts owing to the presence of endogenous Class II AP endonucleases and dRPase.

The assays described here, in addition to their specificity, have other features which improve significantly on other AP endonuclease assays. These assays are rapid and simple, and the

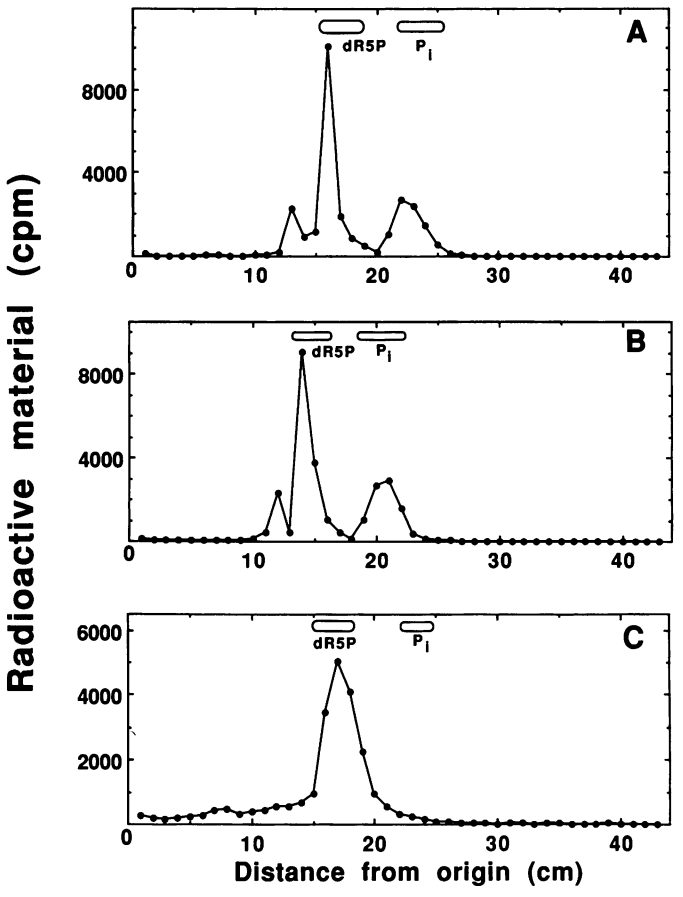

Figure 6: Chromatography of the products of enzyme-dependent reactions. AP polymer in 25 μl reaction buffer was treated with saturating amounts of enzyme followed by the indicated post-treatment: *A*, exonuclease III followed by alkali; *B*, endonuclease IV followed by alkali; *C*, endonuclease III followed by endonuclease IV. The Norit-nonadsorbed material from these reactions was chromatographed on paper as described (15). The positions of the marker compounds are indicated by ovals. P_i: orthophosphate.

results are easily quantitated. The linear ranges of the polymer-based assays are much broader than that of the plasmid nicking assay (11), while the sensitivities of the two methods are about equal.

One disadvantage of the polymer-based assay is the need to make fresh ^{32}P-labeled substrates periodically. The preparation is simple, however, and can be done on an approximately monthly basis without significant interference by the products of radioactive decay.

A second disadvantage of the assays described here is that special reagent enzymes are required that are commercially unavailable. For the Class II assay, only uracil glycosylase is required. This enzyme is abundant in wild-type *E. coli*, however, and is easily purified to a state where it is free of contaminating nucleases (31). A single preparation from ~ 100 g of *E. coli* has been sufficient in our hands for a large number of assays over >5 years. For the Class I assay, a 3′-diesterase is required as a reagent enzyme. Endonuclease IV is easily obtained in homogeneous form in a rapid purification (14) from an overproducing strain of *E. coli* (18). Exonuclease III, which also has the appropriate 3′-diesterase activity (13), is commercially available.

Linn et al. (32) have pointed out that at least four types of AP endonucleases might exist, depending on the possible incision positions adjacent to AP sites. To date, nearly all known AP endonucleases have been identified as either class I AP lyases or class II AP endonucleases. Spiering and Deutsch (33) reported a putative Class III AP endonuclease from *Drosophila*. Such an enzyme could be distinguished from Class I and Class II activities by chromatography of the products released by the enzyme. These products would be expected to behave quite differently from dR5P or ddR5P on chromatography. However, paper chromatography would not easily distinguish between Class I AP lyases and theoretical enzymes that cleave DNA hydrolytically 3′ to AP sites to leave 3′-dR5P termini, because dR5P is poorly separated from ddR5P (22). These two products could be distinguished by the reactivity of the latter toward low-molecular weight thiols (22,34). Alternatively, synthetic oligonucleotides containing AP site analogs (35) could be used. These oligonucleotides involve complex organic synthesis, however, and therefore are probably not suitable for routine use.

At least three Class II AP endonucleases have been purified to homogeneity from mammalian sources: calf thymus (36), HeLa cells (37), and human placenta (38). The latter enzyme has been proposed to incise AP sites by either a Class I or Class II mechanism, but not both (38). It would be useful to return to these enzymes with the assays described here in order to confirm their mechanism of action and to determine their relationship to each other and to the enzyme from human T-lymphoblasts[3].

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