

# Abasic site binding by the human apurinic endonuclease, Ape, and determination of the DNA contact sites

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## ABSTRACT

The mutagenic and lethal effects of abasic sites in DNA are averted by repair initiated by 'class II' apurinic (AP) endonucleases, which cleave immediately 5' to abasic sites. We examined substrate binding by the human AP endonuclease, Ape protein (also called Hap1, Apex or Ref-1). In electrophoretic mobility-shift experiments, Ape bound synthetic DNA substrates containing single AP sites or tetrahydrofuran (F) residues. No complexes were detected with single-stranded substrates or unmodified duplex DNA. In EDTA, the concentration of Ape required to shift 50% of duplex F-DNA was ~50 nM, while the addition of 10 mM MgCl<sub>2</sub> nearly eliminated detectable F-DNA·Ape complexes. Filter-binding studies demonstrated a half-life of ~50 s at 0°C for F-DNA·Ape complexes in the presence of EDTA, and <15 s after the addition of Mg<sup>2+</sup>. The DNA recovered from F-DNA·Ape complexes was intact but was rapidly cleaved upon addition of Mg<sup>2+</sup>, which suggests that these protein-DNA complexes are on the catalytic pathway for incision. Methylation and ethylation interference experiments identified DNA contacts critical for Ape binding, and Cu-1,10-phenanthroline footprinting suggested an Ape-induced structural distortion at the abasic site prior to cleavage.

## INTRODUCTION

Abasic sites in DNA arise via spontaneous or mutagen-induced hydrolysis of the N-glycosylic bond, or through the repair activity of DNA glycosylases (1,2). If left unrepaired, apurinic/aprimidinic (AP) sites are potentially lethal or mutagenic (3). To cope with the deleterious consequences of AP sites, organisms possess AP endonucleases that initiate the repair of these DNA lesions (2). Most notably, bacteria and yeast strains deficient in AP endonuclease activity display increased spontaneous mutation rates driven by AP site formation (4-7).

'Class II' AP endonucleases initiate repair by catalyzing the hydrolysis of the 5'-phosphodiester of an abasic site to generate

a 3'-OH group and a 5'-abasic residue (2). These enzymes also generate 3'-OH groups by removing fragmentary 3'-termini that arise from free radical attack on DNA, or from spontaneous or protein-catalyzed β-elimination reactions at AP sites. Class II AP endonucleases form two protein families based on homology to *Escherichia coli* exonuclease III or endonuclease IV (2).

Using synthetic DNA substrates containing different AP site analogs, we have found that exonuclease III and the homologous human AP endonuclease, Ape (8; also known as Hap1, Apex and Ref1; 9-11), have near-identical substrate specificities (12,13) that differ in key ways from the specificities of endonuclease IV and the related Apn1 protein of *Saccharomyces cerevisiae* (14). These findings corroborate other studies indicating differences in substrate preference between exonuclease III or mammalian AP endonuclease and endonuclease IV (15-18). Collectively, the data indicate (i) branching or oxidation at the 4-carbon inhibit exonuclease III and Ape strongly, but inhibit endonuclease IV and Apn1 weakly or not at all; (ii) stereospecific effects of different phosphorothioate diastereomers positioned on the 5' side of a tetrahydrofuran (F) residue. For all the enzymes, incision is reduced significantly by a mismatch immediately 5' to F and only slightly affected by the base opposite F. Whether these effects result from altered DNA binding or from changes in the rate of the incision step remains unknown.

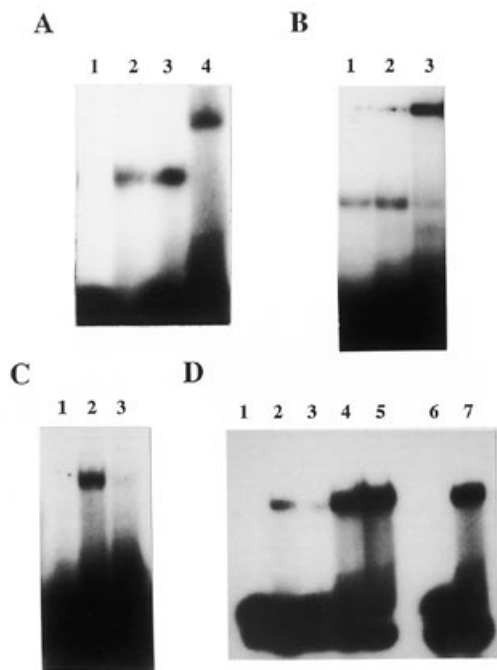
A key question of long standing has been the way in which repair proteins such as Ape engage damaged DNA. Here we present methods for detecting complexes of Ape protein with abasic DNA, and analyze the DNA contact sites in complexes of Ape with an abasic residue. Together with results from enzymatic analysis (13) and structural studies (19,20), this new work helps establish a framework for unraveling the mechanism of damage recognition and incision by these critical DNA repair enzymes.

## MATERIALS AND METHODS

### Materials

[γ-<sup>32</sup>P]ATP (3000 Ci/mmol) was purchased from DuPont NEN (Boston, MA), T4 polynucleotide kinase from New England Biolabs (Beverly, MA), and uracil-DNA glycosylase (UDG) from Gibco-BRL (Gaithersburg, MD). An oligonucleotide containing a single F (tetrahydrofuran, an AP site analog) residue was

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**Figure 1.** Specificity of Ape binding to DNA containing abasic sites and effects of  $Mg^{2+}$  and EDTA. (A) EMSA analysis of different Ape preparations. Binding reactions were performed with duplex DNA substrates containing F-DNA. Lane 1, DNA alone; lane 2, 28 nM Ape protein previously purified from HeLa cells (22); lane 3, 28 nM Ape protein newly purified from HeLa cells (13); lane 4, 28 nM purified GST-Ape fusion protein (13). (B) Presence of Ape in protein-DNA complexes. Binding reactions were performed as above (see also Materials and Methods), except the buffer contained 1 mM EDTA. Lane 1, 8 nM HeLa Ape; lane 2, 8 nM recombinant Ape from *E.coli*; lane 3, 8 nM recombinant Ape from *E.coli*, plus 1  $\mu$ l Ape-specific antiserum (8). (C) Destabilization of F-DNA·Ape complexes by  $Mg^{2+}$ . Lane 1, DNA alone; lane 2, Ape (28 nM; 13) and F (5 nM) in SBC buffer; lane 3, Ape and F in SBC buffer containing 10 mM  $MgCl_2$ . (D) Stabilization of F-DNA·Ape or AP-DNA·Ape complexes by EDTA. Lane 1, F-DNA alone; lane 2, Ape (28 nM), and F-DNA (5 nM) in SBC buffer; lanes 3–5, 2.8, 28 or 280 nM of Ape, and F-DNA (5 nM) in SBC containing 1 mM EDTA; lanes 6 and 7, AP-DNA (5 nM; following UDG treatment) without Ape or with 28 nM Ape in SBC containing 1 mM EDTA. All binding reactions were performed for 5 min at 0°C.

synthesized as described previously (12,13,21). A uracil-containing 18mer (U at position 10) was obtained from Operon Technologies, Inc. (Emeryville, CA).

### Purification of Ape

Highly purified (>95% purity; ref. 22) native Ape protein from HeLa cells was used unless otherwise specified. HeLa Ape of >80% purity (13) was used for some initial experiments and is so indicated in the Figure 1 legend. Recombinant (glutathione *S*-transferase)-Ape (GST-Ape) fusion protein (>90% purity) was isolated from *E.coli* bearing plasmid pGEX-Ape essentially as described (13), but without Factor Xa cleavage. Recombinant native-size Ape (>95% purity) was isolated using chromatography on DEAE-Bio-Gel agarose (BioRad) and phosphocellulose (Whatman) essentially as described (13).

### Incision analysis

Incision activity of Ape at the synthetic AP sites was determined in 10  $\mu$ l reactions in 50 mM HEPES-KOH (pH 7.5), 50 mM KCl, 100  $\mu$ g/ml BSA, 10 mM  $MgCl_2$ , 0.05% Triton X-100 performed as described in (13).

### Electrophoretic mobility shift assay (EMSA)

Ape protein was incubated with 5'- $^{32}P$ -labeled duplex DNA substrates (13) for 5 min at 0°C in 10  $\mu$ l SBC buffer (50 mM HEPES-KOH, pH 7.5, 50 mM KCl, and 10% glycerol) unless otherwise noted. The amount of residual single-stranded oligonucleotide present in these reactions was ~5% of the total substrate. Typically, 10 ng Ape protein (28 nM final concentration) was mixed with 0.05 pmol duplex DNA (5 nM final concentration). Binding reactions were resolved in non-denaturing polyacrylamide gels (8% acrylamide, 0.1% bis-acrylamide, 2.5% glycerol; 14 cm  $\times$  16 cm  $\times$  0.8 mm) in 20 mM Tris-HCl, pH 7.5, 10 mM sodium acetate, pH 7.5, 0.5 mM EDTA, and electrophoresis was performed at 4°C for ~2 h at 20 mA. The gels were dried and autoradiographed to identify the location of bound and unbound DNA. The percent of complexed DNA substrate was determined by excising bound and free DNA, and assaying the gel slices for  $^{32}P$  content using a Beckman LS1801 scintillation counter [percent bound = 100%  $\times$  (c.p.m. of protein-DNA complex) / (c.p.m. of protein-DNA complex + c.p.m. of unbound duplex DNA)].

For antibody 'supershift' experiments, Ape-specific rabbit antiserum (8,22) was added 2 min after the addition of Ape to DNA binding mixtures, and the incubation was continued at 0°C for an additional 20 min prior to electrophoresis. For competition reactions, a 10-fold excess of competitor DNA was mixed with end-labeled DNA substrates prior to the addition of Ape protein for DNA binding analysis.

Duplex DNAs with a centrally located uracil base in one strand were treated with 1 U UDG for 5 min at 37°C to generate a hydrolytic AP site. The resulting DNA was used directly in binding experiments; UDG did not detectably bind this DNA substrate under our conditions.

### Filter binding studies

Nitrocellulose filters (0.45 mm; Schleicher & Schuell) were pre-treated with 0.4 M KOH for 40 min at room temperature, washed thoroughly with distilled water and stored at 4°C in SBC buffer. Binding reactions in 10  $\mu$ l SBC supplemented with 1 mM EDTA contained 0.01 pmol duplex DNA and various amounts of Ape protein. After a 2 min incubation at 0°C, the samples were applied rapidly to filters (23) and washed twice with 500  $\mu$ l SBC containing 1 mM EDTA. The filters were then dried and assayed for bound  $^{32}P$  as described above. To determine the half-life of the protein-DNA complexes, a 100-fold excess of unlabeled competitor DNA was added with or without 10 mM  $MgCl_2$  and the percentage of bound DNA measured at the time points indicated.

### Analysis of DNA in protein-DNA complexes

Following non-denaturing gel electrophoresis, the gels were immediately autoradiographed at -80°C to identify the location of free DNA and protein-DNA complexes. The DNA containing regions were then excised from the gel and submerged into 500  $\mu$ l elution buffer (0.3 M sodium acetate, pH 7.0, 1 mM EDTA) pre-heated to 65°C to inactivate Ape, followed by incubation at

65°C for 15 min and at room temperature overnight to elute the DNA. In some experiments, the gel slices were incubated in SBC supplemented with 10 mM MgCl<sub>2</sub> for 1 min at 37°C, then returned to the sodium acetate/EDTA buffer and processed as described above. After the overnight incubation, residual gel pieces were removed by centrifugation at 14 000 g for 5 min and the supernatants retained. The DNA was precipitated by the addition of 10 µg yeast tRNA and 2.5 vol 100% ethanol, and incubation at -80°C for 15 min. The precipitated DNA was collected by centrifugation at 14 000 g for 30 min, resuspended in formamide gel loading buffer (23), and analyzed under denaturing conditions in 20% polyacrylamide gel containing 7 M urea (13).

### Interference and footprinting studies

The oligonucleotide strand to be analyzed was 5'-<sup>32</sup>P-labeled and annealed to unlabeled complementary DNA (13). In methylation interference studies, 1.25 pmol of this duplex DNA was methylated by dimethylsulfate treatment (23), incubated with Ape protein, and the binding reactions resolved by non-denaturing gel electrophoresis. Bound and unbound DNA was extracted from the respective gel slices, treated with 1 M piperidine (23), and analyzed on denaturing gels. In ethylation interference experiments, 1.25 pmol substrate DNA was treated with ethylnitrosourea (24), and the free and bound DNA analyzed for ethylation content by heat and alkali treatment (25).

*In situ* footprinting with 1,10-phenanthroline-copper ion was performed with 5'-<sup>32</sup>P-labeled DNA as described by Kuwabara and Sigman (26).

## RESULTS

### Binding of Ape to duplex DNA substrates

The initial studies determined whether Ape could bind abasic sites in DNA sufficiently stably to allow detection of DNA-protein complexes using conventional assays. Ape protein was isolated from HeLa cells and incubated with a synthetic DNA substrate containing a tetrahydrofuran residue (F, an AP site analog not sensitive to β-elimination; ref. 21). After incubation of Ape and DNA in SBC buffer (containing ≤ 0.5 mM residual MgCl<sub>2</sub> from the kinase reaction), EMSA revealed that Ape protein formed detectable complexes with duplex oligonucleotides bearing F (Fig. 1A). Ape never formed complexes detectable by EMSA with unmodified duplex DNA (data not shown). Positioning a guanine or cytosine opposite F, which may have different structural effects (27–29), did not dramatically affect (<2-fold) the amount of F-DNA · Ape complexes formed (data not shown). Under these conditions (SBC buffer), 5–10% of the available F-DNA substrate was shifted upon incubation with 10 ng of two different Ape preparations from HeLa cells (Fig. 1A, lanes 2 and 3). This determination omits the 'smear' (8–27% of the total DNA substrate; see Fig. 1A), which likely represents protein-DNA complexes that dissociated during electrophoresis. Thus, this method probably underestimates the amount of bound DNA.

A sample of recombinant GST-Ape fusion protein (~67 kDa) isolated from *E. coli* also showed binding to F-containing DNA, but yielded a complex of significantly slower mobility (lane 4 in Fig. 1A), consistent with the larger size of the fusion protein. GST protein (not fused to Ape) purified in the same manner (see Materials and Methods) did not form detectable complexes with

F-DNA (data not shown). Purified recombinant Ape protein (not fused to GST) isolated from *E. coli* formed complexes with F-DNA that had the same mobility as those formed by Ape from HeLa cells (Fig. 1B, lanes 1 and 2), and Ape-specific antibodies (8,22) supershifted the complexes formed by recombinant Ape (Fig. 1B). Thus, the observed protein · F-DNA complexes are formed by Ape and not by a contaminating protein.

Competition experiments were used to determine the selectivity of Ape binding to F-DNA. A 10-fold molar excess of unlabeled duplex competitor DNA containing F reduced the amount of EMSA complexes with the F substrate by 8-fold, while unmodified duplex DNA competitor caused only a slight decrease (11%) in the amount of Ape-DNA complexes detected. Single-stranded F-DNA was without effect in competition experiments.

### Effect of Mg<sup>2+</sup> on Ape-DNA binding

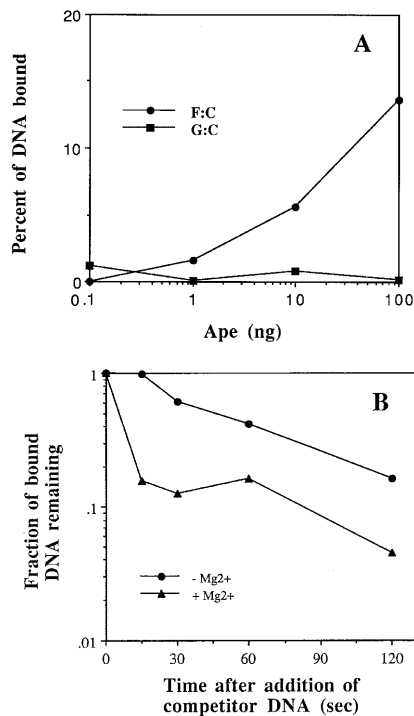
Because Mg<sup>2+</sup> stimulates Ape endonuclease activity (30) and low amounts of Mg<sup>2+</sup> were present in SBC (see above), we determined the effect of this metal on DNA binding. Addition of 10 mM MgCl<sub>2</sub> reduced detectable Ape binding to F-DNA, an effective substrate for cleavage (13), to <5% of that detected in SBC (Fig. 1C). In contrast, the addition of 1 mM EDTA increased the formation of F-DNA · Ape complexes >3-fold (at 10 ng of Ape) over SBC conditions (Fig. 1D, compare lanes 2 and 4). EDTA also stabilized Ape binding to DNA with a regular AP site generated by UDG (Fig. 1D, lanes 6 and 7), with binding efficiency similar to that for F in the same duplex (compare Fig. 1D, lane 4). However, F was used for interference and footprinting experiments (see below), because AP sites are prone to spontaneous cleavage via β-elimination (1,3,21).

Filter binding could also be used to detect Ape-DNA complexes in the presence of EDTA. An increasing fraction of F-containing DNA was trapped on nitrocellulose filters as the amount of Ape was increased from 0.1 to 100 ng per assay, while control DNA without abasic sites was not bound detectably (Fig. 2A). Filter-binding allowed us to determine the half-life of F-DNA · Ape complexes. In one set of experiments, after binding incubations, unlabeled F-DNA was added in a 100-fold excess over the labeled substrate with simultaneous addition of EDTA to 1 mM or MgCl<sub>2</sub> to 10 mM, and the amount of complex remaining was measured by filtration at specific times thereafter. These studies indicate half-lives of ~50 s for the F-DNA · Ape complexes in EDTA and <15 s in MgCl<sub>2</sub> (Fig. 2B). The results also indicate that a minor fraction of more stable complexes may persist in the presence of Mg<sup>2+</sup> (Fig. 2B).

### Intact DNA in Ape-DNA complexes

Pre-incision of duplex F-DNA with a catalytic amount of endonuclease IV virtually eliminated (≥95%) F-DNA · Ape complex formation even in the presence of EDTA (data not shown), which indicates that the complexes observed in EMSA are not due to product binding. When the DNA in F-DNA · Ape complexes (accounting for >70% of the total DNA; Fig. 3A) was isolated, >95% was present as the uncleaved 18mer in reactions with EDTA, and >83% in reactions in SBC buffer (Fig. 3B). For the latter, some incision may have occurred during DNA isolation. Intact DNA was also found within the 'smear' region of EMSA gels (Fig. 3A and B), consistent with the relative instability of F-DNA · Ape complexes even in the presence of EDTA (Fig. 2B). When gel slices containing F-DNA · Ape





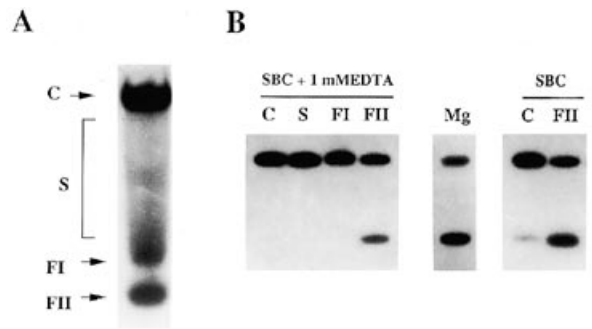
**Figure 2.** Filter-binding assay of Ape–DNA complexes. (A) Specificity for abasic DNA. Increasing amounts of Ape protein (0.28, 2.8, 28, and 280 nM) were incubated for 5 min at 0°C with 1 nM duplex DNA containing either F opposite C (F:C, circles) or a G:C base pair (G:C, squares), and subjected to filtration over nitrocellulose (see Materials and Methods). (B) Stability of F–DNA · Ape in EDTA or Mg<sup>2+</sup>. Binding reactions in SBC with 1 mM EDTA contained 1 nM F–DNA and 28 nM Ape protein and were incubated for 2 min at 0°C, at which time (defined as t = 0) an aliquot was added containing a 100-fold excess of unlabeled competitor F–DNA and sufficient MgCl<sub>2</sub> to achieve a final concentration of 10 mM (+Mg<sup>2+</sup>) or EDTA to a final concentration of 1 mM (–Mg<sup>2+</sup>). These additions increased the volume of the binding reactions <10%. At the indicated times, samples were removed for immediate assay by filtration over nitrocellulose. The experiment was replicated three times; data from a typical run are shown.

complexes were soaked briefly in Mg<sup>2+</sup> containing buffer at 37°C, the 18mer substrate was rapidly converted to the 9mer product (Fig. 3B). When F–DNA · Ape binding was performed in buffer containing 1 mM EDTA and MgCl<sub>2</sub> then added (10 mM) together with a 100-fold excess of unlabeled competitor F–DNA for 1 min at 0°C, the initial fraction of substrate DNA bound (37.3%) was close to the fraction cleaved (42.6% of the substrate converted to product). Thus, the complexed material is on the catalytic pathway for incision by Ape.

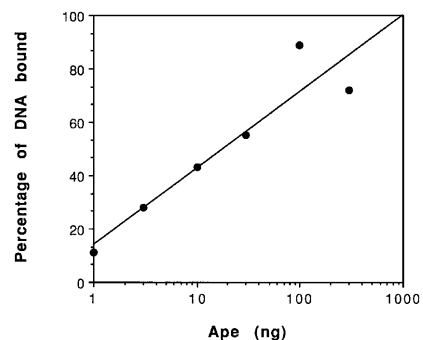
We determined the concentration dependence of Ape binding to duplex F–DNA substrate (FI, not FII; see Fig. 3A) in EDTA, independent of incision. Under these conditions, EMSA revealed that nearly all of the F–DNA could be bound (as in Fig. 3A), with half-maximal binding at ~50 nM Ape (Fig. 4).

### Interference and footprinting studies

For incision of F–DNA, Ape requires >4 bp of duplex DNA on the 5' side of the lesion and >3 bp on the 3' side (13). To identify potential base contacts in F-containing DNA, methylation interference experiments were conducted using a pair of 23mer substrates (Fig. 5A). For the strand containing the abasic site,



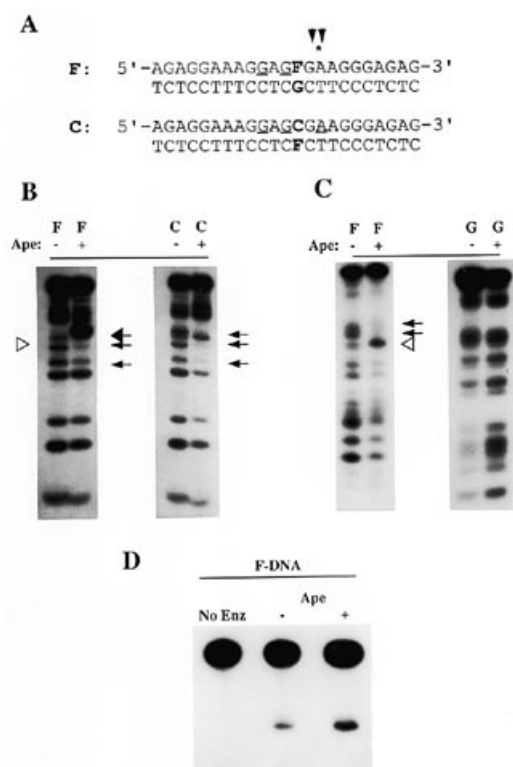
**Figure 3.** Analysis of DNA in complex with Ape protein. (A) EMSA of F–DNA and Ape. Ape protein (280 nM) was incubated with 5'-<sup>32</sup>P-labeled duplex F–DNA (5 nM) in SBC buffer containing 1 mM EDTA, and the binding reaction electrophoresed in a non-denaturing gel. The positions of the protein–DNA complexes (C), a smear of DNA (S), the uncleaved duplex DNA (FI), and single-stranded or incised DNA (FII) are indicated. (B) Nature of the DNA in Ape binding reactions. Binding reactions were performed in the indicated buffer (SBC buffer without or with 1 mM EDTA), and resolved on a non-denaturing gel as in (A). The DNA was then isolated from the gel slices of the indicated electrophoretic positions and analyzed on a denaturing gel (see Materials and Methods). The upper band is due to the intact 18mer substrate, while the lower band corresponds to the 9mer incision product. The lane labeled 'Mg' shows DNA that was isolated from a gel slice containing F–DNA · Ape complexes (C) formed in 1 mM EDTA, then soaked for 1 min at 37°C in SBC buffer containing 10 mM MgCl<sub>2</sub>, immediately heated at 65°C for 5 min, recovered and analyzed on a denaturing gel.



**Figure 4.** Concentration dependence of Ape binding to F–DNA. 5'-<sup>32</sup>P-labeled duplex F–DNA (5 nM) was incubated with 1 (2.8 nM), 3, 10, 30, 100 and 300 ng of recombinant Ape protein from *E.coli* (>95% purity) in SBC buffer containing 4 mM EDTA. Binding reactions were resolved on non-denaturing gels, and the percentage of bound DNA was determined as described in Materials and Methods using FI (see Fig. 3) as unbound DNA.

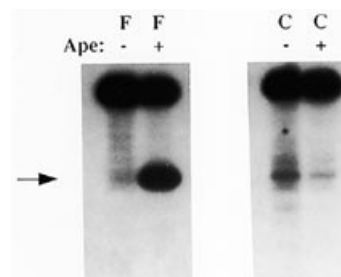
methylation of guanines located 1 or 3 bp 5' of the F residue prevented complex formation (Fig. 5B). Unexpectedly, methylation of an adenine residue 2 bp 3' of the abasic site stimulated Ape binding (Fig. 5B). Analysis of the strand opposite the AP lesion showed that methylations at 2 bp 5', or 1 or 3 bp 3' of the abasic site (Fig. 5B) interfered with Ape binding.

To identify critical contact sites for Ape along the phosphodiester backbone of duplex F–DNA, ethylation interference studies were undertaken (24). Ethylation obstructed Ape binding only at the phosphates two or three positions 3' of the abasic site on the damaged strand ('F' in Fig. 5C); no obvious ethylation interference was detected for the undamaged DNA strand ('G' in Fig. 5C). Strikingly, a significant amount of cleaved DNA was present in the ethylated F–DNA · Ape complexes formed in the presence of



**Figure 5.** Alkylation interference analysis of Ape binding to F-DNA. (A) Nucleotide sequence of the DNA substrates. The strand indicated (F, the abasic site-containing strand of the upper duplex; or C, the non-abasic strand of the lower duplex) was 5'-<sup>32</sup>P-labeled. The location of the F residue and the base opposite are shown in bold. Underlined positions indicate sites of methylation interference; the asterisk indicates a position where methylation enhanced Ape binding. The vertical arrowheads indicate sites of ethylation interference (see C). (B) Methylation interference assays. EMSA were performed with partially methylated DNA substrates (see Materials and Methods), and both free (-Ape) and complexed DNA (+Ape) were analyzed from the same lane. The recovered DNA was treated with piperidine and electrophoresed on a denaturing gel, followed by autoradiography. Sites of interference are indicated by the smaller arrowheads, and correspond to the underlined positions in panel A. The site of methylation enhancement of Ape binding is indicated by the larger arrowhead, corresponding to the asterisk in (A). The open arrowhead indicates the position of the F residue. (C) Ethylation interference assays. DNA was partially ethylated, subjected to Ape binding and electrophoresis as for (B), isolated and treated with heat and alkali before analysis on sequencing gels (see Materials and Methods). Symbols as for (B), with the arrows indicating sites of ethylation interference [overhead arrowheads in (A)]. The F-containing strand or the intact G-containing strand (G) of the upper duplex was 5'-end-labeled for these experiments. (D) Cleavage of ethylated F-DNA upon incubation with Ape in EDTA. Partially ethylated F-DNA (100 000 c.p.m.) was incubated with Ape (280 nM) and subjected to EMSA. Free DNA was isolated from a gel lane of a sample not incubated with Ape (No Enz), or free (-) and complexed (+) DNA from a lane of a sample incubated with Ape, then electrophoresed on a denaturing gel.

EDTA (Fig. 5C and D). Since incised DNA was not present in the protein-DNA complexes formed in the methylation interference studies, which employed the same oligonucleotides (Fig. 5B), this effect appears to be ethylation dependent. The cleaved DNA did not arise from the ethylation, electrophoresis or DNA recovery procedures per se, but was strongly enhanced by incubation with Ape protein (Fig. 5D). The unexpected cleavage in the presence of EDTA appeared to occur at the normal cleavage site for Ape and comprised ~5% of the total DNA for both the bound and



**Figure 6.** Cu-1,10-phenanthroline footprinting. Binding reactions were performed with F-DNA (100 000 c.p.m.) and Ape (280 nM), and electrophoresed in a non-denaturing polyacrylamide gel (see Materials and Methods). *In situ* footprinting was performed on the gel as described by (26). The gel was autoradiographed following treatment, and free (-Ape) and bound (+Ape) DNA eluted from the appropriate gel slices (see Materials and Methods). Either the F-containing strand (F) or the non-abasic strand (C) was 5'-<sup>32</sup>P-labeled. The position of the abasic site is indicated by an arrow, as determined by the parallel electrophoresis of the partial Ape-incised F-DNA substrate (not shown).

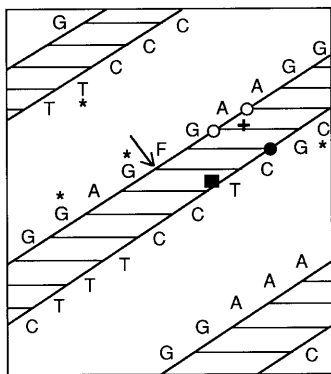
unbound fractions (Fig. 5D). However, random ethylation did not significantly enhance the rate of Ape turnover at 37°C (data not shown).

Cu-1,10-phenanthroline footprinting experiments further characterized the interaction of Ape with F-DNA. This reagent is sensitive to DNA conformational changes, particularly localized unwinding, brought on by association with proteins (31). *In situ* Cu-1,10-phenanthroline footprinting of F-DNA-Ape complexes and free DNA in EMSA gels revealed that Ape binding generates a hypersensitive site at F (Fig. 6, left). Since >95% of the complexed DNA was intact prior to treatment with the cleavage reagent (Fig. 3), it is unlikely that the cleaved product results from Ape catalyzed incision. Furthermore, 0.05 mM CuSO<sub>4</sub> alone did not support Ape-mediated cleavage (data not shown). In contrast to the abasic strand, some protection by Ape was seen for the undamaged strand opposite F, at a site that is hypersensitive to Cu-1,10-phenanthroline in the absence of protein (Fig. 6, right). This hypersensitivity may reflect some single-stranded character of the complementary strand opposite F, which becomes protected in Ape-DNA complexes.

## DISCUSSION

A previous study revealed several structural features of DNA substrates that affect Ape endonuclease activity (13). Meanwhile, a crystal structure was determined for exonuclease III (19) and used to model a proposed structure for the homologous Ape protein (20). However, structures for these enzymes bound to substrate DNA molecules have not been reported. We have addressed the issue of how AP endonucleases engage their substrate by defining contact sites of Ape protein with a stable abasic site in DNA.

Our findings show that binding and incision by Ape protein can be separated by relatively simple procedures. Two independent methods (EMSA and filter binding) demonstrated a clear preference of Ape for binding double-stranded DNA substrates with an F residue or a regular AP site compared to undamaged DNA. These complexes are evidently on the catalytic pathway, since F-DNA bound by Ape in the presence of EDTA is rapidly incised upon addition of MgCl<sub>2</sub>. Complex formation is not merely due to the sequence context, because EMSA revealed Ape



**Figure 7.** Major and minor groove interactions of Ape at an abasic site. A planar representation of the substrate is shown, in which the positions of the residues are projected onto the surface of a cylinder, which is then unrolled onto a flat surface. The base pairs are connected by solid lines that indicate the minor groove; the major groove corresponds to the blank diagonals. \*, sites of interfering guanine methylation; ■, site of interfering adenine methylation; +, site of enhancing adenine methylation; ○, site of interfering phosphate ethylation; ●, site of Ape protection against Cu-1,10-phenanthroline; →, Ape incision site and site of hypersensitivity to Cu-1,10-phenanthroline upon Ape binding. The T residues marked with \* and ■ correspond to the positions of G and A residues, respectively, in duplex 'C' of Figure 5. See text for discussion.

that bound a 51mer duplex (32) containing a central, UDG-generated AP site, or an F-site, with the same affinity as seen for the F substrate examined here (R.A.O. Bennett, D.M.W., D. Wong and B.D., submitted). Thus, the complexes observed in this work are appropriate for studying the details of the interaction of Ape with its substrate.

Ethylation interference indicated important contacts for Ape at phosphates two and three residues to the 3' side of F in the abasic strand (Fig. 7). Methylation interference experiments showed Ape contacts both the abasic and the undamaged DNA strand for 2–3 bp on either side of an F moiety, with major groove interactions indicated by the interfering effects of specific guanine methylations (Fig. 7). The enhancement of binding by methylation of an adenine 2 bp 3' of the abasic site is more unusual. One possibility is that Ape binding causes a structural distortion mimicked by adenine methylation (presumably at the N3 position in the minor groove), such as the base extrusion observed for T4 endonuclease V (33). However, a mismatch (C opposite A) at this position, expected to promote extrusion (34), did not detectably affect Ape binding (unpublished data).

Experiments probing the F-DNA·Ape complex with Cu-1,10-phenanthroline more directly indicate a structural distortion in the DNA exerted by Ape binding. Those experiments demonstrated that the target phosphodiester (5' of the abasic site) becomes strongly hypersensitive to Cu-1,10-phenanthroline cleavage in the complex. For the homologous protein exonuclease III, Mol *et al.* (19), based on the protein crystal structure, proposed possible DNA distortion upon binding.

The partial cleavage of F-DNA after ethylation may also be related to structural distortion, possibly of the abasic 5' phosphodiester upon ethylation. Alternatively, ethylation of this phosphodiester may simply alleviate the requirement for metal in Ape catalysis. In this regard, it is noteworthy that the ~5% cleavage observed (Fig. 5C) is similar to the fraction of ethylation expected for one phosphodiester out of the 44 present in the DNA

molecule. The (difficult) construction of site-specific ethylations might be necessary to address this possibility.

Complexes of Ape with F-DNA were found to be short-lived, which is not unexpected for a repair enzyme that needs to cleave its target sites and move on [Ape has a turnover number of ~500 min<sup>-1</sup> (13)]. Addition of Mg<sup>2+</sup>, a cofactor that stimulates Ape endonuclease activity (30), was found to shorten this half-life dramatically, whereas EDTA increased the amount and stability of F-DNA·Ape complexes. In the simplest interpretation, the addition of Mg<sup>2+</sup> promotes incision, which generates an even more unstable enzyme-product complex that rapidly dissociates. Indeed, no complexes of Ape were observed using EMSA when F-DNA was pre-incised by endonuclease IV (unpublished data).

Our studies have demonstrated refined analysis of the manner in which an AP endonuclease engages its target site in the context of undamaged DNA. The approach opens the way for further examination of the DNA structural determinants that mediate specific Ape binding to an abasic site. This information will be valuable for interpretation of eventual crystal structures for Ape or other AP endonucleases in complex with an abasic site in DNA.

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## REFERENCES

- Lindahl, T. (1993) *Nature* **363**, 709–715.
- Demple, B. and Harrison, L. (1994) *Annu. Rev. Biochem.* **63**, 915–948.
- Loeb, L.A., and Preston, B.D. (1986) *Annu. Rev. Genet.* **20**, 201–230.
- Ramotar, D., Popoff, S.C., Gralla, E.B., and Demple, B. (1991) *Mol. Cell. Biol.* **11**, 4537–4544.
- Xiao, W., and Samson, L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2117–2121.
- Kunz, B.A., Henson, E.S., Roche, H., Ramotar, D., Nunoshiba, T., and Demple, B. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 8165–8169.
- Wilson III, D.M., Bennett, R.A.O., Marquis, J.C., Ansari, P. and Demple, B. (1995) *Nucleic Acids Res.* **23**, 5027–5033.
- Demple, B., Herman, T., and Chen, D.S. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11450–11454.
- Robson, C.N., and Hickson, I.D. (1991) *Nucleic Acids Res.* **19**, 5519–5523.
- Seki, S., Hatsushika, M., Watanabe, S., Akiyama, K., Nagao, K., and Tsutsui, K. (1992) *Biochim. Biophys. Acta* **1131**, 287–299.
- Xanthoudakis, S., Miao, G., Wang, F., Pan, T.-C.E., and Curran, T. (1992) *EMBO J.* **11**, 3323–3335.
- Takeuchi, M., Lillis, R., Demple, B., and Takeshita, M. (1994) *J. Biol. Chem.* **269**, 21907–21914.
- Wilson III, D.M., Takeshita, M., Grollman, A.P. and Demple, B. (1995) *J. Biol. Chem.* **270**, 16002–16007.
- Demple, B., Harrison, L., Wilson III, D.M., Bennett, R.A.O., Takagi, T. and Ascione, A.G. (1997) *Environmental Health Perspectives*. In Press.
- Kow, Y.W. (1989) *Biochemistry* **28**, 3280–3287.
- Sanderson, B.J.S., Chang, C.-N., Grollman, A.P., and Henner, W.D. (1989) *Biochemistry* **28**, 3894–3901.
- Håring, M., Rudiger, H., Demple, B., Boiteux, S., and Epe, B. (1994) *Nucleic Acids Res.* **22**, 2010–2015.
- Ide, H., Tedzuka, K., Shimizu, H., Kimura, Y., Purmal, A.A., Wallace, S., and Kow, Y.W. (1994) *Biochemistry* **33**, 7842–7847.

- 19 Mol, C.D., Kuo, C.-F., Thayer, M.M., Cunningham, R.P., and Tainer, J.A. (1995) *Nature* **374**, 381–386.
- 20 Barzilay, G., Mol C.D., Robson, C.W., Walker, L.J., Cunningham, R.P., Tainer, J.A., and Hickson, I.D. (1995) *Nature Struct. Biol.* **2**, 561–568.
- 21 Takeshita, M., Chang, C.-N., Johnson, F., Will, S., Grollman, A.P. (1987) *J. Biol. Chem.* **262**, 10171–10179.
- 22 Chen, D.S., Herman, T., and Demple, B. (1991) *Nucleic Acids Res.* **19**, 5907–5914.
- 23 Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G., and Struhl, K. (1987) *Current Protocols in Molecular Biology*. John Wiley and Sons, NY.
- 24 Siebenlist, U., and Gilbert, W. (1980) *Proc Natl. Acad. Sci. USA* **77**, 122–126.
- 25 Manfield, I., and Stockley, P.G. (1994) *DNA–Protein Interactions: Principles and Protocols*, Humana Press Inc., NJ, pp. 125–139.
- 26 Kuwabara, M.D., and Sigman, D.S. (1987) *Biochemistry* **26**, 7234–7238.
- 27 Vesnaver, G., Chang, C.N., Fisenberg, M., Grollman, A.P., Breslauer, K.J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3614–3618.
- 28 Cuniassse, P., Fazakerley, G.V., Guschlbauer, W., Kaplan, B.E., and Sowers, L.C. (1990) *J. Mol. Biol.* **213**, 303–314.
- 29 Goljer, I., Kumar, S., and Bolton, P.H. (1995) *J. Biol. Chem.* **270**, 22980–22987.
- 30 Kane, C.M., and Linn, S. (1981) *J. Biol. Chem.* **256**, 3405–3414.
- 31 Spassky, A., and Sigman, D.S. (1985) *Biochemistry* **24**, 8050–8056.
- 32 Singhal, R.K., Prasad, R., and Wilson, S.H. (1995) *J. Biol. Chem.* **270**, 949–957.
- 33 Vassylyev, D.G., Kashiwagi, T., Mikami, Y., Ariyoshi, M., Iwai, S., Ohtsuka, and Morikawa, K. (1995) *Cell* **83**, 773–782.
- 34 Klimasauskas, S., and Roberts, R.J. (1995) *Nucleic Acids Res.* **23**, 1388–1395.