Endonuclease III Interactions with DNA Substrates. 1. Binding and Footprinting Studies with Oligonucleotides Containing a Reduced Apyrimidinic Site†

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ABSTRACT: The binding of endonuclease III from *Escherichia coli* to damaged DNA has been studied using gel shift and footprinting assays. Oligonucleotides containing a reduced apyrimidinic (AP) site were used since reduction of the AP site blocks the β-elimination reaction catalyzed by the enzyme and yields a noncleavable substrate. The K_{obs} for a 13-mer carrying a centrally located reduced AP site is $(2 \times 10^6)-(2 \times 10^7)$ M^{-1}, while the K_{obs} for a 13-mer with no damage is $(4.5 \times 10^3)-(3.2 \times 10^4)$ M^{-1} (approximately a 500-fold difference). Larger oligonucleotides would not enter a gel when endonuclease III was bound so that binding constants to oligonucleotides longer than 13 base pairs could not be determined directly. Competition assays suggest that the K_{obs} measured for both damaged and undamaged 13-mers is a minimum value and that the K_{obs} for larger oligonucleotides could be an order of magnitude greater. Fluorescence quenching on related 19-mers yielded a specific binding constant for the 19-mer carrying a centrally located reduced AP site for 4×10^7 M^{-1} and a nonspecific binding constant to an undamaged 19-mer of approximately 10^5 M^{-1} [Xing, D., Dorr, R., Cunningham, R. P., & Scholes, C. P. (1995) *Biochemistry 34,* 2537−2544]. Several footprinting reagents were used to determine the size and location of the endonuclease III binding site on damaged oligonucleotides. When endonuclease III is bound to a 25-mer or 39-mer duplex containing a centrally located reduced AP site, it protects 9−11 nucleotides on each strand from cleavage by deoxyribonuclease I and five nucleotides on each strand from cleavage by (methidiumpropyl-EDTA)Fe(II), indicating an extremely small binding site. Protection from hydroxyl radical cutting is only observed on the damaged strand, indicating that endonuclease III binds more tightly or closely to this strand. Also, endonuclease III does not protect guanine N7's in the major groove from methylation by dimethyl sulfate. Taken together, the footprinting studies imply that endonuclease III may bind along the minor groove side of the helix.

Endonuclease III from *Escherichia coli* is a DNA repair enzyme with both DNA *N*-glycosylase activity and AP[1] (apurinic/apyrimidinic) endonuclease activity. The DNA *N*-glycosylase activity cleaves a variety of ring-damaged, ring-rearranged, and ring-contracted pyrimidines formed by UV irradiation or active oxygen species (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1985; Boorstein et al., 1989; Dizdaroglu et al., 1993). The endonuclease activity cleaves the phosphodiester backbone of DNA 3′ to AP sites via a *syn* β-elimination reaction (Mazumder et al., 1991), leaving a baseless 3′ α,β-unsaturated aldose terminus.

The gene for endonuclease III has been cloned, sequenced, and overexpressed (Cunningham & Weiss, 1985; Asahara et al., 1989), allowing endonuclease III to be overproduced and large quantities to be purified to homogeneity (Asahara et al., 1989). Biophysical and biochemical characterizations have established that endonuclease III is a 4Fe-4S protein (Cunningham et al., 1989).

Endonuclease III has been crystallized (Kuo et al., 1992a) and the structure determined to 2.0 Å resolution (Kuo et al., 1992b). Endonuclease III is elongated and bilobal with a deep cleft separating two domains of similar size. One domain contains a unique, sequence-continuous, six-helix configuration. The other domain consists of the 4Fe-4S cluster and four α-helices, one formed at the amino terminal end and three by residues in the carboxyl terminal end, to form a Greek-key helix bundle. One face of the protein has a large positive electrostatic potential and has been implicated as the potential binding surface for damaged DNA.

An important step in understanding the mechanism by which endonuclease III recognizes and repairs damaged sites on DNA is to characterize the binding of endonuclease III to damaged DNA. Proteins that bind to specific sites on DNA can be grouped into two categories: those that bind to specific sequences and those that bind to specific structural elements or distortions. The binding of sequence-specific regulatory proteins has been well characterized by a number of binding and footprinting methods [reviews by Rhodes

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[1] Abbreviations and definitions: AP, apurinic/apyrimidinic; Ches, (cyclohexylamino)methanesulfonic acid; DMS, dimethyl sulfate; DNase I, bovine deoxyribonuclease I; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HE, Hepes−EDTA; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MPE-Fe(II), (methidiumpropyl-EDTA)Fe(II); OH•, hydroxyl radical; rAP, reduced AP; RNase, ribonuclease; TBE, Tris-borate−EDTA; Tris, tris(hydroxymethyl)-aminomethane; K_{obs}^{ns}, binding constant of endonuclease III to an undamaged 13-mer; K_{obs}^{sp}, binding constant of endonuclease III to a 13-mer carrying a reduced AP site.

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(1990), Tullius (1989), and Sauer (1991)].

These same techniques have been applied, to a limited extent, to studying the binding of structure-specific DNA repair enzymes to DNA, with the main limitation being the synthesis of substrates which are recognized but not cleaved by the repair enzyme in question. Binding and footprinting studies have been carried out for *E. coli* DNA photolyase (Sancar et al., 1987; Husain, et al., 1987), *E. coli* formamidopyrimidine-DNA glycosylase (Castaing et al., 1992; Tchou et al., 1993), and the UvrABC complex of *E. coli* (Van Houten et al., 1987), and binding experiments have been performed for T4 endonuclease V (Seawell et al., 1980) and the xeroderma pigmentosum group A complementing protein (Jones & Wood, 1993).

In this paper, we discuss similar experiments for endonuclease III. Oligonucleotide duplexes containing a single reduced AP site in the center of the duplex have been synthesized. These oligomers are recognized by endonuclease III but not cleaved. Quantitative gel shift assays have enabled us to determine binding constants for endonuclease III bound to oligonucleotides containing a reduced AP site. Competition gel shift assays have enabled us to determine the relative binding of endonuclease III to damaged and undamaged oligonucleotides. DNase I, MPE-Fe(II) OH•, and DMS footprinting data have enabled us to determine the size and location of the endonuclease III binding site. These results in conjunction with the crystal structure and spectroscopic techniques will aid in understanding the mechanism by which endonuclease III recognizes and repairs damaged DNA.

MATERIALS AND METHODS

Endonuclease III. Endonuclease III from *E. coli* was prepared and purified to homogeneity as described in Asahara et al. (1989), with minor modifications. The protein concentration was determined from absorbance at 280 nm using an extinction coefficient of 31.5 cm^{-1} mM^{-1} as described by Gill and von Hippel (1989).

Oligonucleotide Substrates. Several oligonucleotides of varying lengths were synthesized as substrates. Oligomers of 13, 25, or 39 nucleotides containing a single uracil in the center of each strand and their complementary strands containing an adenine in the center were synthesized on an Applied Biosystems Model 381A DNA synthesizer. Uracil was removed with uracil-DNA glycosylase to produce an apyrimidinic (AP) site which was reduced with sodium borohydride to yield a reduced apyrimidinic (rAP) site. Each oligomer was combined with its complementary strand to form the stable duplexes: (U•A)-13-mer, d(GCGCAGUCAGCCG)•d(CGGCTGACTGCGC) (rAP•A)-13-mer, d(GCGCAG(rAP)CAGCCG)•d(CGGCTGACTGCGC) (U•A)-25-mer, d(GCTCATGCGCAGUCAGCCGTACTCG)•d(CGAGTACGGCTGACTGCGCATGAGC) (rAP•A)-25-mer, d(GCTCATGCGCAG(rAP)CAGCCGTACTCG)•d(CGAGTACGGCTGACTGCGCATGAGC) (U•A)-39-mer, d(GCAGATGGCTCATGCGCAGUCAGCCGTACTCGGATCGCG)•d(CGCGATCCGAGTACGGCTGACTGCGCATGAGCCATCTGC) (rAP•A)-39-mer, d(GCAGATGGCTCATGCGCAG(rAP)CAGCCGTACTCGGATCGCG)•d(CGCGATCCGAGTACGGCTGACTGCGCATGAGCCATCTGC) See Fu et al. (1992) for complete details regarding oligomer synthesis and purification.

Gel Shift Assays. Each sample contained 10 μM (U•A)-13-mer or (rAP•A)-13-mer, 0−50 μM endonuclease III, 100 mM KCl, 50 mM potassium phosphate (pH 6.6), and 10% glycerol and was incubated at 0 °C for 30 min prior to loading 10 μL onto a 20% nondenaturing polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing HE buffer (100 mM Hepes-NaOH, 2 mM EDTA, pH 8.3). A Biorad Miniprotean II gel system was used with the inner and outer buffer chambers filled with HE buffer and equilibrated to 4 °C prior to loading the samples. The gels were run at 160 V for 3 h and stained with ethidium bromide or Coomassie blue.

Quantitative Gel Shifts. Each sample contained 10 nM ^{32}P-end-labeled (rAP•A)-13-mer, 0−50 μM endonuclease III, 100 mM KCl, 50 mM potassium phosphate (pH 6.6), and 15% glycerol and was incubated at 0 °C for 30 min prior to loading 10 μL onto a 20% nondenaturing polyacrylamide gel (29:1 acrylamide:bisacrylamide) containing HE buffer. The gels were run as described above, except the voltage was lowered to 100 V (15−20 mA) to minimize complex smearing. The dried gels were quantitated on a β-etagen β−scope 603.

Competitive Gel Shifts. Each sample contained 1 μM ^{32}P-end-labeled (rAP•A)-13-mer and 1 or 2 μM endonuclease III. The cold competitor oligomers were added to yield concentrations of 1−100 μM. Each sample also contained 100 mM KCl, 50 mM potassium phosphate (pH 6.6), and 20% glycerol. Samples were incubated at 0 °C for 30 min. Competition was analyzed on 15% nondenaturing polyacrylamide gels (79:1 acrylamide:bisacrylamide) containing HE buffer. Gels were run under the same conditions as with the quantitative gel shifts and quantitated on the β-scope.

DNase I Footprinting. Aliquots (10 μL) containing 40 nM ^{32}P-end-labeled (rAP•A)-25-mer or (rAP•A)-39-mer, 0 or 5−20 μM endonuclease III (in 125 mM KCl, 25 mM Hepes-NaOH (pH 7.6), 50% glycerol), 14 mM Tris-HCl (pH 8), 5 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM DTT, and 0.1 mg/mL BSA (nuclease free from Boehringer-Mannheim) were incubated for 30−60 min at room temperature; 1 μL of 0.03−1 unit of DNase I (RNase free from Boehringer-Mannheim) was added to each aliquot. The reaction was allowed to proceed for exactly 1 min at room temperature and promptly quenched with 10 μL of 20 mM EDTA and 0.1 mg/mL proteinase K. Endonuclease III was degraded by proteinase K at 53 °C for 30 min. Samples were phenol extracted, ethanol precipitated, resuspended in 5 μL of formamide dye, heat denatured, and analyzed on a 20% denaturing polyacrylamide gel (19:1 acrylamide: bisacrylamide) containing TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, 8.3 M urea, pH 8.3).

MPE-Fe(II) Footprinting. Aliquots (8 μL) containing 63 nM ^{32}P-end-labeled (rAP•A)-25-mer, 0 or 13 μM endonuclease III (in 250 mM KCl, 50 mM Hepes-NaOH (pH 7.6) without glycerol), 62.5 mM KCl, and 12.5 mM Hepes-NaOH (pH 7.6) were incubated for approximately 1 h at room temperature. Equal volumes of 1 mM methidium-propyl-EDTA (gift from B. Van Houten, University of Vermont; synthesized by P. B. Dervan, Caltech) and 2 mM $(NH_4)_2Fe(SO_4)_2$ (99.997% pure from Aldrich) were combined and diluted to yield a 100 μM MPE-Fe(II) solution. MPE was stored at −20 °C. The $(NH_4)_2Fe(SO_4)_2$ and MPE-Fe(II) solutions were made fresh prior to each experiment. One microliter each of 100 μM MPE-Fe(II) and 100 mM

sodium ascorbate (Sigma; solution made fresh) were added to each DNA or DNA-endonuclease III aliquot. Reactions were carried out for 10 min at 23 °C and quenched at −70 °C. Samples were further prepared and analyzed as in the DNase footprinting experiments.

OH• Footprinting. Samples each contained 0 or 5 μM endonuclease III (in 250 mM KCl, 50 mM Hepes-NaOH (pH 7.6) without glycerol) and 1 μM ^{32}P-end-labeled (rAP•A)-25-mer. All reagents were made fresh and kept on ice prior to use; 3 μL each of 1.5% H_2O_2, 100 mM ascorbate, and 1 mM Fe-EDTA (2 mM $(NH_4)_2Fe(SO_4)_2$, 4 mM EDTA) were quickly mixed, and 3 μL of the mixture was immediately added to 7 μL of each sample. The reactions were quenched after 2 min with 5 μL of 50% glycerol. Samples were further purified and analyzed as in the above footprinting experiments.

Methylation Protection. Dimethyl sulfate (1 or 2 μL) (Aldrich) was added to 200 μL aliquots containing 10 nM ^{32}P-end-labeled (rAP•A)-25-mer and 10 μM endonuclease III. The reactions proceeded for 2−20 min at room temperature and were quenched by the addition of 50 μL of 5 M ammonium acetate, 1 μg of calf thymus DNA, and 1 mL of 100% ethanol and incubation at −70 °C for 30 min. After ethanol precipitation, the samples were resuspended in 100 μL of 10% piperidine (Aldrich) and incubated at 95 °C for 30 min. The samples were dried, resuspended in 5 μL of formamide dye, heat denatured, and analyzed as in the other footprinting experiments.

RESULTS

Reduced AP Sites as a Model for AP Sites. In order for an oligonucleotide duplex containing a modified site to be used as a model system for binding studies with repair enzymes, the modified site must be recognized specifically but not cleaved by the enzyme in question. It has been documented by Jorgensen et al. (1987) that reduced AP sites are not cleaved by endonuclease III, which is as expected, since the β-elimination reaction can not take place if the reactive open chain aldehyde of an AP site is reduced to an alcohol. We have also confirmed that endonuclease III does not cleave the reduced AP sites in our oligonucleotides. When endonuclease III is incubated with oligomer duplexes of the same sequence containing either an AP site or a reduced AP site, there is complete cleavage of the AP site and no cleavage of the reduced AP site, respectively, as monitored by anion exchange HPLC analysis (data not shown). Although endonuclease III does not cleave the oligomers containing reduced AP sites, endonuclease III does recognize and bind specifically to the reduced AP sites, as illustrated by gel shift assays and footprinting experiments discussed throughout the rest of this paper.

The reduced AP site can be made quantitatively to yield one stable, uniform product. Degradation of the oligomer strand, containing the reduced AP site, with nuclease P1 and calf intestinal alkaline phosphatase, and subsequent HPLC analysis of the resultant nucleosides show that there are no side reactions at any of the DNA bases (data not shown). The reduced AP site oligomer duplexes, including the 13-mers, also form stable duplexes, where the T_m of the (rAP•A)-13-mer is ~45 °C at millimolar concentrations. Due to these characteristics, the reduced AP site oligomer duplexes readily

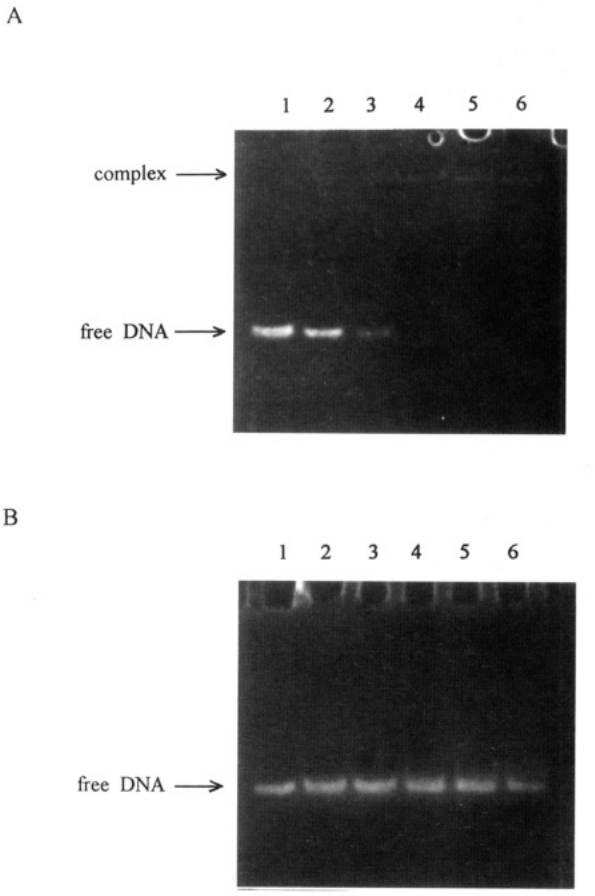

FIGURE 1: Gel shift assay of endonuclease III with modified and unmodified DNA. (A) (rAP•A)-13-mer. (B) (U•A)-13-mer. Lanes 1−6 contain the following ratios of endonuclease III to oligonucleotide duplex: 0:1, 0.5:1, 1:1, 2.5:1, 3.5:1, and 5:1, respectively. In A, the lower band corresponds to free DNA and the upper band corresponds to the endonuclease III−DNA complex. In B, all bands correspond to free DNA; no complex formation between endonuclease III and the unmodified DNA is observable.

lend themselves to being used in the binding and footprinting experiments that follow.

Gel Shift Assays. Formation of a stable DNA−protein complex between endonuclease III and rAP•A)-13-mer is observed by electrophoresis on a nondenaturing polyacrylamide gel (Figure 1A). As increasing amounts of endonuclease III are added to a set amount of the rAP oligomer duplex, the free DNA band disappears and the DNA−protein complex band increases in intensity. The composition of each band was identified by staining the gel with either ethidium bromide to locate the DNA or Coomassie blue to locate the protein. Ethidium bromide stains the lower band for DNA, while both ethidium bromide and Coomassie blue stain the upper band for DNA and protein. Only one complex band is observed. Since endonuclease III is a monomeric protein and each oligomer contains only one damaged site, we assume that a 1:1 complex is formed.

When endonuclease III is incubated with the unmodified DNA oligomer (U•A)-13-mer, no gel shift of the free DNA (no complex formation) is observed (Figure 1B). Thus, the interaction between endonuclease III and the reduced AP site is a specific interaction.

We also attempted to perform gel shift assays on the longer oligomer duplexes (rAP•A)-25-mer and (rAP•A)-39-mer. Surprisingly, complexes between endonuclease III and these longer oligomers did not enter the gel very well. Although

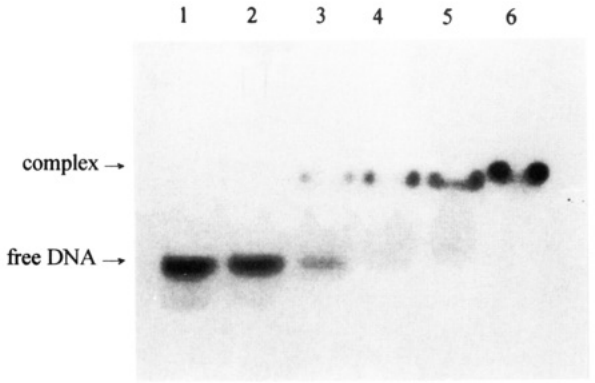

FIGURE 2: Quantitative gel shift assay of endonuclease III with (rAP•A)-13-mer. Each lane contains 10^{-8} M (rAP•A)-13-mer. Lanes 1−6 contain increasing concentrations of endonuclease III (0.5×10^{-9}, 5×10^{-8}, 5×10^{-7}, 5×10^{-6}, and 5×10^{-5} M^{-1}, respectively). The lower bands correspond to free DNA, while the upper bands correspond to the endonuclease III−DNA complex. The binding constant is determined as $[\text{endonuclease III}]^{-1}$, when the DNA is half-bound and half-free, to yield a binding constant of $(2 \times 10^6)-(2 \times 10^7)$ M^{-1}. Band intensities were quantitated on a β-scope.

the longer oligomers would add more negative charge to the complex, the bulkier complexes would be more physically hindered from entering the gel matrix. Howev :r, lowering the cross-linking or percent acrylamide did not make a difference. Since endonuclease III is a very basic protein with an isoelectric point of 10, we changed the pH of the gel from pH 8.3 (Hepes−EDTA buffer) to pH 10 (Ches buffer) to neutralize the positive charge of endonuclease III; however, this too did not improve the ability of the larger complexes to enter the gel.

Quantitative Gel Shift Assays. The binding constant for endonuclease III binding to (rAP•A)-13-mer was determined from quantitative gel shift assays as depicted in Figure 2. When the concentration of bound DNA in the DNA−protein complex is equal to the concentration of free DNA in solution, and the protein concentration is significantly greater than the DNA concentration, the binding constant is equal to the inverse of the protein concentration as described in Fried (1989). In lane 3 of Figure 2, there is more free DNA than bound DNA, whereas in lane 4, the DNA is mostly bound. The DNA would be half-bound and half-free at a protein concentration between the protein concentrations in lane 3 (5×10^{-8} M) and lane 4 (5×10^{-7} M); therefore, the binding constant for endonuclease III binding to (rAP•A)-13-mer is 2×10^6 to 2×10^7 M^{-1}, compared to the somewhat higher 4×10^7 M^{-1} for the rAP-19-mer as determined by fluorescence quenching (Xing et al., 1995).

This binding constant is relatively low compared to binding constants for DNA binding proteins and even somewhat lower when compared to most other DNA repair enzymes such as formamidopyrimidine-DNA glycosylase from *E. coli* with a K_{obs} of 4×10^9 M^{-1} for binding to a 25-mer containing a reduced AP site (Castaing et al., 1992) or DNA photolyase from *E. coli* with a K_{obs} of $(5 \times 10^7)-(2 \times 10^8)$ M^{-1} for binding to plasmid DNA (Sancar et al., 1987). However, the xeroderma pigmentosum group A complementing protein binds to damaged DNA with a binding constant of only 3×10^6 M^{-1} (Jones & Wood, 1993), which is very similar to our binding constant of $(2 \times 10^6)-(2 \times 10^7)$ M^{-1} for the binding of endonuclease III to (rAP•A)-13-mer. Although the larger 25-mer and 39-mer oligomer−protein complexes could not be readily analyzed by the above gel shift assays, we expected the binding constant for endonuclease III binding to these larger oligomers to be somewhat higher. Therefore, the relative binding of endonuclease III to (rAP•A)-25-mer and (rAP•A)-39-mer as compared to (rAP•A)-13-mer was probed by competition gel shift assays.

Competition Gel Shift Assays. Because the binding between endonuclease III and the unmodified 13-mer is not very strong, the complexes are not stable enough to persist upon entering a gel matrix and thus can not be detected by gel shift assays (see Figure 1). Therefore, to derive a relative nonspecific binding constant for endonuclease III, competition gel shift assays were carried out where (rAP•A)-13-mer is end-labeled with ^{32}P.

To test the method, we used cold (rAP•A)-13-mer as competitor against labeled (rAP•A)-13-mer as substrate. When the concentrations of cold and labeled oligomer were equal, one-half of the labeled oligomer that was originally complexed was displaced from the complex by the unlabeled oligomer. These results are in agreement with theoretical calculations.

To determine the relative nonspecific binding of endonuclease III to unmodified DNA, cold (U•A)-13-mer was used as the competitor against labeled (rAP•A)-13-mer substrate (Figure 3). When enough competitor is added to displace one-half of the labeled substrate from the complex, the ratio of the specific binding constant to the nonspecific binding constant is equal to 2[competitor]/[substrate] (Lin & Riggs, 1972; Fried, 1989). When the concentration of (U•A)-13-mer is 100 times the concentration of (rAP•A)-13-mer, less than one-half of the (rAP•A)-13-mer is displaced from the complex with endonuclease III (last lane in panel A or B of Figure 3); therefore, endonuclease III binds to (rAP•A)-13-mer at least 200 times better than to (U•A)-13-mer.

Even when enough competitor can not be added to displace half of the substrate from the complex, the relative binding constants can be determined by another method (Terry et al., 1983). The concentration of the competitor is plotted against the inverse of the concentration of the substrate that is in the DNA−protein complex (Figure 3C). From this graph, the binding constant for endonuclease III binding to the unmodified 13-mer is calculated to be between 4.5×10^3 M^{-1} (for $K_{obs}^{sp} = 2 \times 10^6$ M^{-1}) and 3.2×10^4 M^{-1} (for $K_{obs}^{sp} = 2 \times 10^7$ M^{-1}), such that endonuclease III binds to reduced AP sites 440−630 times better than to unmodified regions of the oligonucleotide. Our fluorescence measurements indicate the binding constant on (rAP•A)-19-mer was 400 times larger than on (U•A)-19-mer (Xing et al., 1995).

From our data, we were unable to derive binding constants for endonuclease III binding to (rAP•A)-25-mer or (rAP•A)-39-mer. However, we could determine qualitatively that endonuclease III binds to both (rAP•A)-25-mer and (rAP•A)-39-mer better than to (rAP•A)-13-mer (Figure 4). Therefore the binding constants for endonuclease III binding to longer oligonucleotides containing a rAP site are greater than $(2 \times 10^6)-(2 \times 10^7)$ M^{-1}, as also seen by fluorescence quenching experiments using a rAP•A-19-mer (Xing et al., 1995).

DNase I Footprinting. The DNase I footprint for endonuclease III bound to (rAP•A)-39-mer is shown in Figure 5. When the modified strand containing the reduced AP site is labeled, a footprint of nine nucleotides centered around the reduced AP site is observed (Figure 5A). When the complementary unmodified strand is labeled, a footprint of

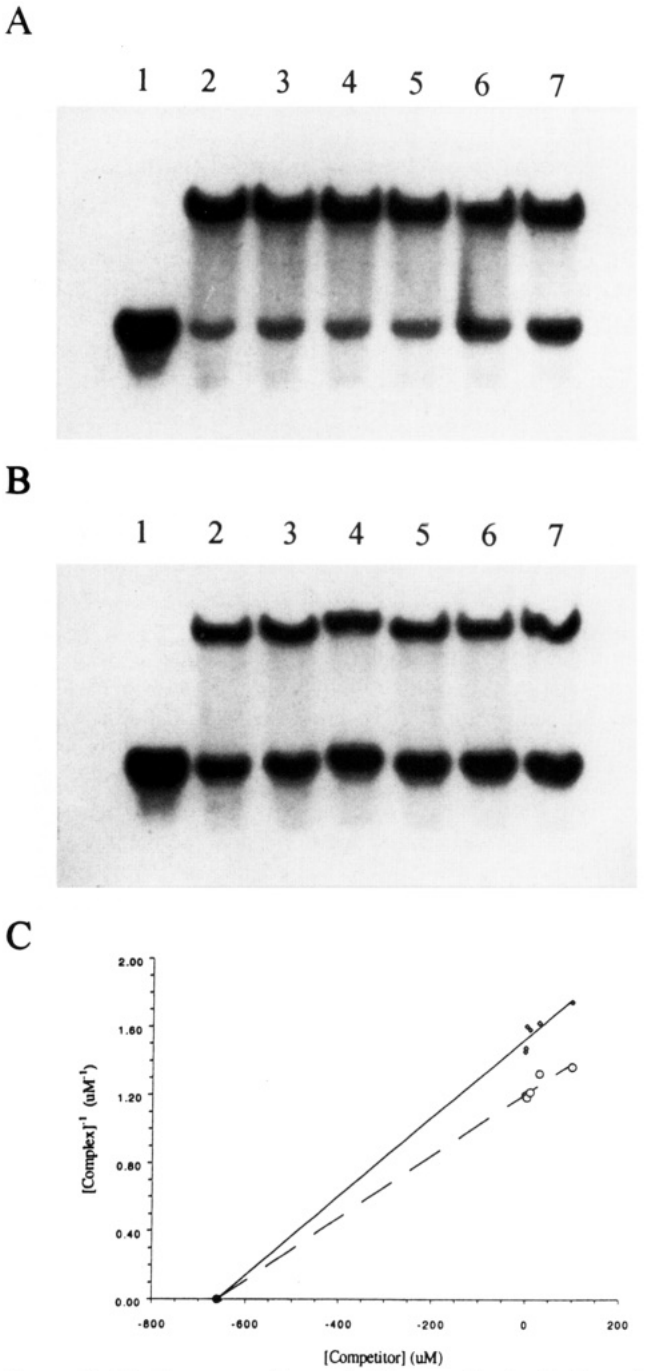

FIGURE 3: Binding competition gel shift assay for the binding of endonuclease III to (U•A)-13-mer as a competitor to (rAP•A)-13-mer. For each gel, the concentration of ^{32}P-labeled (rAP•A)-13-mer was kept constant at 1 μM and the concentration of endonuclease III was kept constant at (A) 1 μM or (B) 1.5 μM. Lane 1 contains only labeled (rAP•A)-13-mer. Lanes 2−7 contain labeled (rAP•A)-13-mer, endonuclease III, and increasing concentrations of unlabeled competitor (U•A)-13-mer (0, 1, 4, 10, 40, and 100 μM, respectively). (C) The inverse concentration of the labeled complex (endonuclease III bound to (rAP•A)-13-mer) is plotted as a function of the competitor (U•A)-13-mer concentration for two different endonuclease III concentrations (—, 1 μM; − − −, 1.5 μM). The association constant, K_{obs}^{ns}, for endonuclease III binding to the competitor (U•A)-13-mer is derived from the x-intercept (660 μM) by using the following equation: $-x = (1/K_{obs}^{ns})(1 + K_{obs}^{sp}[S])$, where [S] is the concentration of the labeled substrate (rAP•A)-13-mer (1 μM) and K_{obs}^{sp} is the association constant of endonuclease III binding to this substrate [$(2 \times 10^6)-(2 \times 10^7)$ M^{-1}]. The binding constant of endonuclease III binding to (U•A)-13-mer is $(4.5 \times 10^3)-(3.2 \times 10^4)$ M^{-1}. Endonuclease III binds to (rAP•A)-13-mer 440−630 times better than to the unmodified (U•A)-13-mer.

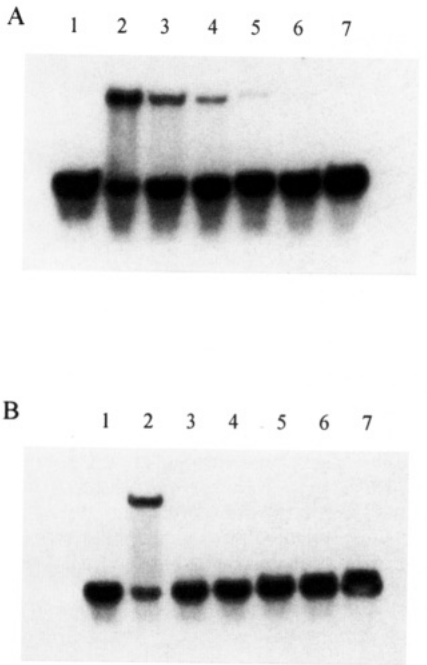

FIGURE 4: Binding competition gel shift assay to compare relative binding of endonuclease III to (rAP•A)-13-mer and (rAP•A)-39-mer. (A) Unlabeled (rAP•A)-13-mer as a competitor to labeled (rAP•A)-13-mer. (B) Unlabeled (rAP•A)-39-mer as a competitor to labeled (rAP•A)-13-mer. Lane 1 contains only labeled (rAP•A)-13-mer. On both gels, the concentration of labeled oligomer is kept constant at 1 μM, the concentration of endonuclease III is kept constant at 1 μM, and the concentration of unlabeled competitor increases from lanes 2 to 7 (0, 1, 4, 10, 40, and 100 μM, respectively). Unlabeled (rAP•A)-39-mer competes with labeled (rAP•A)-13mer better than does unlabeled (rAP•A)-13mer, indicating that endonuclease III binds to (rAP•A)-39mer better than to (rAP•A)-13mer.

10−11 nucleotides, extending from one nucleotide 5′ to the adenine opposite the reduced AP site and continuing in the 3′ direction, is observed (Figure 5B). The footprint on each strand is staggered by several nucleotides relative to the other strand

such that the nucleotides protected on each strand are across the minor groove from one another. This pattern of protection is characteristic of DNase I cutting within the minor groove.

The intensity of the bands created by DNase I cleavage varied, even in the absence of endonuclease III, presumably due to local variations in the DNA structure, especially near the reduced AP site. Thus, when defining the footprint, it was important not only to compare the intensity of the bands within each lane but also to compare each corresponding band's intensity in the presence and absence of endonuclease III. On the modified strand, the footprint for eight of the nine nucleotides is fairly clean, whereas the last nucleotide on the 5′ end of the footprint is considerably less protected. On the unmodified strand, the footprint is strongest for the seven nucleotides on the 5′ end of the footprint and weaker for the three to four nucleotides at the 3′ end of the footprint.

DNase I yields a comparable footprint for endonuclease III binding to (rAP•A)-25-mer (data not shown). The

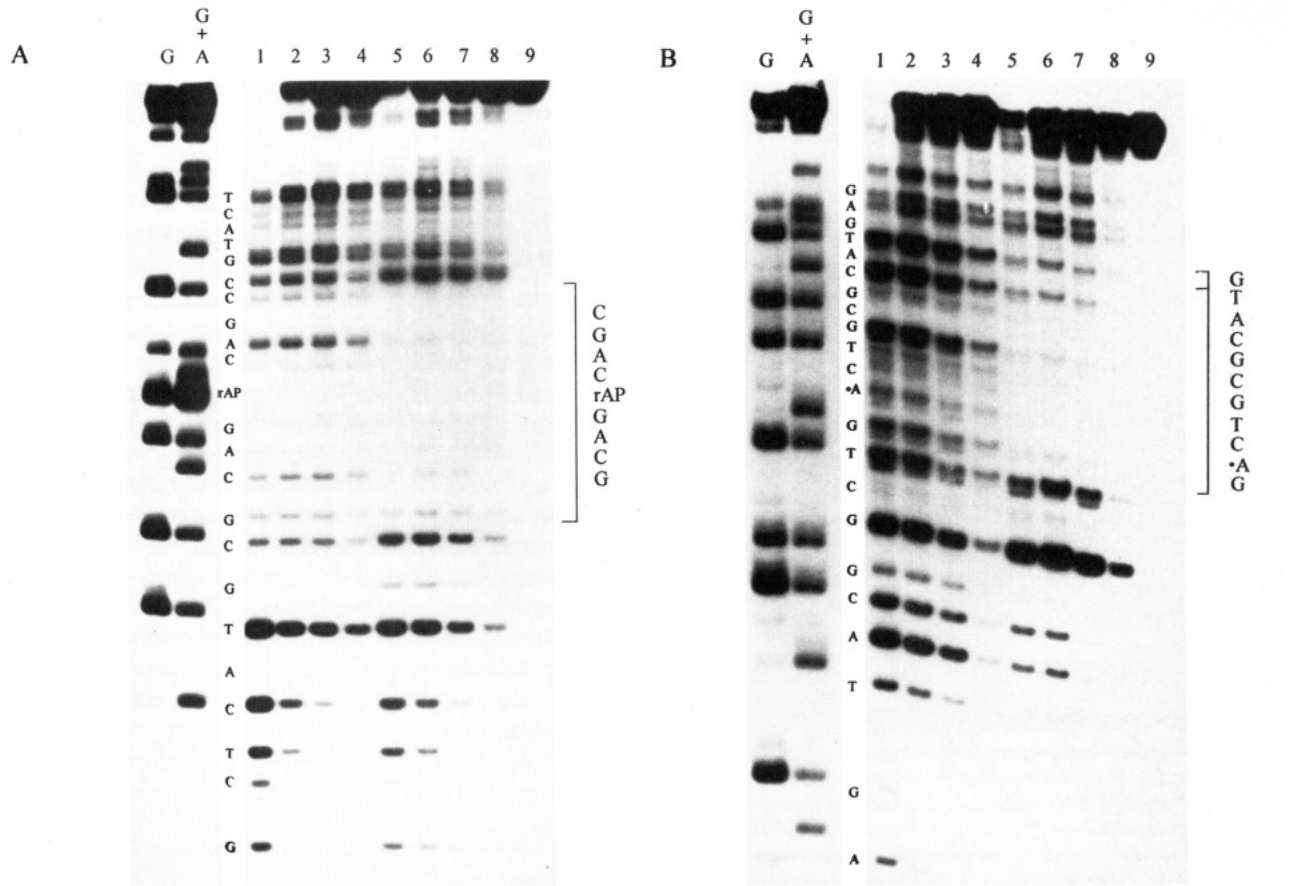

FIGURE 5: DNase I footprint of endonuclease III. (A) DNA strand containing the reduced AP site (modified strand). (B) Complementary DNA strand (unmodified strand). Lanes G and G + A are Maxam−Gilbert sequencing lanes. Lanes 1−4 contain (rAP•A)-39-mer and decreasing concentrations of DNase I (1.0, 0.3, 0.1, and 0.03 unit, respectively). Lanes 5−8 contain (rAP•A)-39-mer, endonuclease III, and decreasing concentrations of DNase I as in lanes 1−4. Lane 9 contains (rAP•A)-39-mer as a control for background cutting. The concentration of DNA is 40 nM in all lanes, and the concentration of endonuclease III is 20 μM in lanes 5−8. The footprints (in brackets) are nine nucleotides on the modified strand and 10−11 nucleotides on the complementary strand. In B, •A is the nucleotide opposite the reduced AP site.

footprint on the modified strand of the 25-mer is identical to that of the 39-mer, with the exception that, because the oligonucleotide is smaller, the bands just outside the footprint are enhanced. The footprint on the complementary unmodified strand of the 25-mer starts in the same position on the 5′ end but extends to the 3′ end of the strand. As with the modified strand, there is enhanced cutting just outside the footprint on the 5′ side. This enhanced cutting of bands just outside the (rAP•A)-25-mer footprint was useful in defining the boundaries of the footprint. No DNase I footprint was observed for the unmodified oligonucleotide (U•A)-25-mer (data not shown), a further indication that the binding of endonuclease III to reduced AP sites is a specific interaction.

(Methidiumpropyl-EDTA)Fe(II) and OH• Footprinting. The MPE-Fe(II) footprint of endonuclease III bound to (rAP•A)-39-mer is shown in Figure 6. Endonuclease III protects a seven-base pair region of the modified DNA, five nucleotides on each strand which are shifted by two nucleotides, from cleavage by MPE-Fe(II)-generated hydroxyl radicals. Therefore, the endonuclease III binding site is no larger than seven base pairs for specific binding. Protection of the modified strand extends from one nucleotide 5′ to the reduced AP site to three nucleotides 3′ to the reduced AP site, encompassing five nucleotides. On the complementary unmodified strand, protection extends from one nucleotide 5′ to the adenine opposite the reduced AP site to three nucleotides 3′ of the adenine, totaling five nucleotides.

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G C T C A T G C G C A G rAP C A G C C G T A C T C G
C G A G T A C G C G T C  A  G T C G G C A T G A G C
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According to Van Dyke and Dervan (1983a), MPE-Fe(II) footprinting underestimates the region of binding by one to two nucleotides on the 5′ side of the binding site and overestimates the region of binding by one to two nucleotides on the 3′ side of the binding site. If the MPE-Fe(II) footprint on each strand is shifted by one nucleotide in the 5′ direction, the binding site of endonuclease III consists of five base pairs of DNA with the reduced AP site exactly in the center. The size of the five- to seven-base pair endonuclease III binding site closely matches the positively charged 24 Å diameter surface groove of endonuclease III, which has already been proposed as the likely binding site on endonuclease III (Kuo et al., 1992b). As noted in Xing et al. (1995), the size of the site for nonspecific binding to high molecular weight DNA is approximately 12 base pairs.

MPE-Fe(II) footprints could only be detected when certain measures were taken. The ^{32}P that end labels the oligonucleotides slowly degrades the DNA by randomly cutting at each nucleotide to produce background cutting, which can interfere with the cutting from the footprinting reagents. Cutting from ^{32}P can be minimized, if the time of the entire

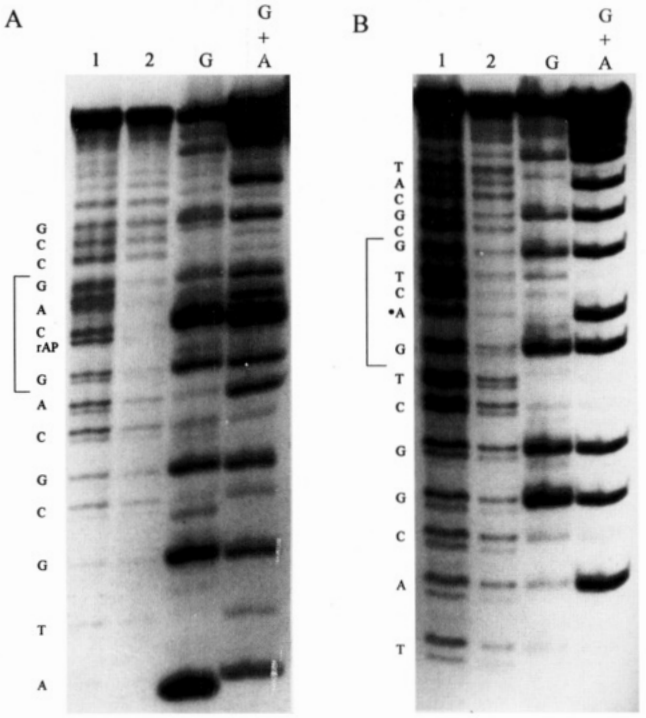

FIGURE 6: MPE-Fe(II) footprint of endonuclease III. (A) DNA strand containing the reduced AP site (modified strand). (B) Complementary DNA strand (unmodified strand). Lanes G and G + A are Maxam−Gilbert sequencing lanes. Lane 1 contains (rAP•A)-25-mer cut with MPE-Fe(II), while lane 2 contains (rAP•A)-25-mer cut with MPE-Fe(II) and protected by endonuclease III. The DNA concentration is 50 nM in each lane, and the endonuclease III concentration is 10 μM in lane 2. The footprints (in brackets) are only five nucleotides on each strand. In B, •A is the nucleotide opposite the reduced AP site. The intensities of the bands were quantitated on a β-scope to determine the footprint.

footprinting procedure is minimized; therefore, to successfully obtain a MPE-Fe(II) footprint, the entire experiment including phosphorylation with ^{32}P, purification, footprinting, and running the gel was completed within 2 days. ^{32}P degrades DNA much more rapidly in a dry state than in solution; therefore, the ^{32}P-labeled DNA was never allowed to sit dry for any period of time. These steps may be necessary to obtain footprints for other proteins that bind to DNA with low binding constants.

The footprint from the solution-generated OH• (data not shown) was identical to the MPE-Fe(II) footprint on the modified strand but too weak on the complementary unmodified strand to be observed. This observation suggests that endonuclease III may bind more tightly or closely to the modified strand. Addition of a gel purification step after footprinting to separate complexed DNA from free DNA (Dixon et al., 1991) did not yield a stronger footprint. Therefore, the weak hydroxyl radical footprints are probably due to the low binding constant for endonuclease III binding to (rAP•A)-25-mer.

Methylation Protection. Endonuclease III does not protect any of the guanines of (rAP•A)-25-mer from methylation by dimethyl sulfate and subsequent depurination and cleavage by piperidine (data not shown); therefore, either endonuclease III does not bind on the major groove side of the DNA or it does not bind tightly enough to protect the major groove from attack by DMS. Unfortunately, we could not methylate adenine N3 to a high enough level to be observed, and therefore we could not use methylation protection to determine whether or not endonuclease III protects the minor groove. Heating the DNA in the piperidine solution causes cleavage at the reduced AP site. Interestingly, when endonuclease III is complexed to the DNA, the rAP site is partially protected from cleavage by piperidine. This observation indicates that endonuclease III is bound to the DNA during the methylation footprinting experiments and does not just protect the DNA from methylation in the major groove.

Interaction of Endonuclease III with (rAP•C)-oligomers. The DNase I footprints for endonuclease III bound to (rAP•C)-39-mer were identical to those for endonuclease III bound to (rAP•A)-39-mer (data not shown). The only difference between the 39-mers was that in the (rAP•C)-39-mer a cytosine on the unmodified strand was opposite the reduced AP site, whereas in the (rAP•A)-39-mer an adenine on the unmodified strand was opposite the reduced AP site. Cuniasse et al. (1990) determined that the structure of DNA containing abasic sites is dependent on the base opposite the AP site. Guanine or adenine opposite an abasic site stacks within the helix, thymidine exchanges between being stacked and being extrahelical, and cytosine opposite an abasic site was found to be extrahelical. Regardless of any structural variations that may exist between the oligomers containing rAP•A or rAP•C sites, there appears to be no qualitative difference in the recognition of these sites by endonuclease III.

DISCUSSION

Endonuclease III binds to DNA containing reduced AP sites in a specific manner as demonstrated by gel shift assays and footprinting experiments. The binding constant for endonuclease III binding to (rAP•A)-13-mer is fairly low [K_{obs} = $(2 \times 10^6)-(2 \times 10^7)$ M^{-1}] when compared to binding constants for other systems. The binding constant may be low for a number of reasons. (1) The quantitative gel shift assays were performed on a small oligonucleotide (13-mer). A comparable 25-mer and 39-mer did compete for endonuclease III better than the 13-mer, indicating that the binding constants for endonuclease III binding to these longer oligomers are somewhat higher than $(2 \times 10^6)-(2 \times 10^7)$ M^{-1}. (2) The inability of a covalent complex such as a Schiff base to form between endonuclease III and a reduced AP site may lower the binding constant as compared to that for endonuclease III binding to an AP site. Despite this fact, endonuclease III does bind to the 13-mer containing the reduced AP site ~500 times stronger than to the equivalent unmodified oligomer, indicating that even though the interaction between endonuclease III and (rAP•A)-13-mer may not be particularly strong, it is a very specific interaction. (3) The small footprints for endonuclease III indicating a small binding site (~five to seven base pairs) suggest that there are probably few contact sites between endonuclease III and the damaged DNA. (4) Repair enzymes (and perhaps other enzymes) may not bind to DNA as tightly as regulatory "binding" proteins do because their functions are to recognize and repair damaged DNA, not to recognize and bind to a specific region of DNA. Tight binding may actually be counterproductive to catalysis by repair enzymes.

The DNase I, MPE-Fe(II), and OH• footprints for endonuclease III binding to oligonucleotides each containing a reduced AP site are relatively small as compared to footprints

for other proteins such as regulatory proteins and restriction enzymes, which are sequence-specific. The DNase I footprint for endonuclease III bound to oligomers each containing a reduced AP site is nine nucleotides on the strand containing the reduced AP site and 10−11 nucleotides on the complementary strand. DNase I footprints for regulatory proteins are usually at least twice this size on both strands. On the other hand, DNA photolyase, another DNA repair enzyme, was reported as having a DNase I footprint of 16 nucleotides on the damaged strand and 12 nucleotides on the complementary strand.

There are many interpretations of DNase I footprinting data available, each of which try to correlate the nucleotides protected from DNase I cleavage to the nucleotides that are actually bound by the protein providing protection. From the discrepancies in these interpretations, the correlation is probably dependent on the exact positioning of the enzyme relative to the DNA (i.e., major groove binding, minor groove binding, or binding along the DNA backbone).

For this reason, we could not determine the exact positioning of endonuclease III bound to the DNA. A crystal structure of the complex is in progress and should be able to define this point. The studies with reduced AP sites will be especially important since the ability to form stable endonuclease III−damaged DNA complexes will allow for cocrystallization of a complex for X-ray analysis.

From the MPE-Fe(II) and OH• footprinting data, the binding site of endonuclease III can be no larger than five nucleotides on both strands. Although the DNase I footprints for endonuclease III are a little smaller and located about the damaged nucleotide somewhat differently than those for DNA photolyase, the MPE-Fe(II) footprints for both enzymes are strikingly similar. The MPE-Fe(II) footprint for endonuclease III covers one nucleotide 5′ to the reduced AP site and three nucleotides 3′ to the reduced AP site on the modified strand, while the MPE-Fe(II) footprint for DNA photolyase covers one nucleotide 5′ to the thymine dimer and three nucleotides 3′ to the thymine dimer on the modified strand. On the complementary strand, the MPE-Fe(II) footprint for endonuclease III covers one nucleotide 5′ and three nucleotides 3′ to the adenine across from the reduced AP site and the MPE-Fe(II) footprint for DNA photolyase covers one nucleotide 5′ and three nucleotides 3′ to the adenine dinucleotide across from the thymine dimer. Husain et al. (1987) pointed out that this remarkably small MPE-Fe(II) footprint yielded a binding site that was to their knowledge the smallest binding site ever reported for a DNA binding protein. Recently, Tchou et al. (1993) reported a OH• footprint for formamidopyrimidine-DNA glycosylase of only five nucleotides centered around a tetrahydrofuran site in a 23-mer DNA duplex. Our results indicate that endonuclease III also binds an extremely small region of only five to seven base pairs. It will be interesting to see if other DNA repair enzymes also have such small binding sites.

The MPE-Fe(II) footprints of endonuclease III and DNA photolyase are very similar to those of small drug molecules which interact with DNA in the minor groove (Van Dyke & Dervan, 1983a,b). In addition, we have shown that endonuclease III does not protect the major groove from methylation by DMS. Therefore, endonuclease III may protect the DNA helix along the minor groove as has been shown for T4 endonuclease V (Iwai et al., 1994).

We were only able to obtain OH• footprints for the modified strand and not for the unmodified strand, indicating that endonuclease III may bind more tightly or closely to the strand containing the reduced AP site. Our observation suggests that endonuclease III may bind along the DNA backbone rather than in the groove, thus protecting one strand more than the other. The other strand would be more solvent accessible to OH•'s in solution. MPE-Fe(II) must intercalate to cut and therefore would be more readily excluded than the OH•'s. Similar results were reported by Tchou et al. (1993) for formamidopyrimidine-DNA glycosylase, where formamidopyrimidine-DNA glycosylase protected five nucleotides on the modified strand of a DNA duplex from OH• cleavage but did not protect the unmodified strand. Tighter or closer binding to the modified strand may be a common element in the binding of repair enzymes to damaged DNA.

From our studies and those of DNA photolyase and formamidopyrimidine-DNA glycosylase, it appears that the recognition and binding of repair enzymes may be uniquely different from binding of regulatory proteins. This observation is not so surprising since each class of proteins perform very different functions; DNA repair enzymes recognize anomalies in DNA structure and repair these anomalies, whereas regulatory proteins regulate transcription and translation events by recognizing and binding to specific DNA sequences. For regulatory proteins it is advantageous to bind tightly to DNA. For repair enzymes, tight binding may be counterproductive. To effectively repair damaged DNA, it may be more advantageous for the enzymes to have fast on/off rates for efficient turnover. Regulatory proteins, being sequence-specific, make numerous contacts with DNA over an extensive binding region to maximize sequence specificity. DNA repair enzymes must recognize a damaged site, often a single nucleotide, regardless of sequence. Thus a smaller binding site with fewer DNA−protein contacts would lower the possibility of sequence dependence.

It is fairly well understood how sequence-specific proteins recognize and bind DNA (Seeman et al., 1976; Steitz, 1990). However, it is not yet completely understood how "structure-specific" proteins such as nucleases and repair enzymes recognize and bind to DNA. The only nonsequence-specific protein whose interaction with DNA has been elucidated in detail, to our knowledge, is DNase I (Suck & Oefner, 1986). DNase I binds in the minor groove and discriminates on the basis of the width of the minor groove. It is doubtful that the mechanism of recognition for repair enzymes with such site-specific recognition is that simple. A cocrystal structure should help elucidate the mechanism of recognition for endonuclease III. Since the footprints of endonuclease III and DNA photolyase are so similar, the interpretations from this work and the cocrystal structure may be applicable to other DNA repair enzymes.

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REFERENCES

Asahara, H., Wistort, P. M., Bank, J. F., Bakerian, R. H., & Cunningham, R. P. (1989) *Biochemistry 28*, 4444−4449.

Boorstein, R. J., Hilbert, T. P., Cadet, J., Cunningham, R. P., & Teebor, G. W. (1989) *Biochemistry 28*, 6164−6170.
Breimer, L. H., & Lindahl, T. (1985) *Biochemistry 24*, 4018−4022.
Castaing, B., Boiteux, S., & Zelwar, C. (1992) *Nucleic Acids Res. 20*, 389−394.
Cuniasse, Ph., Fazakerley, G. V., Guschlbauer, W., Kaplan, B. E., & Sowers, L. C. (1990) *J. Mol. Biol. 213*, 303−314.
Cunningham, R. P., & Weiss, B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 474−478.
Cunningham, R. P., Asahara, H., Bank, J. F., Scholes, C. P., Salerno, J. C., Surerus, K., Münck, E., McCracken, J., Peisach, J., & Emptage, M. H. (1989) *Biochemistry 28*, 4450−4455.
Demple, B., & Linn, S. (1982) *J. Biol. Chem. 257*, 2848−2855.
Dixon, W. J., Hayes, J. J., Levin, J. R., Weidner, M. F., Dombroski, B. A., & Tullius, T. D. (1991) *Methods Enzymol. 208*, 380−413.
Dizdaroglu, M., Laval, J., & Boiteux, S. (1993) *Biochemistry 32*, 12105−12111.
Fried, M. G. (1989) *Electrophoresis 10*, 366−376.
Fu, W., O'Handley, S., Cunningham, R. P., & Johnson, M. K. (1992) *J. Biol. Chem. 267*, 16135−16137.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
Husain, I., Sancar, G. B., Holbrook, S. R., & Sancar, A. (1987) *J. Biol. Chem. 262*, 13188−13197.
Iwai, S., Maede, M., Shimada, Y., Hori, N., Murata, T., Morioka, H., & Ohtsuka, E. (1994) *Biochemistry 33*, 5581−5588.
Jones, C. J., & Wood, R. D. (1993) *Biochemistry 32*, 12096−12104.
Jorgensen, T. J., Kow, Y. W., Wallace, S. S., & Henner, W. D. (1987) *Biochemistry 26*, 6436−6443.
Katcher, H. L., & Wallace, S. S. (1983) *Biochemistry 22*, 4071−4081.
Kuo, C. F., McRee, D. E., Cunningham, R. P., & Tainer, J. A. (1992a) *J. Mol. Biol. 227*, 347.
Kuo, C. F., McRee, D. E., Fisher, C. L., O'Handley, S. F., Cunningham, R. P., & Tainer, J. A. (1992b) *Science 258*, 434−440.
Lin, S.-Y., & Riggs, A. D. (1972) *J. Mol. Biol. 72*, 671−690.
Mazumder, A., Gerlt, J. A., Absalon, M. J., Stubbe, J., Cunningham, R. P., Withka, J., & Bolton, P. H. (1991) *Biochemistry 30*, 1119−1126.
Rhodes, D. (1990) in *Protein Function-a Practical Approach* (Creighton, T. E., Ed.) pp 177−198, IRL Press, Oxford.
Sancar, G. B., Smith, F. W., Reid, R., Payne, G., Levy, M., & Sancar, A. (1987) *J. Biol. Chem. 262*, 478−485.
Sauer, R. T. (1991) *Methods Enzymol. 208*.
Seawell, P. C., Simon, T. J., & Ganesan, A. K. (1980) *Biochemistry 19*, 1685−1691.
Seeman, N. C., Rosenberg, J. M., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 804−808.
Steitz, T. A. (1990) *Q. Rev. Biophys. 23*, 205−280.
Suck, D., & Oefner, C. (1986) *Nature 321*, 620−625.
Tchou, J., Michaels, M. L., Miller, J. H., & Grollman, A. P. (1993) *J. Biol. Chem. 268*, 26738−26744.
Terry, B. J., Jack, W. E., Rubin, R. A., & Modrich, P. (1983) *J. Biol. Chem. 258*, 9820−9825.
Tullius, T. D. (1989) *Annu. Rev. Biophys. Biophys. Chem. 81*, 213−237.
Van Dyke, M. W., & Dervan, P. B. (1983a) *Biochemistry 22*, 2373−2377.
Van Dyke, M. W., & Dervan, P. B. (1983b) *Nucleic Acids Res. 11*, 5555−5567.
Van Houten, B., Gamper, H., Sancar, A., & Hearst, J. E. (1987) *J. Biol. Chem. 262*, 13180−13187.
Xing, D., Dorr, R., Cunningham, R. P., & Scholes, C. P. (1995) *Biochemistry 34*, 2537−2544.

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