*932–941* *Nucleic Acids Research, 1998, Vol. 26, No. 4* 

# Multiply damaged sites in DNA: interactions with *Escherichia coli* endonucleases III and VIII

**Lynn Harrison[+], Zafer Hatahet, Andrei A. Purmal[§] and Susan S. Wallace***

Department of Microbiology and Molecular Genetics, Markey Center for Molecular Genetics, University of Vermont, Burlington, VT 05405, USA

Received November 7, 1997; Revised and Accepted December 16, 1997

## ABSTRACT

**Bursts of free radicals produced by ionization of water in close vicinity to DNA can produce clusters of opposed DNA lesions and these are termed multiply damaged sites (MDS). How MDS are processed by the *Escherichia coli* DNA glycosylases, endonuclease (endo) III and endo VIII, which recognize oxidized pyrimidines, is the subject of this study. Oligonucleotide substrates were constructed containing a site of pyrimidine damage or an abasic (AP) site in close proximity to a single nucleotide gap, which simulates a free radical-induced single-strand break. The gap was placed in the opposite strand 1, 3 or 6 nt 5′ or 3′ of the AP site or base lesion. Endos III and VIII were able to cleave an AP site in the MDS, no matter what the position of the opposed strand break, although cleavage at position one 5′ or 3′ was reduced compared with cleavage at positions three or six 5′ or 3′. Neither endo III nor endo VIII was able to remove the base lesion when the gap was positioned 1 nt 5′ or 3′ in the opposite strand. Cleavage of the modified pyrimidine by endo III increased as the distance increased between the base lesion and the opposed strand break. With endo VIII, however, DNA breakage at the site of the base lesion was equivalent to or less when the gap was positioned 6 nt 3′ of the lesion than when the gap was 3 nt 3′ of the lesion. Gel mobility shift analysis of the binding of endo VIII to an oligonucleotide containing a reduced AP (rAP) site in close opposition to a single nucleotide gap correlated with cleavage of MDS substrates by endo VIII. If the strand break in the MDS was replaced by an oxidized purine, 7,8-dihydro-8-oxoguanine (8-oxoG), neither endo VIII cleavage nor binding were perturbed. These data show that processing of oxidized pyrimidines by endos III and VIII was strongly influenced by the position and type of lesion in the opposite strand, which could have a significant effect on the biological outcome of the MDS lesion.**

## INTRODUCTION

Early experiments, in which the yields of certain types of free radical-induced DNA damage were measured, showed that ionizing radiation produced ~1000 single-strand breaks (ssb) per cell per lethal event (1), while $H_2O_2$ was estimated to introduce 400 000 ssb/cell/lethal event (2). Both agents produce more sites of base damage than ssb (2) and, in addition, ionizing radiation produces double-strand breaks (dsb) (3) and crosslinks (4). In order to explain why ionizing radiation requires fewer lesions to cause lethality, taking into account its ability to produce dsb, it was proposed (2) that the spatial distribution of individual lesions in the DNA molecule alters the ability of the damage to be repaired. Ionizing track structure simulations have clearly demonstrated that even low energy electrons, such as are produced by γ- or X-rays, introduces multiple lesions within the DNA molecule (5). It has been estimated that a single energy deposition can produce 2–5 ionizations in a 1–4 nm diameter site (6), which would place the ionizations within a helical turn on the DNA, forming a multiply damaged site (MDS). Since a single hydrogen peroxide molecule produces a single radical upon reaction with a metal ion, clustering of damage is unlikely (2). Thus the survival of a cell exposed to ionizing radiation damage may depend in large part on its ability to repair MDS.

Many different types of MDS can be produced by ionizing radiation. Two ssb closely opposed will give rise to a dsb, which has been well characterized as a lethal lesion (7). However, due to the relative amounts of the different types of lesions produced, MDS containing two sites of base damage, a site of base damage and an abasic site near a strand break or two abasic sites, are likely to be formed. Complex lesions, containing more than two sites of damage or more than one site of damage on a single strand are also formed (8) and their nature, in large part, depends on the quality of the ionizing radiation used (5).

Individually, sites of base damage, abasic sites and ssb are repaired by base excision repair (BER) (9). Pyrimidine base damage formed by free radicals is recognized by the pyrimidine-specific DNA glycosylases, the prototypic enzymes in *Escherichia coli* being endonuclease (endo) III and endo VIII. In addition to removing the modified pyrimidine by cutting the *N*-glycosyl

*To whom correspondence should be addressed. Tel: +1 802 656 2164; Fax: +1 802 656 8749; Email: swallace@zoo.uvm.edu

Present addresses: [+]Department of Molecular and Cellular Physiology, Louisiana State Medical Center, Shreveport, LA 71130, USA and [§]Pentose Pharmaceuticals Inc., 45 Moulton Street, Cambridge, MA 02138, USA

bond, the associated lyase activity of these enzymes cleaves the DNA backbone, either by β elimination in the case of endo III (10) or by β,δ elimination in the case of endo VIII (11). Furthermore, both enzymes cleave at sites of base loss with a 10-fold greater efficiency than at a damaged pyrimidine site (Hatahet and Wallace, unpublished observations). Sites of base loss are also recognized by the 5′-apurinic endonucleases (AP) (9). Finally, ssb produced by ionizing radiation most often have blocked 3′-termini (12) and therefore must be processed by the phosphatase or phosphodiesterase activities of the 5′-AP endonucleases prior to replication (13–15).

With respect to MDS, the question arises as to whether the damage-specific enzymes are capable of recognizing and cleaving at the site of the damage in the presence of a closely opposed lesion. Incision at the lesions in both strands could give rise to a dsb, the lethal event presumed to be responsible for the high cytotoxic efficiency of ionizing radiation. To address this question we placed thymine glycol (Tg), dihydrothymine (DHT) or an abasic site opposite a ssb and Tg or DHT opposite a purine lesion, 8-oxoG, and asked whether the presence of the lesion in the opposing strand affected recognition of the lesion in the target strand. All three target lesions, Tg, DHT and AP sites, are recognized by endos III (16) and VIII (11,17). The MDSs chosen are biologically relevant, since both Tg and DHT are radiolysis products of DNA thymine (18), the latter being formed only under anaerobic conditions, and 8-oxoG is a commonly produced purine lesion (19,20). Also, sites of base loss and ssb are produced at equal frequencies by ionizing radiation (21). Finally, ssb formed by ionizing radiation *in vitro* contain 5′ P residues and, 50% of the time, 3′ P residues (12), which is the model ssb used here. These lesions also fall into different categories with respect to their interaction with DNA polymerases and, accordingly, their potential cytotoxicity and mutagenicity. For example, DHT (22) and 8-oxoG (23) are readily by-passed by DNA polymerases and thus are not cytotoxic lesions. However, 8-oxoG readily mispairs with adenine (23), leading to G→T transversions (24), while DHT faithfully pairs with adenine and is not mutagenic (25). In contrast to these and other radiation-modified DNA bases that retain their intact ring structure, Tg blocks DNA polymerases (26,27) and is a lethal lesion (28,29). Abasic sites (30,31) and strand breaks also block DNA polymerases and are potentially lethal lesions. However, when by-pass of abasic sites occurs, DNA polymerases most often insert purines, thus leading to mutations (32).

Previous studies have examined the repair of closely opposed DHT (33) or abasic site residues (34). In this work we have examined MDS in which the repair enzyme recognizes only one lesion in the MDS, allowing us to determine whether the opposing lesion inhibited the enzyme.

A closely opposed purine lesion did not inhibit cleavage of a pyrimidine lesion by endos III or VIII, even when they were as closely spaced as 3 nt. However, if the closely opposed lesion was a strand break at the position of the next nucleotide, the pyrimidine lesions and AP sites were poorly incised. If, however, the opposed strand break was positioned 3 or 6 nt apart a dsb was formed. These data have been correlated with the ability of endo VIII to bind to DNA containing reduced AP (rAP) sites in opposition to a single nucleotide gap or 8-oxoG.

## MATERIALS AND METHODS

### Oligonucleotides

Strand A oligonucleotides (see Table 1) containing Tg or DHT were prepared as described in Purmal *et al.* (35) and Hatahet *et al.* (36). Oligonucleotides containing uracil, 8-oxoG or 5′- and 3′-phosphate termini were synthesized in the Department of Microbiology and Molecular Genetics, University of Vermont, or purchased from Operon Technologies. The oligonucleotides were purified by electrophoresis through a 12% polyacrylamide–7 M urea gel, electroeluted from the excised gel fragment into 8 M ammonium acetate and desalted using a NAP 5$^{TM}$ column (Pharmacia).

**Table 1.** Oligonucleotides

| Strand | Damage | Sequence | Position of damage relative to X in strand A |
|---|---|---|---|
| A | **X** = Tg, AP, DHT, rAP or U | 5′-ATTCCAGACTGTCAATAACACGG**X**GGACCAGTCGATCCTGGGCTGCAGGAATTC-3′ | |
| B | | 3′-TAAGGTCTGACAGTTATTGTGCCACCTGGTCAGCTAGGACCCGACGTCCTTAAG-5′ | |
| B | 1 nt gap | 3′-TAAGGTCTGACAGTTAT GTGCCACCTGGTCAGCTAGGACCCGACGTCCTTAAG-5′ | 6 nt 5′ |
| B | 1 nt gap | 3′-TAAGGTCTGACAGTTATTGT CCACCTGGTCAGCTAGGACCCGACGTCCTTAAG-5′ | 3 nt 5′ |
| B | 1 nt gap | 3′-TAAGGTCTGACAGTTATTGTGC ACCTGGTCAGCTAGGACCCGACGTCCTTAAG-5′ | 1 nt 5′ |
| B | 1 nt gap | 3′-TAAGGTCTGACAGTTATTGTGCCA CTGGTCAGCTAGGACCCGACGTCCTTAAG-5′ | 1 nt 3′ |
| B | 1 nt gap | 3′-TAAGGTCTGACAGTTATTGTGCCACC GGTCAGCTAGGACCCGACGTCCTTAGG-5′ | 3 nt 3′ |
| B | 1 nt gap | 3′-TAAGGTCTGACAGTTATTGTGCCACCTGG CAGCTAGGACCCGACGTCCTTAAG-5′ | 6 nt 3′ |
| B | **Y** = 8oxoG | 3′-TAAGGTCTGACAGTTATT**Y**TGCCACCTGGTCAGCTAGGACCCGACGTCCTTAAG-5′ | 5 nt 5′ |
| B | **Y** = 8oxoG | 3′-TAAGGTCTGACAGTTATTGT**Y**CCACCTGGTCAGCTAGGACCCGACGTCCTTAAG-5′ | 3 nt 5′ |
| B | **Y** = 8oxoG | 3′-TAAGGTCTGACAGTTATTGTGCCACCT**Y**GTCAGCTAGGACCCGACGTCCTTAAG-5′ | 4 nt 3′ |
| B | **Y** = 8oxoG | 3′-TAAGGTCTGACAGTTATTGTGCCACCTG**Y**TCAGCTAGGACCCGACGTCCTTAAG-5′ | 5 nt 3′ |

The double-stranded substrates were produced by annealing strand A with strand B as described in Materials and Methods. For substrates with a nucleotide gap, two oligonucleotides were used to form strand B. The oligonucleotides forming the gap had 5′ or 3′ phosphate termini (see Fig. 1).

### Enzymes

Endos III (37) and VIII (38) were prepared as described previously. Uracil DNA glycosylase (UDG) was purchased from US Biochemical and T4 polynucleotide kinase was purchased from Boehringer Mannheim.

### Preparation of duplex substrates

Oligonucleotides (3–5 pmol) were 5′-labeled with $^{32}$P using 1 U T4 polynucleotide kinase, 50 µCi [γ-$^{32}$P]ATP (6000 Ci/mmol, 10 mCi/ml; NEN Dupont) in 25 µl supplied reaction buffer at 37°C for 30 min. The enzyme was heat inactivated at 65°C for 15 min. Unincorporated [γ-$^{32}$P]ATP was removed following purification of the oligonucleotide using a NENSORB™ 20 cartridge (NEN Dupont). The oligonucleotide, eluted in 50% ethanol, was dried and resuspended in double distilled water at 100 fmol/µl. Annealing of the complementary oligonucleotide (Table 1) was performed using a 1.2-fold molar excess of the unlabeled strand (strand B) at concentrations of 30–200 fmol/µl in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl. To generate the DNA duplex containing a single nucleotide gap the two oligonucleotides used to form strand B were each annealed at 1.2-fold molar excess to strand A. The hybridization reaction was heated to 75°C for ~2 min and slowly cooled to room temperature. This procedure was carried out over ~2 h. The final substrate had a specific activity of ~100–200 d.p.m./fmol.

To obtain a labeled AP site-containing double-stranded substrate strand A containing uracil was labeled, purified and annealed, as described above, at 200 fmol/µl duplex DNA. UDG (0.5 U) was incubated with 900 fmol duplex DNA in 5 µl 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl for 30 min at 37°C. To determine if the uracil had been removed a small aliquot of the resulting substrate was boiled for 30 min and the products visualized after electrophoresis through a 12% polyacrylamide–7 M urea gel. Approximately 90% of the substrate was fragmented after boiling.

### Cleavage reaction conditions

Duplex substrate (100 fmol unless otherwise noted) was mixed with endo VIII (5–80 nM final concentration) or endo III (0.5–50 nM) in 5 µl 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl on ice and then incubated at 37°C for 15 min. Reactions were stopped on ice by addition of 5 µl formamide, 0.03% bromophenol blue, 0.03% xylene cyanol. Samples were subjected to electrophoresis through a 12% polyacrylamide–7 M urea gel at 1500 V for ~2 h in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8) and then dried. The reaction products were visualized by autoradiography of the gel and quantitated using a BioRad Molecular Imager.

### Preparation of duplex binding substrates

Strand A containing uracil (11.5 pmol) was incubated with 0.5 U UDG in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA in 5 µl at 37°C for 75 min. To this was added 95 µl 100 mM sodium borohydride and the reaction incubated at room temperature for 20 min. The oligonucleotide was purified using a NENSORB™ 20 cartridge. After elution the oligonucleotide was dried and resuspended at 250 fmol/µl in double distilled water and stored at –20°C. To ensure that uracil was removed and the AP site was reduced the oligonucleotide was labeled and annealed to an undamaged strand

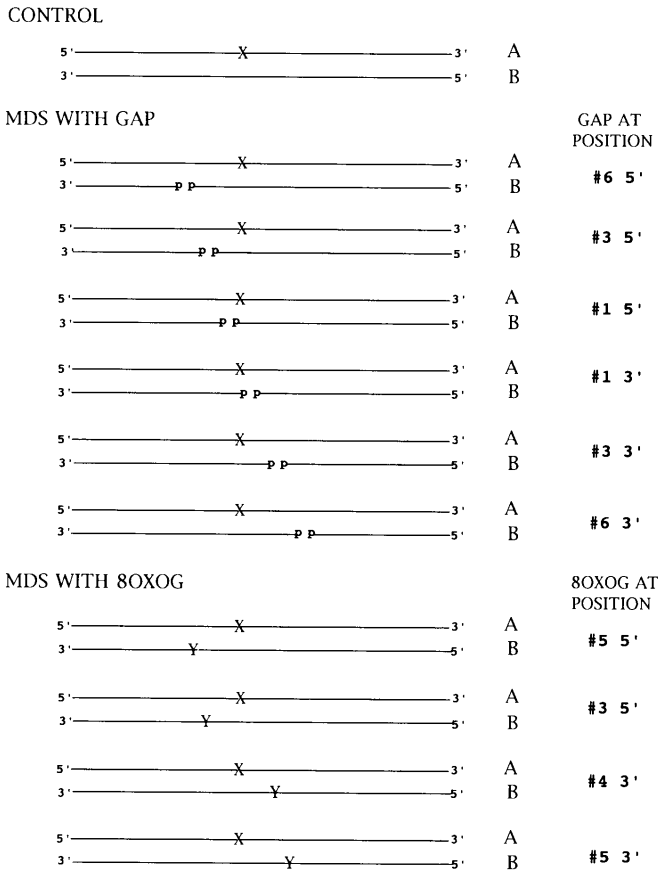



**Figure 1.** Diagrammatic representation of control and MDS substrates. The oligonucleotides used to form the substrates are described in Table 1. The control substrate has a single site of damage in strand A, while MDS substrates contain one site of damage in strand A and one in strand B. X corresponds to either a DHT, Tg, AP or rAP site. Y represents an 8-oxoG residue. P is a phosphate moiety attached to either the 3′- or 5′-terminus of strand B of the oligonucleotides which form the single nucleotide gap. The position of damage in strand B is relative to X in strand A.

B, as described above, at 15 fmol/µl. Approximately 8 fmol duplex DNA containing a putative rAP site were boiled in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl for 30 min. Also, ~8 fmol were incubated with ~18 ng endo IV in 5 µl 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl. Analysis of these samples showed that the boiled sample was not fragmented, while the endo IV-treated sample was cleaved ~95%, thus indicating that the AP site was reduced. For electrophoretic mobility shift analysis with endo VIII the substrate was annealed as described above at 5 fmol/µl.

### Gel electrophoretic mobility shift analysis

rAP site-containing duplex substrates (5 fmol) were mixed on ice with endo VIII (1–20 nM final concentration) in 5 µl 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 3.2% glycerol and incubated for 5 min at 20°C. After addition of 1 µl 20% glycerol, 25 mM Tris–HCl, pH 7.5, samples were subjected to electrophoresis through a 15% polyacrylamide gel containing 2.5% glycerol and 0.5× TBE. The buffer used was 0.5× TBE and electrophoresis was performed at 4°C at 200–300 V for ~3 h.

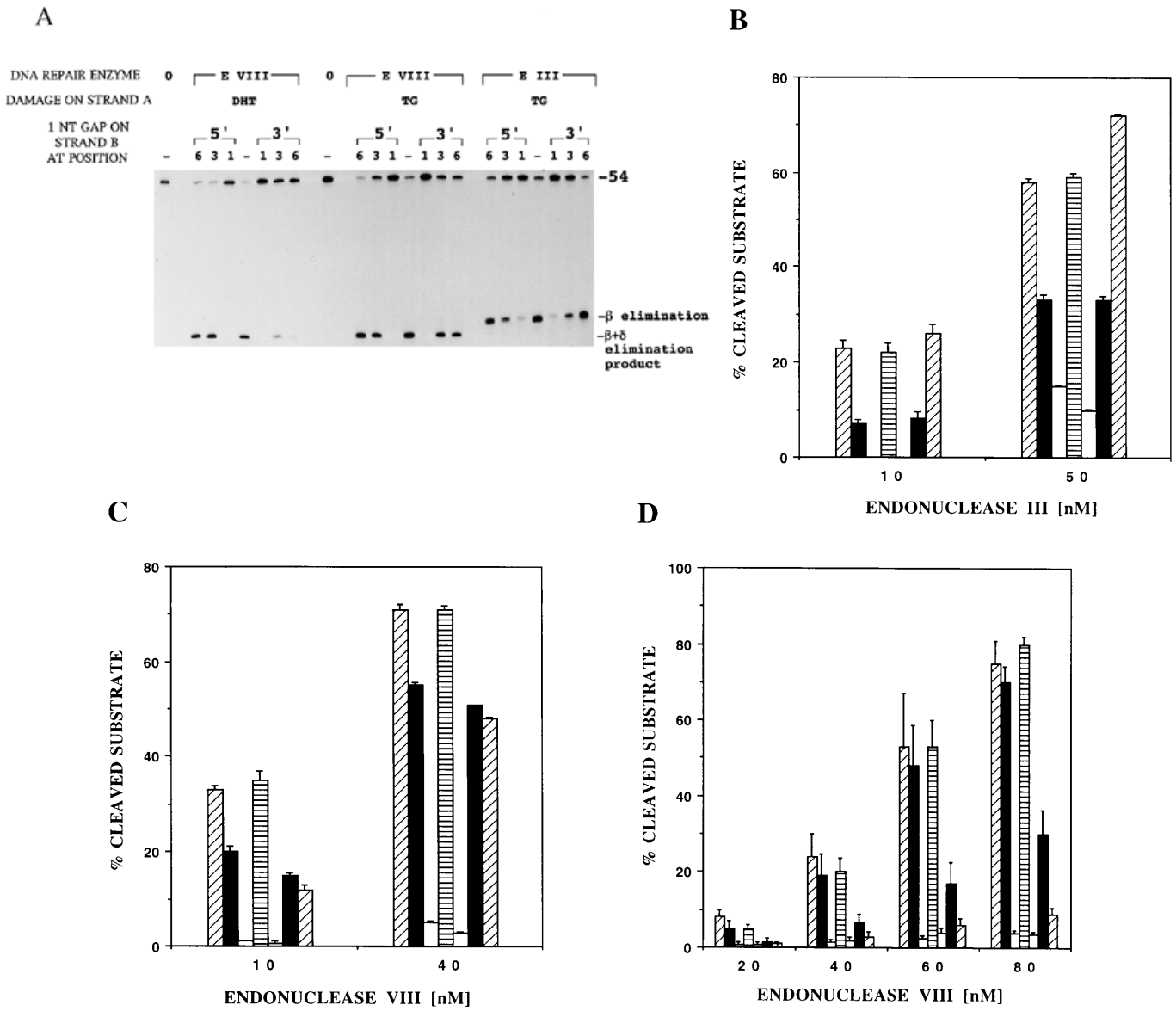



**Figure 2.** Reaction of endos III and VIII with sites of base damage closely opposed to a nucleotide gap. Strand A was 5′-labeled with $^{32}$P and contained either DHT or Tg. Duplex substrates (20 nM; see Fig. 1) were treated with either endo III (10 or 50 nM) or endo VIII (10–80 nM) under standard reaction conditions. Samples were subjected to electrophoresis through a 12% polyacrylamide–7 M urea gel. Aliquots of 40 (for reaction with Tg) or 80 nM (for reaction with DHT) endo VIII and 50 nM endo III were used to generate the reaction products seen in (**A**). A BioRad molecular imager was used to quantitate the percent of cleaved substrate. The average percent cleaved substrate for three reactions with standard errors are shown in (**B**), (**C**) and (**D**) for endo III with Tg-containing substrates, endo VIII with Tg-containing substrates and endo VIII with DHT-containing substrates respectively. The open bar, filled bar and stippled bar represent substrates with single nucleotide gaps at positions one, three and six respectively; the horizontal bar represents the control substrate containing a single site of damage with no opposing gap. Bars to the left of the control correspond to substrates with nucleotide gaps 5′ of the damage in strand A and bars to the right of the control are for substrates with the nucleotide gap 3′ of the damage in strand A.

## RESULTS

### Removal of a site of base damage closely opposed to a single nucleotide gap

Strand A (54mer) containing a DHT or Tg at position 24 (Table 1) was labeled with $^{32}$P and annealed to strand B (as described in Materials and Methods) to form duplex substrates with a site of base damage closely opposed to a single nucleotide gap (Fig. 1). Following incubation with endo VIII or endo III strand A was cleaved (Fig. 2A), leaving the expected reaction products: after removal of the site of base damage endo III catalyzed a β elimination reaction resulting in a 23mer with an attached α,β unsaturated aldehyde moiety, while endo VIII catalyzed a β followed by a δ elimination reaction producing a 23mer with an attached 3′-phosphate group, which migrates faster than the β elimination product during electrophoresis. DNA containing Tg with a single nucleotide gap positioned 1 nt 5′ or 3′ of the site of damage in the complementary strand was a poor substrate for both endo III and endo VIII (Fig. 2A). Similar results were observed for endo VIII cleavage of a DHT-containing substrate (Fig. 2A). Increasing the amount of the DNA glycosylase increased cleavage of the substrates with the gap at position one

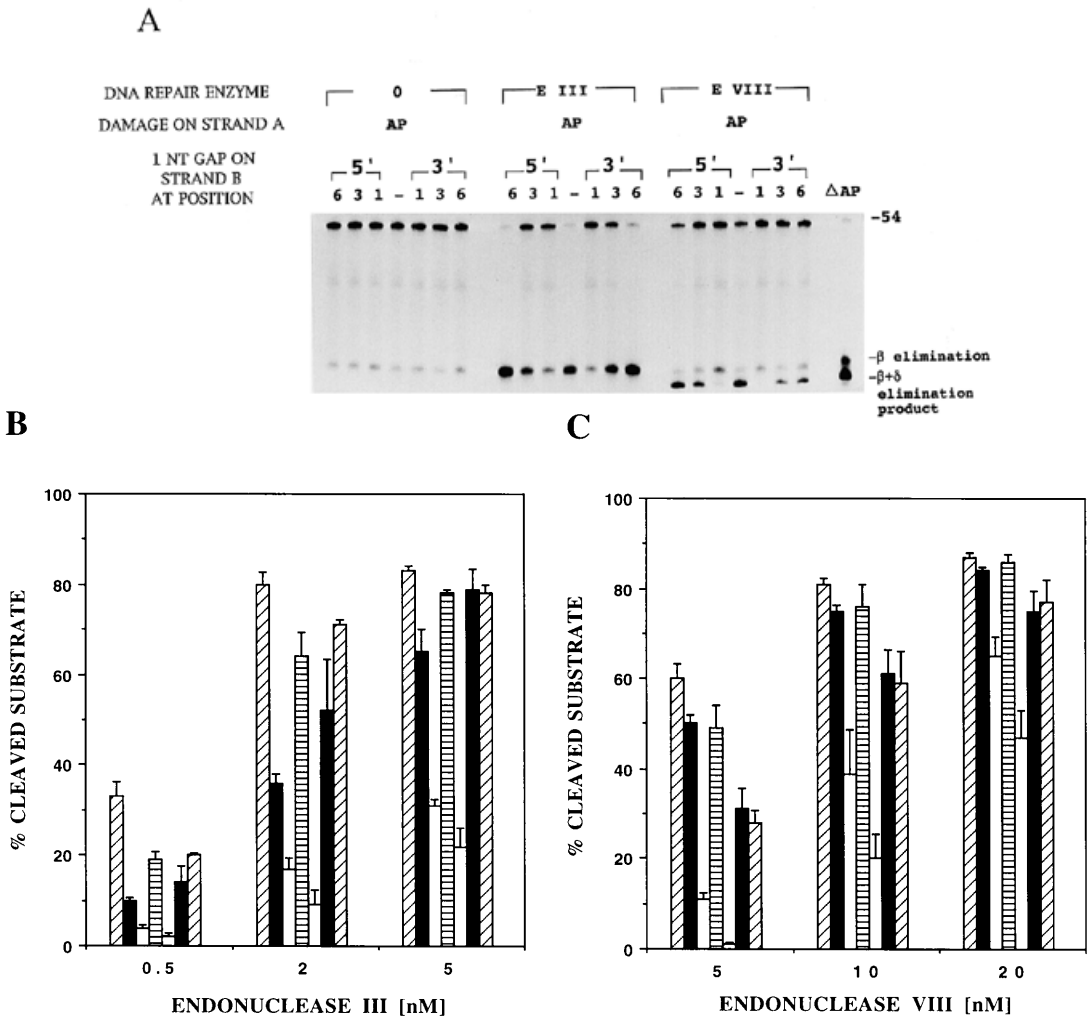



**Figure 3.** Reaction of endos III and VIII with an AP site closely opposed to a nucleotide gap. Strand A was 5′-labeled with $^{32}P$ and contained an AP site. Duplex substrates (20 nM; see Fig. 1) were treated with either endo III (0.5–5 nM) or endo VIII (5–20 nM) under standard conditions. The reaction products generated by incubating 2 nM endo III or 5 nM endo VIII with the AP site-containing substrates are shown in (**A**). ΔAP shows fragmentation of the control substrate after it was boiled for 30 min. The average percent cleaved substrate and the standard errors for three reactions are shown in (**B**) and (**C**) for reactions with endo III and endo VIII respectively. The open bar, filled bar and stippled bar represent substrates with single nucleotide gaps positioned 1, 3 and 6 nt from the AP site respectively; the horizontal bar represents the control substrate containing the single AP site with no opposing gap. Bars to the left of the control correspond to the substrates with nucleotide gaps 5′ of the damage in strand A and bars to the right of the control correspond to substrates with the nucleotide gap 3′ of the damage in strand A.

on strand B (see Fig. 1), but cleavage was still limited, 5% for endo VIII and 15% for endo III, under the conditions examined (Fig. 2B–D). Increasing the distance between the gap and the Tg, however, increased the efficiency of endo III cleavage. In fact, cleavage was equivalent to that of the control when the gap was at position six 5′ or 3′ of Tg (Fig. 2B). A similar increase in substrate cleavage was seen for endo VIII with DHT or Tg if the gap was 5′ of the site of base damage on the opposing strand. However, endo VIII activity was inhibited to a greater extent if the opposing gap was 3′ of the site of base damage. If the gap was positioned 6 nt away from the modified pyrimidine, cleavage was equal to or less than that of the substrate with the gap 3 nt 3′ of the base damage (Fig. 2C and D).

### Cleavage at an abasic site closely opposed to a single nucleotide gap

To determine if the AP lyase activity of endo VIII and endo III was altered by the presence of a nucleotide gap in close proximity to the AP site, strand A of the MDS substrate contained an AP site at position 24 and was 5′-labeled with $^{32}P$ (Fig. 1). As seen in Figure 3A, endo VIII or endo III activity was substantially lower when the substrate contained a gap 1 nt 5′ or 3′ of the AP site than the control substrate or when the gap opposite was further away. Increasing the enzyme concentration substantially increased cleavage of these substrates by either enzyme (Fig. 3B and C). When 20 nM endo VIII was used 65% fragmentation was achieved if the gap was at position one 5′ of the AP site. This cleavage was much greater than when the MDS contained DHT or Tg at the same position on strand A, when approximately equal levels of control cleavage were compared. As was found for MDS containing Tg or DHT, the efficiency of endo VIII cleavage of the AP site was affected more by the presence of the gap if it was placed 3′ or opposite the AP site rather than 5′. However, only the distance between the two opposing lesions and not the orientation of the opposing gap appeared to affect endo III activity (Figs 2B and 3B).

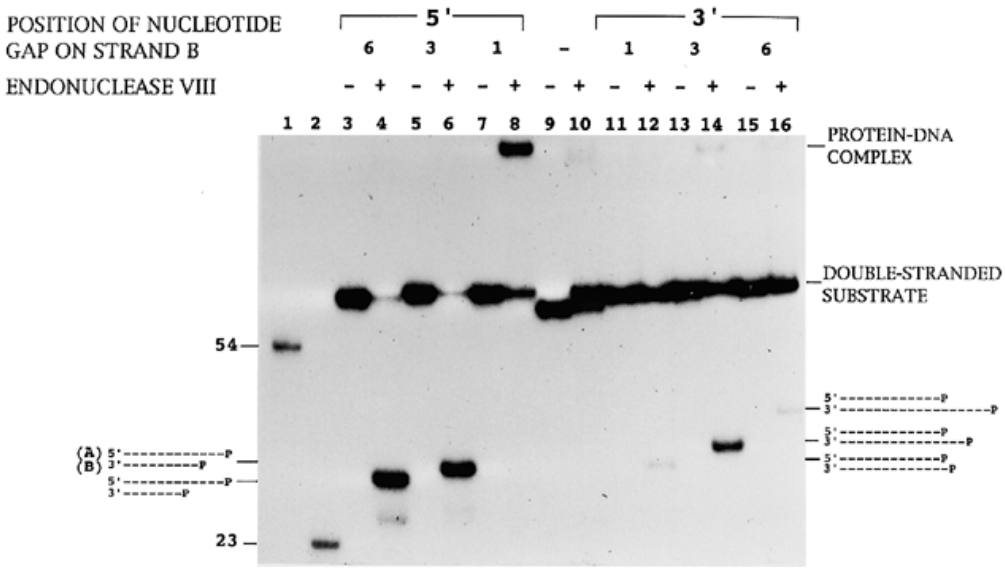



**Figure 4.** Analysis under non-denaturing conditions of reaction products generated by endo VIII activity on MDS substrates. Endo VIII (80 nM) was incubated under standard conditions with 18 nM control or MDS substrates containing DHT in strand A and a single nucleotide gap in strand B. Samples were subjected to electrophoresis in a 15% non-denaturing polyacrylamide gel. After the gel was dried autoradiography was used to visualize the reaction products.

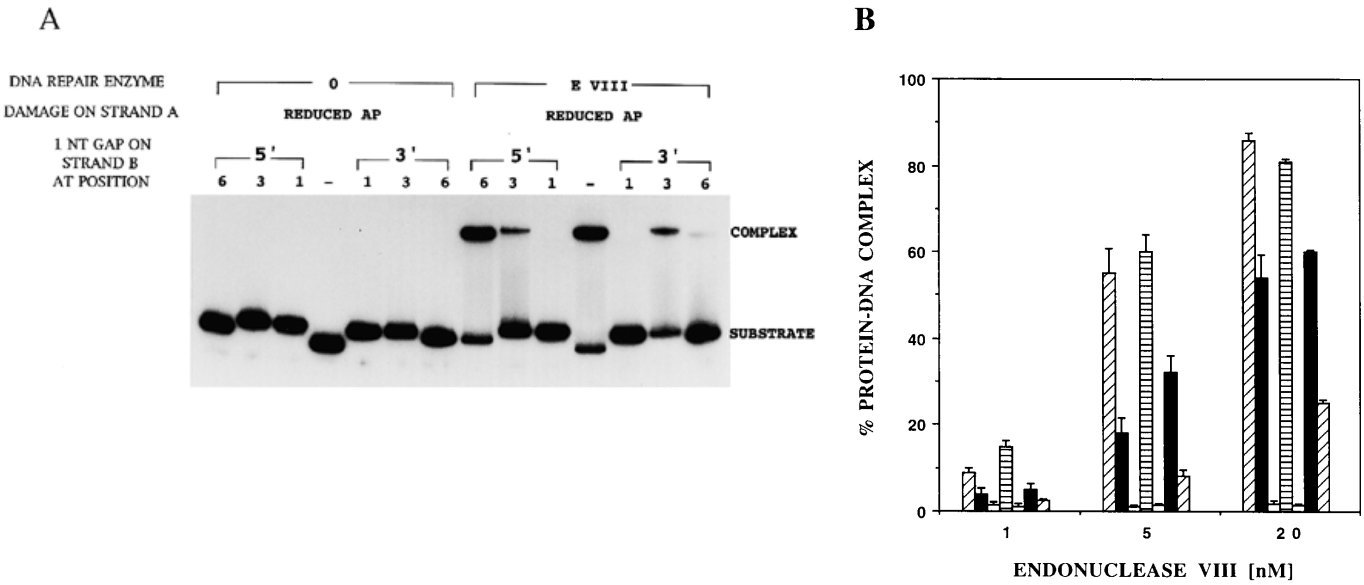



**Figure 5.** Endo VIII binding to a rAP site closely opposed to a single nucleotide gap. Control and MDS substrates (1 nM) were incubated with 1–20 nM endo VIII in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 3.2% glycerol for 5 min at 20°C. Samples were subjected to electrophoresis in a 15% non-denaturing polyacrylamide gel. (**A**) An example of a gel showing the products of 0 or 5 nM endo VIII binding reactions. Triplicate reactions were performed and the averages of each set with standard errors are shown in (**B**). The open bar, filled bar and stippled bar represent substrates with single nucleotide gaps 1, 3 and 6 nt from the rAP site in strand A respectively; the horizontal bar represents the control substrate containing the rAP site with no opposing gap. Bars to the left of the control correspond to substrates with nucleotide gaps 5′ of the damage in strand A and bars to the right of the control are for substrates with the nucleotide gap 3′ of the damage in strand A.

### Does cleavage of the site of base damage in a MDS result in a double-strand break?

To test this possibility strand A of a duplex substrate containing a DHT was 5′-labeled with $^{32}$P. Reactions were carried out as for Figure 2 using 18 nM substrate and 80 nM endo VIII. The products of the reaction (Fig. 4) were visualized following electrophoresis through a non-denaturing gel. The double-stranded structure of the DNA was maintained during electrophoresis. The control double-stranded substrate (lane 9), containing DHT but no gap opposite, had a faster mobility on the non-denaturing gel than a DHT-containing substrate with a nucleotide gap in strand B (Fig. 4, lanes 3, 5, 7, 11, 13 and 15). When the control substrate was treated with endo VIII (lane 10) two bands were detected, one the size of the untreated control substrate and the other the size of the untreated MDS substrate. The latter band was more intense and corresponded to dsDNA containing a gap in strand A, due to removal of DHT by endo VIII. As can be seen in Figure 2D, under these reaction conditions 80% of strand A containing DHT was cleaved by endo VIII.

Addition of endo VIII to MDS substrates with DHT opposite a gap resulted in breakage of the duplex substrate (Fig. 4, lanes 4, 6, 12, 14 and 16). The breakage products had different electrophoretic mobilities and migrated more slowly than a 23mer. As the single nucleotide gap was moved towards the 5′-end of strand B and the 3′-end of strand A in the MDS substrate mobility of the breakage products decreased. We believe this to be due to an increase in size of the 3′-end of fragment B that is annealed to the 5′-labeled 23mer of strand A. A dsb was not produced by endo VIII treatment of the control substrate (lane 10).

Endo VIII did not cleave the MDS substrate containing a single nucleotide gap at position one 5′ of the DHT (Fig. 4, lane 8), although it did form a complex with the DNA. This complex had a similar mobility to faint bands (~1% of the substrate) detected in lanes corresponding to the endo VIII-treated control substrate and the MDS substrate with the gap at position three or six 3′ of the DHT (Fig. 4, lanes 10, 14 and 16). To determine whether endo VIII had removed the DHT from the substrate containing an opposed gap 1 nt 5′ of the DHT the reaction was repeated and the products boiled and analyzed on a denaturing gel. Strand A did not fragment (data not shown), indicating that the DHT was still present. Similar binding was observed for a substrate with the gap at position one but which had a T in place of the DHT on strand A (data not shown). It is possible that endo VIII bound to an altered DNA structure caused by the presence of the opposing gap or to the gap itself.

### Does a decrease in cleavage of the substrate correspond to an alteration in the binding of endo VIII to the MDS substrate?

To test this supposition strand A of the duplex substrate containing a reduced AP site and labeled with $^{32}$P was incubated with endo VIII and binding determined by gel shift analysis. Endo VIII has previously been shown to bind strongly to a rAP site (38). Endo VIII is unable to cleave the DNA, as cleavage is accomplished by β,δ elimination, which requires an aldehydic AP site. Figure 5 shows binding of endo VIII to MDS substrates with a rAP site closely opposed to a single nucleotide gap. Binding was drastically reduced by the presence of a gap at position one 5′ or 3′ of the rAP site (Fig. 5A and B). Binding increased as the distance between the gap and rAP site increased to the 5′-side of the rAP. However, binding to the substrate was reduced when the gap was at position six versus position three 3′ of the rAP site. A crude equilibrium disassociation constant ($K_d$) was estimated by determining the concentration of endo VIII required to bind 50% of the rAP substrate. No significant binding to MDS substrates containing a gap at position one could be detected even at concentrations of 50 nM endo VIII (data not shown). The approximate $K_d$ values for MDS substrates with gaps at the remaining positions are shown in Table 2.

### Endo VIII cleavage of a site of pyrimidine damage closely opposed to an oxidized purine lesion

Strand A, containing either a DHT or Tg at position 24, was 5′-labeled with $^{32}$P and annealed to each of the four different B strands that contained a single 8-oxoG (see Table 1 and Fig. 1) or a B strand with no damage. This generated five substrates: control (containing only a site of pyrimidine damage) or MDS with an opposing 8-oxoG 4 or 5 nt 3′, or 3 or 5 nt 5′ of a DHT or Tg. As shown in Figure 6A and B, removal of the Tg or DHT by endo VIII was not altered by the close proximity of the 8-oxoG in the opposite strand. A similar result was found for endo III cleavage of a Tg closely opposed to an 8-oxoG lesion (data not shown).

To determine whether endo VIII binding to a rAP site opposite 8-oxoG correlated with the ability of endo VIII to cleave the pyrimidine lesion opposite 8-oxoG, binding substrates were generated with a rAP site in strand A and no damage or an 8-oxoG 3 or 5 nt 5′, or 4 or 5 nt 3′ of the rAP site in strand B (Fig. 1 and Table 1). As was observed for cleavage of pyrimidine lesions opposite 8-oxoG (Fig. 6A and B), the binding of endo VIII to the rAP site opposite 8-oxoG was identical for all the substrates (Fig. 6C).

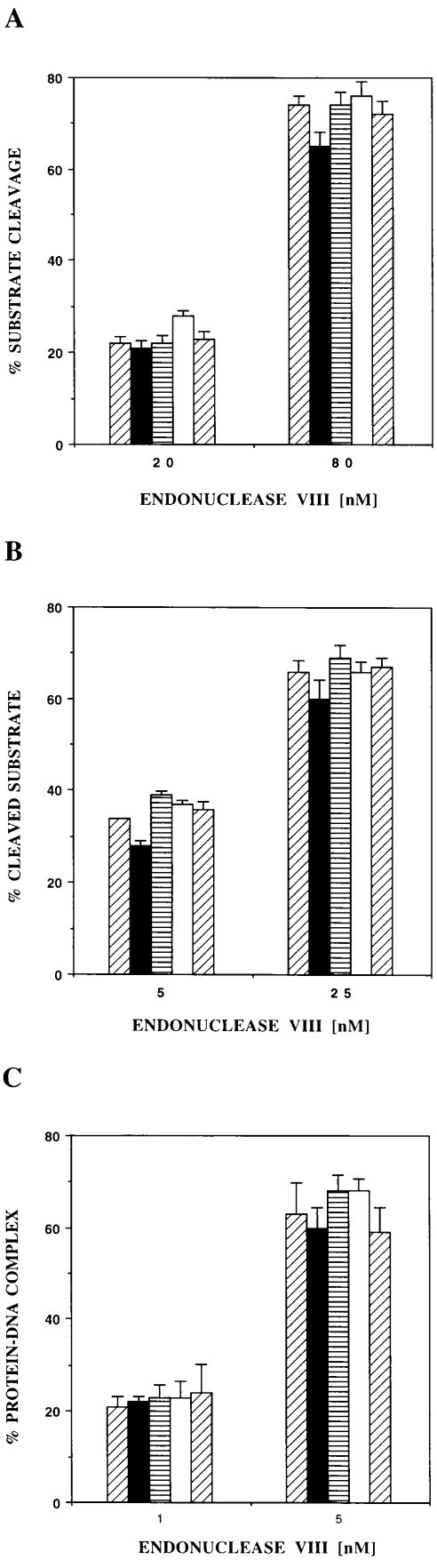
**Table 2.** Binding of endo VIII to MDS substrates containing a rAP site opposite a ssb[a]

| Position of gap in strand B relative to rAP in strand A | Approximate $K_d$ (nM) |
|---|---|
| 6 nt 5′ | 4 |
| 3 nt 5′ | 19 |
| 1 nt 5′ | ND[b] |
| Control | 4 |
| 1 nt 3′ | ND |
| 3 nt 3′ | 15 |
| 6 nt 3′ | 60 |

[a]Binding reactions (see Materials and Methods) were performed (in triplicate) using 1 nM substrate and 1, 5 or 20 nM endo VIII. Reactions were also carried out with 50 nM endo VIII. The amount of bound versus total substrate in the reaction was quantitated using a BioRad Molecular Imager. Percent protein–DNA complex was plotted against concentration of endo VIII and the concentration at which 50% of the substrate was bound to endo VIII was determined. For substrates containing a gap 6 nt 3′ of the rAP site the line was extrapolated to estimate the $K_d$. Endo VIII did not bind to the rAP site when the gap was at position one in the MDS.
[b]ND, not detected.

## DISCUSSION

Studies of ionizing radiation track structure (5,6) and assessments of the number of lesions required to induce one lethal event/cell have indicated that certain agents, such as ionizing radiation and bleomycin A2, induce complex DNA lesions (2). Experimental evidence suggests that repair of such complex lesions can result in an increase in DNA damage. For example, incubation of γ-irradiated plasmid DNA with human cell extracts under scavenging conditions considered to be equivalent to those in the cell (200 mM Tris–HCl) resulted in an increase in ssb in the plasmid that were distinct from heat-labile sites (sites of base loss) (40). Also, the level of DNA dsb has been found to increase in irradiated cells that were allowed time to repair DNA damage (41–43). Consistent with these observations, endo VIII formed dsb in substrates containing DHT and an opposing ssb 3 or 6 nt away (Fig. 4). However, endos III and VIII were unable to remove Tg if a ssb was situated 1 nt 5′ or 3′ of or opposite the base damage (Fig. 2). Similarly, removal of DHT by endoVIII was inhibited by the ssb at position one (Fig. 2). The ssb still inhibited the activity of the repair enzymes if it was 3 nt away, although to a lesser extent (Fig. 2). Previously, repair of closely opposed DHT residues by endo III was shown to generate a ssb not a dsb if the two sites of base damage were 1 or 3 bp apart (33). Our




observation accounts for this result (33), since removal of the first DHT by endo III would have generated a ssb 1 or 3 nt from or opposite the second DHT, which would have inhibited further action by endo III. Endo III treatment of a substrate containing two opposed DHT 5 or 7 nt apart was also shown to form a dsb (32), although this reaction was slow and required a high concentration of endo III. The decreased ability of endo III to remove a DHT compared with Tg opposite a ssb, when the ssb was >5 nt away, may be attributed to the higher $K_m$ of endo III for DHT compared with Tg (Hatahet and Wallace, unpublished observations). Endo VIII removal of DHT was also inhibited to a greater extent than removal of Tg (Fig. 2C and D) in MDS with a ssb at positions three or six 3′ of the base damage. Endo VIII, like endo III, binds and cleaves Tg better than DHT (Hatahet and Wallace, unpublished observations).

AP sites in the MDS examined here were more susceptible to cleavage, especially by endo VIII, even when the gap was 1 nt 5′ or 3′ of the AP site (Fig. 3). This was also noted for endo III by Chaudhry and Weinfeld (33). However, their assay did not detect inhibition of dsb formation when the AP sites were 1 nt apart compared with >3 nt apart, suggesting that endo III removal of an AP site was not affected by a ssb at position one, which is contrary to our results. An explanation for this difference may be the high enzyme concentration and long incubation times used by Chaudhry and Weinfeld (33). Their result does infer, however, that given enough time and enzyme the inhibition of endo III seen in Figure 3B could be overcome and result in enhanced dsb formation. Bleomycin and neocarzinostatin also produce complex DNA lesions with AP sites opposite ssb. This MDS represents only a small proportion of bleomycin-induced DNA damage but accounts for the majority of neocarzinostatin-induced damage (44). The structure of the bleomycin MDS is unknown, however, neocarzinostatin produces an AP site 2 nt 5′ of a strand break (45). High concentrations of endo III were also required to cleave these complex lesions *in vitro* (45).

DNase I footprinting suggests that endonuclease III protects ~9–11 bases on the rAP site strand and the complementary strand in a duplex 39mer, with four bases 5′ and 3′ of the rAP site protected (46). In other words, the footprint is reasonably symmetrical around the lesion site. In contrast, examination of the DNase I footprint of endo VIII with a rAP site (11) shows that binding of the enzyme is asymmetrical, predominantly 3′ of the damage site, with contact sites primarily on the damaged strand. These observations are in keeping with the effect of a closely opposed ssb on the abilities of endos III and VIII to remove a base

**Figure 6.** The effect of a closely opposed 8-oxoG on cleavage of a pyrimidine lesion and binding to a rAP site by endo VIII. Control or MDS substrates (20 nM) containing either DHT or Tg on strand A and no damage or an 8-oxoG on strand B (see Fig. 1) were incubated with increasing amounts of endo VIII under standard reaction conditions. The averages and standard errors of triplicate reactions are shown in (**A**) and (**B**) for DHT- and Tg-containing substrates closely opposed to 8-oxoG respectively. Binding reactions were performed under standard conditions with 1 nM control or MDS substrate containing a rAP site on strand A and no damage or an 8-oxoG on strand B. (**C**) The average and standard error of percent protein–DNA complex from three independent reactions. The filled bar, open bar and stippled bar represent DNA molecules with the 8-oxoG 3, 4 and 5 nt from the target lesion respectively; the horizontal line bar represents the control substrate containing the rAP site with no opposing gap. Bars to the left of the control correspond to the substrates with 8-oxoG 5′ of the site of pyrimidine damage or rAP site and bars to the right of the control are for substrates with the 8-oxoG 3′ of the site of pyrimidine damage or rAP site.

lesion, i.e. the opposed ssb 5′ or 3′ of the target damage inhibited endo III equally if they were equidistant from the damage in strand A, but ssb positioned 3′ of the target damage inhibited endo VIII to a greater extent than those positioned equidistant or 5′ of the target damage (Fig. 2). Gel mobility shift analysis of endo VIII binding to DNA containing a rAP site opposite a ssb (Fig. 5) also correlated with the cleavage asymmetry except when the AP site was separated from the opposed gap by 1 nt. In this case no binding to the rAP site was found but cleavage of the AP site was observed. Taken together with the footprinting data, the binding and cleavage data suggest that the enzyme may contact the nucleotide 'lost' at the gap six bases 3′ of the rAP site or its pairing partner in strand A. A similar situation has been found for cleavage of two closely opposed AP sites by human AP endonuclease (APE). Activity was inhibited if the AP sites were positioned 1 and 3 nt 5′ of each other (34) and methylation interference studies using one AP site have indicated that APE contacts the DNA at position one and three 5′ of the AP site on the damaged strand (47). Alternatively, since endo VIII preferentially cleaves double-stranded damaged substrates (17) and the single nucleotide gap in the MDS substrates may result in an altered DNA conformation (Fig. 4), binding of the enzyme to the MDS substrates could have been disrupted by the presence of the single-stranded region or a perturbation in the DNA conformation. Computational simulations of Tg and DHT (47) and NMR studies of a Tg (49) or an abasic site (50) in DNA do not show an alteration in the B-DNA backbone structure. However, AP sites and Tg lesions cause disruption of the local conformation of the adjacent base pairs. In the case of Tg, computational studies suggest that the $C^5$ methyl group is pseudoaxial with respect to the plane of the base and distorts the base pair 5′ of the Tg lesion (48). NMR studies show that both Tg (50) and the deoxyribose of AP sites (49) can 'flip out' of the B-DNA structure. Based on these structural observations it might be expected that MDS containing a Tg or an AP site might exert a greater effect on enzyme cleavage than those with a DHT. However, the trends of the cleavage patterns for MDS containing each of the three lesions were similar. The principal difference was that a MDS containing an abasic site was cleaved by endo VIII to a greater extent than a damaged base when the opposing ssb was at position one.

Positioning of an 8-oxoG in the complementary strand in close proximity to a Tg or DHT did not inhibit DNA cleavage by endo III or VIII. Binding of endo VIII to a rAP site was also not perturbed by the presence of an opposing 8-oxoG. If these results hold true in the cell, a Tg lesion closely opposed to an 8-oxoG could be readily converted into an 8-oxoG closely opposed to a ssb. Work in progress also indicates that this MDS can be cleaved by Fpg to form a dsb if the initial lesions are ≥3 nt apart.

It seems likely, therefore, that when a site of base damage is closely opposed to another site of base damage (this work and ref. 33) one of the sites of base damage can be readily removed to generate a MDS with a site of base damage near a ssb. Such lesions can also be generated in DNA by ionizing radiation. Closely opposed abasic sites can also be converted to an AP site opposing a ssb (32). Removal of the remaining site of base damage or AP site is then dependent upon its distance from the ssb (this work and ref. 33). It appears clear that two enzymes with similar substrate specificities, endo III and endo VIII, are affected differently by the position of the ssb. When the ssb is 1 nt 5′ or 3′ of the site of base damage or AP site the enzymes are inhibited to the greatest extent (Fig. 2). Exonuclease III, the major class II AP endonuclease of *E.coli*, also demonstrated reduced cleavage of this substrate (34). If replication of this MDS substrate is attempted it is likely that replication could be blocked on the strands containing a ssb, abasic site or thymine glycol (for a review see ref. 51). In *E.coli*, where the replication complex contains two polymerase holoenzyme units which replicate both strands in concert (52), such a MDS could either block the complex and kill the cell or the holoenzyme could reinitiate downstream, generating a gap requiring post-replication recombination repair. No genetic alterations would be expected from replicating a strand containing DHT (24). However, if an opposing ssb is introduced 3 or 6 nt away by either a hydroxyl radical during irradiation or initiation of repair of a site of base damage or AP site, further action by the BER pathway to remove the DHT could form a dsb (Fig. 4). Thus a non-lethal lesion, such as DHT or 8-oxoG, could be converted into a lethal dsb. The question remains whether the ssb can be repaired prior to cleavage of the opposing site of base damage or AP site. If this occurred, a dsb would not be formed and the MDS lesions would be repaired sequentially as single lesions. This question is currently under investigation.

## ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grant R37CA33657 awarded by the National Cancer Institute. Lynn Harrison was the recipient of a Lake Champlain Cancer Research Organization Postdoctoral Fellowship awarded by the Vermont Cancer Center. The authors are grateful to Dr Michael Weinfeld for helpful discussions.

## REFERENCES

1 Elkind,M.M. and Redpath,J.L. (1977) In Becker,F.F. (ed.), *Cancer, Comprehensive Treatise*. Plenum Press, NY. Vol. 6, pp. 51–99.
2 Ward,J.F., Evans,J.W., Limoli,C.L. and Calabro-Jones,P.M. (1987) *Cancer*, **55**, 105–112.
3 Bradley,M.O. and Kohn,K.W. (1979) *Nucleic Acids Res.*, **7**, 793–804.
4 Cress,A.E., Kurath,K.M., Stea,B. and Bowden,G.T. (1990) *J. Cancer Res. Clin. Oncol.*, **116**, 324–330.
5 Goodhead,D.T. (1994) *Int. J. Radiat. Biol.*, **61**, 7–17.
6 Brenner,D.J. and Ward,J.F. (1992) *Int. J. Radiat. Biol.*, **61**, 737–748.
7 Iliakis,G. (1991) *BioEssays*, **13**, 641–648.
8 Ward,J.F. (1998) *Prog. Nucleic Acid Res. Mol. Biol.*, **35**, 95–125.
9 Demple,B. and Harrison,L. (1994) *Annu. Rev. Biochem.*, **63**, 915–948.
10 Bailly,V. and Verly,W.G. (1987) *Biochem. J.*, **242**, 565–572.
11 Jiang,D., Hatahet,Z., Melamede,R.J., Kow,Y.W. and Wallace,S.S. (1997) *J. Biol. Chem.*, **272**, 32230–32239.
12 Henner,W.D., Rodriguez,L.O., Hecht,S.M. and Haseltine,W. (1983) *J. Biol. Chem.*, **258**, 711–713.
13 Kow,Y.W., Faundez,G., Melamede,R.J. and Wallace,S.S. (1991) *Radiat. Res.*, **126**, 357–366.
14 Bernelot-Moens,C. and Demple,B. (1986) *Nucleic Acids Res.*, **17**, 587–600.
15 Demple,B., Johnson,A. and Fung,D. (1986) *Proc. Natl. Acad. Sci.USA*, **83**, 7731–7735.
16 Breimer,L.H. and Lindahl,T. (1984) *J. Biol. Chem.*, **259**, 5543–5548.
17 Melamede,R.J., Hatahet,Z., Kow,Y.W., Ide,H. and Wallace,S.S. (1994) *Biochemistry*, **33**, 1255–1264.
18 Teoule,R. (1987) *Int. J. Radiat. Biol.*, **51**, 573–589.
19 Nackerdien,Z., Olinski,R. and Dizdaroglu,M. (1992) *Free Radical Res. Commun.*, **16**, 259–273.
20 Dizdaroglu,M. (1985) *Biochemistry*, **24**, 4476–4481.
21 Hutchinson,F. (1985) *Prog. Nucleic Acid Res. Mol. Biol.*, **32**, 115.
22 Ide,H., Petrullo,L.A., Hatahet,Z. and Wallace,S.S. (1991) *J. Biol. Chem.*, **266**, 1469–1477.
23 Shibutani,S., Takeshita,M. and Grollman,A.P. (1991) *Nature*, **349**, 431–434.

24 Cheng,K.D., Cahill,D.S., Kasai,H., Nishimura,S. and Loeb,L.A. (1992) *J. Biol. Chem.*, **267**, 166–172.
25 Evans,J., Macabee,M., Hatahet,Z., Courcelle,J., Bockrath,R., Ide,H. and Wallace,S.S. (1993) *Mutat. Res.*, **299**, 147–156.
26 Clark,J.M. and Beardsley,G.P. (1986) *Nucleic Acids Res.*, **14**, 737–749.
27 Clark,J.M. and Beardsley,G.P. (1987) *Biochemistry*, **26**, 5398–5403.
28 Moran,E. and Wallace,S.S. (1985) *Mutat. Res.*, **146**, 229–241.
29 Hayes,R.C. and LeClerc,J.E. (1986) *Nucleic Acids Res.*, **14**, 1045–1061.
30 Sagher,D and Strauss,B. (1983) *Biochemistry*, **22**, 4518–4526.
31 Paz-Elizur,T., Takeshita,M. and Livneh,Z. (1997) *Biochemistry*, **36**, 1766–1773.
32 Shibutani,S., Takeshita,M. and Grollman,A.P. (1997) *J. Biol. Chem.*, **272**, 13916–13922.
33 Chaudhry,M.A. and Weinfeld,M. (1995) *J. Mol. Biol.*, **249**, 914–922.
34 Chaudhry,M.A. and Weinfeld,M. (1997) *J. Biol. Chem.*, **272**, 15650–15655.
35 Purmal,A.A., Kow,Y.W. and Wallace,S.S. (1994) *Nucleic Acids Res.*, **22**, 72–78.
36 Hatahet,Z., Purmal,A. and Wallace,S.S. (1993) *Nucleic Acids Res.*, **21**, 1563–1568.
37 Asahara,H., Wistort,P.M., Bank,J.F., Bakerian,R.H. and Cunningham,R.P. (1989) *Biochemistry*, **28**, 4444–4449.
38 Jiang,D., Hatahet,Z., Blaisdell,J.O., Melamede,R.J. and Wallace,S.S. (1997) *J. Bacteriol.*, **179**, 3773–3782.
39 Hatahet,Z., Kow,Y.W., Purmal,A.A. and Cunningham,R.P. and Wallace,S.S. (1994) *J. Biol. Chem.*, **269**, 18814–18820.
40 Hodgkins,P.S., Fairman,M.P. and O'Neill,P. (1996) *Radiat. Res.*, **145**, 24–30.
41 Bonura,T., Smith,K.C. and Kaplan,H.S. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4265–4269.
42 Dugle,D.L., Gillespie,C.J. and Chapman,J.D. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 809–812.
43 Ahnstrom,G. and Bryant,P.E. (1982) *Int. J. Radiat. Biol.*, **41**, 671–676.
44 Povirk,L.F. and Houlgrave,C.W. (1988) *Biochemistry*, **27**, 3850–3857.
45 Povirk,L.F, Houlgrave,C.W. and Han,Y. (1988) *J. Biol. Chem.*, **263**, 19263–19266.
46 O'Handley,S., Scholes,C.P. and Cunningham,R.P. (1995) *Biochemistry*, **34**, 2528–2536.
47 Wilson,D.M., Takeshita,M. and Demple,B. (1997) *Nucleic Acids Res.*, **25**, 933–939.
48 Miaskiewicz,K., Miller,J., Ornstein,R. and Osman,R. (1995) *Biopolymers*, **35**, 113–124.
49 Goljer,I., Kumar,S. and Bolton,P.H. (1995) *J. Biol. Chem.*, **270**, 22980–22987.
50 Kung,H.C. and Bolton,P.H. (1997) *J. Biol. Chem.*, **272**, 9227–9236.
51 Hatahet,Z. and Wallace,S.S. (1997) In Hoekstra,M.F. and Nickoloff,J.A. (eds), *DNA Damage and Repair Biochemistry, Genetics and Cell Biology*. Humana Press, NJ, in press.
52 Kelman,Z. and O'Donnell,M. (1995) *Annu. Rev. Biochem.*, **64**, 171–200.