Several oxidative DNA-damaging agents, including ionizing radiation, can generate multiply damaged sites in DNA. Among the postulated lesions are those with abasic sites located in close proximity on opposite strands. The repair of an abasic site requires strand scission by a repair endonuclease such as human apurinic/apyrimidinic endonuclease (Ape) or exonuclease III in Escherichia coli. Therefore, a potential consequence of the "repair" of bistranded abasic sites is the formation of double-strand breaks. To test this possibility and to investigate the influence of the relative distance between the two abasic sites and their orientation to each other, we prepared a series of oligonucleotide duplexes containing abasic sites at defined positions either directly opposite each other or separated by 1, 3, or 5 base pairs in the 5′- or 3′-direction. Analysis following Ape and exonuclease III treatment of these substrates indicated a variety of responses. In general, cleavage at abasic sites was slower in duplexes with paired lesions than in control duplexes with single lesions. Double-strand breaks were, however, readily generated in duplexes with abasic sites positioned 3′ to each other. With the duplex containing abasic sites set 1 base pair apart, 5′ to each other, both Ape and exonuclease III slowly cleaved the abasic site on one strand only and were unable to incise the other strand. With the duplex containing abasic sites set 3 base pairs apart, 5′ to each other, Ape protein was unable to cleave either strand. These data suggest that closely positioned abasic sites could have several deleterious consequences in the cell. In addition, this approach has allowed us to map bases that make significant contact with the enzymes when acting on an abasic site on the opposite strand.

The cytotoxic effects of many agents are believed to be the result of damage to DNA. In addition to producing isolated DNA base and deoxyribose lesions, several important antineoplastic agents, including ionizing radiation and drugs such as bleomycin and neocarzinostatin, can generate clustered lesions or locally multiply damaged sites (LMDS) located within one or two helical turns (1–5). Such LMDS may be critical lesions in the DNA as they present an additional challenge to the repair machinery of the cell.

One type of LMDS, the DNA double-strand break, has been the subject of considerable study and is considered to contribute significantly to cytotoxicity (6, 7). However, the biological consequences of other types of LMDS, such as 2 modified bases or two abasic sites close to each other, require further investigation. A great deal will depend on if and how these lesions are recognized by the initial damage recognition enzymes. In the case of abasic sites, the enzymes that recognize these lesions and cleave the DNA have been classified according to mechanism. The major apurinic/apyrimidinic (AP) DNA repair endonuclease in human cells is Ape (8) or HAP1 (9). The major AP endonuclease of Escherichia coli is the multifunctional enzyme exonuclease III (10). Both enzymes, which share several structural and functional similarities, cleave the phosphodiester bond in duplex DNA immediately 5′ to the abasic site and are thus classified as class II AP endonucleases (11).

E. coli endonuclease III, on the other hand, cleaves DNA 3′ to an abasic site via a β-elimination mechanism and has therefore been classified as a class I AP endonuclease or AP lyase. An important potential outcome of cleavage of closely opposed abasic sites would be the production of double-strand breaks.

Data regarding the enzymatic fate of abasic sites as components of more complex lesions are relatively sparse. Recent physicochemical measurements of an oligonucleotide duplex with a bistranded abasic site were suggestive of a structure in which the lesion forms an extrahelical loop (12). This may explain the earlier observation that abasic sites on opposite strands reduce the efficiency of strand cleavage by Ape (13) and exonuclease III (14) and that bistranded abasic sites site-specifically placed in shuttle vectors and transfected into E. coli and COS-7 simian cells appear to be highly mutagenic (15). All these studies, however, were restricted to stable synthetic analogues of deoxyribose, such as tetrahydrofuran groups, placed directly opposite each other. Povirk and Houlgrave (16), on the other hand, examined the enzymatic cleavage of abasic sites generated within the complex lesions induced by bleomycin and neocarzinostatin. They observed that considerably higher concentrations of E. coli exonuclease III (but not endonuclease III) were required to effect DNA cleavage at these lesions in comparison to the more spatially isolated abasic sites generated by heat depurination.

We have previously modeled LMDS using plasmid DNA constructs containing site-specific bistranded dihydrothymine lesions and abasic sites and studied the response of the glycosylase and AP lyase activities of E. coli endonuclease III toward such damage (17). With substrates containing modified bases set 1 and 3 bp apart, the enzyme was able to remove only one of the dihydrothymines and cleave the resulting abasic site, thereby generating a single-strand break. Treatment of the
substrates containing two abasic sites, however, readily yielded double-strand breaks. It was thus possible to infer that the glycosylase activity of endonuclease III, but not the AP lyase activity, is inhibited by the presence of a closely positioned break in the opposite strand. In this work, we have extended our modeling of LMDS by preparing oligonucleotide duplexes with bistranded natural abasic sites (i.e. 2-deoxy-d-erythro-pentofuranose) set various distances apart and have examined the reactivity of human Ape protein and E. coli exonuclease III with this type of clustered lesion.

EXPERIMENTAL PROCEDURES

**Enzymes**—T4 polynucleotide kinase was supplied by U. S. Biochemical Corp. Uracil-DNA glycosylase was purchased from Life Technologies, Inc. and Epicentre Technologies. (One unit of uracil-DNA glycosylase catalyzes the release of 1 nmol of free uracil from 1 μg of DNA in 1 h at 37 °C.) Endonuclease III (fraction IV) was purified from strain AN993, carrying the pHT1 plasmid (kindly provided by Dr. R. P. Cunningham, State University of New York, Albany, NY) according to the procedure of Asahara et al. (18). Exonuclease III was obtained from Life Technologies, Inc. (One unit of exonuclease III produces 1 nmol of acid-soluble nucleotide from sonicated DNA in 30 min at 37 °C.) Recombinant human Ape protein (in the form of the glutathione S-transferase fusion protein (GST-Ape) and the purified protein isolated after clipping the fusion protein with Factor Xa) was generously provided by Drs. David Wilson III and Bruce Demple (Harvard University). The purification of the enzyme is fully described by Wilson et al. (13). One unit of enzyme cleaves or releases 1 pmol of damaged sites/min from a synthetic substrate (19) under standard conditions (20, 21). The concentration of the original stock of GST fusion protein was 500 μg/ml (100,000 units/ml). For our purposes, the enzyme was diluted to 7 μg/ml in enzyme reaction buffer (50 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 1.0 mM CaCl₂, and 10 mM dithiothreitol) in a final volume of 10 μl.

**Synthesis, Purification, and Labeling of Oligonucleotides**—Oligonucleotides (23- or 28-mers) containing deoxyuridine (dU) at various positions (see Table I) were synthesized by the DNA Synthesis Core Facility at the University of Alberta. Oligonucleotides were purified according to Sambrook et al. (22). Briefly, after the electrophoresis of synthetic oligonucleotides on a 20% polyacrylamide gel containing 7 M urea, the bands containing the oligonucleotides were located, using fluorescent Merck Silica Gel 60 F₂₅₄ thin-layer chromatography plate (Brinkmann Instruments) and UV illumination from above in a Brinkmann Chromato-Vue (Model CC 20) apparatus, and excised. The gel slices were crushed, and the oligonucleotides were eluted in a buffer containing 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate at 37 °C for 12 h in a shaker incubator. The solution was filtered through a Millex HV filter (0.45 μm) and washed with a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 0.5 M ammonium acetate, and 10 mM magnesium acetate using a 0.1% SDS, 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and thus create abasic sites at these positions.

To assess the extent of uracil removal, the oligonucleotide duplexes were subjected to alkali or endonuclease III treatment to induce quantitative cleavage at the abasic sites. (An example of NaOH and endonuclease III analysis for duplex F+A is shown in Fig. 5C.) In accordance with the observation of Erdal et al. (23), the efficiency of enzymatic release of uracil was dependent on sequence context. In duplexes F+A, F+K, G+A, H+K, and M+N, the uracils were resistant to uracil-DNA glycosylase and therefore required the use of 3 units of enzyme to effect >90% removal. The subsequent percent cleavage of abasic sites after incubation of these substrates with Ape protein and exonuclease III was normalized according to the available AP sites in each substrate.

**Interaction of Ape with Single AP Sites**—Oligonucleotide duplexes B+A, F+K, G+K, H+K, and M+N containing a single AP site at various positions (Table I) served as controls. These substrates were incubated with 70 pg of Ape protein for various periods of time, and the percent cleavage at the AP sites was determined after electrophoresis of reaction products on a polyacrylamide gel (see “Experimental Procedures”). The rate of cleavage in these substrates is shown in Fig. 2. With all substrates except H+K, the enzyme rapidly cleaved the oligonucleotide, resulting in >50% cleavage after 5 min of incubation. The cleavage rate of duplex H+K was slow, probably due to the close proximity of the AP site to the 3′-terminus of the oligonucleotide. When cleavage of a longer duplex (M+N; Table I) containing an AP site in the same sequence context as in duplex H+K but farther from the 3′-terminus was examined, the rate of scission matched closely that of the other substrates.

**Interaction of Ape with Bistranded AP Sites Directly Opposite or 1 and 3 bp Apart, 3′ to Each Other**—Substrates C+A (AP sites directly opposite each other), D+A (AP sites 1 bp apart), and E+A (AP sites 3 bp apart) were incubated with Ape protein, and the degree of oligonucleotide incision was determined at various time points. Two reactions were carried out with each duplex, with only one strand labeled in each reaction, thus permitting the monitoring of cleavage of both strands. Hydrolysis of the strands of duplex E+A is shown as an example in Fig. 1, where the labeled strand is marked with an asterisk. The kinetics of cleavage of these substrates are presented in Fig. 3A. Substrate B+A, with a single abasic site, is included for comparison. Although cleavage rates were universally slower for the abasic sites in duplexes with bistranded lesions than for the control, there appeared to be no strong influence of distance between the lesions.

**Interaction of Ape with Bistranded AP Sites 1, 3, and 5 bp Apart, 5′ to Each Other**—The rate of incision of substrates F+A (AP sites 1 bp apart), G+A (3 bp apart), and H+A (5 bp apart) resulting from incubation with 70 pg of Ape protein is shown in Fig. 3B. There is clearly a marked difference between the enzyme response to bistranded lesions in this orientation compared with lesions displaced 3′ to each other. In substrate F+A, strand F was not cleaved even after 1 h of incubation, whereas only ~30% of strand A could be cleaved during this incubation period. Even more striking was the inability of Ape to incise

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**Table I**

| Duplex | Sequence* |
|--------|-----------|
| B+A    | C: 5'-AGA GGA TAT GTA TGT AAGT *GAG AG-3' |
| C+A    | A: 3'-TCT CCT ATA CA* X ACA TAC CTC TC-5' |
| D+A    | A: 3'-TCT CCT ATA CA* X ACA TAC CTC TC-5' |
| E+A    | F: 5'-AGA GGA TAT GTA XGT AAGT AGG-3' |
| G+A    | A: 3'-TCT CCT ATA CA* X ACA TAC CTC TC-5' |
| H+A    | H: 5'-AGA GGA TAT GTA XGT XAG GAG AG-3' |
| F+K    | F: 5'-AGA GGA TAT GTA XGT XGT AAGT AGG-3' |
| G+K    | G: 5'-AGA GGA TAT GTA TGX ATG GAG AG-3' |
| H+K    | H: 5'-AGA GGA TAT GTA XGT XAG GAG AG-3' |
| M+L    | K: 3'-TCT CCT ATA CAT ACA TAC CTC TC-5' |
| M+N    | M: 5'-AGA GGA TAT GTA XGT XAG GAG AGG T-3' |
| N: 3'-TCT CCT ATA CAT ACA TAC CTC TC-5' |

* X designates a dU or an abasic site.
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FIG. 3. Kinetics of cleavage at the abasic site in oligonucleotide duplexes containing bistranded abasic sites. Duplexes were treated with 70 pg of GST-Ape fusion protein and analyzed as described under "Experimental Procedures." The 32P-labeled strand in each duplex is marked with an asterisk. A, duplexes (23-mers) in which the abasic sites lie directly opposite each other (C+A) or 3’ to each other (D+A and E+A). B, duplexes (23-mers) in which the AP sites lie 5’ to each other. The percent cleavage of the single abasic site in strand A of duplex B-A is included for comparison. C, duplexes (28-mer) in which the AP sites lie 5 base pairs apart, 5’ to each other. The percent cleavage of the single abasic site in strand M of duplex M+N is included for comparison.

either AP site in substrate G+A. When the distance between the abasic sites was extended to 5 bp in substrate H+A, we observed a near-normal rate of cleavage for strand A, but no scission of strand H. The slow cleavage of strand H in the control substrate, H+K (Fig. 2), suggested that the lack of strand H cleavage in duplex H+A was in part attributable to the proximity of the abasic site in strand H to the 3’-terminus of the oligonucleotide. This was confirmed when incubation of Ape with the longer duplex, M+L, resulted in substantial cleavage of strand M, although notably still less than scission of strand L, or strand M in the control duplex M+N (Fig. 3C).

The refractory nature of the abasic sites in duplexes F+A and G+A was further evaluated by incubation with 210 and 700 pg of the enzyme (data not shown). Incubation of substrate F+A with 210 pg of Ape protein resulted in ~70% cleavage of strand A, but 700 pg of the enzyme were required to cleave strand F to ~40% after 1 h of incubation. Incubation of substrate G+A with 700 pg of the enzyme resulted in 30–35% cleavage of AP sites in each strand.

Comparison of Clipped Ape Versus GST-Ape Fusion Protein—To rule out the possibility that the inability of the enzyme to cleave AP sites in substrates F+A and G+A was an artifact due to interference from the glutathione S-transferase component of the fusion protein, the cleavage of these substrates was re-examined using the clipped (GST-free) protein. The substrates, including the control substrate B+A, were incubated with an equal number of units (0.014 units) of GST-Ape and clipped Ape protein for 30 min, and the percent cleavage was determined. The results (Fig. 4) indicate little difference in response between the two forms of the enzyme.

Interaction of Exonuclease III with Single and Bistranded Abasic Sites—We carried out a similar analysis with E. coli exonuclease III in which the substrates were treated with increasing quantities of enzyme. To suppress the exonucleolytic activity of the enzyme, CaCl2 was substituted for MgCl2 in the incubation buffer (14). Nonetheless, some exonucleolytic activity was still evident, especially with the higher concentrations of exonuclease III. Examples of the responses are presented in Fig. 5 (A and B). Fig. 5A shows a comparison of cleavage of strand A with three different complementary oligonucleotides, whereas Fig. 5B contrasts the incision of the abasic sites in duplex F+A. Fig. 5C shows the result of cleavage of strand F by exonuclease III (En) or NaOH (Alk) used to measure the induction of abasic sites. (Endonuclease III cleavage generates 3’-termini with 4-hydroxy-2-pentenal phosphate groups, whereas NaOH generates these termini as well as termini with unmodified 3’-phosphate groups, hence the doubling of products seen in the Alk lane.)

Analysis of the percent cleavage of the various oligonucleotide duplexes (Fig. 6) demonstrated the following. (i) The rates of scission of all the bistranded lesion-containing duplexes were slower than those of the controls, but varied greatly from one duplex to the next. (ii) Direct opposition of the abasic site (duplex C+A) did not lead to a sharp reduction in the cleavage of either abasic site. (iii) Of those constructs with lesions lying 3’ to each other, the most marked effect was seen with duplex D+A, where the abasic sites were 1 bp apart. (iv) Of the constructs with lesions lying 5’ to each other, the most resistant to enzyme-induced scission was duplex F+A (abasic sites 1 bp apart), with the abasic site in strand F being even more resistant than the opposing abasic site.

DISCUSSION

Of the wide variety of DNA lesions generated by ionizing radiation, double-strand breaks are considered to be the predominant lesion responsible for cell lethality (6). However, in addition to the double-strand breaks initially induced by radiation, double-strand breaks can arise after the cessation of irradiation (24–26) and are most likely due to repair of closely opposed damaged or missing bases or damaged/missing bases opposite
single-strand breaks. Theoretical calculations (e.g. Refs. 27 and 28) and recent experimentation (e.g. Refs. 1 and 3) have indicated that a single deposition of energy from ionizing radiation can lead to the formation of two or more DNA lesions (single-strand break or base damage/loss) within one to two helical turns.

In an earlier study (17), we examined the capacity of E. coli endonuclease III, a well characterized DNA glycosylase/AP lyase, to generate double-strand breaks in substrates containing bistranded base lesions (dihydrothymine). The present study has focused on the major AP endonuclease in human and E. coli cells and their response to bistranded abasic sites. Our data clearly indicate that, for both enzymes, the oligonucleotide constructs elicited a marked variation in response, which was dependent on the distance between the abasic sites and their orientation to one another. If these in vitro observations reflect the repair responses in human and bacterial cells, this class of locally multiply damaged site would have several deleterious consequences. First, the bistranded abasic sites in most of the configurations that were examined, especially those oriented 3' to each, gave rise to double-strand breaks when treated with either of the AP endonucleases. Thus, in the cell, such lesions would be anticipated to contribute to lethality caused by double-strand breaks. Second, the reaction of duplex F+A with

**Fig. 5. Examples of exonuclease III-catalyzed scission of lesion-containing duplexes.** The 32P-labeled duplexes (labeled strands marked with an asterisk) were incubated with increasing units of exonuclease III as described under “Experimental Procedures.” The number of enzyme units is indicated at the top of each lane. After the reaction, the products were analyzed on 16% polyacrylamide gels. A, comparison of cleavage of single versus bistranded lesions (marked schematically with an X) and the influence of the orientation of the two AP sites. Strand A was cleaved to a labeled 11-mer. Further exonucleolytic digestion produced the bands under the 11-mer. B, comparison of scission of the two strands in duplex F+A. Lane M contains oligo(dT) size markers. C, cleavage of strand F by endonuclease III (En) or NaOH (Alk) to evaluate the level of uracil removal by uracil-DNA glycosylase.

Ape and exonuclease III generated a single-strand break opposite an endonuclease-resistant abasic site. Third, abasic sites with the configuration seen in duplex G+A are both resistant to the human AP endonuclease. Since unrepaired AP sites represent a potent class of premutagenic lesions (29), the latter configuration of bistranded abasic sites must be considered to have a high mutagenic potential unless cells can respond to such endonuclease-resistant abasic sites by an alternative mechanism (e.g. the SOS response in E. coli).

In addition to contributing to our understanding of the possible biological consequences of bistranded lesions, this investigation has afforded information regarding the physical interactions between the endonucleases and DNA. The strategy of changing the location of one abasic site while keeping the other constant (strand A or L) in each duplex has allowed us to map at least some of the bases on the opposite strand that strongly interact with the enzymes when acting on the constantly positioned abasic site (Fig. 7). This is a similar approach to the “missing thymine site interference assay” used by Devchand et al. (30) to map thymines essential for binding of the lac repressor to the lac operator. Exonuclease III and Ape cleave abasic sites in double-stranded DNA far more efficiently than in single-stranded substrates (31, 32); thus, reduction of activity toward a substrate with abasic sites could be due to either partial denaturation of the substrate or loss of interaction with a specific base. Data on denaturation caused by paired abasic sites, although limited, do suggest that there is sufficient denaturation to alter enzyme activity. For example, paired abasic
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sites set 1 and 3 base pairs apart cause sufficient destabilization to allow the single strand-specific nuclease, S1 nuclease, to induce double-strand breaks (33). The analysis of a duplex with abasic sites situated directly opposite each other demonstrated a melting temperature 12 °C lower than the duplex lacking an abasic site (12). Partial denaturation may therefore be responsible for the modest decrease in the rate of incision by both enzymes of duplexes C+A, D+A, and E+A (Figs. 3A and 6). In the case of duplexes F+A and G+A with Ape and duplex F+A with exonuclease III, the resistance of the substrates to cleavage is much more severe, suggesting a strong interaction with the missing base. Inspection of the B-form of the DNA helix reveals that the bases missing in duplexes F+A and G+A are situated close to each other across the minor groove. (Those in duplexes D+A and E+A face each other across the major groove.) In their model of exonuclease III/DNA interaction based on x-ray crystallographic data, Mol et al. (34) proposed two minor groove binding domains that would make significant contact with the DNA: the first involving Arg-90 and Lys-36 interacting with the phosphate backbone while Tyr-63 stacks with the sugar ring of the deoxyribose and the second being the βC-βC1 loop. While no x-ray crystallographic model has been published for Ape, we would predict a similar interaction with possibly even more extensive contact with bases in the minor groove given the greater resistance to Ape than to exonuclease III demonstrated by duplex G+A. A recent methylation interference analysis of Ape interaction with a synthetic oligonucleotide duplex carrying an abasic site on one strand displayed a substantial interference signal from an adenine two nucleotides 5’ to the abasic site (equivalent to the guanine lying between the two thymines marked in Fig. 7) (35). If this is due to alkylation of N-3 of adenine, it would also be strongly indicative of binding within the minor groove.

The x-ray crystallography of exonuclease III (34) also indicated that the enzyme has a relatively large positively charged DNA-binding face (55 Å long). It is therefore possible that subtle differences in DNA charge and hydrophobicity due to sequence context close to the abasic site could influence DNA binding and may account for the unexpected preferential cleavage of strand A in duplex F+A (Fig. 6). Alternatively, sequence context may influence the degree to which the two abasic sites can protrude into the enzyme’s active site for cleavage. Again, we anticipate that similar properties would account for the same strand-specific cleavage of this duplex shown by Ape (Figs. 3B and 4).

It is worth noting that many of the more potent chemicals that produce complex lesions bind to the minor groove and abstract hydrogen atoms from deoxyribose carbon atoms on opposite strands that protrude into the minor groove. Neocarzinostatin, for example, generates a lesion very similar to the product of duplex F+A after Ape or exonuclease III cleavage, i.e. a single-strand break opposite an abasic site situated 2 bases 5’ to the strand break (36). In this case, the abasic site is oxidized to a 2-deoxyribonolactone moiety (37). It remains to be seen if the resistance of the abasic site to exonuclease III (38) is due to its oxidation or to the presence of a strand break as seen with the product of duplex F+A.

Finally, the relatively slow rate of Ape-catalyzed scission of strand H in duplex H+K, where the abasic site lies 7 bases from the 3’-terminus, in comparison to strand M in duplex M+N (Fig. 2), where the abasic site lies 12 bases from the 3’-terminus, implies that the enzyme interacts with at least 7 bases 3’ to the abasic site on the same strand. Wilson et al. (13) have previously shown an absolute requirement for 3 base pairs on the 3′-side of the lesion.

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