Efficiency of Incision of an AP Site within Clustered DNA Damage by the Major Human AP Endonuclease†

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ABSTRACT: A major DNA lesion induced by ionizing radiation and formed on removal of oxidized base lesions by various glycosylases is an apurinic/apyrimidinic site (AP site). The presence of an AP site within clustered DNA damage, induced following exposure to ionizing radiation or radiomimetic anticancer agents, may present a challenge to the repair machinery of the cell, if the major human AP endonuclease, HAP1, does not efficiently incise the AP site. In this study, specific oligonucleotide constructs containing an AP site located at several positions opposite to another damage [5,6-dihydrothymine (DHT), 8-oxoG, AP site, or various types of single strand breaks] on the complementary strand were used to determine the relative efficiency of the purified HAP1 protein in incising an AP site(s) from clustered DNA damage. A base damage (DHT and 8-oxoG) on the opposite strand has little or no influence on the rate of incision of an AP site by HAP1. In contrast, the presence of either a second AP site or various types of single strand breaks, when located one or three bases 3′ to the base opposite to the AP site, has a strong inhibitory effect on the efficiency of incision of an AP site. The efficiency of binding of HAP1 to an AP site is reduced by ∼1 order of magnitude if a single strand break (SSB) is located one or three bases 3′ to the site opposite to the AP site on the complementary strand. If the AP site and either a SSB or a second AP site are located at any of the other positions relative to each other, a double strand break may result.

DNA base lesions can be generated in cells by reactive oxygen species, radiation, or radiomimetic agents, such as bleomycin, a drug commonly used in chemotherapy. In particular, it has been proposed that radiation produces a significant proportion of DNA damage as clustered DNA damage (*1*−*3*). Clustered DNA damage exists when a combination of two or more damaged bases, apurinic/apyrimidinic (AP)[1] sites, or single strand breaks are produced within about one or two helical turns of the DNA by a single radiation track. Indeed, for high-linear energy transfer (LET) radiation, more than 50% of single (SSB) and double strand breaks (DSB) have a neighboring base lesion or an abasic site within approximately 20−30 bases (*4*, *5*). Recently, it has been demonstrated experimentally that significant levels of clustered DNA damage are induced in mammalian cells by ionizing radiation (*6*). Single hydroxyl radicals are also known to produce tandem damage, in which two vicinal base lesions are formed on the same strand (*7*−*9*). It is thought that clustered damages are difficult to repair and are relevant therefore to the biological severity of radiation (*1*−*3*, *10*). Indeed, 10−15% of lung cancers can be attributed to exposure to radon, the decay daughters of which produce high-LET radiation (*11*). The efficiencies of cleavage of radiation-induced base lesions [e.g., 8-oxo-7,8-dihydroguanine (8-oxoG), thymine glycol, and 5,6-dihydrothymine (DHT) or an AP site] when present within clustered DNA damage were previously determined using either the prokaryotic Nth, Nei, or Fpg proteins, the yeast and human OGG1 proteins (*12*−*19*), or nuclear extracts (*16*, *19*).

Significant inhibition of incision of an AP site by the major human AP endonuclease, HAP1 (also known as APE and Ref-1), occurs in the presence of a neighboring AP site, especially when one AP site is located one and three bases 3′ to the base opposite to the other AP site (*12*). This asymmetric inhibition of incision of an AP site by a neighboring AP site could be biologically important in that it minimizes formation of a DSB (*12*). AP sites are both cytotoxic and premutagenic as a single lesion, if not repaired. AP sites make up one of the major groups of DNA lesions that are induced by ionizing radiation and chemical oxidants and are also formed on removal of alkylated or oxidized bases by various glycosylase enzymes involved in the eukaryotic base excision repair (BER) pathway (*20*−*24*). For instance, the OGG1 and Nth proteins are able to remove their respective oxidized bases by cleavage of the *N*-glycosidic bond (*25*, *26*). The resulting AP site may be subsequently

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[1] Abbreviations: AP, apurinic/apyrimidinic; 8-oxoG, 7,8-dihydro-8-oxo-2′-deoxyguanine; DHT, 5,6-dihydrothymine; SSB, single strand break; DSB, double strand break; LET, linear energy transfer; HAP1, human AP endonuclease 1; UDG, uracil DNA glycosylase; BER, base excision repair.

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cleaved on its 3′ side through the AP lyase (β-elimination) action of these proteins, to give a SSB with 3′-α,β-unsaturated aldehyde and 5′-phosphate termini. This 3′ fragmented sugar terminus is then removed from DNA by incision on the 5′ side of the sugar remnant by HAP1, leading to the loss of a base and a SSB with 5′-phosphate and 3′-OH termini. These termini are substrates for DNA polymerases, which catalyze gap filling, with the major role in BER being performed by DNA polymerase β. AP endonucleases also cleave intact AP sites by making a single nick 5′ to the AP site, to give 3′-OH and 5′-deoxyribose phosphate (dRP) termini. This latter terminus is subsequently removed by the action of dRPase proteins, such as DNA polymerase β itself, which incise 3′ to the dRP terminus to give a 5′-phosphate terminus (*21*, *27*).

HAP1 is the functional homologue of the *Escherichia coli* exonuclease III protein. This 35.5 kDa protein acts as a monomer, and its catalytic action can be eliminated by substitution of a variety of amino acids in the active site, such as His309, Asp210, or Asn212, present within conserved motifs in this family of AP endonucleases (*28*−*30*). Importantly for this study, mutation of Asp210 to Asn (D210N) eliminates AP endonuclease activity but stabilizes binding of the mutant to AP sites, whereas an Asn212 to Ala (N212A) mutant loses both its AP site binding and cleavage functions.

In this study, specific oligonucleotides have been constructed containing an AP site at a known position opposite to a second lesion (DHT, 8-oxoG, an AP site, or a variety of SSBs) on the complementary strand. The influence of the presence of the second lesion within the clustered damage on the efficiency of incision of the AP site by HAP1 has been assessed. The kinetic constants (K_M, k_{cat}, and specificity constants) have been determined for the incision of an AP site within various types of clustered damages by HAP1, to assess whether the effect of an opposing lesion on the incision of the AP site by HAP1 is associated with a change in the lesion recognition step (binding) and/or in the catalytic activity of the enzyme. The D210N mutant protein has been used to determine its binding efficiency with oligonucleotides containing clustered damage. The findings from this study extend significantly the earlier studies of Chaudhry and Weinfeld (*12*) and contribute to the models being developed on the orchestration of the processing of clustered DNA damage in cells (*10*, *31*).

MATERIALS AND METHODS

Oligonucleotides. All oligonucleotides were purchased from Genosys or Glen Research. The sequences of the various oligonucleotides containing 8-oxoG, uracil (subsequently converted to an AP site as described later), various types of SSBs, or DHT are presented in Table 1. To assist the reader, the nomenclature used to relate the relative position of the two lesions, one on each strand, is based on assignment of a positive or negative number to each residue in the oligonucleotide. This number refers to the separation, in base pairs, of one lesion on strand 1 located 5′ (negative number) or 3′ (positive number) to the base opposite to the AP site (X) on strand 2, as shown schematically in Figure 1.

To obtain a probe containing a hydroxy SSB with 3′- and 5′-hydroxy termini, two oligonuclotides were used to form strand 1 with a gap at position Y as shown in Table 1. The oligonucleotide 5′ of gap Y contains a 3′-hydroxy terminus, whereas the oligonucleotide 5′ of Y contain a 5′-hydroxy terminus. Likewise, to obtain a probe containing a $\beta-\delta$ SSB with 3′- and 5′-phosphate termini, two oligonucleotides were used to form strand 1 with a gap at position Y. The oligonucleotide 5′ of gap Y contains a 3′-phosphate terminus, whereas the 3′ oligonucleotide contains a 5′-phosphate terminus. Strand 2 in each case contained uracil at a fixed position. This residue was excised to create an AP site (see below).

To create an AP site opposite to 8-oxoG, DHT, or the various types of SSBs on the complementary strand, the uracil-containing strands were 5′-end-labeled with ^{32}P by incubation with 10 units of T4 polynucleotide kinase (Gibco BRL) and 50 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, 10 mCi/mL, NEN Dupont) in 25 μL of the recommended buffer for 30 min at 37 °C. For an AP site opposite to another AP site on the complementary strand, strand 1, containing uracil at the various positions, was 5′-end-labeled with ^{32}P. Following purification on a 12% denaturing polyacrylamide gel (see below for details), the labeled oligonucleotides were hybridized with a 1.5-fold molar excess of the respective non-radiolabeled complementary strands, as described previously (*16*, *18*). That the annealing was efficient was verified by migration on a native 10% polyacrylamide gel (see below for details), which was also used for purification. To convert the uracil site into an AP site, the ^{32}P-labeled double-stranded oligonucleotides (5−7 pmol) containing uracil were treated with 1 unit of uracil-DNA-glycosylase (UDG) (Gibco BRL) in 50 μL of buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA] for 30 min at 37 °C. The extent of conversion of uracil into an AP site was >98% as verified by denaturing PAGE as described below for the SSB assay, after treatment of the oligonucleotide with 1 ng of either HAP1, Nth, or Fpg protein.

Purified Proteins. The purified HAP1 protein and its catalytic mutant D210N were expressed in *E. coli* and purified as described by Rothwell et al. (*28*). The purified Nth and Fpg proteins were generous gifts from R. Wood (Imperial Cancer Research Fund, South Mimms, U.K.) (*32*) and Jacques Laval (CNRS UMR 8335, Institut Gustave Roussy, Villejuif, France) (*33*), respectively.

Cleavage Assay for SSB Analysis. The double-stranded oligonucleotides (10 000 cpm, 200 fmol) were incubated with various amounts of the HAP1 protein, as specified in the figure legends, in 5 μL of incubation buffer [20 mM HEPES (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, and 0.2 mM EDTA] for 30 min at 37 °C. Subsequently, 5 μL of the denaturing stop solution [98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, and 2 mM EDTA (pH 8.0)] was added to each of the samples, which were then subjected to electrophoresis on a 12% denaturing PAGE gel containing 8 M urea in 1× TBE [89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA (pH 8.3)] for 90 min at a constant power of 85 W. The dried gel was subsequently exposed to a Bio-Rad PhosphorImager screen for visualization of cleaved and full-length DNA fragments using phosphorimaging (Bio-Rad, Molecular Imager FX). Quantification was achieved using Quantity One software (Bio-Rad) to determine the incision

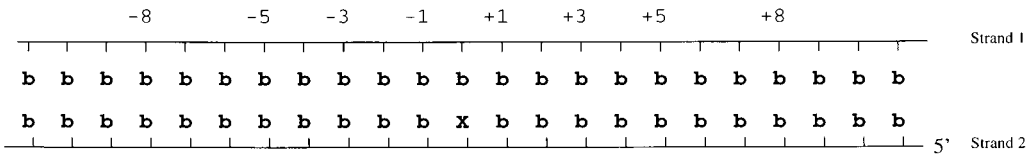


FIGURE 1: Schematic representation of the double-stranded oligonucleotides (b represents a nucleobase) showing the numbering system used to identify the position of the second lesion relative to the position of the AP site (X).

Table 1: Oligonucleotide Sequences and Nomenclature for Relative Positions of the Two Lesions[a]

Positions	Sequence	Strand
	8-oxoG/AP*	
U=AP* -5	5′-ctcttagtca ggaa**U**atgtc tctatgctgg gagcaaaggc-3′	(1)
-3	5′-ctcttagtca ggaata**U**gtc tctatgctgg gagcaaaggc-3′	
-1	5′-ctcttagtca ggaatatg**U**c tctatgctgg gagcaaaggc-3′	
+1	5′-ctcttagtca ggaatatgtc **U**ctatgctgg gagcaaaggc-3′	
+3	5′-ctcttagtca ggaatatgtc tc**U**atgctgg gagcaaaggc-3′	
+5	5′-ctcttagtca ggaatatgtc tcta**U**gctgg gagcaaaggc-3′	
X=G or **8-oxoG**	3′-gagaatcagt ccttataca**G** agatacgacc ctcgtttccg-5′	(2)
	DHT/AP*	
T=DHT -5	5′-ctcttagtca ggaa**T**atgta tctatgctgg gagcaaaggc-3′	(1)
-3	5′-ctcttagtca ggaata**T**gta tctatgctgg gagcaaaggc-3′	
-1	5′-ctcttagtca ggaatatg**T**a tctatgctgg gagcaaaggc-3′	
+1	5′-ctcttagtca ggaatatgta **T**ctatgctgg gagcaaaggc-3′	
+3	5′-ctcttagtca ggaatatgta tc**T**atgctgg gagcaaaggc-3′	
+5	5′-ctcttagtca ggaatatgta tcta**T**gctgg gagcaaaggc-3′	
C	5′-ctcttagtca ggaatatgta tctatgctgg gagcaaaggc-3′	
U=AP*	3′-gagaatcagt ccttataca**U** agatacgacc ctcgtttccg-5′	(2)
	AP/AP*	
U=AP* -16	5′-ctc**U**tagtca ggaatatgta tctatgctgg gagcaaaggc-3′	(1)
-12	5′-ctcttag**U**ca ggaatatgta tctatgctgg gagcaaaggc-3′	
-5	5′-ctcttagtca ggaa**U**atgta tctatgctgg gagcaaaggc-3′	
-3	5′-ctcttagtca ggaata**U**gta tctatgctgg gagcaaaggc-3′	
-1	5′-ctcttagtca ggaatatg**U**a tctatgctgg gagcaaaggc-3′	
+1	5′-ctcttagtca ggaatatgta **U**ctatgctgg gagcaaaggc-3′	
+3	5′-ctcttagtca ggaatatgta tc**U**atgctgg gagcaaaggc-3′	
+5	5′-ctcttagtca ggaatatgta tcta**U**gctgg gagcaaaggc-3′	
+8	5′-ctcttagtca ggaatatgta tctatgc**U**gg gagcaaaggc-3′	
+14	5′-ctcttagtca ggaatatgta tctatgctgg gag**U**aaaggc-3′	
C	5′-ctcttagtca ggaatatgta tctatgctgg gagcaaaggc-3′	
AP	3′-gagaatcagt ccttataca**U** agatacgacc ctcgtttccg-5′	(2)
	SSB/AP*	
Y=SSB -5	5′-ctcttagtca ggaa**Y**atgtN tctatgctgg gagcaaaggc-3′	(1)
-3	5′-ctcttagtca ggaata**Y**gtN tctatgctgg gagcaaaggc-3′	
-1	5′-ctcttagtca ggaatatg**Y**N tctatgctgg gagcaaaggc-3′	
+1	5′-ctcttagtca ggaatatgtN **Y**ctatgctgg gagcaaaggc-3′	
+3	5′-ctcttagtca ggaatatgtN tc**Y**atgctgg gagcaaaggc-3′	
+5	5′-ctcttagtca ggaatatgtN tcta**Y**gctgg gagcaaaggc-3′	
+8	5′-ctcttagtca ggaatatgtN tctatgcYgg gagcaaaggc-3′	
C	5′-ctcttagtca ggaatatgtN tctatgctgg gagcaaaggc-3′	
AP*	3′-gagaatcagt ccttataca**U** agatacgacc ctcgtttccg-5′	(2)

[a] **U** represents uracil which is converted into an AP site by UDG. **Y** represents a hydroxy SSB or a β−δ SSB where N is adenine or cytosine, respectively. Asterisks denote the ^{32}P-labeled strand.

efficiency of HAP1 for a single AP site or when present within clustered damage. The intensity of the band representing the labeled, cleaved strand was expressed as a percentage of the total intensity of the bands for the cleaved and intact strands. The efficiencies for incision of an AP site within clustered damages were compared with that for incision of a single AP site in the control oligonucleotide at the same concentration of HAP1. This comparison permitted assessment of the effect of the second lesion, present on the unlabeled strand, on the efficiency of incision of an AP site on the labeled strand. The errors represent standard errors of the mean from at least three independent experiments.

Assessment of DSBs Induced by HAP1. The experiments were performed as described above for the analysis of SSBs, except that the samples were run on a 10% native polyacrylamide gel in 1× TBE for 3 h at a constant voltage of 300 V

(7.8 V/cm). The reactions were stopped by the addition of 5 μL of a nondenaturing solution [40% sucrose, 0.025% bromophenol blue, 0.025% xylene cyanol, and 5 mM EDTA (pH 8.0)]. The gels were dried and band intensities quantified as described above.

Kinetic Measurements. For kinetic measurements, different concentrations of the substrates (5−1000 nM) were incubated in 5 μL of reaction buffer for 10 min at 37 °C with various concentrations of HAP1, as described previously (*18*, *19*). Briefly, the concentration of the cleaved, labeled strand relative to that of the intact strand was determined as described above for the SSB assay with denaturing PAGE. The initial reaction rate, obtained from the concentration of cleaved DNA per minute for a constant concentration of HAP1, was plotted versus the substrate concentration using a double-reciprocal dependence assay based on Michaelis−Menten kinetics. Each point represents the standard error of the mean for at least three independent experiments using six to eight different substrate concentrations. The values of K_M and V_{max} were obtained from the linear regression analysis of the Lineweaver−Burk plots, using the method of weighted least squares, with the ordinate of each point being weighted by its reciprocal.

Electrophoretic Mobility Shift Assays (EMSAs). To assess the ability of HAP1 to bind to an oligonucleotide containing an AP site opposite to a $\beta-\delta$ SSB or an AP site at the various positions, binding reactions were performed by incubating ^{32}P 5′-end-labeled oligonucleotides (10 000 cpm, 0.5 nM) with 2.5 ng of catalytic mutant protein D210N-HAP1 in 5 μL of binding buffer [20 mM HEPES (pH 7.9), 100 mM KCl, 1 mM DTT, 1 mM PMSF, 0.2 mM EDTA, and 20% glycerol] for 20 min at 37 °C. The samples were then loaded directly onto a 5% polyacrylamide gel as described in ref *34*. After electrophoresis at a constant current of 35 mA in 0.7× TAE [4.7 mM Tris-HCl, 2.3 mM sodium acetate, and 0.7 mM EDTA (pH 7.9)] for 2 h, gels were dried and exposed to a phosphorimager screen, and band intensities were quantified as described above.

RESULTS

Effect of a Neighboring Base Lesion on the Incision of an AP Site by HAP1. The influence of a neighboring base lesion, DHT or 8-oxoG, on the efficiency of incision of an AP site by HAP1 was determined when the base lesions were located one, three, or five bases 3′ or 5′ of the base opposite to the AP site on the complementary strand (designated positions −5 to 5 in Table 1). The presence of DHT at any of the tested positions has no significant effect on incision of the AP site on the complementary strand by HAP1 (Figure 2A). In contrast, the presence of 8-oxoG has a small inhibitory effect on the incision of an AP site by HAP1, particularly at position 3 (Figure 2B).

Effect of a Second AP Site on the Incision of an AP Site by HAP1. With an oligonucleotide containing a single AP site, HAP1 is more efficient than either Nth or OGG1 at converting this AP site into a SSB. As shown in Figure 3, the inhibitory effect of the presence of a second AP site at position 1 or 3 is more than 1 order of magnitude larger than that seen when the AP site is at any of the other studied positions. Inhibition of incision is also seen when the second

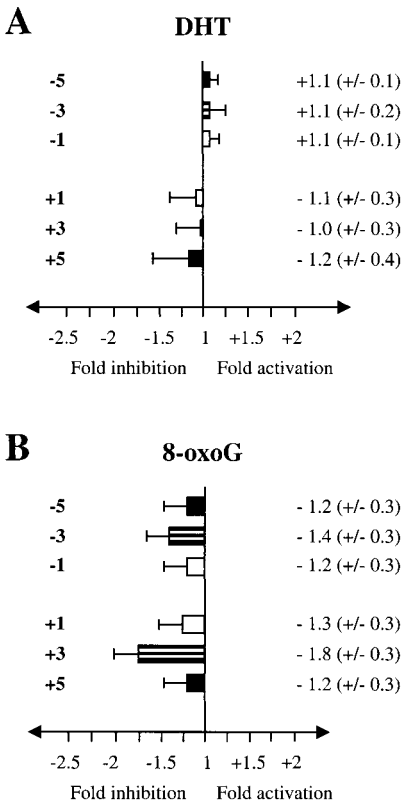


FIGURE 2: Influence of either DHT (A) or 8-oxoG (B) on the efficiency of incision of an AP site on the labeled, complementary strand by HAP1 (1−5 pg), expressed as fold inhibition or activation by comparison with the control oligonucleotide containing an AP site. The error bars represent the standard deviation from three independent experiments.

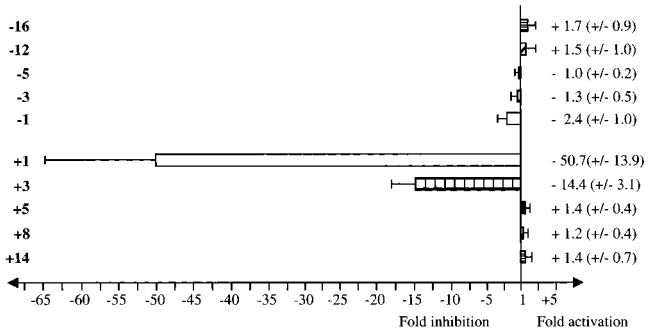


FIGURE 3: Influence of a second AP site on the efficiency of incision of an AP site on the labeled, complementary strand by HAP1 (1−5 pg), expressed as fold inhibition or activation by comparison with the control oligonucleotide containing an AP site. The error bars represent the standard deviation from five independent experiments.

AP site is at position −1. The inhibitory effect (Figure 3) is asymmetric. Since a second AP site at any of the other positions does not have a significant effect on the incision of an AP site by HAP1, this may reflect the processive action of the AP endonuclease enzyme (*35*).

Influence of a Neighboring SSB on the Incision of an AP Site by HAP1. The main inhibitory effect of the presence of a neighboring hydroxy SSB on the incision of an AP site by HAP1 (Figure 4A) occurs when the hydroxy SSB is at position 1 or 3 on the complementary strand. When the hydroxy SSB is at position −1, −3, or 5, smaller inhibitory effects are still apparent. The profile of this inhibitory effect on interlesion distance is asymmetric, similar to that seen

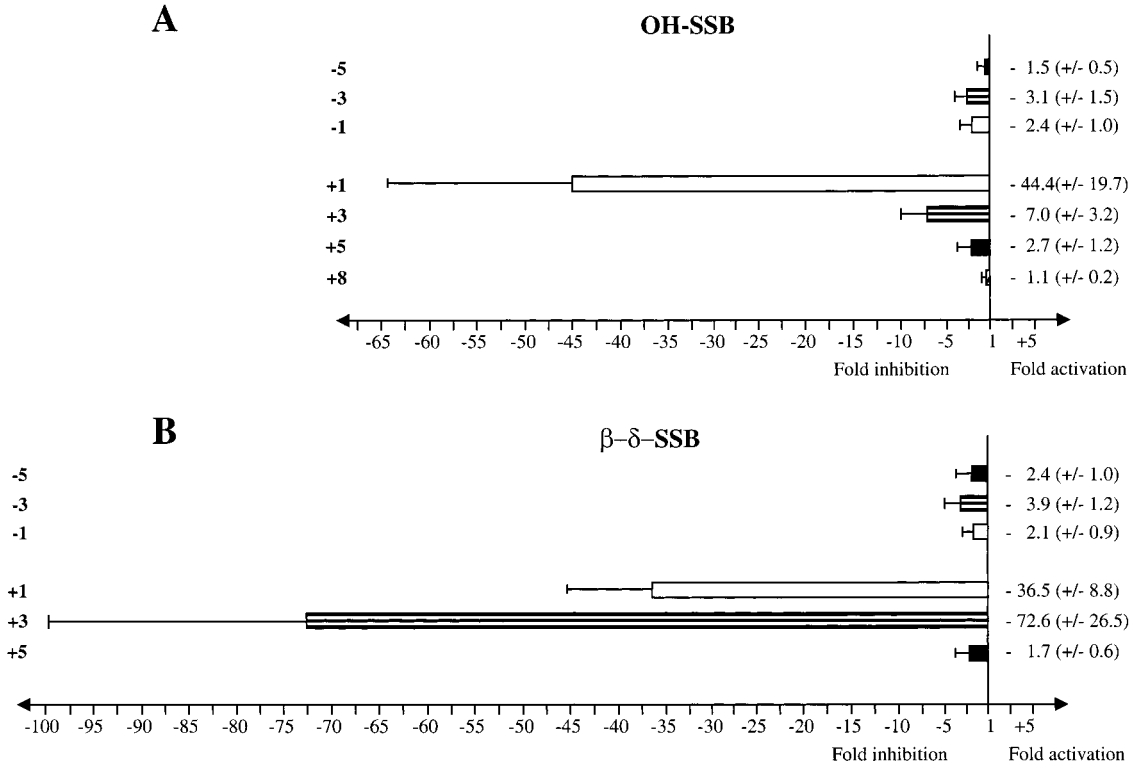


FIGURE 4: Efficiency of HAP1 (1−5 pg) in incising an AP site in the vicinity of (A) a hydroxy SSB or (B) a $\beta-\delta$ SSB at the various positions (Table 1) from the AP site on the labeled strand, expressed as the fold inhibition or activation by comparison with the control oligonucleotide containing an AP site. The error bars represent the standard deviation from nine (A) or seven (B) independent experiments.

with a second AP site (Figure 3). For comparison, a $\beta-\delta$ SSB was tested for its influence on the efficiency of incision by HAP1 of the AP site on the complementary strand. As shown in Figure 4B, the inhibitory effect on the incision of an AP site is large when a $\beta-\delta$ SSB is at position 1 or 3. Smaller inhibitory effects of a $\beta-\delta$ SSB are also seen at the other tested positions. As with a second AP site or a hydroxy SSB, the profile of this inhibitory effect is asymmetric. The inhibitory effect of a $\beta-\delta$ SSB at position 3 is greater than that at position 1. The extent of inhibition of a $\beta-\delta$ SSB at position 1 is comparable to that of a hydroxy SSB (Figure 4A) or an AP site (Figure 3) at the same position, whereas the effect of a $\beta-\delta$ SSB is far greater than that seen with a hydroxy SSB or an AP site at position 3.

Effect of a SSB on the Formation of a DSB. If a neighboring, pre-existing SSB were present in the complementary strand within 6 bp (*36*), conversion of an AP site into a SSB by HAP1 would lead to the formation of a DSB. Hence, the reduction in the level of DSB formation as a function of the position of a neighboring $\beta-\delta$ SSB relative to that of the AP site (Figure 5) reflects the extent of inhibition of incision of the AP site. On the basis of the protein concentration required to induce a DSB in 50% of the oligonucleotide molecules, the formation of a DSB is ~1 and ~2 orders of magnitude less efficient when the $\beta-\delta$ SSB is at positions 1 and 3, respectively, to the AP site, relative to that for formation of DSB when the $\beta-\delta$ SSB is at any of the other tested positions.

Kinetics for Incision of an AP Site by HAP1 in the Presence of a Neighboring Lesion. To gain further insight into the mechanism of the inhibitory effect on incision of an AP site by HAP1 in the presence of a second AP site, a hydroxy SSB or a $\beta-\delta$ SSB, the kinetic constants K_M, k_{cat},

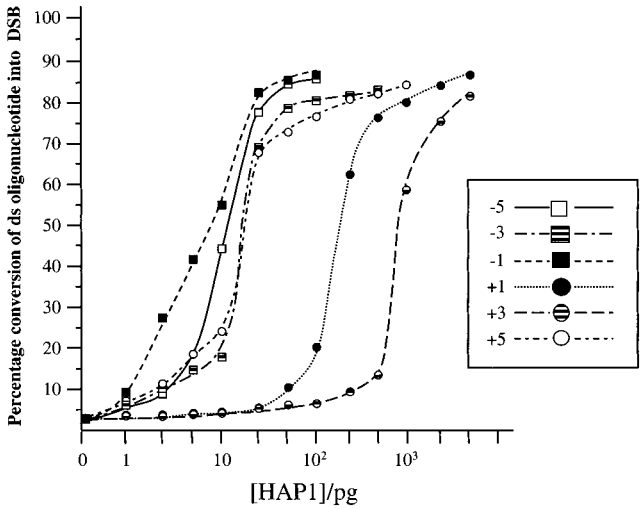


FIGURE 5: Dependence of the percentage yield of double-stranded oligonucleotide converted into a DSB on the concentration of HAP1 following incubation of oligonucleotides containing an AP site on the labeled strand and a $\beta-\delta$ SSB at the various positions on the complementary strand with various concentrations of HAP1 for 30 min at 37 °C.

and specificity constants were determined with oligonucleotides containing an AP site on the labeled strand opposite to one of these other lesions on the complementary strand at the positions shown in Figure 6. The presence of a second AP site at position −1, 1, or 3 significantly reduces the specificity constant in comparison with that of the control, for incision of an AP site. When a second AP site is at position 1, this decrease in the specificity constant is associated with an increase in the value of K_M, whereas when an AP site is at position 3, the decrease is mainly associated with a decrease in the value of k_{cat}.

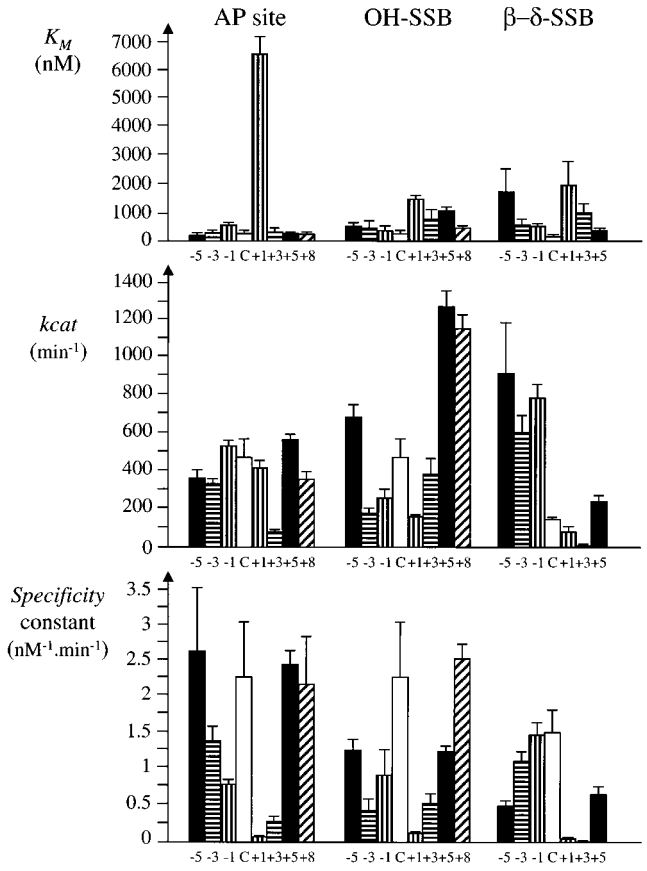


FIGURE 6: Variation of K_M, k_{cat}, and the specificity constants for incision of the AP site by HAP1 on the interlesion separation with either an AP site, a hydroxy SSB, a $\beta-\delta$ SSB, or the normal base (C) on the nonlabeled, complementary strand of the oligonucleotides (5−1000 nM). The oligonucleotides containing an AP site, a $\beta-\delta$ SSB, or a hydroxy SSB on the nonlabeled strand at the various positions were incubated with the following concentrations of HAP1 protein: an AP site or a hydroxy SSB at position 1 with 25 pg (141.25 pM) of HAP1, an AP site at position 3 with 10 pg (56.5 pM) of HAP1, a hydroxy SSB or a $\beta-\delta$ SSB at position −3 with 5 pg (28.5 pM) of HAP1, and a $\beta-\delta$ SSB at positions 1 and 3 with 50 pg (282.5 pM) or 1 ng (5.65 nM) of HAP1. All the other oligonucleotides as well as the control oligonucleotides (C) were incubated with 2.5 pg (14.125 pM) of HAP1.

The presence of a hydroxy SSB at positions −3 to 3 significantly reduces the specificity constant in comparison with that of the control. This decrease in the specificity constant corresponds to both an increase in the value of K_M, especially for the hydroxy SSB at position 1 or 3, and a decrease in the value of k_{cat}, especially for the hydroxy SSB at positions −3 to 1, as shown in Figure 6. The presence of a $\beta-\delta$ SSB at positions 1 and 3 has a larger effect on the specificity constant than that seen with a hydroxy SSB or a second AP site at the corresponding positions. This effect of a $\beta-\delta$ SSB corresponds to an increase in the value of K_M and a decrease in the value of k_{cat}. It should be noted that the base opposite to the AP site is cytosine in the oligonucleotides containing a second AP site or a hydroxy SSB, and adenine for the oligonucleotides containing a $\beta-\delta$ SSB. The kinetics values for the control oligonucleotides in Figure 6 are not significantly different.

Efficiency of Binding of an AP Site Opposite to a Neighboring Lesion by the D210N Mutant Protein. The effect of a second AP site or $\beta-\delta$ SSB on the level of binding to an AP site on the labeled strand was determined by EMSA using the HAP1 mutant protein, D210N, which binds to but does not incise an AP site within an AP-containing oligonucleotide (*28*). This protein was used to determine whether variations in the efficiency of binding to the oligonucleotides contribute significantly to the inhibitory effect seen on incision of the AP site in the presence of a lesion on the complementary strand. As shown in Figure 7A, the D210N mutant (2.5 ng) forms a single retarded complex with the control oligonucleotide (C) containing a single AP site. With a second AP site at any of the positions from position −5 to 8, the DNA−protein complex comigrates with that formed with the control oligonucleotide, but the efficiency of binding is dependent on the interlesion separation of the AP sites. An additional, weak complex is present with the oligonucleotides containing a neighboring AP site at position −3 or 8. It is assumed that this additional band is due to nonspecific binding of the mutant protein to these oligonucleotides, although it was not seen in the control oligonucleotide. The quantity of DNA−protein complex formed as a function of the amount of protein added is shown in Figure 7B. A decrease, compared with that of the control, in the amount of DNA−protein complex was observed with the oligonucleotide containing a second AP site, especially when the second AP site is located at positions −5 to 3 to the AP site on the labeled strand. The amount of HAP1 protein required to complex 50% of the oligonucleotide when a second AP

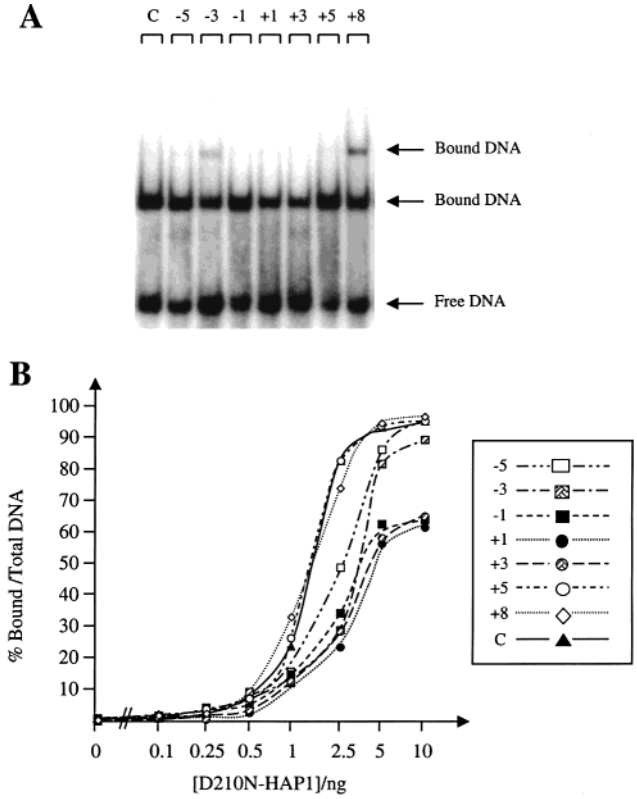


FIGURE 7: (A) Gel profiles using EMSA showing the binding of the mutant protein D210N-HAP1 (2.5 ng) to ^{32}P-labeled oligonucleotides containing an AP site as a single lesion (control probe, 0.5 nM, lane C) or an AP site opposite to a second AP site at positions −5 to 8 on the complementary strand. (B) The extent of binding of D210N-HAP1 to oligonucleotide containing either a single or two closely opposed AP sites at positions −5 to 8 as a function of the protein concentration. The percentage of the protein−DNA complex was determined from the relative amounts of bound and free probe with respect to the total amount of probe for each oligonucleotide.

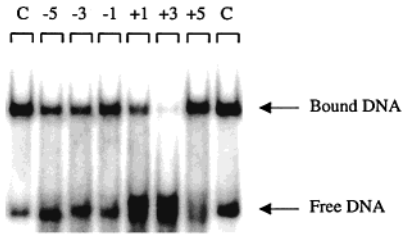


FIGURE 8: Gel profiles using EMSA showing the binding of the mutant protein D210N-HAP1 (2.5 ng) to ^{32}P-labeled oligonucleotides containing an AP site as a single lesion (control probe, 0.5 nM, lanes C) or an AP site opposite to a $\beta-\delta$ SSB at positions from −5 to 5 on the complementary strand.

site is located at positions −5 to 3 is approximately twice that required when the AP site is located at position −5, 5, or 8. The apparent binding constant of the mutant protein with the control oligonucleotide containing a single AP site was estimated to be $\sim 1 \times 10^8$ M^{-1}. Therefore, the efficiency of binding of the mutant protein to an AP site is reduced by a factor of only 2, even in the presence of a second, neighboring AP site.

The binding efficiency of the D210N catalytic mutant (2.5 ng) was also determined with an AP site either as a single lesion (C) or in the vicinity of a $\beta-\delta$ SSB at the various positions on the complementary strand of the oligonucleotides. In Figure 8, the gel shift shows that protein binding to the AP site is influenced by the presence of a $\beta-\delta$ SSB on the complementary strand, particularly at positions 1 and 3. The extent of formation of the DNA−protein complex depends on the interlesion distance between the AP site and the $\beta-\delta$ SSB. The efficiency of binding of the D210N protein to an AP site in the presence of a $\beta-\delta$ SSB is similar, within a factor of 2, with that of the control, except when the $\beta-\delta$ SSB is at position 1 or 3. With these latter oligonucleotides, the binding efficiency is reduced by ∼1 order of magnitude compared with that of the control.

DISCUSSION

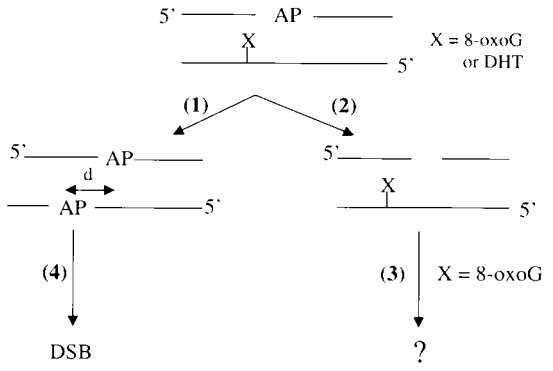
This study assesses the influence of various types of neighboring DNA lesions on the incision of an AP site by purified HAP1, to gain insights into the initial steps of the processing of clustered DNA damage. The presence of an opposite base lesion (DHT or 8-oxoG) only slightly modifies, if at all, the efficiency of incision of an AP site by HAP1. In contrast, significant inhibition of incision of an AP site by HAP1 occurs in the presence of a neighboring AP site, especially when the second AP site is at position 1 or 3. This asymmetric effect of the influence of a second AP site on the incision of an AP site by HAP1 is consistent with previous findings (*12*). The efficiency of binding of a mutant HAP1 protein to the AP site is only slightly reduced, even in the presence of a second, neighboring AP site. The specificity constant for incision of the AP site when opposite adenine in the control oligonucleotides (Figure 6) is similar to those for incision of synthetic AP sites by HAP1 (*37*, *38*), consistent with a lack of sequence specificity for recognition of an AP site by HAP1 (*39*).

In contrast to the data for base lesions, it was confirmed that SSBs, both hydroxy SSBs and $\beta-\delta$ SSBs, show a large inhibitory effect on incision of an AP site by HAP1, particularly when the SSB is located at position 1 or 3. A $\beta-\delta$ SSB with phosphate termini causes maximal inhibition at position 3, whereas a hydroxy SSB, with hydroxy termini, or an AP site causes maximal inhibition at position 1. It is inferred that the phosphate termini of the $\beta-\delta$ SSB may contribute to the extent of inhibition of incision of the AP site by HAP1, involving differences in stereochemical and/or electrostatic interactions between HAP1 and DNA. The DNA−HAP1 interaction may be influenced by the charge of the phosphate group, since the HAP1 surface has a significant region of negative electrostatic potential and the active center, where the AP site sits, is generally negatively charged (*39*). The asymmetric profile for inhibition of incision of an AP site by HAP1 on the position of a $\beta-\delta$ SSB is consistent with the reduced efficiency of conversion of the clustered damage into a DSB, especially when the $\beta-\delta$ SSB is at position 1 or 3 (Figure 5). This inhibition of DSB formation may correspond with an increase in the lifetime of the AP site when opposite to a $\beta-\delta$ SSB.

There is a marked effect of altering the position of the SSB on the extent of inhibition of HAP1 cleavage at the AP site. The major reduction in the catalytic activity of HAP1, by a factor greater than 1−2 orders of magnitude when compared with that of the control, is seen when a $\beta-\delta$ SSB is at position 1 or 3. This inhibition of the catalytic activity for incision of an AP site is reflected in a greatly reduced affinity for binding of the mutant D210N-HAP1 protein to an AP site, when the $\beta-\delta$ SSB is at position 1 or 3. From structural studies of binding of HAP1 to DNA containing an AP site (*39*, *40*), it was proposed that specific amino acid residues of HAP1 make contact with the two or three phosphate groups on the 5′ side of the AP site and that three amino acid residues make contact on the complementary strand with three consecutive phosphates 5′ to the base opposite to the AP. HAP1 also binds two phosphate groups on the 3′ side of the AP site. This footprint for HAP1 binding to an AP site is similar to the kinetically derived footprint of HAP1 binding to an AP site, extending four bases 5′ and three bases 3′ to the AP site (*38*). The importance of the interactions of HAP1, particularly with a lesion 5′ to the base opposite to the AP site, would be consistent with the asymmetric effects reported here and results previously reported with double-stranded DNA containing AP sites (*12*).

An additional factor, which may contribute to the reduced catalytic activity of HAP1, is the effect a second lesion present on the complementary strand may have on the extent of distortion of the DNA induced when HAP1 binds to the AP site. A single AP site causes a 30−35° kink when the AP site is bound to HAP1 (*40*, *41*), and it was suggested that HAP1 is a structure-specific nuclease that detects and binds DNA that can adopt this particular kinked conformation. Distortions in the structure of DNA around the AP site, caused by a second lesion, may reduce the recognition and/or binding affinity. NMR characterization of clustered, double-stranded AP sites indicated that if one of the AP sites is at any of the positions 5′ to the base opposite to the other AP site, the AP sites are smoothly aligned with the sugar−phosphate backbone (*42*). In contrast, when the second AP sites is at position 1 or 3, the AP sites take up extrahelical locations, perhaps making recognition by HAP1 easier (*42*). Therefore, the reduced catalytic activity of HAP1 for excision of an AP site in the presence of a $\beta-\delta$ SSB would be

Scheme 1: Pathways of Processing Clustered DNA Damage Containing an AP Site



consistent with reduced binding affinity, together with any distortions in the oligonucleotides caused by the second lesion, particularly when located at position 1 or 3.

Inhibition of HAP1 AP endonuclease activity by neighboring lesions may have evolved to prevent DSB formation. Indeed, significant inhibition of HAP1 occurs in the presence of a neighboring AP site (*12*) or SSB, especially when the second lesion is located at position 1 or 3. However, if the SSB or second AP site is located at any of the other studied positions, the induction of a DSB by HAP1 is not significantly affected (pathway 4 of Scheme 1). Therefore, the relative positions of the lesions within clustered DNA damage may have significant consequences on their processing. For instance, if either 8-oxoG or DHT is located within a few base pairs of an AP site on the complementary strand within clustered DNA damage, it is predicted that the AP site should be preferentially converted into a SSB (pathway 2 in Scheme 1), in competition with the less efficient excision of the base lesion by glycosylases to give an AP site (*16*, *19*) (pathway 1 in Scheme 1). Conversion of the AP site into a SSB in pathway 2 would minimize formation of a DSB. Whether the intermediate damage formed in pathway 2 leads to a DSB is as yet not known, since enzymatic excision of 8-oxoG (pathway 3 in Scheme 1) is inhibited in the presence of a SSB (*16*, *19*), allowing time for repair of the SSB prior to significant processing of the base lesion. However, some types of radiation-induced clustered DNA damage were recently shown to be converted into additional DSBs in bacteria (*31*). However, if two AP sites, one on each strand, are induced within a clustered damage, it is shown that they will be converted into a DSB (pathway 4 in Scheme 1), unless the second AP site is at position 1 or 3.

In summary, the efficiency of incision of an AP site within a region of clustered DNA damage is significantly reduced by the presence of a second AP site or SSB, particularly when located at position 1 or 3. If an AP site and either a SSB or a second AP site is positioned at any of the other positions within clustered DNA damage, a DSB may result. This would be predicted to contribute significantly to the genotoxic effects of ionizing radiation.

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