Efficiency of Repair of an Abasic Site within DNA Clustered Damage Sites by Mammalian Cell Nuclear Extracts†

Martine E. Lomax,* Siobhan Cunniffe, and Peter O'Neill

Radiation and Genome Stability Unit, Medical Research Council, Harwell, Didcot, Oxfordshire OX11 0RD, U.K.

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ABSTRACT: Ionizing radiation induces clustered DNA damage sites which have been shown to challenge the repair mechanism(s) of the cell. Evidence demonstrating that base excision repair is compromised during the repair of an abasic (AP) site present within a clustered damage site is presented. Simple bistranded clustered damage sites, comprised of either an AP-site and 8-oxoG or two AP-sites, one or five bases 3′ or 5′ to each other, were synthesized in oligonucleotides, and repair was carried out in xrs5 nuclear extracts. The rate of repair of an AP-site when present opposite 8-oxoG is reduced by up to 2-fold relative to that when an AP-site is present as an isolated lesion. The mechanism of repair of the AP-site shows asymmetry, depending on its position relative to 8-oxoG on the opposite strand. The AP-site is rejoined by short-patch base excision repair when the lesions are 5′ to each other, whereas when the lesions are 3′ to one another, rejoining of the AP-site occurs by both long-patch and short-patch repair processes. The major stalling of repair occurs at the DNA ligase step. 8-OxoG and an AP-site present within a cluster are processed sequentially, limiting the formation of double-strand breaks to <4%. In contrast, when two AP-sites are contained within the clustered DNA damage site, both AP-sites are incised simultaneously, giving rise to double-strand breaks. This study provides new insight into understanding the processes that lead to the biological consequences of radiation-induced DNA damage and ultimately tumorigenesis.

Ionizing radiation can induce clustered DNA damage, which is defined as two or more individual lesions within one or two helical turns of the DNA helix, through passage of a single radiation track. The lesions induced within clustered damage sites are the same as those produced during oxygen metabolism by the cell [oxidized base lesions, abasic (AP)[1] sites, and single-strand breaks (SSB)], but a unique feature of ionizing radiation is that these lesions are induced in clusters. Clustered DNA damage sites arise as the energy deposited by ionizing radiation is heterogeneous, with direct ionization and formation of radicals within the DNA together with indirect DNA damage produced by the ionization of water molecules in the hydration shell of DNA. The complexity and yield of radiation-induced clustered DNA damage increase with the increasing ionization density of the radiation (*1*, *2*), and track structure simulations demonstrate that for sparsely ionizing radiation approximately 30−40% of double-strand breaks (DSB) are associated with other lesions. This value increases to more than 90% with densely ionizing radiation (*3*, *4*). Experimentally, it has been shown that non-DSB clustered damage sites are induced by γ-radiation in mammalian cells in yields that are 4−8 times that of prompt DSB (*5*, *6*). A small subclass of these non-DSB clustered damage sites have been shown to be converted into DSB post-irradiation in both prokaryotic (*7*) and eukaryotic cells (*8*). The induction of clustered DNA damage is thought to contribute to the biological consequences of radiation. Indeed, 10−15% of all deaths from lung cancer can be attributed to exposure to radon (*9*).

The base excision repair (BER) pathway has evolved for the repair of small oxidative lesions such as those introduced by endogenous reactive oxygen species (reviewed in ref *10*) and is largely responsible for the repair of many lesions induced by ionizing radiation. BER is initiated by the action of a DNA glycosylase to excise the damaged base, and the resulting AP-site is incised by an AP endonuclease. DNA polymerase β inserts the missing nucleotide into the repair gap, and the ligase III−XRCC1 complex seals the nick to complete short-patch repair. If ligation cannot take place following addition of the missing nucleotide, there is a polymerase switch to DNA polymerase δ/ϵ and the long-patch base excision repair pathway is initiated, with the addition of two to six nucleotides and concomitant strand displacement. The resulting flap is removed by flap endonuclease 1 (FEN1) in a PCNA-dependent manner, and repair is completed with ligation by ligase 1.

Using synthetically engineered DNA clustered damaged sites containing two lesions (one on each strand) known to be produced by ionizing radiation, several groups have established that the processing of lesions within a clustered DNA damage site by DNA glycosylases and/or human AP endonuclease 1 (HAP1) is impaired (reviewed in refs *11*−*13*). For instance, an AP-site or a SSB retards the excision of 7,8-dihydro-8-oxoguanine (8-oxoG) by nuclear extracts, hOGG1, or formamidopyrimidine DNA glycosylase (Fpg) if present up to five bases 3′ or 5′ on the opposite

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* To whom correspondence should be addressed. Telephone: 44 1235 841000. Fax: 44 1235 841200. E-mail: m.lomax@har.mrc.ac.uk.

[1] Abbreviations: AP, abasic; 8-oxoG, 7,8-dihydro-8-oxoguanine; SSB, single-strand break; DSB, double-strand break; HAP1, human AP endonuclease 1; BER, base excision repair.

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Table 1:[a]

Position	Sequence	Strand
-5	5′-ctcttagtcaggaa**Y**atgtctctatgctgggagcaaaggc	1
	3′-gagaatcagtccttataca**X**agatacgaccctcgtttccg	2
-1	5′-ctcttagtcaggaatatg**Y**ctctatgctgggagcaaaggc	1
	3′-gagaatcagtccttataca**X**agatacgaccctcgtttccg	2
+1	5′-ctcttagtcaggaatatgtc**Y**ctatgctgggagcaaaggc	1
	3′-gagaatcagtccttataca**X**agatacgaccctcgtttccg	2
+5	5′-ctcttagtcaggaatatgtctcta**Y**gctgggagcaaaggc	1
	3′-gagaatcagtccttataca**X**agatacgaccctcgtttccg	2
-12	5′-aatccgagccctcttag**Y**caggaatatgtctctatgctgggagcaaaggctctcc	1
	3′-ttaggctcgggagaatcagtccttataca**X**agatacgaccctcgtttccgagagg	2
+8	5′-aatccgagccctcttagtcaggaatatgtctctatgc**Y**gggagcaaaggctctcc	1
	3′-ttaggctcgggagaatcagtccttataca**X**agatacgaccctcgtttccgagagg	2
control 1	5′-ctcttagtcaggaatatgtctctatgctgggagcaaaggc	
	3′-gagaatcagtccttataca**Y**agatacgaccctcgtttccg	2
control 2	5′-ctcttagtcaggaatatgtctctatgctgggagcaaaggc	
	3′-gagaatcagtccttataca**X**agatacgaccctcgtttccg	2
control 3	5′-aatccgagccctcttag**Y**caggaatatgtctctatgctgggagcaaaggctctcc	1
	3′-ttaggctcgggagaatcagtccttatacagagatacgaccctcgtttccgagagg	

[a] **X** represents 8-oxoG or an AP-site (following conversion of uracil to an AP-site as described in Materials and Methods). **Y** represents an AP-site (following conversion of uracil to an AP-site as described in Materials and Methods). −5 and −1 are the positions on the complementary strand of the **X** base 3′ from the **Y** base. +1 and +5 are the positions on the complementary strand of the **X** base 5′ from the **Y** base. Control 1 and control 3 are control oligonucleotides containing an AP-site as a single lesion. Control 2 is the control oligonucleotide containing 8-oxoG as a single lesion.

strand (*14*−*16*), thus minimizing the level of formation of a DSB. However, a strong asymmetric effect was observed upon formation of DSB through the incision of an AP-site by HAP1 when the AP-site is opposite from another AP-site or SSB (*17*, *18*). Only when an AP-site is positioned up to three bases 5′ to another AP-site or a SSB on the opposite strand is the formation of DSB significantly retarded.

Recent work has demonstrated that the repair of both a HAP1-SSB (a single-strand break created by the incision of an AP-site by HAP1) and 8-oxoG present within a clustered damage site is compromised, compared with the efficiency of repair of the single lesions (*19*). The major retardation of repair was shown to occur at the ligation stage involving the ligase III−XRCC1 complex, thereby resulting in initiation of a long-patch repair process when the two lesions present within the clustered damage site are 3′ to each other. The study presented here was designed to extend the previous findings by investigating how clustered damage sites (containing an AP-site opposite 8-oxoG or two opposing AP-sites) are processed by the whole BER pathway, using cell extracts. The results show that a striking difference is seen in the way clustered damage sites containing different lesions are processed. For example, an AP-site and 8-oxoG on opposing DNA strands are predominantly repaired sequentially, limiting the formation of DSB, whereas the incision of two opposing AP-sites (the initial step of their repair) occurs simultaneously, leading to the formation of a large number of DSBs, a highly mutagenic and potentially lethal lesion (reviewed in refs *20* and *21*). Poor efficiency of repair of lesions within a clustered damage site can lead to the generation of mutations (*22*, *23*) and contribute to the detrimental health consequences of ionizing radiation.

MATERIALS AND METHODS

Substrate Oligonucleotides. The oligonucleotides were purchased as HPLC-purified species from Genosys. The sequences of the double-stranded oligonucleotides are presented in Table 1. Strand 1 contains an AP-site at variable positions (position **Y**) opposite from the fixed lesion on strand 2. Strand 2 contains 8-oxoG or an AP-site at a fixed position (position **X**). In the control oligonucleotides containing a single lesion, the lesion is on strand 2. It has been determined that the efficiency of rejoining of the AP-site is independent of the sequence context of strands 1 and 2 (see Figure 1S of the Supporting Information). The nomenclature of the relative positions of the two lesions in the clustered DNA damage site was developed by David-Cordonnier *et al.* (*17*). A positive or negative number has been assigned to each residue in the oligonucleotide. This number refers to the separation, in base pairs, of one lesion on strand 1 located 5′ (positive number) or 3′ (negative number) opposite from the lesion on strand 2.

Preparation of 5′-End-Labeled Oligonucleotides. Oligonucleotide (0.2 μg) was 5′-end-labeled using 10 units of T4 polynucleotide kinase (Invitrogen, Paisley, U.K.) with 25 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, 10 mCi/mL, Perkin-Elmer) in 20 μL of buffer [70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, and 1 mM β-2-mercaptoethanol] for 30 min at 37 °C. Following purification on a 12% denaturing

polyacrylamide gel, the labeled oligonucleotide was hybridized with a 2-fold excess of the purified nonradiolabeled complementary strand. Efficient annealing of the oligonucleotides was verified on a 12% native polyacrylamide gel.

Preparation of an AP-Site. The purified double-stranded oligonucleotides that contained a uracil residue(s) were treated with 1 unit of uracil DNA glycosylase (UDG) (Invitrogen) in 100 μL of buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM EDTA] for 30 min at 37 °C to produce an AP-site. The AP-site-containing oligonucleotides were used immediately in the repair assays.

Preparation of Nuclear Extracts. The nuclear extracts were prepared as previously described (*14*) from Ku_{80} deficient xrs5 cells (*24*) to avoid possible interference from the binding of Ku to termini of linear DNA (*25*) and SSB (*26*). Briefly, the cells were harvested in exponential phase, and the pelleted cells were resuspended in an equal volume of buffer [10 mM HEPES (pH 7.9), 100 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM DTT] and incubated on ice for 15 min. The cells were lysed by drawing the cell suspension into a 0.5 μm diameter needle 10 times, and the nuclei were collected by a brief centrifugation at 12000*g* and 4 °C. The nuclear proteins were extracted by incubation in $^2/_3$ volume of high-salt buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF] for 30 min with agitation on ice. Following centrifugation for 10 min at 12000*g* and 4 °C, the supernatant was dialyzed twice over a total period of 16 h against 1 L of buffer [20 mM HEPES (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, and 0.5 mM PMSF]. The protein concentration was determined using the Bradford colorimetric technique and was found to be between 9.5 and 13.3 mg/mL. Aliquots of nuclear extracts were stored at −80 °C.

Repair Assays. The double-stranded oligonucleotides (10 000 cpm, 2 fmol) were incubated with either 1 μg (for analysis of the AP-site-containing strand) or 5 μg (for analysis of the 8-oxoG-containing strand) of xrs5 nuclear extracts in 5 μL of repair buffer [70 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 4 mM ATP, 40 mM phosphocreatine, 1.6 μg/mL phosphocreatine kinase, and dATP, dCTP, dGTP, and dTTP (0.1 mM each)] at 37 °C for 0, 1, 5, 15, 30, and 60 min. The efficiency of the AP endonuclease present in the nuclear extract is such that a few seconds at room temperature is sufficient for most of the AP-site contained in the oligonucleotide to be incised (see the 0 min time points in Figures 1 and 4). The concentrations of extract were optimized from titration studies (data not shown). To stop the reactions, 5 μL of denaturing stop solution (98% formamide, 2 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol) was added. The samples were then subjected to electrophoresis on a 12% denaturing polyacrylamide 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 exposed to a Bio-Rad PhosphorImager screen for visualization of repair products using phosphorimaging technology (Bio-Rad, Molecular Imager FX) and quantified with Quantity One software (Bio-Rad, Hercules, CA). When the time dependence of the repair of the AP-site is followed, the intensity of the bands representing either single-stranded DNA, single-stranded DNA with one, two, or five bases added (before ligation; see the Results), or rejoined DNA [ligation of the AP-site following addition of the missing base(s)] is expressed as a percentage of the total intensities for all bands. When the repair of the strand containing 8-oxoG is examined, the intensity of the band representing the cleaved strand is expressed as a percentage of the total intensities for the bands representing the cleaved and intact strands. The efficiencies of repair of an AP-site or of incision at 8-oxoG within clustered damage sites were compared with that for the repair of the respective single lesions in the control oligonucleotides. The errors represent standard deviations of the mean from at least three experiments.

Different preparations of the xrs5 nuclear extract had slightly different activities for the repair of an AP-site. To account for interpreparation differences of the nuclear extracts, the relative amount of rejoining of an AP-site within a clustered damage site was always compared with that of a control oligonucleotide containing an AP-site as a single lesion, using the same extract preparation within each experiment.

Assessment of DSB Induction during Repair. Repair assays were carried out as described above, but the reactions were stopped by the addition of 5 μL of native stop solution (40% sucrose, 5 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene cyanol). The products of the repair assay were then subjected to electrophoresis on a 12% native polyacrylamide gel in 1× TBE [89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA (pH 8.3)] for 2 h at a constant voltage of 300 V. The dried gel was exposed to a Bio-Rad PhosphorImager screen for visualization of repair products using phosphorimaging technology (Bio-Rad, Molecular Imager FX) and quantified with Quantity One software (Bio-Rad). The intensity of the band representing the DSB strand is expressed as a percentage of the total intensities for the bands resulting from the DSB and intact strands.

RESULTS

Effect of 8-OxoG on the Efficiency of Repair of an AP-Site. (i) The efficiency of repair of the AP-site depends on the interlesion separation from 8-oxoG. To investigate the repair of an AP-site when present within a clustered DNA damage site containing 8-oxoG, the oligonucleotide strand containing the AP-site was 5′-end-labeled with ^{32}P and treated with nuclear extract for various times (0−60 min). Figure 1A shows that when an AP-site is present as a single lesion (AP control) the intact 40mer band is initially (within 1 min) incised to give a 20mer band as a result of the cleavage of the AP-site. The resulting SSB is then repaired over the course of 60 min (Figure 1A,B) to restore the intact, repaired 40mer band (rejoined DNA). If 8-oxoG is present on the complementary strand, the initial cleavage of the AP-site still occurs within 1 min to produce a band of 14, 18, 20, or 24 bases depending on the position of the AP-site on the various oligonucleotides (see Table 1). When 8-oxoG is present in the +1 position with respect to the AP-site, rejoining is impaired relative to that of the AP control oligonucleotide, with the initial rate of rejoining (over the first 15 min) being reduced by 2-fold (Figure 1B). The initial rates of rejoining of the AP-site when 8-oxoG is present in the −1 and −5 positions are similar to that of the AP control oligonucleotide (reduced by 1.2-fold). However, at 60 min there is a reduction

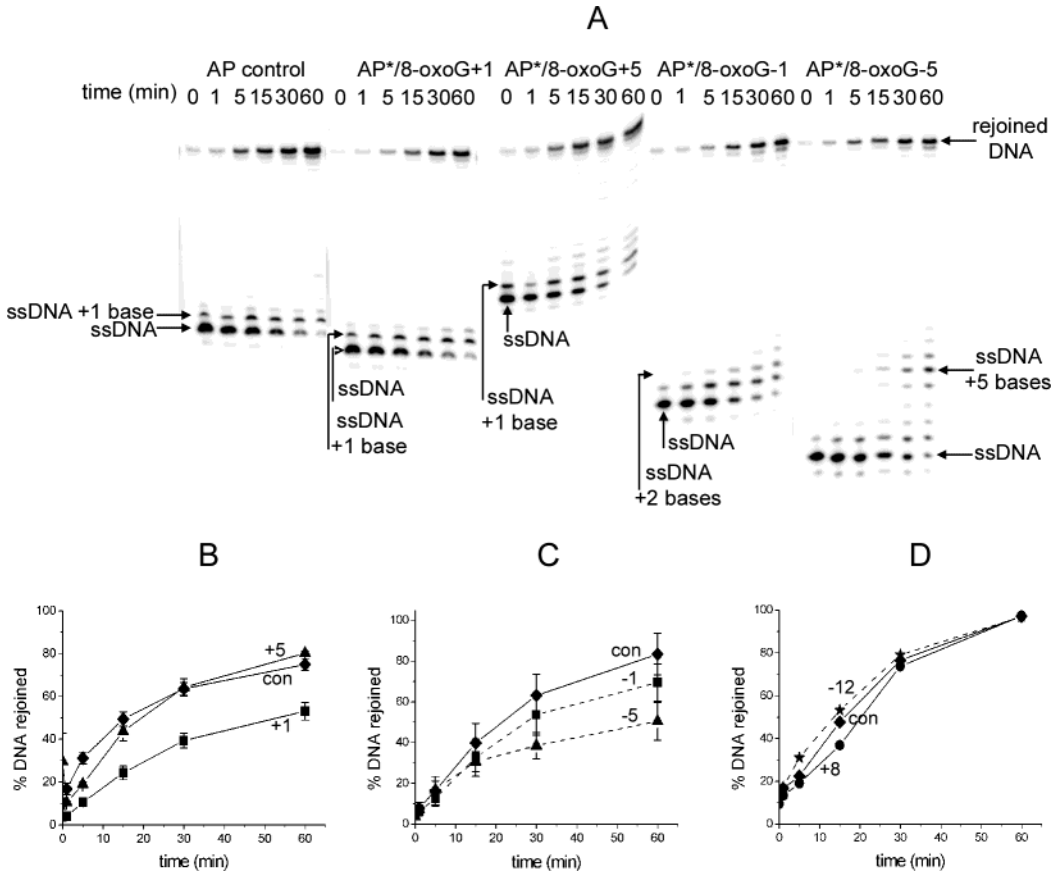

FIGURE 1: Repair of an AP-site is impaired by the presence of 8-oxoG. (A) Representative denaturing polyacrylamide gel showing the rejoining of an AP-site in the absence and presence of 8-oxoG at positions +1, +5, −1, and −5. The oligonucleotide strand containing an AP-site was 5′-end-labeled with ^{32}P and incubated with xrs5 nuclear extracts for the times shown. The ssDNA band represents the single-stranded DNA band before rejoining of the induced AP-site. ssDNA +1, +2, or +5 base bands represent single-stranded DNA following addition of the respective number of bases but before the DNA nick is sealed, seen as restoration of the 40mer band. (B−D) Time scale for the rejoining of the AP-site: (—◆—) AP-site control (no 8-oxoG), (—■—) oligonucleotide with 8-oxoG at position +1, (—▲—) oligonucleotide with 8-oxoG at position +5, (- - -■- - -) oligonucleotide with 8-oxoG at position −1, (- - -▲- - -) oligonucleotide with 8-oxoG at position −5, (—●—) oligonucleotide with 8-oxoG at position −12, and (- - -★- - -) oligonucleotide with 8-oxoG at position +8. The efficiency of the HAP1 present in the nuclear extract is such that a few seconds at room temperature is sufficient for most of the AP-site contained in the oligonucleotide to be incised (see the 0 min time point). To assist the reader, lines have been drawn on the graphs between the points only to guide the eye and do not represent fitted curves.

in the amount of rejoining of the AP-site by 1.7-fold when 8-oxoG is present in the −5 position, compared to the control. No effect on the rate of rejoining of the AP-site was seen when 8-oxoG was in the +5 position (Figure 1B) or when the interlesion distance was increased to +8 or −12 residues (Figure 1D).

(ii) Repair of an AP-site occurs by long- and short-patch BER depending on the orientation with respect to 8-oxoG. During rejoining of the AP-site in the control oligonucleotide by xrs5 nuclear extracts, an intermediate band (ssDNA +1 base) was seen (Figure 1A), corresponding to the addition of one base by the DNA polymerase(s) present in the nuclear extract prior to ligation. This intermediate band accumulates over the first 15 min, and then its level is reduced slightly as SSB repair (ligation) occurs (Figure 2A). This sequence of events is consistent with the occurrence of short-patch BER which is also seen when 8-oxoG is present at positions +1 and +5 with respect to the AP-site (Figure 1A). With 8-oxoG present in the +5 position with respect to the AP-site, the rate of accumulation and the steady state level of the ssDNA +1 base band are similar to those for the AP control oligonucleotide (Figure 2A). However, when 8-oxoG is present in the +1 position, the intermediate band (ssDNA +1 base) still accumulates over the first 15 min, but its level reaches a steady state higher than that seen with the control oligonucleotide containing an AP-site (Figure 2A). From the similar initial rates of accumulation of this intermediate band, it is suggested that the presence of 8-oxoG does not inhibit the polymerase(s), presumably polymerase β, but the higher level of accumulation when 8-oxoG is at the +1 position with respect to the AP-site indicates that subsequent ligation is retarded during short-patch repair. That the ligation step is retarded by 8-oxoG opposite from an AP-site at position +1 is compatible with the retardation of the ligase III−XRCC1 complex by 8-oxoG during the rejoining of a HAP1-SSB when the two lesions are one base 5′ to each other within a clustered DNA damage site (*19*). Short-patch repair of the AP-site in the presence of 8-oxoG was verified by substituting a dideoxy version of the dNTP present a few bases downstream of the repair gap (ddTTP for AP, ddCTP for AP/8-oxoG+1, and ddGTP for AP/8-oxoG+5). In all cases, the rejoining occurred at the same rate independent of the presence of the ddNTP or the corresponding dNTP (data not shown), thus ruling out chain elongation.

In contrast to the repair of an AP-site in the positive orientation with respect to 8-oxoG occurring by short-patch

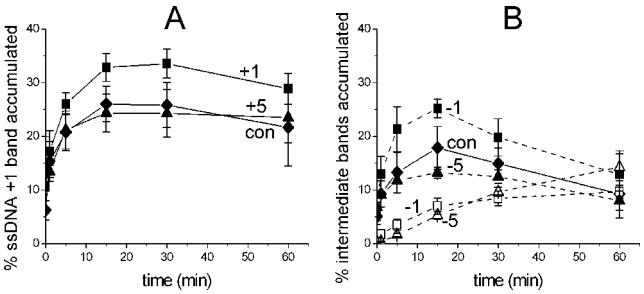

FIGURE 2: Accumulation of intermediate bands during repair of an AP-site in the presence of 8-oxoG, following addition of one, two, or five nucleotides. (A) (—◆—) AP-site control (no 8-oxoG), SSB +1 nucleotide; (—■—) oligonucleotide with 8-oxoG at position +1, SSB +1 nucleotide; and (—▲—) oligonucleotide with 8-oxoG at position +5, SSB +1 nucleotide. (B) (—◆—) AP-site control (no 8-oxoG), SSB +1 nucleotide; (- - -■- - -) oligonucleotide with 8-oxoG at position −1, SSB +1 nucleotide; (- - -□- - -) oligonucleotide with 8-oxoG at position −1, SSB +2 nucleotides; (- - -▲- - -) oligonucleotide with 8-oxoG at position −5, SSB +1 nucleotide; and (- - -△- - -) oligonucleotide with 8-oxoG at position −5, SSB +5 nucleotides. To assist the reader, lines have been drawn on the graphs between the points only to guide the eye and do not represent fitted curves.

BER, the repair of the AP-site proceeds, at least in part, by a long-patch repair process when 8-oxoG is in the −1 and −5 positions with respect to the AP-site. Evidence for long-patch repair can be seen from the addition of up to two and six bases, respectively (Figure 1A). For instance, when 8-oxoG is in the −5 position, the band corresponding to the addition of one base (ssDNA +1 base) is initially the most intense band but the band corresponding to the addition of five bases (ssDNA +5 bases) accumulates to the highest level at 60 min. Weaker intermediate bands of a long-patch repair process can also be seen. The accumulation of the intermediate band corresponding to addition of one base may reflect a contribution of short-patch BER during the repair of the AP-site, when 8-oxoG is present in the negative orientation. Alternatively, accumulation of a band following the addition of one base could reflect a slow "handover" from the DNA polymerase involved in short-patch BER to the polymerase(s) involved in long-patch repair. When 8-oxoG is present at positions −1 and −5 with respect to the AP-site, the ssDNA +1 base band accumulates at an initial rate similar to that of the intermediate band in the absence of 8-oxoG (Figure 2B), showing that the initial rate for addition of the first base by the polymerase(s) is not significantly different.

Incision at 8-OxoG Is Impaired by the Presence of an AP-Site. To investigate incision at 8-oxoG, the oligonucleotide strand containing 8-oxoG was 5′-end-labeled with ^{32}P and visualized as a band of 40 bases that was reduced to 20 bases if incision at 8-oxoG had occurred. Approximately 5−8% (the control values vary with different preparations of nuclear extract; see Materials and Methods) of 8-oxoG is incised for an incubation period of 60 min at the concentration of nuclear extract that was used, when no damage is present on the opposite strand (control 8-oxoG oligonucleotide) (Figure 3A,B). This level of incision is unaffected by the presence of an AP-site in the −1 or −5 position (Figure 3B). However, the level of incision at 8-oxoG determined at 60 min is reduced by 1.6- or 3.1-fold when an AP-site is in the +1 or +5 position, respectively (Figure 3A).

To determine if incision at 8-oxoG occurs at the same time as incision of the AP-site or whether the two lesions are processed sequentially, the products from the repair assay were run on a native polyacrylamide gel. Evidence of DSB formation was not seen when either the strand containing an AP-site or the strand containing 8-oxoG was 5′-end-labeled with ^{32}P (Figure 3E), demonstrating that the two lesions are processed sequentially. For correct repair of the SSB arising from incision of the AP-site, thymine would be the first base to be incorporated into the repair gap, since adenine is located on the template strand in the repair gap. Therefore, to investigate which of the two lesions is cleaved initially, ddTTP was substituted for dTTP so that the DNA polymerase(s) present in the nuclear extract could incorporate the missing thymine base at the site of the SSB but ligation would not take place. If the AP-site had to undergo complete repair before incision at 8-oxoG could occur, then incorporation of ddTTP would result in a persistent nick and incision at 8-oxoG would not take palce. When ddTTP is substituted for dTTP, the degree of incision at 8-oxoG is reduced from 5−8 to 1.5−3.5%, consistent with persistence of the nick, when an AP-site is present one or five bases 3′ or 5′ to 8-oxoG on the opposite strand (Figure 3C,D), confirming that the AP-site is generally incised and rejoined prior to incision at 8-oxoG.

The Presence of an AP-Site Does Not Impair the Incision of a Second AP-Site, Except at Position +1. When the clustered damage site consists of two opposing AP-sites, the rejoining of either the fixed AP-site (lesion **X** in Table 1) or the variable AP-site (lesion **Y** in Table 1) may be investigated, depending on which oligonucleotide strand was end-labeled with ^{32}P. Data have been shown for only the rejoining of the fixed AP-site as the results are the same regardless of which oligonucleotide strand was labeled with ^{32}P. Figure 4A shows that when an AP-site is present as an isolated lesion, the AP-site is rapidly converted into a SSB and then repaired to a final level of 78% during the 60 min incubation, similar to that seen in Figure 1.

The presence of a second AP-site in the negative orientation (in position −1 or −5 with respect to the fixed AP-site) resulted in the persistence of SSB arising from the rapid incision of the AP-site on the labeled strand (Figure 4A), demonstrating that the incision of an AP-site is not retarded by the presence of a second AP-site but rejoining of the resulting SSB is inhibited when the AP-sites are 3′ to each other (see below). When a second AP-site is in the +5 position, the AP-site is rapidly incised as if it were present as an isolated lesion; however, a small amount of rejoining does take place (up to 18% during the 60 min incubation), suggesting that incomplete incision of the opposing AP-site on the nonlabeled strand occurs in the +5 position. An AP-site present in the +1 position results in rapid incision (within 1 min) of ∼60% of the AP-site on the labeled strand, seen as a greater amount of the intact 40mer remaining compared with that seen with the control oligonucleotide (fixed AP-site) (Figure 4A). The amount of intact 40mer band, corresponding to ∼40% of the initial oligonucleotide, decreases slowly with time in the nuclear extract, although 20% of the oligonucleotide still remains intact at 60 min.

Since little, if any, restoration of the 40mer band was seen regardless of which oligonucleotide strand was end-labeled with ^{32}P, it is proposed that mainly DSBs are formed during the processing of the two AP lesions present within a clustered damage site. To test this hypothesis, the products

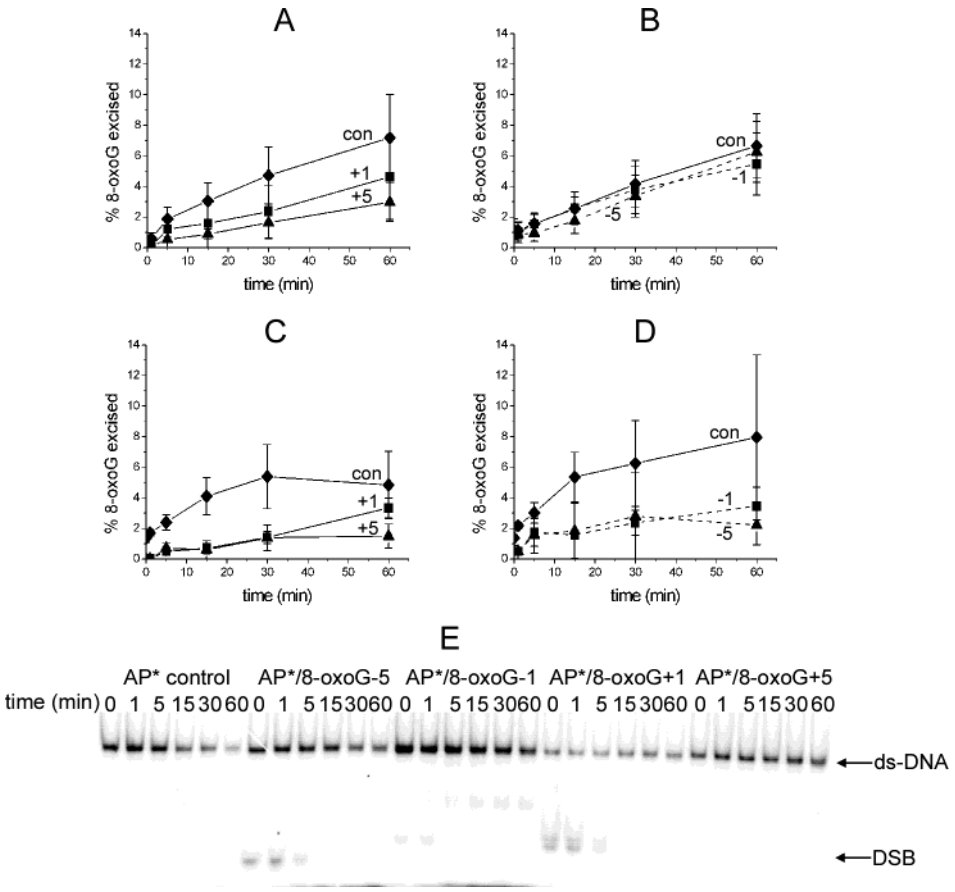

FIGURE 3: Effect of an AP-site on the rate of excision of 8-oxoG when oligonucleotides were incubated in xrs5 nuclear extracts for various times. The ^{32}P-5′-end-labeled oligonucleotide strand contained 8-oxoG. (A and B) Excision of 8-oxoG and (C and D) excision of 8-oxoG when dTTP was substituted with ddTTP: (—◆—) 8-oxoG control (no AP-site), (—■—) oligonucleotide with an AP-site at position +1, (—▲—) oligonucleotide with an AP-site at position +5, (- - -■- - -) oligonucleotide with an AP-site at position −1, and (- - -▲- - -) oligonucleotide with an AP-site at position −5. To assist the reader, lines have been drawn on the graphs between the points only to guide the eye and do not represent fitted curves. (E) Representative native polyacrylamide gel demonstrating that a band due to DSB is not observed when a clustered damage site, consisting of an AP-site on the strand opposite from 8-oxoG, is incubated with xrs5 nuclear extracts under conditions of repair.

of the repair assay were run on a native polyacrylamide gel. Figure 4C shows a large number of DSBs are indeed formed when two AP lesions are opposing each other. The percentage of clustered AP-sites converted into DSBs is shown in Figure 4B. Approximately 90% of the oligonucleotides are converted into DSBs when the two AP-sites are in the −5, −1, and +5 positions. Conversely, only 40−60% of the oligonucleotides are converted into DSBs over the 60 min incubation when the AP-sites are +1 to each other (Figure 4B). These observations are compatible with the levels of intact 40mer seen in Figure 4A, indicating that a proportion of the AP-sites remain intact so that incision of both AP lesions within the clustered damage site does not always occur, reflecting the lower levels of DSBs that are seen.

To investigate if any repair of the DSB formed by incision of the two AP-sites takes place, the repair assays were repeated using nuclear extracts prepared from the xrs5 parental cell line CHO-K1, which are competent in DSB rejoining. The results presented in panels D and E of Figure 4 demonstrate that the yields of DSB are independent of the presence of Ku_{80} and that repair of the DSB does not take place at the concentration of extract that is used. Interestingly, the observations with the oligonucleotide containing the AP-sites +1 to each other in the CHO-K1 extracts (Figure 4D,E) are very similar to those with the xrs5 extracts, showing that only 50−60% of the clustered damage sites are converted to DSBs. In addition, the presence or absence of Ku_{80} in the extracts does not significantly affect the incision of the AP lesions within the clustered damage site or the repair of an AP lesion when present as a single lesion.

DISCUSSION

Ionizing radiation induces a number of DNA lesions, including SSBs, DSBs, sugar modifications, and modified bases, and the induction of some of these lesions in clusters is thought to contribute to the detrimental biological effects of radiation. This study demonstrates that the processing of an AP-site and 8-oxoG or two AP-sites when present on opposing DNA strands within a clustered damaged site by the BER pathway may be compromised, depending on the orientation and interlesion separation of the two lesions. The major findings of this study using nuclear extracts are (i) 8-oxoG impairs the repair of an AP-site when it is positioned one base 5′ or one or five bases 3′ with respect to the AP-site, (ii) 8-oxoG and AP-sites within a clustered damage site are processed sequentially with the AP-site being repaired prior to incision at 8-oxoG, and (iii) two opposing AP-sites are not repaired by the BER pathway but are preferentially converted into a DSB, presumably by the action of AP endonuclease, except in the +1 position.

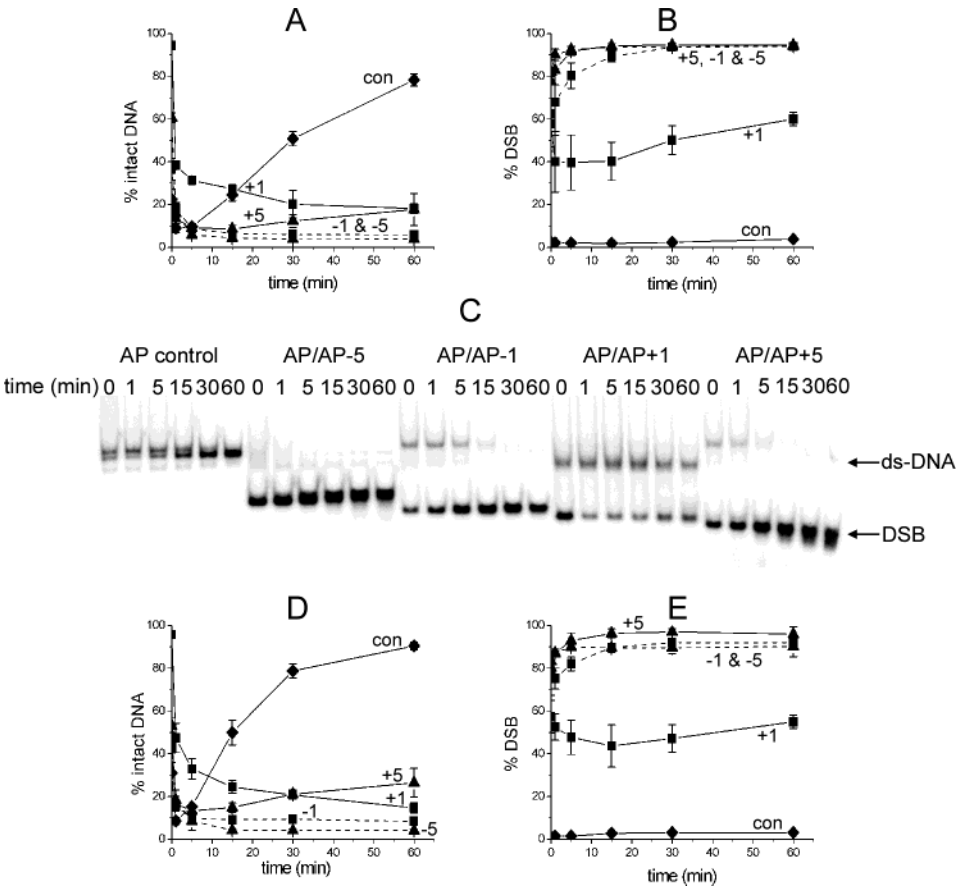

FIGURE 4: Presence of an AP-site does not impair the incision of a second AP-site. (A) Intact DNA following incubation of an oligonucleotide containing two opposing AP-sites with xrs5 nuclear extract. (B) Formation of DSB following incubation of an oligonucleotide containing two opposing AP-sites with xrs5 nuclear extract. (D) Intact DNA following incubation of an oligonucleotide containing two opposing AP-sites with CHO-K1 nuclear extract. (E) Formation of DSB following incubation of an oligonucleotide containing two opposing AP-sites with CHO-K1 nuclear extract: (—◆—) AP-site control (no 8-oxoG), (—■—) oligonucleotide with 8-oxoG at position +1, (—▲—) oligonucleotide with 8-oxoG at position +5, (- - -■- - -) oligonucleotide with 8-oxoG at position −1, and (- - -▲- - -) oligonucleotide with 8-oxoG at position −5. The efficiency of the HAP1 present in the nuclear extract is such that a few seconds at room temperature is sufficient for most of the AP-site contained in the oligonucleotide to be incised (see the 0 min time point). To assist the reader, lines have been drawn on the graphs between the points only to guide the eye and do not represent fitted curves. (C) Representative native polyacrylamide gel demonstrating that a band due to DSB is observed when a clustered damage site consisting of an AP-site on the strand opposite from 8-oxoG is incubated with xrs5 nuclear extracts under conditions of repair.

Previous studies have demonstrated that 8-oxoG reduces the efficiency of repair of a HAP1-SSB on the opposite strand even when separated by up to five bases (by nuclear extract) (*19*). An AP-site when positioned one base 3′ to either 8-oxoG or thymine glycol on the opposing strand also retards repair of the AP-site (*27*). In the study presented here, evidence that the presence of 8-oxoG at positions +1, −1, and −5 with respect to the AP-site results in retardation of the rejoining of the SSB arising from the rapid incision of the AP-site is presented. Retardation of the DNA ligase (following addition of the missing base) present in the xrs5 nuclear extract is proposed to be mainly responsible for this stalled repair of the AP-site when 8-oxoG is on the opposing strand (Figures 1 and 2), since the rate of addition of one base into the gap resulting from incision of the AP-site is the same in the presence or absence of 8-oxoG. This proposal is consistent with recent findings demonstrating that retardation of the ligase III−XRCC1 complex is the major influence on the reduced level of rejoining of a HAP1-SSB when present within a clustered damage site containing 8-oxoG by short-patch repair (*19*). Interestingly, the repair of the SSB arising from the incision of the AP-site by nuclear extracts shows some small differences when compared with the findings for repair of a HAP1-SSB, as discussed later.

The pathway for repair of the AP-site in the presence of 8-oxoG depends on the orientation of the two lesions with respect to each other. For instance, when 8-oxoG and the AP-site are 5′ with respect to one another, the AP-site is repaired by short-patch BER, as occurs with an AP-site present as an isolated lesion. In contrast, the AP-site is repaired by long-patch BER when 8-oxoG is in the 3′ orientation with respect to the AP-site, although short-patch BER may compete. The relative contributions of the long- and short-patch BER pathways will reflect the weakened ability of the ligase(s) involved in short-patch BER to compete effectively with recruitment of protein(s) involved in initiation of long-patch BER. Indeed, it has been observed previously that in the absence of DNA ligase, DNA polymerase β may insert additional nucleotides (*19, 28*), although the observed long-patch repair is not due to chain elongation (see the Results). The recognition of the SSB, resulting from cleavage of the AP-site, by DNA ligase could offer an explanation for the asymmetry of the repair mechanism for an AP-site in the proximity of an opposing 8-oxoG. For

instance, DNA ligase III binds to four nucleotides 5′ and 14 nucleotides 3′ to the SSB (*29*). As the binding of DNA ligase III is asymmetric with respect to the SSB, any distortion in the DNA would need to have a greater effect on the binding of DNA ligase III when the SSB is 3′ rather than 5′ with respect to the 8-oxoG.

That the repair of the AP-site and 8-oxoG occurs sequentially, with the AP-site being repaired before 8-oxoG is excised, serves to minimize the potential to form DSB (<4% DSB formation observed). The reduced efficiency of excision of 8-oxoG when an AP-site is one or five bases 5′ to 8-oxoG on the opposing strand (Figure 3) is consistent with retardation of incision at 8-oxoG by glycosylases when an AP-site or a SSB is on the opposing DNA strand (*14*, *16*, *30*, *31*). This retardation seen with AP-sites is due to the rapid conversion of the AP-site into a SSB. In the nuclear extract, the AP-site is converted rapidly (within 1 min) into a SSB (Figure 1A) which should cause retardation of the incision at 8-oxoG (Figure 3A). Base lesions, AP-sites, and SSBs may also disrupt the stability of the DNA duplex and impair the binding and/or recognition by enzymes involved in the BER pathway to damaged DNA. Indeed, the efficiency of binding of Fpg to 8-oxoG is reduced when a SSB is on the opposing DNA strand (*31*).

As mentioned earlier, the repair of an AP-site within a clustered damage site containing 8-oxoG is slightly different from that of a HAP1-SSB in a similar clustered damage site (*19*), even though the AP-site is converted rapidly (within 1 min) into a HAP1-SSB, presumably by incision of the AP-site by AP endonuclease in the xrs5 nuclear extract (see Figure 2S of the Supporting Information). The differences seen during the repair of an AP-site in this study and a HAP1-SSB (*19*) in the context of a clustered damage site are as follows: (i) A larger impairment of the efficiency of repair of a HAP1-SSB is compared with that of an AP-site by 8-oxoG. (ii) The HAP1-SSB is repaired mainly by long-patch processes when 8-oxoG is 3′ to HAP1-SSB, whereas for the corresponding cluster containing an AP-site and 8-oxoG, repair of the AP-site occurs by both long- and short-patch repair processes. (iii) Six nucleotides are inserted into the repair patch for HAP1-SSB (*19*), whereas insertion of five nucleotides predominates for an AP-site when at position −5 with respect to the 8-oxoG. BER is a coordinate process orchestrated by various proteins (reviewed in refs *32* and *33*). With an AP-site, AP endonuclease will be the first protein to be recruited to the AP-site which remains bound to the resulting SSB until DNA polymerase β binds and fills the SSB gap. DNA polymerase β has been shown to bind DNA ligase 1 and XRCC1 which also forms a complex with DNA ligase III. When the double-stranded oligonucleotide containing a HAP1-SSB is repaired, the HAP1-SSB is initially present as a naked SSB so that AP endonuclease is not required during the processing of the SSB. PARP is also known to bind naked SSB (*34*), bind XRCC1, and recruit DNA polymerase β and ligase III (*35*). Whether PARP is involved in the repair of the AP-site is as yet unknown. Therefore, the two different lesions (HAP1-SSB and AP-site) may recruit different base excision repair proteins which have different sensitivities to the distortion caused by a nearby 8-oxoG lesion. In addition, the different ligases and polymerases involved in BER could have differing efficiencies.

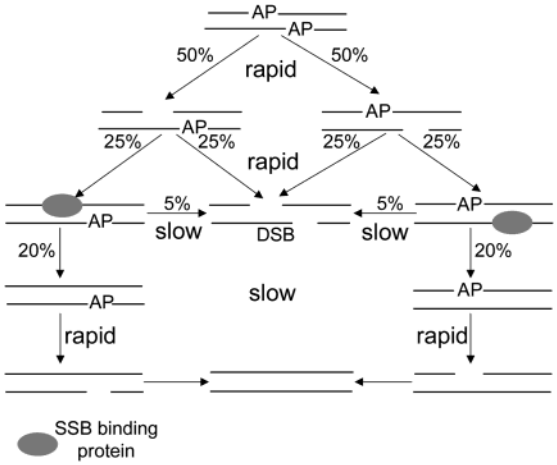

FIGURE 5: Schematic representation of the repair of two opposing AP-sites in the +1 position. There is an equal chance of either of the AP-sites within the cluster being incised first. Fifty percent of the double-stranded oligonucleotide containing a single AP-site and a SSB (resulting from incision of the first AP-site) will rapidly form DSBs through the incision of the second AP-site. Attempted repair of the SSB will then occur, initiated by binding of a repair protein to the SSB. The binding of this protein may retard the incision of the second AP-site, and conversely, the second AP-site may retard the repair of the SSB, resulting in a competition between SSB repair and AP-site incision. Thus, there will be a slow formation of further DSB together with a slow rejoining of the SSB. Once the SSB is rejoined, the remaining AP-site can be incised, followed by repair of the arising SSB.

The observed conversion of a clustered DNA damage site containing two AP-sites into DSB (>85% efficiency), within 5 min in nuclear extracts even when the repair enzymes for an AP-site are present, is consistent with the observation that two closely opposing uracil residues are ultimately converted into DSB when a plasmid construct containing the clustered damage is transformed into *Escherichia coli* (*36*). Only when the AP-sites are separated by one base in the 5′ orientation (+1 position) is the yield of DSB reduced, since 40% of the double-stranded oligonucleotide remains intact, even though one of the two AP-sites within the clustered damage site is converted to a SSB per oligonucleotide (Figures 4 and 5). Following the incision of one of the AP-sites (within 1 min) to give a SSB opposite the second AP-site, it is proposed as shown in Figure 5 that a protein binds to this SSB to initiate its repair in competition with rapid incision of the second AP-site by AP endonuclease. The protein bound to the SSB could prevent incision of the second AP-site, reflecting the known retardation of HAP1 incision of an AP-site when at position +1 with respect to a SSB (*17*, *18*). A slow incision of the remaining AP-site into additional DSBs over the course of 60 min occurs in competition with rejoining of the SSB (Figure 5). If the SSB is repaired prior to incision of the second AP-site, the second AP-site would only be rapidly incised once the SSB is rejoined. This process would give an oligonucleotide containing only a single SSB. The slow formation of this second SSB over 60 min (Figure 4B,D) is consistent with the time scale for rejoining of the original SSB (see Figure 1B) followed by rapid incision of the AP-site on the opposite strand to give the second SSB.

Two groups have recently shown that clustered DNA damage sites lead to up to 6 times more mutations in wild-type *E. coli* than composite lesions (*22*, *23*), and these mutations were found to be predominately due to stalled

replication. An implication of the stalled repair of clustered DNA lesions is that the lesions may still be present at replication. AP-sites are noncoding lesions, and purines are preferentially inserted opposite AP-sites, with adenine being preferred over guanine (*37*, *38*). Similarly, adenine (not cytosine) is preferentially incorporated opposite 8-oxoG by replicative polymerases (*39*). Incorrect incorporation of bases can lead to transversions and hence mutations; for example, 8-oxoG is known to be highly mutagenic, leading to G·C → A·T transversions (reviewed in refs *13* and *40*). The conversion of two closely opposing AP-sites into a DSB could be viewed as the conversion of two potentially mutagenic lesions into a potentially lethal lesion, since one DSB present within a cell can result in its death (*41*), or alternatively, a DSB can lead to chromosomal aberrations and genomic instability (reviewed in refs *20* and *21*).

This study presents evidence demonstrating that clustered DNA damage sites induced by ionizing radiation must be considered when assessing the biological consequences of exposure to ionizing radiation. It has been established that DSBs are not the only important and biologically significant lesion caused by ionizing radiation, as base lesions, AP-sites, and SSBs can be formed in clusters over short regions of DNA that compromise the BER pathway. The processing of clustered DNA damage sites is dependent not only on the lesions that are contained within the cluster but also on the interlesion separation. Impairment in the processing of DNA clustered damage sites can lead to mutation induction, chromosomal aberrations, genomic instability, and ultimately tumorigenesis.

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SUPPORTING INFORMATION AVAILABLE

Rejoining of the AP-site is independent of the sequence context of the AP-site (Figure 1S), and the AP-site is incised by an AP endonuclease in the xrs5 nuclear extract (Figure 2S). This material is available free of charge via the Internet at http://pubs.acs.org.

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