Characterization of complex apurinic/aprimidinic-site clustering associated with an authentic site-specific radiation-induced DNA double-strand break

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Radiation lethality is largely attributed to radiation-induced DNA double-strand breaks (DSBs). A range of structural complexity is predicted for radiation-induced DSBs. However, this lesion has never been analyzed in isolation at the molecular level. To address this problem, we have created authentic site-specific radiation-induced DSBs in plasmid DNA by triplex-forming oligonucleotide-targeted ^{125}I decay. No significant difference in DSB yield was observed after irradiation in the presence or absence of the radical scavenger DMSO, suggesting that DSB formation is a result of the direct effect of the radiation. A restriction fragment terminated by the DSB was isolated and probed with the *Escherichia coli* DNA repair enzyme endonuclease IV (endo IV), which recognizes apurinic/aprimidinic (AP) sites. Enzymatic probing demonstrated clustering of AP sites within 10 bases of the ^{125}I -targeted base in the DNA duplex. Our results suggest scavengeable radicals may not play a large role in the generation of AP sites associated with DSB formation, because at least 30% of all fragments have endo IV-sensitive sites, regardless of irradiation conditions. An internal control fragment recovered from the ^{125}I linearized plasmid did not exhibit endo IV sensitivity in excess of that observed for a similar fragment recovered from an undamaged plasmid. Thus, AP site clustering proximal to the DSB resulted from the ^{125}I decays responsible for DSB formation and was not due to untargeted background irradiation.

DNA damage clustering | multiply damaged site | enzymatic probing | high linear energy transfer | triplex-forming oligonucleotide

Radiation causes a myriad of cellular and molecular effects, but the effect by which it produces cell death is thought to be largely a function of DNA damage (1, 2). Although ionizing radiation causes a variety of lesions in DNA, double-strand breaks (DSB) are implicated as being principally responsible for radiation lethality (3–10). Therefore, insights into the molecular mechanisms of radiation lethality depend on understanding radiation-induced DSB structural characteristics and the aspects of these structures that lead to cytotoxicity.

Typically, as ionizing radiation linear energy transfer (LET) increases, the cytotoxicity of the radiation increases. Cells exposed to low-LET radiation typically exhibit a linear-quadratic survival curve with an initial shoulder that is characteristic of a cell's radiation sensitivity and DSB repair capacity. In contrast, cells exposed to high-LET radiation present a nearly linear survival curve (11). The lack of a shoulder in high-LET survival curves is thought to reflect high structural complexity and decreased repairability of the DSBs produced (4, 12–15). In contrast, low-LET radiation (e.g., x-rays and γ -rays) is predicted to produce DSBs with a range of structural complexity, of which only a small subset with high structural complexity may actually be responsible for the majority of cytotoxic effects (4, 10, 13, 16). Thus, knowledge of DSB structural features produced by high-LET-like processes may provide generalized insights into the molecular mechanisms responsible for radiation cytotoxicity.

Biological and computational studies have been used to investigate the structural complexities of radiation-induced DSBs (17–20).

In most biological studies, DSBs have been produced by beam irradiation, thus the sequence at the break point is not known, and DSB structural features that may influence repair cannot be, and have not been, directly determined. Although plasmid DNA and oligonucleotides have been subjected to ligand-mediated targeting and positioned radionuclide incorporation, respectively, to achieve controlled production of radiation-induced DSBs, the breaks have primarily been treated as biophysical phenomena, and structural properties that might affect repair have not been determined (21–25). Such an analysis is needed, particularly in light of the observation that the yield and distribution of base damage produced in association with radiation-induced DSBs may play an important role in DSB repair (26). However, a radiation-induced DSB has never been isolated and analyzed at the molecular level. To address this problem, we have created an isolated radiation-induced DSB and analyzed its molecular structure by enzymatic probing. A model site-specific high-LET-like DSB was produced by ^{125}I decay in a plasmid bound by a ^{125}I -labeled triplex-forming oligonucleotide (^{125}I -TFO). Triplex-forming oligonucleotide (TFOs) display sequence specificity similar to that of Watson–Crick base pairing and permit targeting of a unique DNA duplex sequence (27, 28).

The decay of ^{125}I results in an average emission of ≈ 20 short-range (several nanometers) Auger electrons of varying energy (most < 1 keV) and a gamma photon, producing a highly positively charged tellurium daughter atom (29). The simultaneous action of the low-energy Auger electrons and charge neutralization of the tellurium daughter atom produces a high-density energy deposition in close vicinity of the decay site. The immediate effect is significant damage in adjacent molecular structures, which results in high-LET-like biological effects when decay occurs within or very near DNA (30, 31).

Decay of ^{125}I within the triplex produces strand breaks in the target plasmid duplex within ≈ 10 bp of the decay site at an efficiency of close to one DSB per decay (24, 32). Therefore, relatively large quantities of DNA with DSBs in a known sequence context can be produced by this approach. Because the sequence position of the damage is predetermined, molecular analysis of the damage site can be attempted. To this end, a short restriction fragment terminated by the DSB end was isolated and probed for apurinic/aprimidinic (AP) sites with the *Escherichia coli* DNA repair enzyme endonuclease IV (endo IV), which hydrolyzes the phosphodiester bond of abasic backbone deoxyribose sugars.

In this study, we demonstrate AP site damage associated with a complex radiation-induced DSB. Our results indicate a high degree of AP site clustering within 8–10 bp of the DSB end. Formation of such clusters does not seem to depend on scavengeable radicals. Further analysis of an internal control fragment from the damaged DNA indicates that the AP site clus-

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Abbreviations: DSB, double-strand break; ^{125}I -TFO, ^{125}I -labeled triplex-forming oligonucleotide; LET, linear energy transfer; TFO, triplex-forming oligonucleotide.

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Fig. 1. The pTC27 target sequence aligned with the ¹²⁵I-TFO. The TFO's 3'-terminal 5-[¹²⁵I]-dC residue is indicated in bold italics. Restriction sites used to obtain fragments for analysis are also indicated.

tering is a direct result of DSB formation and not due to untargeted background radiation.

Materials and Methods

Detailed materials and methods are provided as *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. Briefly, an ^{125}I -labeled 27-mer pyrimidine-motif TFO was synthesized by primer extension as described in ref. 24 and bound to the polypurine TFO-binding site of plasmid pTC27 (Fig. 1). The triplex-containing plasmid was divided equally, and one portion was brought to 2M DMSO. Both samples were stored at -80°C for 1 month to accumulate decays. After damage accumulation, linear plasmid containing a single site-specific DSB was purified (Fig. 6, which is published as supporting information on the PNAS web site), and a short fragment terminated by the ^{125}I -induced DSB was obtained by BglII digestion and purified (33). The DSB-terminated BglII fragment upper and lower strands were then differentially $5'$ or $3'$ ^{32}P -end-labeled and probed for AP sites with endo IV.

Results

Endo IV Probing of the 5' End-Labeled BglII Fragment(s) Upper Strand(s). Initially, DSB-terminated BglII fragments 5'-³²P-end-labeled in the upper strand were investigated (Fig. 2A). Fourteen fragments, representing 14 sites of ¹²⁵I decay-induced breakage, were detected in the damaged DNA, regardless of scavenging conditions during irradiation (Fig. 2B, lanes 3 and 5). For ease of presentation, the bands are numbered as the breaks progress toward the BglII restriction site.

Endo IV treatment revealed significant changes in band density and a distinctive pattern compared with unprobed damaged DNA (Fig. 2*B*). Specifically, a density increase in band A₉ (282% and 269% in (+)/(-) DMSO, respectively) and reduced density at G₁ through A₇ were observed (Fig. 2*C* and *D*). In addition to A₉, a small density increase was consistently noted at position A₈ in samples irradiated in the absence of DMSO.

Increasing density at positions A₈ and A₉, along with the density decreases observed at positions G₁ through A₇, indicates that the quantity of these fragments had increased as a result of cleavage of the longer fragments. Accumulation of fragments A₈ and A₉ unambiguously establishes AP sites at these positions. In contrast, the density of A₁₀ through G₁₄ did not change, indicating that any endo IV-mediated loss of these fragments had either been replaced by cleavage of longer fragments or that these fragments lacked sensitivity to the enzyme. An undamaged 47-mer control oligonucleotide, with the same sequence as the BglII/MblI restriction fragment that spans the ¹²⁵I-TFO target site of the pTC27 plasmid (Fig. 1), was not sensitive to endo IV under identical treatment conditions, indicating damage-specific endo IV cleavage of the ¹²⁵I-TFO damaged fragments (Fig. 2B, lanes 7 and 8). Lane-to-lane loading variability assessed as a function of this control over multiple experiments was an average of $3.7 \pm 2.3\%$ (*Supporting Materials and Methods*).

Analysis of the density changes shows bands G₁ through A₇ were reduced a total of 42% and 38% in the (+) and (-) DMSO samples, respectively, whereas the total density of bands A₈ through G₁₄ were increased 143% and 146% in (+) and (-) DMSO, respectively. This density redistribution demonstrates that fragments resulting from breaks closest to the ¹²⁵I decay site

contain the highest proportion of AP sites. Also, an AP site hotspot may exist at A₉. However, preferential cleavage at A₉ may be due to steric effects of the damaged DNA on endo IV, not to preferential AP site formation at this position (34).

Despite density increases of some bands, there were net total density decreases of $24.39 \pm 0.05\%$ and $27.53 \pm 0.67\%$, respectively, in the endo IV-treated (+) DMSO and (-) DMSO samples (Table 1). This overall loss of total density after endo IV treatment suggests that cleavage also occurs near the labeled 5' end of the fragments, i.e., within the nine bases closest to the BglII terminus.

Endo IV Probing of the 3' End-Labeled BglII Fragment(s) Upper Strand(s). Taq DNA polymerase can incorporate a single untem-

plated dATP to blunt-ended DNA, or misincorporate dATP at 5'-overhanging template ends at high frequency (ref. 35 and data not shown). Therefore, Taq was used to attempt 3' end labeling of the BglII fragment(s) upper strand (Fig. 3A). The BglII fragment's upper strand 3' end constitutes the ¹²⁵I decay-induced DSB end of the fragment and would be expected to be blocked by a nucleotide fragment or a 3'-phosphate, as has been shown for other radiation-induced strand breaks (36–38). If this result were the case, Taq polymerase 3'-end labeling should fail, because Taq requires a 3'-OH to initiate polymerization. Surprisingly, Taq labeled a subset of DSB ends (≤15%), indicating the presence of 3' OH ends on this subset of molecules.

Endo IV treatment of the Taq-labeled upper strands produced a net density loss of $26.70 \pm 0.29\%$ and $20.86 \pm 0.40\%$, respectively, in the (+) DMSO and (-) DMSO samples (Table 1). The graphic representation of the gel (Fig. 3 *B* and *C*) shows no endo IV-dependent increase in any band of either sample, and the only significant density decreases occurred in fragments near the breakage maximum. Therefore, fragments with the highest breakage frequency, occurring closest to the ^{125}I decay site, have the highest proportion of AP sites. Thus, most endo IV-sensitive AP sites in this fragment subset are clustered near the DSB end.

Endo IV Probing of the 3' End-Labeled BglII Fragment(s) Lower Strand(s). The BglII fragment(s) lower strands were labeled at the

restriction cut 3' end by *exo*⁻ Klenow DNA polymerase extension (Fig. 4A). This reaction resulted in a [α -³²P]dCMP terminated four-nucleotide extension of the lower strands.

Table 1. Densitometric change after endo IV treatment

Treatment	Density lost, %*	
	5' end-labeled	3' end-labeled
Endo IV-treated upper strand		
(+) DMSO	24.39 ± 0.05 [†]	26.70 ± 0.29 [‡]
(-) DMSO	27.53 ± 0.67 [‡]	20.86 ± 0.40 [†]
Endo IV-treated lower strand		
(+) DMSO	17.51 ± 0.83 [‡]	36.79 ± 0.78 [†]
(-) DMSO	21.61 ± 0.55 [†]	25.67 ± 1.48 [‡]

*Density lost is the percent total density reduction in the endo IV-treated sample lanes of the gels with respect to the total density observed in the untreated damaged DNA control lanes.

[†]Percentages are shown $\pm$ the SEM for three independent determinations.

[‡]Percentages are shown $\pm$ the SEM for two independent determinations.

Fig. 2. Endo IV probing of DSB-terminated upper strand 5' end-labeled BglIII fragments. (A) The longest model BglIII fragment. An asterisk indicates the 5' ³²P-end label, and the ¹²⁵I-TFO-targeted duplex G is indicated in bold. The subscripted number permits correlation of this sequence to those in *B-D* and Fig. 1. *a*, the site of maximum breakage. (B) A typical 20% denaturing PAGE separation of DSB-terminated upper strands. Lanes: 1 and 2, Maxam and Gilbert "G" and "G+A" sequencing ladders; 3 and 5, ¹²⁵I-DSB-terminated BglIII fragments produced by irradiation in the presence (+) or absence (-) of 2M DMSO, respectively; 4 and 6, endo IV-treated BglIII fragments (+) or (-) DMSO, respectively; 7, undamaged duplex 47-mer control fragment; 8, endo IV-treated 47-mer control. Bands representing break points at nucleotides within the target sequence are numbered according to their position with respect to the longest fragment identified, counting up as the breaks progress toward the BglIII restriction site. (C) Densitometric quantification of bands observed for three experiments, including that shown in *B* for DNA irradiated in the presence of DMSO. Band densities are reported as a percentage of the sum total deconvoluted density of the bands in the untreated-damaged-DNA control lanes and were determined as described in *Supporting Materials and Methods*. ♦, untreated BglIII fragments (*B*, lane 3); △, endo IV-treated fragments (*B*, lane 4). (D) Densitometric quantification of bands observed for two experiments, including that shown in *B* for DNA irradiated in the absence of DMSO. ♦, untreated BglIII fragments (*B*, lane 5); △, endo IV-treated fragments (*B*, lane 6). Error bars represent the standard error of the mean. In some cases, the error is smaller than the label used to indicate the data point.

Fig. 3. Endo IV probing of DSB-terminated upper strand 3' end-labeled (DSB end) BglII fragments. (A) The BglII model fragment. An asterisk indicates the 3' ³²P-end label, and the ¹²⁵I-TFO targeted duplex G (which is also the DSB breakage maximum) is indicated in lowercase bold type. The subscripted number permits correlation of this sequence to those in B and C and Fig. 1. (B) Denitometric quantification of bands observed in two experiments by 20% denaturing PAGE for DNA irradiated in the presence of 2M DMSO. Band density is reported as a percentage of the sum total deconvoluted density of the bands in the untreated-damaged-DNA control lanes. ♦, untreated BglII fragments; △, endo IV-treated fragments. (C) Denitometric quantification of bands observed in three experiments for DNA irradiated in the absence of DMSO. ♦, untreated BglII fragments; △, endo IV-treated fragments. Typical PAGE results are shown in Fig. 7, which is published as supporting information on the PNAS web site. Error bars represent the standard error of the mean. In some cases, the error is smaller than the label used to indicate the data point.

The lower strand damage pattern exhibits higher breakage frequency over a broader range and greater endo IV sensitivity than observed in the upper strand (Fig. 4 *B* and *C*). This result may be due to a slight distance increase between ^{125}I and the lower strand deoxyribose backbone in pyrimidine-motif triplex structures, which would broaden the range of decay effects (39, 40). Apparently, scavengeable radicals do not affect this peak broadening, because the breakage pattern is similar, regardless of irradiation conditions.

Inspection of the fragment density distribution after digestion of the DNA damaged in the presence of DMSO (Fig. 4B) shows a fairly uniform endo IV cleavage sensitivity between fragments T₄ (the DSB maximum) and C₁₀, whereas fragments A₂ and C₃ are more sensitive to endo IV digestion with $\geq 47\%$ of these fragments cleaved by the enzyme. Interestingly, high endo IV sensitivity at A₂ and C₃ is observed only in the (+) DMSO sample; the (−) DMSO irradiated DNA shows similar endo IV sensitivity throughout the DSB fragment spectrum, with those fragments that are most sensitive encompassing the DSB maximum and ranging from T₁ to T₇ (Fig. 4C). These results suggest that AP site damage in the lower strand is tightly clustered around the ¹²⁵I decay site. Further, in this specific circumstance at least, DMSO seems to affect the relative AP site distribution but not yield. This finding is suggested by the convergence of the

in their upper and lower strands. However, because irradiation occurred in a frozen aqueous mixture of plasmid/triplex molecules, the AP site clusters might not be a direct result of the DSB-forming decay event but may reflect nonspecific background damage. To test this hypothesis, we isolated a 49-bp internal control restriction fragment from 1,354 bp upstream of the ^{125}I -TFO-target site of the ^{125}I -linearized plasmids used to generate BglII fragments for endo IV probing. These internal control fragments were essentially insensitive to endo IV cleavage (Fig. 10, which is published as supporting information on the PNAS web site). This result indicates a lack of nonspecific radiation-induced AP site background damage, because the internal control fragments were no more endo IV sensitive than the equivalent fragment from unirradiated plasmid. Therefore, the AP site clusters found in close proximity to the ^{125}I -induced DSBs are a direct consequence of the decays that formed the DSBs.

Discussion

Both low- and high-LET radiation produce temporally and spatially grouped ionizations in the medium with which they interact. More than 20 years ago, Ward hypothesized that this unique mechanism of radiation-derived energy deposition would lead to spatially clustered DNA lesions (12, 42). Such lesions (termed locally multiply damaged sites) have been predicted to occur in numerous forms, including base damage in one or both DNA strands, combinations of base damage and single-strand breaks, simple DSBs, and DSBs with proximal damage (13). Radiation cytotoxicity has largely been attributed to radiation-induced DSBs, with the high per-lesion cytotoxicity of high-LET radiation attributed to more DSB-associated lesion clustering, resulting in DSBs that are more refractory to repair than simple double-stranded discontinuities (4, 26, 43, 44). This idea is further supported by the demonstration that repair enzyme mediated conversion of clustered base lesions to DSBs *in vivo*, which can produce DSBs that may involve proximal base damage, are important contributors to the toxicity of low-LET radiation (45–47).

Consequently, the structural organization of an individual radiation-induced DSB is likely to be an important determinant of its reparability and, thus, the relative yield of complex DSBs (defined here as a double-stranded discontinuity with secondary lesions proximal to the DSB end) per given radiation dose may determine the cytotoxicity of the radiation. A clear understanding of the biochemical mechanisms responsible for radiation cytotoxicity, and the biochemistry required for DSB repair, cannot be achieved without understanding the structure of the lesion with which the repair machinery must interact.

In this report, we have confirmed (with respect to AP site damage) Ward's locally multiply damaged sites hypothesis for radiation-induced DSBs by demonstrating damage clustering associated with the high-LET-like DSB produced by ^{125}I decay. To differentiate the role of indirect effects produced by scavengeable hydroxyl radicals from the direct effects produced by ^{125}I decay, irradiations were conducted in the presence and absence of the radical scavenger DMSO. The total DSB yield was not affected by these conditions, suggesting that most DSBs formed by ^{125}I -TFO targeting under our conditions are the result of direct effects.

To investigate DSB structure and assess for the presence of AP sites proximal to the DSB end, we isolated damaged plasmid DNA that had been linearized by TFO targeted ^{125}I decay and recovered a short restriction fragment terminated by the DSB end. Separate upper and lower strand labeling at the fragment's restriction cut end permitted localization of the DSB maximum to the A-T base pair immediately upstream of the G targeted for Hoogsteen base pairing by the 5- ^{125}I -dC of the TFO. For ease of presentation, upstream refers to positions in both the upper and lower fragment strands to the left (BglII cut side) of the upper strand target G. Because the BglII end of the fragment

pool is readily accessible to the end labeling methods used, this DSB maximum represents the true DSB induction maximum for our ^{125}I -TFO targeting system. Colocalization of the upper and lower strand breakage maxima suggests that many of the fragments terminated at this position ($\approx 16\%$ of the total) may be blunt ended. However, the unequal breakage distribution observed between the upper and the lower strands indicates that, in general, the DSB ends consist of overhangs.

In contrast, labeling the fragment's DSB-damaged end permitted several noteworthy observations, the first of which was the presence of an upper strand fragment subset with 3' OH ends (Fig. 3). The breakage maximum of this subset is shifted one base downstream to the ^{125}I -TFO G₄ target, and the breakage distribution is narrower than that observed by 5' end labeling (Fig. 2). DMSO did not affect the DSB maximum shift to G₄, but its absence did affect the apparent yield of 3' OH termini, with up to 2-fold more Taq-labeled fragments at G₁, A₂, and T₃. This finding is surprising because radiation-induced scavengeable radicals, such as $\cdot\text{OH}$, are known to cause strand breaks with blocked 3' ends (37, 38).

Labeling the lower strand 5' end (DSB end) also suggests labeling of a fragment subset, because the breakage maximum shifted three bases upstream to T₆ compared with BglIII 3' end labeling (compare Figs. 5 and 4). This shift suggests that many of the 5' ends represented by the BglIII 3' end labeling maximum at T₄ (T₄ in Fig. 4 is equivalent to T₃ in Fig. 5) are either blocked by nucleotide fragments or exist with an end morphology (i.e., 3' overhang) that is not conducive to T4 PNK labeling (41). In contrast to upper strand DSB end labeling, lower strand DSB end labeling resulted in a breakage distribution similar to that obtained by lower strand BglIII end labeling. As with the upper strand, DSB end labeling of the lower strand detected a DMSO-dependent effect: uniform breakage distribution around the maximum at T₆ after irradiation in DMSO, as opposed to the reproducible observation of a secondary maximum at T₄ after irradiation in the absence of DMSO (Fig. 5).

Taken together, these observations suggest scavengeable radicals have a role in the formation of specific DSB end structures in our system, even though they do not seem to affect the overall DSB yield. These results suggest that regardless of the effects of scavengeable radicals upon DSB end structures, the chemical reactions that produce these structures are likely to occur after the decay-associated physical events responsible for initial strand scission.

Endo IV probing of the 5'-end-labeled fragments' upper strand (BgIII cut end) revealed a distinctive cleavage pattern (Fig. 2). All of the fragments from G₁ through A₇ displayed significant enzyme sensitivity and $\geq 38\%$ of their sum density was lost. Most notably, both DMSO (+) and (-) samples displayed an increase in signal for fragment A₉, whereas fragment A₈ was unchanged in (+) DMSO and increased in the (-) DMSO samples. Therefore, some of the cleavage events occurring in G₁ through A₇ occur at A₈ and A₉ and establish these positions as unambiguous AP site locations. However, the total signal increase at these positions does not equal the signal lost from fragments G₁ through A₇; therefore, it appears that additional cleavage closer to the labeled 5' end may also be occurring. Under our experimental conditions fragments, as short as 9 nucleotides were resolved, but no new fragments between 27 nucleotides (the position of A₉; Fig. 2) and the 9-nucleotide limit of resolution were observed after endo IV treatment. These results seem to imply that cleavage sites exist within 8 bp of the 5' end of the upper strand. Similar results were obtained regardless of scavenging conditions during irradiation (Table 1); therefore, the cleavage events responsible for the unaccounted loss of density in fragments G₁ through A₇ are not likely to be due to indirect effects. Our control reactions (Figs. 2 and 7) indicate that the endo IV preparation used in our experiments exhibits no detectable nonspecific nuclease or 5'-phosphatase

activities, whereas AP site-specific cleavage has been demonstrated with this enzyme (see *Supporting Materials and Methods*). Therefore, it seems that these fragments have clusters of AP sites near the DSB end and again near the 5'-labeled end with an intervening series of fragments that possess few AP sites.

Although circumstantial in nature, taken together these observations lead us to speculate that high frequency AP site formation near the 5' end of the upper strand may result from charge migration originating from the base damaging events that take place during strand break formation at the DSB maximum. Such an explanation also appears to be consistent with the likely charge migration characteristics of the upper strand sequence (48). Therefore, the endo IV-dependent loss of $\geq 38\%$ of the fragments within 4 bases of the upper strand DSB maximum suggests that these fragments possess AP sites clustered in this region, in addition to AP sites that may be forming farther upstream.

Production of AP site clusters proximal to the DSB end seems to be largely a result of the direct effect. This conclusion is supported by the results of endo IV probing of the 3' end-labeled upper strand (Fig. 3), where fragments near the break maximum were most sensitive to endo IV. Furthermore, no accumulation of fragments consistent with endo IV cleavage at a distance from the DSB end was observed, as would be expected if AP sites occur at a high frequency near the label at the upper strand 3' end.

Endo IV probing of 3' end-labeled (BglII end) or 5' end-labeled (DSB end) lower strands produced results similar to those obtained with the upper strands. Again, the overall lower strand endo IV sensitivity as a function of total band density lost was similar to that observed for the upper strand (Table 1), and the lack of accumulation of new fragments between T₁ and positions 9 bp from the 3' end of the lower strand suggests the formation of AP sites within 8 bp of the 3' end, possibly through charge migration (Fig. 4). As with endo IV treatment of the

DSB-end-labeled upper strand, endo IV treatment of the DSB-end-labeled lower strand is consistent with AP sites clustering near the DSB end (Fig. 5).

Based on known limitations for endo IV AP site cleavage efficiency that arise from sequence context or cleavage of locally multiply damaged sites in which two AP sites are closely opposed on opposite strands (34, 49), the enzymatic probing analysis of the ¹²⁵I-DSB-terminated BglII restriction fragments examined in this work suggests that at least 50% of the upper and lower strands contain AP sites, and that many fragments are likely to contain undetected lesions and multiple lesions.

Our study shows AP sites occur frequently within 8 to 10 bp of the DSB end, and this clustering is a direct result of the decays responsible for DSB formation. In addition, AP site formation under our irradiation conditions only modestly depends on scavengeable radicals. We have also conducted a companion enzymatic probing study by using the purine and pyrimidine base damage-specific enzymes, Fpg and endonuclease III, in conjunction with GC/MS, to identify specific base lesions. In that study, we detected DSB-associated base damage clustering that depends on scavengeable radicals, and we have observed damage distributions and frequencies similar to those presented here for AP site clustering (unpublished data). Those data, in conjunction with the data related here for AP site clustering, provide a detailed molecular analysis of the structural configuration of a highly complex radiation-induced DSB with biological properties similar to those of high-LET radiation. Using this data to construct model substrates with structural characteristics reflecting those of authentic radiation-induced DSBs should permit a more comprehensive and realistic evaluation of the biochemistry required to repair such lesions.

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