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

Kamal Datta, Ronald D. Neumann, and Thomas A. Winters*

Nuclear Medicine Department, The Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, MD 20892

Edited by Richard B. Setlow, Brookhaven National Laboratory, Upton, NY, and approved June 10, 2005 (received for review May 12, 2005)

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/apyrimidinic (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 scavengable 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 radionucide 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 (125I-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/apyrimidinic (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 scavengable radicals. Further analysis of an internal control fragment from the damaged DNA indicates that the AP site clus-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: DSB, double-strand break; TFO, triplex-forming oligonucleotide; LET, linear energy transfer; TFO, triplex-forming oligonucleotide.

*To whom correspondence should be addressed. E-mail: twinters@mail.cc.nih.gov.
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 125I-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 single site-specific DSB was obtained by BglII digestion and purified (33). The DSB-terminated BglII fragment upper and lower strands were then differentially 5’ or 3’ 32P-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). This reaction resulted in a [32P]dCMP termination at the BglII 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 ± 0.29% and 20.86 ± 0.40%, respectively, in the (+) DMSO and (−) DMSO samples (Table 1). The graphic representation of the gel 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 125I 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 terminal four-nucleotide extension of the lower strands.

![Table 1. Densitometric change after endo IV treatment](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Density lost, %*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo IV-treated upper strand</td>
<td></td>
</tr>
<tr>
<td>(+) DMSO</td>
<td>24.39 ± 0.05†</td>
</tr>
<tr>
<td>(−) DMSO</td>
<td>26.70 ± 0.29‡</td>
</tr>
<tr>
<td>Endo IV-treated lower strand</td>
<td></td>
</tr>
<tr>
<td>(+) DMSO</td>
<td>27.53 ± 0.67†</td>
</tr>
<tr>
<td>(−) DMSO</td>
<td>20.86 ± 0.40‡</td>
</tr>
</tbody>
</table>

*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 ± the SEM for three independent determinations.

‡Percentages are shown ± the SEM for two independent determinations.
for three experiments, including that shown in DMSO, respectively; 4 and 6, endo IV-treated BglII fragments (Methods control lanes and were determined as described in total deconvoluted density of the bands in the untreated-damaged-DNA presence of DMSO. Band densities are reported as a percentage of the sum the BglII restriction site. (Methods the longest fragment identified, counting up as the breaks progress toward the target sequence are numbered according to their position with respect to treated 47-mer control. Bands representing break points at nucleotides within respect to untreated 47-mer control fragment; 8, endo IV-treated fragments. (b) Densitometric quantification of bands observed for 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) Densitometric 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, scavenging 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 T\(_4\) (the DSB maximum) and C\(_{10}\), whereas fragments A\(_2\) and C\(_3\) are more sensitive to endo IV digestion with \(\geq47\%\) of these fragments cleaved by the enzyme. Interestingly, high endo IV sensitivity at A\(_2\) and C\(_3\) 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 T\(_1\) to T\(_7\) (Fig. 4C). These results suggest that AP site damage in the lower strand is tightly clustered around the \(^{125}\)I 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

Fig. 2. Endo IV probing of DSB-terminated upper strand \(^3\)′-end-labeled BglII fragments. (a) The longest model BglII fragment. An asterisk indicates the 5′-32P-end label, and the \(^{125}\)I-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 BglII fragments produced by irradiation in the presence (+) or absence (−) of 2M DMSO, respectively; 4 and 6, endo IV-treated BglII 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 BglII restriction site. (c) Densitometric quantification of bands observed for three experiments, including that shown in B for DNA irradiated in the absence 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 BglII 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 BglII 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 \(^{125}\)I-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) Densitometric 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) Densitometric 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.
endo IV-treated and -untreated band density curves of the (+) DMSO sample between T11 and T14 (Fig. 4B) compared with the band density curves of the (−) DMSO sample, which converge sooner at T8 and then stay relatively parallel until fragment C13. As with the 5′ end-labeled upper strand, the net loss of band density observed after endo IV treatment of the 3′ end (restriction cut end) -labeled lower strand implies the presence of AP sites close to the labeled end (Table 1).

**Endo IV Probing of the 5′ End-Labeled BglII Fragment(s) Lower Strand(s).** Investigation of AP site formation proximal to the BglII fragments’ DSB end was completed by analysis of the lower strand labeled at the DSB terminated 5′ end (Fig. 5A). As with upper strand DSB-end labeling (3′ end), lower strand DSB-end labeling (5′ end) appeared to label a subset of ends. As observed for the 3′ end-labeled lower strand, the DSB pattern of the 5′ end-labeled fragments displayed peak broadening compared with upper strand end labeling (Fig. 5B and C). However, as seen to a lesser extent after upper strand damaged-end labeling, lower strand 5′ end labeling produced a break pattern with the DSB maximum shifted with respect to the maximum obtained by labeling the fragments’ BglII cut end. This shift reflects the individual DSB end’s relative ability to support phosphorylation by T4 PNK after dephosphorylation by calf intestinal alkaline phosphatase (CIAP). Not only does this finding demonstrate formation of 5′ phosphorylateable fragments upstream from the 125I-TFO target site, it indicates that many of the 5′ ends of the fragments representing the DSB maximum obtained by 3′ end labeling of the lower strand are blocked to labeling by T4 PNK. This blockage may be due to 5′ nucleotide fragments that block CIAP and T4 PNK, or it may be a function of DSB end morphology because T4 PNK does not label 5′ recessed ends efficiently (ref. 41 and data not shown).

The endo IV digestion pattern of the 5′ end-labeled lower strand is different from that of the 3′ end-labeled lower strand (Fig. 5B and C). Most notably, the relative net density loss after endo IV treatment is essentially independent of scavenging conditions (Table 1). These results further support 5′-end labeling of only a fragment subset. In addition to the overall reduction in endo IV sensitivity in these samples, those fragments most sensitive to endo IV cleavage were more narrowly distributed about the DSB maxima than was observed for the 3′ end-labeled lower strand.

**AP Site Clustering is a 125I-DSB-Dependent Event.** As a whole, the enzymatic probing data show a high degree of AP site clustering in fragments formed within 4 base pairs upstream and downstream of the DSB maxima, with up to 50% containing AP sites.
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 125I-TFO-target site of the 125I-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 125I-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 multiply damaged sites (12, 42). Such lesions (termed locally damage) Ward's locally multiply damaged sites hypothesis for DSBs.

DSBs are a direct consequence of the decays that formed the 125I-TFO targeting system. Colocalization of the upper and lower strand breakage maxima suggests that many of the fragments terminated at this position (~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 fragment strand subset with 3′ OH ends (Fig. 3). The breakage maximum of this subset is shifted one base downstream to the 125I-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 scavengable radicals, such as -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 was shifted three bases upstream to T6 compared with BglII 3′ end labeling (compare Figs. 5 and 4). This shift suggests that many of the 5′ ends represented by the BglII 3′ end labeling maximum at T6 (T6 in Fig. 4 is equivalent to T5 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 BglII 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 scavengable 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 scavengable 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 (BglII cut end) revealed a distinctive cleavage pattern (Fig. 2). All of the fragments from G1 through A7 displayed significant enzyme sensitivity and ±38% of their sum density was lost. Most notably, both DMSO (+) and (−) samples displayed an increase in signal for fragment A8, 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, most fragments, as short as 9 nucleotides were resolved, but no new fragments between 27 nucleotides (the position of A7, 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 strand. 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 BglIII restriction fragments examined in this work suggests that at least 50% of the upper and lower strands contain AP sites, and that many fragments may 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 DSB-end responsible for DSB formation. In addition, AP site formation under our irradiation conditions only modestly depends on scavengable 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 scavengable 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.

We thank Dr. Igor Panyutin for helpful discussions and critical reading of the manuscript and Dr. Ludwig Feinendegen for his insightful discussions and his support.