Interstrand Cross-Links: A New Type of γ-Ray Damage in Bromodeoxyuridine-Substituted DNA†

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ABSTRACT: Interstrand cross-links (ICL) represent one of the most toxic types of DNA damage for dividing cells. They are induced both by natural products (e.g., psoralens + UVA) and by several chemical agents, some of which are used in chemotherapy (e.g., carboplatin and mitomycin C). Although repair mechanisms exist for interstrand cross-links, these lesions can induce mutations, chromosomal rearrangements, and cell death. Here, we report, for the first time, the formation of ICL by γ-rays in brominated DNA. It is well established that the radiosensitization properties of bromodeoxyuridine (BrdUrd) result primarily from the electrophilic nature of the bromine, making it a good leaving group and leading to the irreversible formation of a uridinyl radical (dUrd$^{\bullet}$) or uridinyl anion (dUrd^{-}) upon addition of an electron. We observe that the radiolytic loss of the bromine atom is greatly suppressed in double-stranded compared to single-stranded DNA. We have used a model DNA containing a bulge, formed by five mismatched bases, and have observed a linear dose−response for the formation of strand breaks on the single-stranded regions of both the brominated strand and the opposite nonbrominated strand. Surprisingly, we have observed the formation of interstrand cross-links exclusively in the mismatched region. Thus, we propose that the radiosensitization effects of bromodeoxyuridine in vivo will almost certainly be limited to single strand regions such as found in transcription bubbles, replication forks, mismatched DNA, and possibly the loop region of telomeres. Our results suggest that interstrand cross-links may contribute to the radiosensitization effects of BrdUrd. These findings may have profound implications for the clinical use of bromodeoxyuridine as a radiosensitizer, as well as for the development of targeted radiosensitizers.

Interstrand cross-links (ICL)[1] are one of the most toxic types of DNA damage for proliferating cells. For example, the Moustacchi group (*1*) found that 120 cross-links per yeast genome correspond to the DL_{37} (the dose necessary to induce, on average, one lethal hit per cell). There are many examples of DNA cross-linking agents, including nitrogen mustard (*2*), psoralens (*3*−*5*), mitomycin C (*6*), and platinum compounds, several of which are used in cancer radiotherapy (*7*, *8*), chemotherapy (*9*−*11*) (e.g., carboplatin), or phototherapy (e.g., psoralens). The toxicity of DNA cross-links probably results from two mechanisms: they prevent strand separation, thus inhibiting both transcription and replication, and they can induce mutations and DNA rearrangements as a result of repair processes (*12*−*14*).

ICL repair in cells involves both DNA excision repair and recombination, thus increasing the complexity of the repair process (*15*−*17*). Studies on Fanconi anemia, a rare autosomal recessive disorder characterized by sensitivity to ICL agents, have identified seven Fanconi anemia genes involved in cell cycle check points and DNA repair (*18*−*20*).

Very early studies showed that γ-rays and X-rays can induce cross-links between individual DNA molecules (*21*, *22*), and more recently, Kypr's group has demonstrated that UVC light can induce cross-links (*23*−*25*) between the complementary strands of DNA without external chemical agents. However, no studies have reported the induction of interstrand cross-links in brominated DNA by ionizing radiation. Studies over the last 40 years have demonstrated that bromodeoxyuridine (BrdUrd) is able to radiosensitize cells (*26*−*29*). In addition, substitution of BrdUrd for thymidine was found to increase radiation-induced DNA single- and double-stranded breaks in living cells (*30*, *31*) as well as chromosomal aberrations (*32*). Recognized as potential tumor radiosensitizers, halogenated thymidine analogues, BrdUrd, iododeoxyuridine (IdUrd), and fluorodeoxyuridine (FdUrd) (*33*, *34*) have been used in several clinical trials (*35*−*37*) to enhance the response of nonhypoxic tumor cells to radiation (*38*−*40*). Some clinical studies have reported radiosensitization of malignant brain tumors by bromodeoxyuridine (*41*), but in general the clinical results have been disappointing, perhaps in part because of a lack

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[1] Abbreviations: A, adenine; AB, bromodeoxyuridine-substituted oligonucleotide 1; AT, nonbromodeoxyuridine-substituted oligonucleotide 2; B, bromouracil; BrdUrd, 5-bromodeoxyuridine; C, cytosine; CC, semicomplementary oligonucleotide 4; DSc, complementary double strand; DSsc, semicomplementary double strand; dUrd^{-}, deoxyuridinyl anion; dUrd$^{\bullet}$, deoxyuridinyl radical; e^{-}_{aq}, hydrated electron; EDTA, ethylenediaminetetraacetic acid; FdUrd, 5-fluorodeoxyuridine; G, guanine; ICL, interstrand cross-link; IdUrd, 5-iododeoxyuridine; $O_2^{\bullet-}$, superoxide radical; $^{\bullet}$OH, hydroxyl radical; PAGE, polyacrylamide gel electrophoresis; SS, single strand; T, thymine; TA, complementary oligonucleotide 3; UVA, ultraviolet light A; UVC, ultraviolet light C.

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Composition	Strand #	Title and sequence	Abbreviation
oligonucleotide 1/3:		Brominated complementary double stranded oligonucleotide	$DSc_{1/3}$-AB//TA
1 Brominated strand	**1**	5'-C-G-A-G-T-A-C-T-G-C-A-A-B-A-A-C-G-T-G-T-A-C-A-G-C-3'	SS_1-AB
3 Complementary strand	**3**	3'-G-C-T-C-A-T-G-A-C-G-T-T- A -T-T-G-C-A-C-A-C-G-T-C-G-5'	SS_3-TA
oligonucleotide 1/4:		Brominated semi-complementary double stranded oligonucleotide	$DSsc_{1/4}$-AB//CC
1 Brominated strand	**1**	5'-C-G-A-G-T-A-C-T-G-C-A-A-B-A-A-C-G-T-G-T-A-C-A-G-C-3'	SS_1-AB
4 Semi-complementary strand	**4**	3'-G-C-T-C-A-T-G-A-C-G C-C-C-C-C G-C-A-C-A-C-G-T-C-G-5'	SS_4-CC
		Brominated single stranded region	
oligonucleotide 2/3:		Non brominated complementary double stranded oligonucleotide	$DSc_{2/3}$-AT//TA
2 Non brominated strand	**2**	5'-C-G-A-G-T-A-C-T-G-C-A-A-T-A-A-C-G-T-G-T-A-C-A-G-C-3'	SS_2-AT
3 Complementary strand	**3**	3'-G-C-T-C-A-T-G-A-C-G-T-T- A -T-T-G-C-A-C-A-C-G-T-C-G-5'	SS_3-TA
oligonucleotide 2/4:		Non brominated semi-complementary double stranded oligonucleotide	$DSsc_{2/4}$-AT//CC
2 Non brominated strand	**2**	5'-C-G-A-G-T-A-C-T-G-C-A-A-T-A-A-C-G-T-G-T-A-C-A-G-C-3'	SS_2-AT
4 Semi-complementary strand	**4**	3'-G-C-T-C-A-T-G-A-C-G C-C-C-C-C G-C-A-C-A-C-G-T-C-G-5'	SS_4-CC

B: bromouracil; T: thymine; A: adenine; G: guanine; C: cytosine; SS: single strand; DS: double strand; c: complementary; sc: semi-complementary (bulge).

FIGURE 1: Sequences of brominated (strand 1), nonbrominated (strand 2), complementary (strand 3), and semicomplementary (strand 4) oligonucleotides showing the location of the single-stranded bubble (double strand 1/4 and 2/4) and the position of the bromodeoxyuridine (B).

of understanding of the mechanism of radiosensitization by BrdUrd.

Two general biological mechanisms for radiosensitization of cells by BrdUrd have been proposed: BrdUrd incorporation increases DNA damage induced by ionizing radiation, particularly single and double strand breaks (*42*−*44*), and/or influences the rate of DNA repair of sublethal damage (*45*) and/or potentially lethal damage (*46*, *47*). One mechanism of strand breakage by bromouracil probably involves hydrated electrons (e^-_{aq}) (*48*), produced by the radiolysis of water, which can interact with pyrimidine and halogenated bases (*49*). However, thymine and particularly cytosine (*50*−*52*) react with e^-_{aq} to form a thermally stable anion radical that can be potentially converted back to the initial base (*53*) or can be stabilized by transfer of the hydrogen involved in the interaction with the complementary base (*54*). In contrast, BrdUrd is irreversibly destroyed by dissociative attachment of a thermal (*55*) or solvated (*56*−*58*) electron, yielding a reactive uridinyl radical (*59*, *60*). We have proposed that Watson−Crick base pairing can stabilize bromine in bromouracil, thus inhibiting a crucial step in the DNA radiosensitization process (*61*). Some recent theoretical work has suggested that an increased activation barrier for bromine loss in double-stranded DNA may account for its resistance to strand breakage compared to single strand breaks in DNA (*62*).

In the present study, we report the creation of γ-radiation-induced interstrand cross-links which appear exclusively in the mismatched single-stranded brominated region of a double-stranded oligonucleotide. Our results suggested that both DNA breaks and cross-links resulting from the presence of BrdUrd in the DNA of γ-irradiated cells should form primarily in regions of DNA which were single stranded at the time of irradiation. Single-stranded DNA exists under at least five physiological situations: DNA replication (*63*), transcription (*64*), homologous recombination (*65*), in the D-loop region of telomeres (*66*), and in DNA bulges (*67*) and mismatches (*68*).

MATERIALS AND METHODS

5′ End Labeling of Oligonucleotides. The oligonucleotides shown in Figure 1 were purchased from the DNA Synthesis Lab (University of Calgary, Alberta, Canada). [γ-^{32}P]ATP, 111 TBq·mmol^{-1}, and T4 polynucleotide kinase (Amersham Pharmacia Biotech, City, NJ) were used for end-labeling oligonucleotides, which were then purified on a G-50 Sephadex microcolumn, yielding a labeled oligonucleotide which was more than 99% [γ-^{32}P]ATP free.

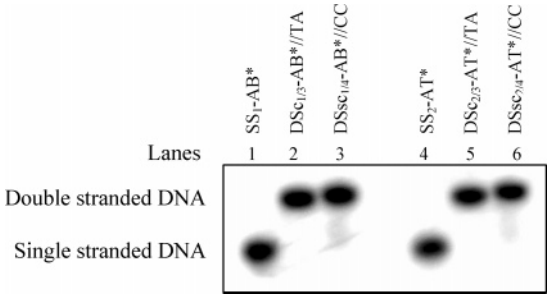

FIGURE 2: Nondenaturing gel electrophoresis of hybridized ^{32}P-labeled oligonucleotides. Lanes: 1 and 4, single-stranded oligonucleotides 1* (SS_1-AB*) and 2* (SS_2-AT*); 2 and 3, hybridized oligonucleotides 1* and 3 ($DSc_{1/3}$-AB*//TA) and 1* and 4* ($DSsc_{1/4}$-AB*//CC) (bubble); 5 and 6, hybridized oligonucleotides 2* and 3 ($DSc_{2/3}$-AT*//TA) and 2* and 4 ($DSsc_{2/4}$-AT*//CC) (bubble). The asterisk indicates the ^{32}P end-labeled strand.

Condition	•OH	e^-_{aq}
Air	2.79	≈ 0
N_2	2.79	2.74
N_2 + iso-butanol	≈ 0	2.74
N_2 + EDTA*	≈ 0*	2.74*

FIGURE 3: Yields (G) of hydroxyl radicals and hydrated electrons in water (μmol·J^{-1}) with γ-irradiation (^{60}Co).

Hybridization of Oligonucleotides. Hybridization of oligonucleotides with their complementary or semicomplementary strand was performed in 60 μL of solution using a 2-fold excess of the nonradioactive strand. Solutions were heated to 80 °C for 5 min and then allowed to slowly cool (2 h) to room temperature.

Verification of Hybridation. To determine the extent of hybridation of the radioactive oligonucleotides (with or without a mismatched bubble), samples were separated by electrophoresis in a nondenaturing polyacrylamide gel. Figure 2 shows the migration of single-stranded oligonucleotides SS_1-AB* and SS_2-AT* (lanes 1 and 4) which migrate faster than double-stranded oligonucleotides (lanes 2, 3, 5,and 6). Complementary (lanes 2 and 5, oligonucleotides $DSc_{1/3}$-AB*//TA and $DSc_{2/3}$-AT*//TA) and semicomplementary (lanes 3 and 6, oligonucleotides $DSsc_{1/4}$-AB*//CC and $DSsc_{2/4}$-AT*//CC) double strands both migrate at almost the same rate (*69*). These results demonstrate that complete hybridization of labeled oligonucleotides with their complementary and semicomplementary strands occurs under our conditions.

Strand Break and Interstrand Cross-Link Detection and Quantification. Denaturing PAGE gel electrophoresis was used to detect interstrand cross-links, as previously described (*70*−*72*). Following γ-irradiation, samples were loaded on a 7 M urea denaturing 20% polyacrylamide gel (35 × 43 cm) and electrophoresed for 2 h at 30 W. A phosphorescent screen was exposed to the gel overnight (12 h) and subsequently analyzed by a fluorescence scanning system, Storm (Molecular Dynamics Inc.), using a 100 μm pixel size. The gels were quantified using ImageQuant 5.0 software (Molecular Dynamics Inc.). Molecular weight ladders were generated by random depurination using formic acid at room temperature followed by cleavage at apurinic sites with piperidine at 90 °C.

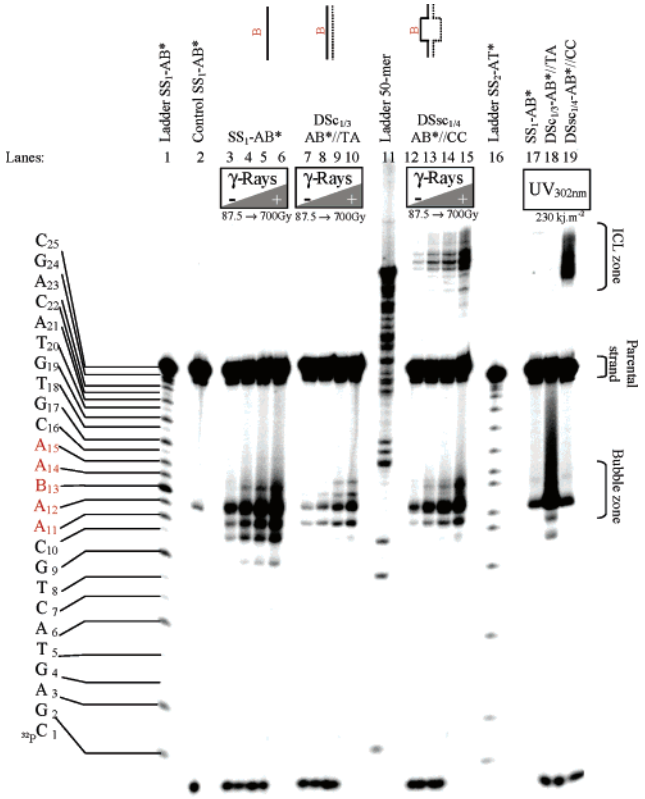

FIGURE 4: Formation of single strand breaks and interstrand cross-links in bromodeoxyuridine-containing oligonucleotides. Oligonucleotides were irradiated with different γ-ray doses (0, 87.5, 175, 350, and 700 Gy) under a nitrogen atmosphere, and damage in the following conformations was measured: single stranded (SS_1-AB*), double stranded ($DSc_{1/3}$-AB*//TA), or double stranded with a single-stranded bubble ($DSsc_{1/4}$-AB*//CC) (nucleotides 11−15 mismatched) were measured. In this experiment, the strands containing a BrdUrd (oligonucleotide SS_1-AB) at position 13 were labeled with ^{32}P, as indicated by an asterisk. UV radiation (lanes 17−20) was used as a standard to generate strand breaks and interstrand cross-links in brominated oligonucleotides.

Experimental Conditions. (A) Free Radical Selection. Radical quenchers and dissolved gases (nitrogen or air) were used to vary the concentrations of the reactive species generated by γ-irradiation (Figure 3). EDTA was chosen as a hydroxyl radical quencher. The rate constants for the reaction of EDTA, pH 9.0 and 4.0, with hydroxy radical (•OH) are 2.0 × 10^9 L·mol^{-1} (*73*) and 4.0 × 10^8 L·mol^{-1}, respectively. The rate constant for the reaction of isobutyl alcohol with •OH radicals is 3.3 × 10^9 L·mol^{-1} (*74*).

To increase the concentration of hydrated electrons, solutions were bubbled with nitrogen to eliminate dissolved oxygen and thus to minimize the capture of electrons by oxygen (reaction 1) (*75*).

$$e^-_{aq} + O_2 \rightarrow O_2^{\bullet -} \qquad k = 2.0 \times 10^{10}\ (\mathrm{L \cdot mol^{-1}}) \quad (1)$$

To eliminate residual dissolved gases, oligonucleotide solutions were bubbled with wet nitrogen gas for 2 min in a glovebox filled with nitrogen gas having a stated purity of 99.998%.

An ISO2 oxygen meter (World Precision Instruments, Saratosa, FL) was used to determine the concentration of dissolved oxygen in solutions. The initial concentration of

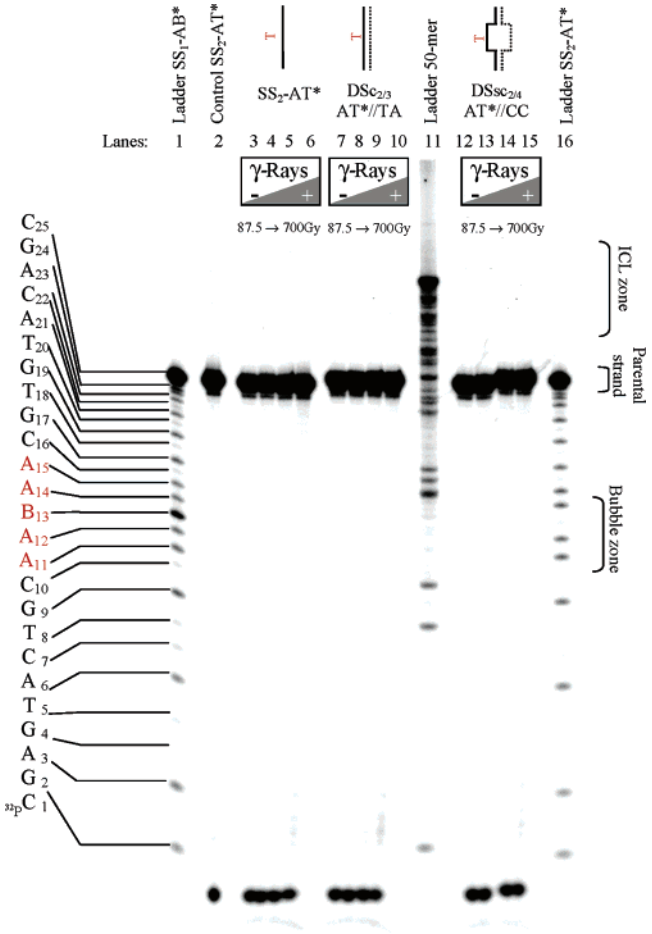

FIGURE 5: Sensitization of nonbrominated oligonucleotides to γ-rays. Oligonucleotides were irradiated with different doses (0−700 Gy) under a nitrogen atmosphere, and the formation of single strand breaks and interstrand cross-links was measured in the following conformations: single stranded (SS_2-AT*), double stranded ($DSc_{2/3}$-AT*//TA), or double stranded with a single-stranded bubble ($DSsc_{2/4}$-AT*//CC) (nucleotides 11−15 mismatched). In this experiment, the nonbrominated oligonucleotide (oligonucleotide 2, SS_2-AT*) was labeled with ^{32}P as indicated by an asterisk.

oxygen in the samples was 0.258 mM at 25 °C. After 1 min of nitrogenation, solutions were free of all measurable oxygen (<3 nM).

(B) γ-Irradiation. Samples were irradiated using a GAMMACELL 220 cobalt 60 irradiation unit from Atomic Energy of Canada with a dose rate of 3.91 Gy·min^{-1}. The energies of the γ-rays are 1.18 and 1.33 MeV.

UV Photolysis. Samples were photolyzed for 3 h using a transluminator (302 nm, UV lamp) with a fluence of 1.3 kJ·m^2·min^{-1} at 302 nm.

RESULTS

γ-Induced Breaks and Cross-Links Involving the Brominated Strand in Single-Stranded, Duplex, or Semiduplex DNA. (A) Strand Breaks. As shown in Figure 4, the presence of bromodeoxyuridine at position 13 sensitized a single-stranded oligonucleotide, SS_1-AB*, to γ-radiation-induced breakage (lanes 3−6) at positions 11, 12, and 13. In contrast, perfectly double-stranded DNA ($DSc_{1/3}$-AB*//TA) quenched the radiosensitization effect of the BrdUrd (lanes 7−10). However, the radiosensitivity was restored when the brominated oligonucleotide, SS_1-AB*, was hybridized to a semicomplementary oligonucleotide, SS_4−CC, resulting in a five base pair mismatched bubble comprising the BrdUrd ($DSc_{1/4}$-AB*//CC, lanes 12−15). γ-Irradiation of a nonbrominated control oligonucleotide (Figure 5) generated a very low level of strand breaks. When the nonbrominated oligonucleotide SS_2-AT (Figure 5) was hybridized to a semicomplementary oligonucleotide SS_4-CC to create a bubble, no interstrand cross-links were observed.

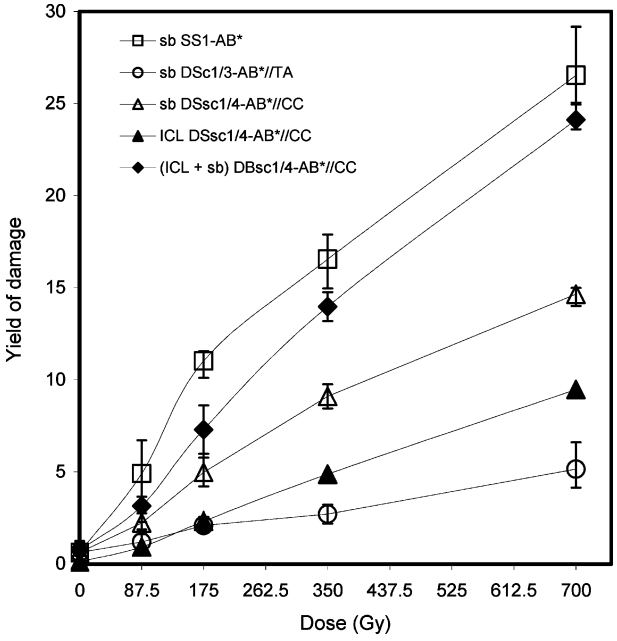

FIGURE 6: Yield of damage as a function of the irradiation dose. Strand breaks (sb), open symbols (□, △, ○), and interstrand cross-links (ICL), solid triangles (▲), of the brominated oligonucleotide SS_1-AB* in a single-stranded form (open squares, □) or hybridized with a nonbrominated complementary ($DSsc_{1/4}$-AB*//TA; open circles: ○) or a semicomplementary strand [$DSsc_{1/4}$-AB*//CC: open triangles, △ (sb); and solid triangles, ▲ (ICL)]. The sum of the damage (strand breaks and interstrand cross-links) for the brominated semicomplementary double-stranded DNA ($DSsc_{1/4}$-AB*//CC) is plotted (solid diamonds).

(B) Interstrand Cross-Link Formation. As shown in Figure 4, a series of slowly migrating bands representing interstrand cross-links appears when a brominated oligonucleotide is hybridized to a semicomplementary oligonucleotide forming a bubble comprising the BrdUrd. Cross-links do not appear either in single-stranded DNA or in the brominated, completely duplex DNA. UV radiation (302 nm) was used to generate a positive control consisting of interstrand cross-linked DNA.

(C) Quantitative Analysis (Figure 6). In Figure 6, the total yield of strand breaks formed in the bubble region (position A_{11} to A_{15}) and the sum of all ICL bands are presented for each DNA structure. We observe that the single-stranded oligonucleotide, SS_1-AB*, is up to 6 times more radiosensitive (Figure 6, open squares) than the same brominated oligonucleotide hybridized to oligonucleotide 3, which gives completely duplex DNA ($DSc_{1/3}$-AB*//TA, open circles). Strand breakage of the semicomplementary double-stranded DNA ($DSc_{1/4}$-AB*//CC, open triangles) is up to 3 times greater than the brominated completely duplex DNA (Figure 6, open circles).

Interstrand cross-links (solid triangles) occurred only in brominated semicomplementary double-stranded DNA. They

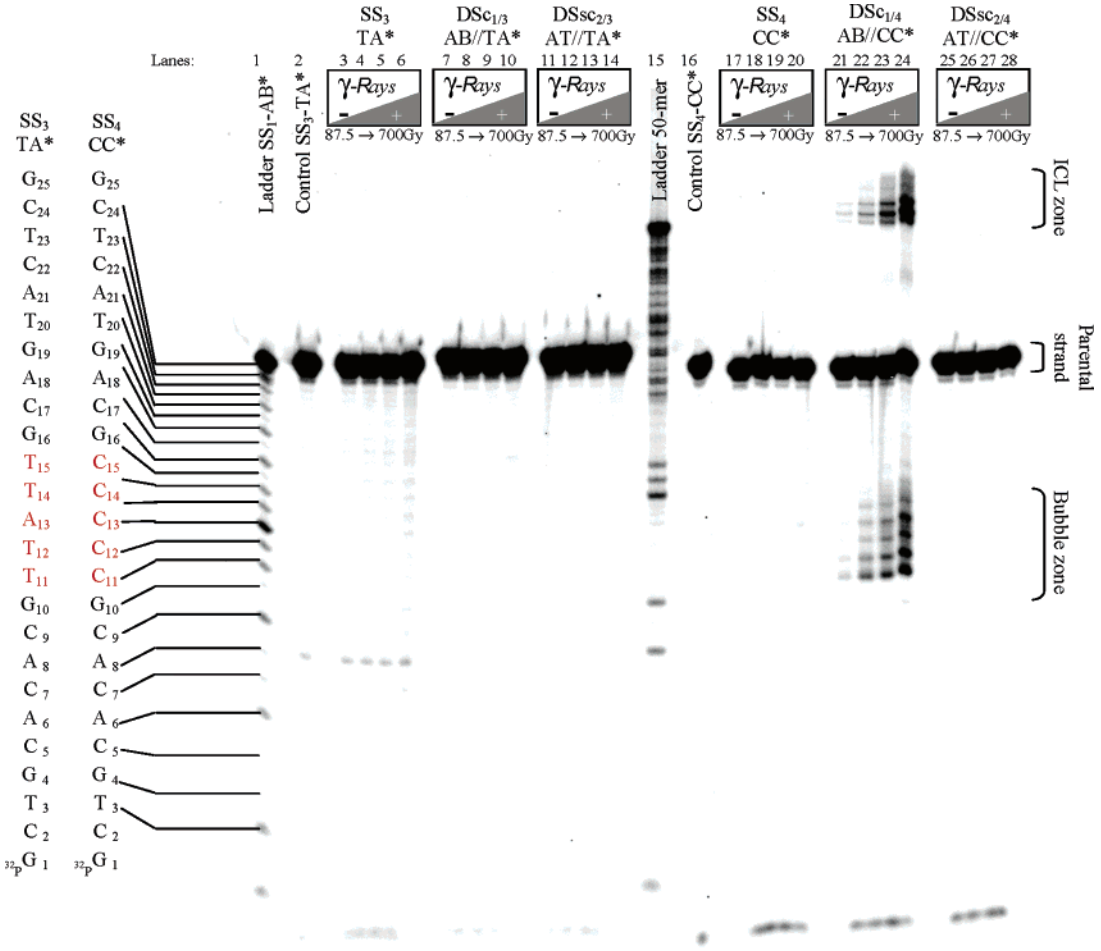

FIGURE 7: Sensitization to γ-rays of a nonbrominated complementary or semicomplementary oligonucleotide by the presence of bromouracil in the opposite strand. Oligonucleotides were irradiated with different doses (0−700 Gy) under a nitrogen atmosphere, and the formation of single strand breaks was measured in the following conformations: single stranded (SS_3-TA* and SS_4-CC*), double stranded complementary ($DSc_{2/3}$-AT//TA* and $DSc_{1/3}$-AB//TA*), or double stranded semicomplementary with a single-stranded bubble ($DSsc_{2/4}$-AT//CC* and $DSsc_{1/4}$-AB//CC*) (nucleotides 11−15 mismatched). In this experiment, the nonbrominated strands (oligonucleotides 3 and 4) were labeled (*) and then hybridized with oligonucleotide 1 [a bromouracil (B) at position 13] or oligonucleotide 2 [which contains a thymine (T) at position 13].

are proportional to the radiation dose and represent about 50% of the number of strand breaks within the five base pair mismatch forming the bubble (Figure 6, open triangles). The sum of ICLs and strand breaks in semicomplementary brominated DNA ($DSc_{1/4}$-AB*//CC) is reported on Figure 6 (♦, solid diamond). Interestingly, this total amount of DNA damage was about equal in the brominated single-stranded oligonucleotide SS_1-AB* and the brominated semicomplementary double-stranded DNA ($DSc_{1/4}$-AB*//CC) following γ-irradiation, suggesting that the uracilyl radical can produce either a strand break or a cross-link but not both.

Damage in the Complementary (SS_3-TA) or Semicomplementary (SS_4-CC) Nonbrominated Strand Hybridized to a Brominated Strand. (A) Strand Breaks. Figure 7 shows the radiosensitivity of the nonbrominated complementary (SS_3-TA*, lanes 2−14) and semicomplementary (SS_4-CC*, lanes 16−28) ^{32}P-labeled oligonucleotides hybridized to each of two oligonucleotides, a brominated (SS_1-AB, lanes 7−10 and 21−24) or a nonbrominated (SS_2-AT, lanes 11−14 and 25−28) oligonucleotide. The presence of BrdUrd in a perfect duplex did not influence the damage to the nonbrominated complementary strand (lanes 7−10 compared to lanes 11 and 14). In contrast, the presence of BrdUrd in the mismatched bubble significantly increased the strand breakage at each base, forming the bubble of the nonbrominated strand (Figure 7, lanes 21−24, bubble zone).

Figure 8 shows the total yield of strand breaks produced in the bubble region (base position 11−15) of the nonbrominated oligonucleotide. We observe that the nonbrominated semicomplemetary strand (SS_4-CC*) is up to 20 times more radiosensitive when hybridized to a brominated strand (SS_1-AB) ($DSsc_{1/4}$-AB//CC*, open triangles) than when hybridized to a nonbrominated strand (SS_2-AT) ($DSsc_{2/4}$-AT//CC*, open circles).

(B) Interstrand Cross-Linking Damage. Radiation-induced interstrand cross-links were only observed if one of the oligonucleotides was brominated and if the brominated base was present in a bubble region. Thus, when the nonbrominated oligonucleotide (SS_4-CC*) was hybridized with a brominated oligonucleotide (SS_1-AB), forming a five base pair mismatched bubble, ICL were observed (Figure 7, lanes 21−24). In Figure 8, we report the sum of all bands corresponding to ICLs. The formation of cross-links (Figure 8, solid triangles) is proportional to the γ-ray dose such that about 10% of the oligonucleotides contain an ICL for a 700 Gy irradiation dose. One would expect that the extent of

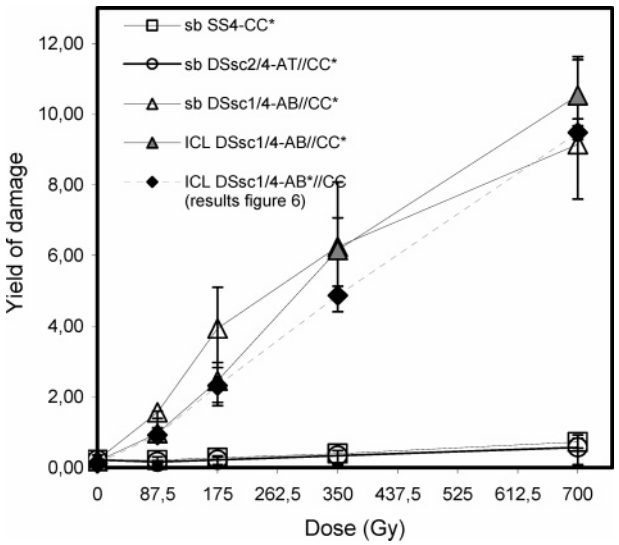

FIGURE 8: Yield of damage as a function of the irradiation dose [strand breaks (sb), open symbols (□, △, ○), and interstrand cross-links (ICL), solid symbols (▲, ◆)] of a ^{32}P-labeled nonbrominated semicomplementary strand (SS_4-CC*) in the single-stranded condition (SS_4-CC*, □) or hybridized with a nonbrominated ($DSsc_{2/4}$-AT//CC*, ○) or a brominated strand ($DSsc_{1/4}$-AB//CC*, △, ▲). ICL formation involving the brominated strand (^{32}P-labeled) hybridized with a semicomplementary strand ($DSsc_{1/4}$-AB*//CC, ◆) was transferred from Figure 6 to allow comparison of the formation of ICL when the nonbrominated strand (SS_4-CC*) or the brominated strand (SS_1-AB*) of the semicomplementary double-stranded DNA ($DSsc_{1/4}$-AB//CC) was ^{32}P-labeled.

cross-link formation should be independent of which oligonucleotide (brominated or nonbrominated) is ^{32}P-labeled. Indeed, this is the case: the same proportion of ICLs is formed when the brominated ^{32}P-labeled oligonucleotide (SS_1-AB*) was hybridized with a nonbrominated semicomplementary strand (SS_4-CC) (data from Figure 6 transposed to data in Figure 8, solid diamonds).

DISCUSSION

The DNA bubble in our model system consists of five mismatched bases in the center of a 25 base pair double-stranded oligonucleotide, with the BrdUrd at position 13 (Figure 1). Thus, the bases on the opposite strands of the single-stranded bubble are constrained to remain in close proximity by the flanking double-stranded portions of the oligonucleotide. We have previously hypothesized that the proximity of the opposite, nonbrominated strand as well as the mobility of the bases in the single-stranded bubble allows the uridinyl radical, formed during γ-irradiation by the interaction of an aqueous electron and BrdUrd, to damage bases on the same or the opposite strand and thus to distribute the damage, albeit unequally, between both strands (*61*).

Interstrand Cross-Links. DNA interstrand cross-links are among the most toxic of DNA lesions. Until recently, most studies on ICLs involved chemical or photochemical agents (e.g., mitomycin C or psoralens), but recently Cadet et al. have shown that UV-C can create an ICL in A-form DNA (*76*). They found that nonadjacent thymines on the hybridized strands form an ICL within dry DNA irradiated with UV-C. The main thymine interstrand lesion observed was the spore photoproduct, while the level of interstrand (6-4) photoproduct and *trans*,*syn*-cyclobutane dimer was significantly lower. Although DNA cross-links are often listed as a potential

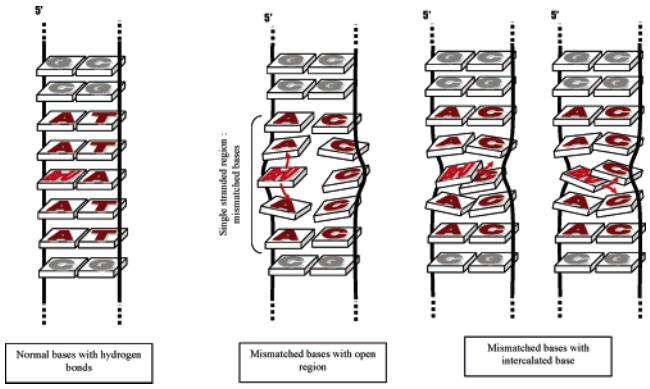

FIGURE 9: Model of damage propagation in a DNA bubble. Multiple mismatched base pairs confer some single-stranded properties to these bases, thus allowing the bases of the two strands to separate or interlace (modified from ref *82*).

lesion formed by ionizing radiation, there are no studies of this lesion in the recent literature. Studies from the 1960s using very high doses (*21*, *22*) provided evidence for γ-irradiated cross-links between individual DNA molecules of genomic DNA, but no comparison of the relative efficiencies of strand break and cross-link formation was possible.

Interaction of Mismatched Bases within a Bubble. In the model DNA used in these experiments, four of the five mismatched base pairs forming the bubble are A-C mismatches, and one is a BrU-C mismatch. It is well documented that A-C mismatch stability depends on the pH of the solution in the case of B-form DNA (*77*). Here, at neutral pH, A-C mismatches can pair with just one hydrogen bond (cytosine N3 and adenine 6-amino proton) or two hydrogen bonds in rare enol tautomeric forms. For the T-C mismatch, several studies have demonstrated the possibility of forming two hydrogen bonds (*78*). Nevertheless, in our case, a BrU-C mismatch could have difficulties making stable hydrogen bonds because four unstable mismatched base pairs surround it. Others studies have shown that multiple mismatches (bubbles) cause a slight bend but also confer greater flexibility and single strand properties (*79*). Some crystallographic studies have observed that multiple mismatched sequences, GAAA, in a nonamer d(GCGAAAGC) can either autohybridize, forming a hairpin loop, or dimerize into a stable "zipper-like duplex"(*80*−*83*). In our case, we propose that the multiple mismatched base pairs may have greater mobility that confers some single strand properties to these bases, thus enhancing base "breathing" of the two strands and allowing the formation of either an intercalation structure or an open region (Figure 9). These structures could explain the damage (radical) transfer from the uridinyl radical to an adjacent base on the same brominated strand or on the nonbrominated hybridized strand that forms the bubble, leading to either a strand break or an ICL.

Biological Implications of Our Results. It is well documented that the presence of BrdUrd in DNA substantially increases single and double strand breaks in cells exposed to ionizing radiation *(*84, *85)*; however, neither intra- nor interstrand cross-links have been reported. It is possible that the single and double strand breaks induced by γ-irradiation may have confounded the detection of interstrand cross-links in cellular DNA. In addition, it is also possible that certain cross-links are enzymatically converted to double strand

breaks prior to harvesting of the DNA and are therefore scored as strand breaks. Nevertheless, our observation that interstrand cross-links appear exclusively in the mismatched single-stranded region of a double-stranded oligonucleotide suggests that it will be worthwhile to search for these lesions in cellular DNA. In vivo, single-stranded regions of double-stranded DNA exist under at least five physiological situations: DNA replication (*63*), transcription (*64*), telomeres (*66*), homologous recombination (*65*), and in DNA bulges (*67*) and mismatches (*68*, *86*). Thus, ICL and strand breaks resulting from the presence of BrdUrd in the parental strand at replication forks during irradiation should be randomly distributed in the genome in asynchronously replicating cells. However, in the case of transcription, these lesions should be targeted to actively transcribed genes independent of the phase of the cell cycle. The D-loop of telomeres contains a single-stranded region which should be susceptible to strand breakage sensitized by BrdUrd. However, our current model system does not allow us to predict whether the D-loop of telomeres will be sensitive to cross-link formation. There is ample evidence that DNA double strand breaks are toxic to cells (*87*−*89*), and the radiosensitization of cells by BrdUrd has been attributed to the additional strand breaks, especially double strand breaks (*28*−*31*) induced by the bromouracil in DNA. Given our findings, the possibility must now be considered that the lethal lesions actually consist of the double strand breaks and interstrand cross-links.

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