Reaching for the Other Side: Generating Sequence-Dependent Interstrand Cross-Links with 5-Bromodeoxyuridine and γ-rays[†]

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ABSTRACT: Interstrand cross-links impede critical cellular processes such as transcription and replication and are thus considered to be one of the most toxic types of DNA damage. Although several studies now point to the existence of γ-radiation-induced cross-links in cellular DNA, little is known about the characteristics required for their creation. Recently, we reported the formation of interstrand cross-links that were specific for mismatched nucleotides within 5-bromo-2′-deoxyuridine-substituted DNA. Given the structural specificity for interstrand cross-link formation, it is likely that open or mismatched regions of DNA in cells may be particularly favorable for cross-link production. Herein, we investigated the effect of the local DNA sequence on the formation of interstrand cross-links, using 5-bromo-2′-deoxyuridine to generate radicals in a mismatched region of DNA. We investigated a total of 12 variations of bases in the mismatched region. The oligonucleotides were irradiated with γ-rays, and interstrand cross-link formation was analyzed by denaturing gel electrophoresis. We found that the efficiency of cross-link formation was highly dependent on the nature of mismatched bases and, on the basis of electrophoretic mobility, observed several distinctive cross-link structures with specific DNA sequences. This study provides new insights into the reactivity of mismatched DNA and the mechanisms leading to interstrand cross-link formation. The potential application of 5-bromo-2′-deoxyuridine-induced interstrand cross-links to the field of DNA repair is discussed.

Among the plethora of DNA lesions produced by ionizing radiation, double-strand breaks are generally considered to be the most toxic (*1*−*3*). However, evidence of the formation of another potentially more toxic type of lesion is now emerging. Interstrand cross-links (ICLs)[1] prevent the separation of the DNA strands, thus blocking several key cellular processes (*4, 5*). Traditionally, ionizing radiation has not been regarded as a cross-linking agent; however, several instances of intrastrand cross-link formation have been reported (*6*−*12*), and recent data from Greenberg and co-workers point to the existence of interstrand cross-links induced by hydroxyl radicals in synthetic DNA (*13, 14*).

Although 5-bromo-2′-deoxyuridine (BrdU), a thymidine analogue that can be incorporated into DNA, is widely used as a marker of replicating cells (*15*−*17*), it was first reported as a photo- and radiosensitizing agent (*18, 19*). When exposed to ionizing radiation, BrdU produces single- and double-strand breaks (*20, 21*), as well as chromosomal aberrations (*22*) that are generally believed to be responsible for its sensitizing properties. Recently, we reported the formation of a new type of BrdU-sensitized damage in synthetic DNA: ICLs that were specific for mismatched nucleotides within BrdU-substituted DNA (*23*). These ICLs were later found to be highly dependent on the presence of B-form DNA, supporting the evidence that the regional structure of DNA is a prerequisite for their formation (*24*) and that these ICLs may only be produced in open or mismatched regions of cellular DNA. Although open regions of DNA transiently exist during transcription and replication and mismatched regions are found in centromeres (*25*−*27*), there are surprisingly few studies asking how these regions are affected by ionizing radiation (*28, 29*). Given the structural prerequisites for ICL formation observed in our studies, it is possible that open regions of DNA may be particularly favorable for ICL production.

Here, we used BrdU, together with ionizing radiation, as a tool to generate radicals in open regions of DNA and to examine how these regions react. To study the reactivity of radicals with different DNA bases in an open region of DNA, we modified the nucleotide sequence surrounding the site of BrdU substitution in both the brominated and the opposite semicomplementary strands. We investigated a total of 12 mismatched oligonucleotides, using three variations of the brominated strand and four variations of the semicomplementary strand. The yield of both ICLs and strand breaks was found to be highly dependent on the identity of the neighboring bases. Furthermore, we observed several distinc-

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[1] Abbreviations: A, adenine; BrdU, 5-bromo-2′-deoxyuridine; ^{Br}U, 5-bromo-2′-deoxyuridine-substituted oligonucleotide; C, cytosine; e_{aq}, solvated electron; EDTA, ethylenediaminetetraacetic acid; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; HPLC, high-performance liquid chromatography; G, guanine; ICL, interstrand cross-link; 2-PrOH, 2-propanol; T, thymine; TEAA, triethylammonium acetate.

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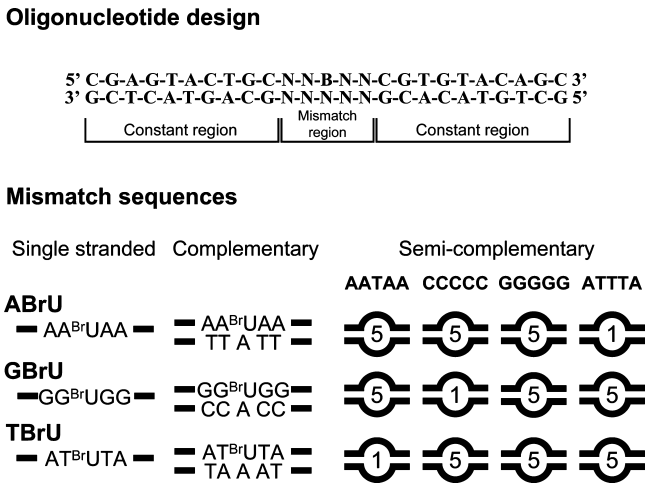

FIGURE 1: Oligonucleotide design. Each 25-mer sequence consisted of a variable central 5 bp region, surrounded by two constant regions. Three variations of the central region of the BrdU-substituted (B) strand were synthesized (AA^{Br}UAA, GG^{Br}UGG, and AT^{Br}UTA) and were either irradiated in single-stranded form or hybridized with their complementary or one of the four semi-complementary strands to form double-stranded or mismatched oligonucleotides, respectively. The mismatch size is indicated for each combination.

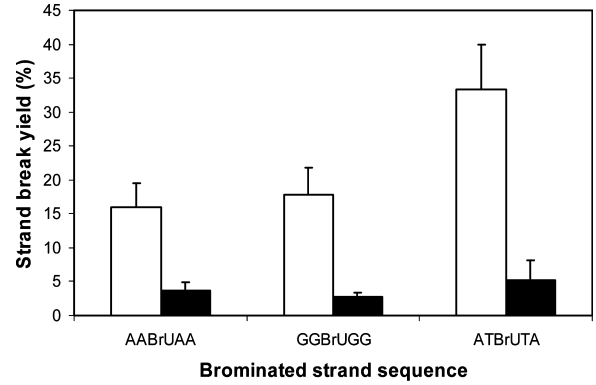

FIGURE 2: Contribution of double strandedness to strand breaks as a function of sequence. The three variations of the brominated strand were irradiated as single-stranded (white) or double-stranded DNA (black). The yield of strand breaks in the brominated region was measured by denaturing gel electrophoresis.

tive ICL structures that were specific for given sequences. This study provides new insights into the reactivity of mismatched DNA and the mechanisms leading to interstrand cross-link formation. Finally, we propose that, given the sequence specificity of BrdU-induced ICLs, this model system may provide a method of producing single ICL structures for use in DNA repair studies.

MATERIALS AND METHODS

Oligonucleotide Design. 5-Bromo-2′-deoxyuridine-modified and nonmodified oligonucleotides were purchased from the University Core DNA Services (University of Calgary, Calgary, AB). Oligonucleotides were designed on the basis of previous sequences used by our group (*23, 24*). Each sequence included two constant regions, with a variable central 5 bp region described in Figure 1. Three variations of the brominated sequence were used (AA^{Br}UAA, GG^{Br}UGG, and AT^{Br}UTA) and were hybridized either with their complementary sequence or with one of the semicomplementary sequences (AATAA, CCCCC, GGGGG, and ATTTA). The choice of hybridized sequences determined the regional conformation of the DNA (double-stranded or mismatched).

Sequence-Dependent Interstrand Cross-Link and Strand Break Formation. Control experiments were performed using single- and double-stranded DNA to verify the hybridization specificity previously observed with AA^{Br}UAA (*30*). For mismatched DNA, each combination of brominated and semicomplementary strands was irradiated and analyzed on the same gel. Also, for each permutation, the production of damage on each DNA strand was investigated by alternatively labeling either the brominated or the semicomplementary strand. A comparison of interstrand cross-link structures was performed using the following guidelines. (1) Oligonucleotides were labeled and irradiated concomitantly and were analyzed together on the same gel by denaturing polyacrylamide gel electrophoresis. (2) For each sequence, an equal amount of radioactivity was loaded on the gel. (3) The structures presented in Figure 4 were taken from a single gel in which the original alignment was conserved.

Experimental Procedures. In each experiment, the 5′ end of one strand was end-labeled with ^{32}P using T4 polynucleotide kinase (Amersham Pharmacia Biotech). Hybridization was carried out by adding a 2-fold concentration of complementary oligonucleotide, heating to 90 °C, and slowly cooling to room temperature for 2.5 h. The hybridization state was confirmed by nondenaturing gel electrophoresis. Prior to irradiation, DNA was bubbled for 1 min with N_2 to remove excess oxygen and then irradiated with 750 Gy in a Gammacell-220 irradiator (^{60}Co, Nordion Canada, dose rate of 5.08 Gy/min) at a final concentration of 0.3 μM in phosphate buffer (10 mM, pH 7.0), using 25 mM EDTA as a hydroxyl radical scavenger, as described by Cecchini et al. (*23, 30*). Strand breaks and interstrand cross-links were assessed by denaturing gel electrophoresis, followed by quantification of damage with ImageQuant, as described by Cecchini et al. (*23*).

Conversion of BrdU to Uracil. Conversion of BrdU to uracil in single-stranded DNA was assessed by high-performance liquid chromatography (HPLC). The three BrdU-substituted sequences (AA^{Br}UAA, GG^{Br}UGG, and AT^{Br}UTA) were irradiated with 750 Gy as single-stranded oligonucleotides in 10 mM phosphate buffer (pH 7.0) and 25 mM EDTA (pH 8.0). These conditions were similar to those used in the gel electrophoresis experiments, except that the DNA concentration was 25 μM rather than 0.3 μM. After irradiation, DNA was digested using 3 units each of snake venom phosphodiesterase (phosphodiesterase I, USB) and nuclease P1 (Sigma) and incubated at 37 °C for 120 min. Alkaline phosphatase (3 units, Roche) was then added, and digestion was continued for 120 min at 37 °C to generate nucleosides. Chloroform extraction was performed to remove proteins prior to injection onto the HPLC system. HPLC analysis was performed using an Alliance system (Waters 2795 or 2690) connected to dual-wavelength UV detectors (Waters 2487) and a Millenium workstation (Waters version 4). The analysis of 2′-deoxyuridine formation was carried out as follows: 150 pmol of digested DNA was separated using a reversed phase column (5 μm ODS-A, 250 mm × 6.0 mm; YMC). The gradient was as follows: 95% solvent A1 and 5% solvent B for 5 min, changing to 85% solvent

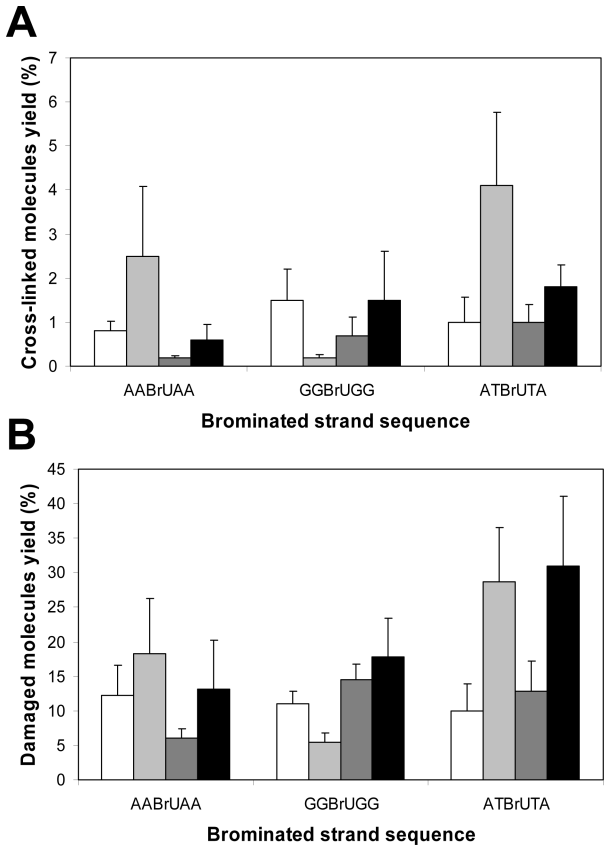

FIGURE 3: Damage yield as a function of sequence in mismatched DNA. Interstrand cross-links and total cross-link plus frank strand break damage (panels A and B, respectively) were measured following irradiation of the 12 combinations. Each variation of the brominated strand was hybridized with each of the four variations of the semicomplementary strand (AATAA, white; CCCCC, light gray; GGGGG, dark gray; and ATTTA, black). Total frank damage corresponds to the sum of interstrand cross-links and strand breaks located on both the brominated and semicomplementary strand.

A1 and 15% solvent B over 45 min (0.25%/min). Solvent A1 was composed of 25 mM triethylammonium acetate (TEAA) (pH 7.0). Solvent B was composed of 95% acetonitrile and 5% H_2O. The flow rate was 1 mL/min, and the column was maintained at a temperature of 20 °C.

RESULTS

Contribution of Secondary Structure to Strand Break Formation as a Function of Sequence. We first investigated the effect of adjacent bases on BrdU-related damage in single- and double-stranded complementary DNA. In agreement with previous results, Figure 2 shows an increase in the number of strand breaks in single-stranded compared to double-stranded DNA containing BrdU (*30*). The yield of strand breaks on the brominated strand, for both single- and double-stranded DNA, was similar for both AA^{Br}UAA and GG^{Br}UGG, whereas there was more damage in AT^{Br}UTA in both hybridization states. However, conversion of BrdU to 2′-deoxyuridine by irradiation of single-stranded DNA was not significantly affected by the surrounding nucleotides (AA^{Br}UAA, 25.3 ± 7.0%; GG^{Br}UGG, 24.9 ± 5.9%; and AT^{Br}UTA 28.0 ± 7.8%). Interstrand cross-links were not found in either hybridization state, regardless of the sequence (not shown).

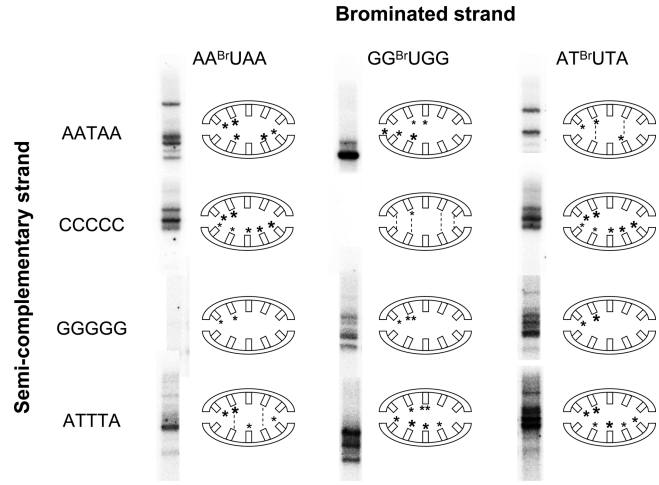

FIGURE 4: Interstrand cross-link structures and strand break location as a function of sequence. For each of the mismatched sequences, the cross-link patterns and the location of strand breaks were reported (only cross-link structures are shown on the left). The size of the asterisk indicates the relative yield of strand break for a particular nucleotide, while dashed lines specify the existence of possible canonical Watson−Crick bonds.

Sequence-Dependent Formation of Interstrand Cross-Links and Strand Breaks in Mismatched DNA. Interstrand cross-links specific for BrdU-substituted mismatched DNA were first reported by our group in 2005 (*23*), using the AA^{Br}UAA//CCCCC mismatched oligonucleotide. Using AA^{Br}UAA//AATAA, we also showed that B-form DNA was necessary for the production of ICLs (*24*). Here, we investigated the effect of several sequences on the production of ICLs and strand breaks, to understand the role of DNA sequence in ICL formation. Figure 3 shows the variation in the yields of ICL and the formation of total frank damage as a function of sequence (panels A and B, respectively). In general, pyrimidines, on either the brominated or the semicomplementary strand, tend to produce more ICLs and strand breaks, while guanine typically does not promote cross-linking. For the three combinations that produced a mismatched region smaller than 5 bp or where internal Watson−Crick bonds could occur, namely, AA^{Br}UAA//ATTTA, GG^{Br}UGG//CCCCC, and AT^{Br}UTA//AATAA, production of ICLs and strands breaks was the lowest, with the exception of AA^{Br}UAA// GGGGG. Table 1 shows the strand location of breaks as a function of DNA sequence. Typically, there was no significant difference between strand breaks produced on each strand (i.e., brominated vs nonbrominated) of a given combination. However, five permutations diverged from this pattern: GG^{Br}UGG//GGGGG and AT^{Br}UTA//ATTTA produced more breaks on the brominated strand, whereas the generation of breaks was more important in the nonbrominated semicomplementary strands of GG^{Br}UGG// AATAA, GG^{Br}UGG//ATTTA, and AA^{Br}UAA//GGGGG. Labeling either the brominated or the semicomplementary strand did not affect the yield of ICLs (not shown). Figure 4 shows the detailed location and frequency of these strand breaks for each strand, as well as the corresponding ICL structures. Several distinct, sequence-dependent structures are observed, with only two permutations producing no cross-links at all: AA^{Br}UAA//GGGGG and GG^{Br}UGG// CCCCC.

Table 1: Localization of Strand Breaks on the Brominated and Semicomplementary Strands

sequence	strand breaks (%)		statistical significance p^a	preferred strand (in bold)
	brominated strand	semicomplementary strand		
AABrUAA	15.90 ± 3.66			
AABrUAA//AATAA	5.60 ± 0.78	5.77 ± 3.39	0.9137	
AABrUAA//CCCCC	6.28 ± 2.85	9.42 ± 3.64	0.2186	
AABrUAA//GGGGG	1.30 ± 0.30	4.53 ± 0.98	0.0004	AABrUAA//**GGGGG**
AABrUAA//ATTTA	8.05 ± 5.87	4.45 ± 0.97	0.3395	
GGBrUGG	17.85 ± 3.89			
GGBrUGG//AATAA	2.90 ± 1.13	6.55 ± 0.08	0.0450	GGBrUGG//**AATAA**
GGBrUGG//CCCCC	2.25 ± 0.49	2.92 ± 0.80	0.3770	
GGBrUGG//GGGGG	8.90 ± 0.71	4.97 ± 1.02	0.0189	**GGBrUGG**//GGGGG
GGBrUGG//ATTTA	3.47 ± 1.86	12.91 ± 2.52	0.0064	GGBrUGG//**ATTTA**
ATBrUTA	33.30 ± 6.66			
ATBrUTA//AATAA	4.85 ± 1.48	4.14 ± 1.89	0.6891	
ATBrUTA//CCCCC	13.18 ± 4.56	11.48 ± 1.64	0.5725	
ATBrUTA//GGGGG	6.35 ± 2.87	5.51 ± 1.02	0.6550	
ATBrUTA//ATTTA	21.14 ± 5.46	8.01 ± 4.20	0.0063	**ATBrUTA**//ATTTA

[a] p determined by a paired Student's t test.

DISCUSSION

Contribution of Secondary Structure to Strand Break Formation as a Function of Sequence. The interaction of ionizing radiation with DNA produces radicals through direct and indirect processes that eventually lead to base lesions and strand breaks (*3*). However, with the exception of charge transfer along the double helix, there is little evidence that the nature of the bases surrounding the initial radical influences the type of lesion produced (*31*−*34*). Even in tandem lesions such as intra- and interstrand cross-links, only the bases immediately adjacent to the initial radical are involved (*3, 8, 35*−*39*). This study provides the first evidence that the identity of the bases adjacent to the initial radical affects both the type (i.e., strand break vs cross-link) and location of lesions produced. Furthermore, we show that, even in single-stranded DNA, the nature of the bases surrounding the BrdU substitution determines the extent of strand breakage following irradiation. We previously reported the formation of strand breaks that were specific for single-stranded oligonucleotides in BrdU-substituted DNA (*30*). As expected, we observed that the same single-strand specificity first described for strand break generation in AA^{Br}UAA also extended to GG^{Br}UGG and AT^{Br}UTA (Figure 2). More intriguing was the observation that the identity of neighboring bases affects the production of strand breaks. Replacement of the DNA bases next to BrdU (replacement of A with T) in AA^{Br}UAA increases the degree of formation of strand breaks in single-stranded AT^{Br}UTA 2-fold. Conversely, replacement of A with G, which have oxidation potentials of 1.56 and 1.29 V at pH 7.0, respectively (*3*), did not affect the production of strand breaks in single- or double-stranded DNA. Thus, the degree of formation of single-strand breaks is increased by the presence of a base with a relatively high electron affinity (T) but is not affected by a base with a low oxidation potential. Given the fact that T is a preferential site of electron addition in normal DNA (*40, 41*), it is possible that T acts as an additional "electron antenna" to capture electrons and subsequently transfer them onto BrdU. However, the level of conversion of BrdU to 2′-deoxyuridine is only slightly (but not significantly) higher for AT^{Br}UTA than for AA^{Br}UAA and GG^{Br}UGG, indicating that this step is not limiting the production of strand breaks, and that generation of other products in AA^{Br}UAA and GG^{Br}UGG may explain the reduction observed in strand break yields. Incidentally, formation of intrastrand cross-links between BrdU and adjacent purines has been observed after UV irradiation (*38*). Although the mechanism for UV sensitization of BrdU-substituted DNA involves the production of two adjacent radicals, only one radical is produced in the case of ionizing radiation. However, it is possible that the same products may be produced by alternative pathways, depending on the radiation source. These results eloquently illustrate that not only the secondary structure but also the surrounding sequence affects the ability of BrdU to damage DNA during irradiation, influencing the type and yield of lesions produced.

Sequence-Dependent Formation of Interstrand Cross-Links and Strand Breaks in Mismatched DNA. (i) ICL and Strand Break Yields. Figure 3A shows that the efficiency of cross-link formation is highly dependent on the nature of the mismatched bases in the brominated as well as the opposite semicomplementary strand. These cross-links are produced with the same yield, whether the brominated or semicomplementary strand is labeled, which indicates that they represent interstrand cross-links, rather than interhelix cross-links. Cross-link preferences increased in the following order: T > A ∼ G and C > T > A > G in the brominated and semicomplementary strands, respectively. Our observation that pyrimidines, regardless of the strand where they were located, produced more total frank damage (Figure 3B) supports the hypothesis that solvated electrons may be attracted to sites containing a large number of electron affinic pyrimidines. Interestingly, cytosine is present on the opposite strand of the two combinations that produced high yields of ICLs, which points to the 5,6-double bond of cytosine as a preferred site of attack for cross-linking (*6, 42*). The size of the mismatch also influences the production of ICLs and strand breaks; the three combinations that produced a mismatched region smaller than 5 bp or where internal Watson−Crick bonds are possible (AA^{Br}UAA//ATTTA, GG^{Br}UGG//CCCCC, and AT^{Br}UTA//AATAA) resulted in less damage than the other sequences. Interestingly, AA^{Br}UAA//GGGGG also produced the least damage of any sequence, possibly because of a particular secondary struc-

ture, as discussed below. Incidentally, the yield of strand breaks produced in GG^{Br}UGG//CCCCC is similar to that produced in the complementary double-stranded counterpart, with no detectable ICL produced. This indicates that a single mismatch in a run of GC base pairs closely resembles complementary DNA. In contrast, the A-T rich sequences, AA^{Br}UAA//ATTTA and AT^{Br}UTA//AATAA, in which the Watson−Crick bonds are surrounded by mismatched base pairs, probably produce a structure close to a typical 5 bp mismatched DNA. More intriguing, however, is the fact that AA^{Br}UAA//GGGGG, the only other sequence to produce no detectable cross-links and only trace levels of strand breaks, cannot form canonical DNA base pairs. Over the years, several reports have been published indicating that stretches of mismatched GA can form sheared base pairs, provided that certain requirements are satisfied regarding the surrounding nucleobases (*26, 43, 44*). However, these sheared base pairs were only reported for alternating GA//AG sequences, with no indication that an AA//GG mismatch can produce such a structure. Another possibility is that wobble G-A bonds are responsible for this behavior. Certainly, the data presented here provide further evidence that BrdU may serve as an internal sensor for probing DNA structure (*24, 45*). Moreover, our results indicate that the reactivity of open or mismatched regions of cellular DNA to radicals, as well as the type of damage that is produced, is probably highly dependent on the nature of bases surrounding the initial radical.

(ii) Strand Break Location and ICL Structures. For BrdU-substituted DNA, the mechanism of single-strand break formation involves attachment of an electron to BrdU, followed by departure of bromide anion, leading to the generation of uracil-5-yl radicals. In turn, these radicals react with DNA bases and sugar moieties to produce ICLs and strand breaks. Our results show that strand breaks are produced on the 5′ side of BrdU and at several positions on the opposite semicomplementary strand, indicating that in mismatched DNA, uracil-5-yl radicals are able to attack multiple sites (Figure 4). It is unclear whether these strand breaks arise from the direct reaction of uracil-5-yl radicals with sugar moieties within the open duplex structure (as far as several bases away) and/or from a multistep reaction involving the formation of base radicals in the proximity of the initial uracil-5-yl radical, followed by transfer to the site of damage. The presence of a relatively long mismatched region in our experimental system is likely to alter the distance of reactive sites normally found in double-stranded DNA, which may permit a diffusion of damage in the proximity of nascent uracil-5-yl radicals.

Although strong evidence exists that mismatched nucleotides disrupt the normal behavior of DNA, such as charge transfer along the double helix (*40, 46*), little is known about how consecutive mismatches react following treatment with ionizing radiation. Thus, it is possible that the inherent flexibility of these DNA regions allows reactions that are not typically seen in complementary DNA. Also, given the fact that uracil-5-yl radicals are good hydrogen atom abstractors [$k_{2\text{-PrOH}} = 4.1 \times 10^7$ dm^3 mol^{-1} s^{-1} (*47*)], mismatched regions of DNA may be particularly favorable for radical transfer to distant bases, followed by strand breakage. Our observation that fewer strand breaks occur on the brominated strand when BrdU is surrounded by guanines (Table 1, GG^{Br}UGG//AATAA and GG^{Br}UGG//ATTTA) suggests that adjacent bases, particularly guanine, interfere with the pathway leading to frank strand breaks. This hypothesis is supported by the decreased yield of strand breaks that is observed when purines are flanking the site of BrdU substitution, and which may be attributable to competitive reactions such as the formation of intrastrand cross-links. Whether this interference involves the intermediate formation of guanine radical cations has not been established. Most often, these radicals do not directly lead to frank strand breaks but give 8-oxo-7,8-dihydroguanine, which is a relatively stable lesion, and other compounds, including 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 2,2,4-triamino-(5*H*)-oxazolone, which are alkali labile lesions (*48*). Previous observations by Gräslund et al. (*49*) indicated an increase in the level of guanine cation radicals in BrdU-substituted oriented fibers, compared to unsubstituted fibers. However, this experiment was conducted in solid state hydrated DNA in the absence of radical scavengers. Thus, these radicals could also arise from either the direct interaction of radiation or hydroxyl radical attack on BrdU.

If we set aside the hypothesis of radical transfer, then the distributions of strand breaks shown in Figure 4 must arise from the physical contact of the uracil-5-yl radical with the damaged sugar moiety. Strikingly, in the case of AA^{Br}UAA//CCCCC and AT^{Br}UTA//CCCCC, bases at the 5′ extremities of the mismatch are those with the higher yield of strand breaks, with strand breaks even being produced outside the mismatch in the case of GG^{Br}UGG//AATAA. If these strand breaks truly reflect the proximity of BrdU, this suggests that the structure of mismatched DNA regions is highly dependent on the sequence. In double-stranded complementary DNA, no strand breaks are observed on the strand opposite the BrdU. Undoubtedly, the presence of a mismatch in our experimental system affects the number and location of nucleotides susceptible to react, especially if mismatched regions of DNA adopt a zipperlike structure rather than a "bubble" configuration (*23, 24, 27, 50*−*52*). In zipperlike DNA structures, the bases interlace with their counterpart on the opposite strand, promoting close base−base contacts that may favor transfer of a radical to the other strand. One of the best indications in favor of this model is the creation of ICLs, where a radical on one strand attacks a nucleotide on the opposite strand to generate a covalent bond.

Without a doubt, the most striking feature of the results presented in this study is the diversity of ICL structures that are obtained when the bases surrounding the BrdU are modified (Figure 4). Overall, 10−12 distinct bands are observed, with several of them present in multiple DNA sequences. Of these, the three-band pattern observed in AA^{Br}UAA//CCCCC, AT^{Br}UTA//CCCCC, and AT^{Br}UTA//ATTTA is perhaps the most intriguing. First, it is present in pyrimidine rich sequences. Second, at least one of these bands is observed with a combination of other bands in four other sequences (AA^{Br}UAA//ATTTA, AT^{Br}UTA//AATAA, AT^{Br}UTA//GGGGG, and GG^{Br}UGG//GGGGG), which indicates that they must represent structures with a higher likelihood of formation. This is supported by the higher ICL yield found in sequences that produce this three-band pattern. Unfortunately, the chemical structure of these cross-links cannot be inferred from their electrophoretic mobility. Nevertheless, valuable information can be extracted from

these data, especially concerning the large number of sites that are involved in ICL formation. If, as discussed above, we dismiss radical transfer, then ICLs can only occur when the radical located at the site of BrdU substitution reacts with one of the five bases on the opposite strand. Because denaturing gel electrophoresis cannot resolve symmetrical structures with the same molecular weight, this predicts that only three different bands should be observed on denaturing gels. Since a greater number of bands are observed, this supports our hypothesis that transfer of a radical to adjacent nucleotides does occur, on both the brominated and the opposite strand, which would greatly increase the number of sites susceptible to being part of an ICL and consequently the number of bands found on the gel. Although radical transfer has not traditionally been considered to be part of the sensitization mechanism, the data presented here certainly raise the question of whether transfer to distant bases is an important part of BrdU sensitization in mismatched DNA. Undoubtedly, the identity of the surrounding bases influences the location of strand breaks, as well as the ICL structures that are produced in open or mismatched regions of DNA. Given the specificity we reported for ICL formation, it is entirely possible that ICL "hot spots" exist in regions of cellular DNA that possess the appropriate sequence and structural characteristics.

5-Bromodeoxyuridine: A Tool for the Study of ICL Repair? In this study, we found that BrdU-substituted DNA, when exposed to ionizing radiation, produces highly sequence-dependent ICLs. These ICLs are so dependent on the nucleotide sequence that 10−12 structures result from a single initial radical. More importantly, we were able to produce a single major ICL structure with GG^{Br}UGG//AATAA. This is of particular interest to the field of ICL repair, which requires the production of single structures for studying how these lesions are processed by DNA repair and replication machinery. Furthermore, because most ICL-inducing agents studied to date produce bulky lesions as well as monoadducts, which can influence repair mechanisms, it would be advantageous to investigate how cells deal with nonbulky DNA cross-link lesions (*53, 54*). However, several of the agents developed over the years to form these types of cross-links cannot be incorporated into cellular DNA (*55*−*57*), and thus, they may not adequately represent repair mechanisms in cells. Finally, as indirect evidence of the existence of BrdU-specific ICLs was recently found in cells (*58, 59*), information regarding its repair in cancer cells would be of great interest in view of future preclinical and clinical studies (*60*).

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