Sequence-Specific Double-Strand Cleavage of DNA by Fe−Bleomycin. 1. The Detection of Sequence-Specific Double-Strand Breaks Using Hairpin Oligonucleotides†

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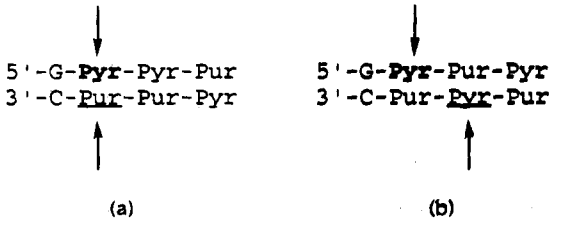
ABSTRACT: A new method is described for the evaluation of sequence-specific double-strand (ds) cleavage of DNA by Fe−bleomycin (BLM). The method uses high-resolution polyacrylamide gel electrophoresis to separate single-strand (ss) and ds-cleavage products derived from hairpin oligonucleotides that have been designed to contain a specific ds-cleavage site. The BLM induced ss/ds-cleavage ratios ranged from ~3.3 to ~5.8 at 4 °C , with the most efficient ds-cleavage involving the thymidines of a 5′-*GT*AC/ 5′-*GT*AC site. Double-strand cleavage was not detected at several sites that were shown to yield significant ss-breaks. A study of the ss/ds-cleavage ratio at the 5′-GTAC/5′-GTAC site revealed that the ratio remained constant over a 70-fold range in concentration of Fe-BLM and extent of DNA degradation. The ss/ds-cleavage ratios at three sites studied were not significantly affected by the presence of "inert" Co(III)-BLM. The results are consistent with the proposal of Steighner and Povirk (1990) that a single molecule of Fe-BLM is responsible for ds-cleavage. The use of these hairpin oligonucleotides has greatly facilitated quantitative analysis of the ds-cleavage process (Absalon et al., 1995).

The bleomycins (BLM)[1] are a family of antitumor antibiotics, isolated from *Streptomyces verticillus*, that are used clinically in the treatment of squamous cell carcinomas, lymphomas of the head and neck, and testicular cancers (Umezawa, 1980; Umezawa et al., 1966). Their mode of cytotoxicity is thought to be related to their ability to bind and degrade DNA, although recent studies have shown that cleavage of RNAs can be efficient and must also be considered a viable target (Carter et al., 1990, 1991a,b; Holmes et al., 1993; Hüttenhofer et al., 1992; Magliozzo et al., 1989). If DNA is, in fact, responsible for BLM's cytotoxicity, then it seems reasonable that double-strand (ds) cleavage, a rare event and one which is probably difficult to repair, would be the major DNA damage responsible for lethality. Previous studies have reported that the ds-cleavage events observed from treatment of BLM are more frequent than expected from a coincident occurrence of closely spaced single-strand (ss) breaks (Lloyd et al., 1978a,b; Mirabelli et al., 1980; Povirk et al., 1977). Reports of the "global" ratios of ss/ds-cleavage vary from 5 to 20 depending upon the choice of DNA and the method of analysis which has included detection of the conversion of supercoiled DNA to cc-DNA and linear DNA using agarose gel electrophoresis, alkaline and neutral sucrose gradient centrifugation, and filter-elution techniques. Information from the "global" ss/ds-cleavage ratios have been used to investigate the stoichiometry of BLM required in the production of a ds-break (Gonikberg & Odintsova, 1987; Povirk, 1983; Povirk et al., 1977), to compare activities of BLM cogeners (Mirabelli et al., 1980; Mirabelli et al., 1985), and to characterize the general form of the lesions which produce a ds-break upon alkaline treatment of DNA incubated with BLM (Povirk & Houlgrave, 1988). No method yet reported, however, has allowed the quantitative description of the ss/ds-cleavage ratio at specific sites of Fe-BLM degradation.

The study of ds-cleavage by BLM on the sequence-specific level has only recently received attention. Experiments using successive native and denaturing sequencing polyacrylamide gel electrophoresis (PAGE) to separate ^{32}P-labeled DNA products of ds-cleavage have allowed Povirk and co-workers to determine the sequences of 30 ds-breaks induced by BLM (Povirk et al., 1989; Steighner & Povirk, 1990). Five sequence patterns were noted: the two predominant types of cleavage are shown below.



One site, shown in bold type, has been designated the primary (1°) site and is a prominent ss-cleavage site as previously defined by numerous investigators (D'Andrea & Haseltine, 1978; Fox, 1990; Kuwahara & Sugiura, 1988; Sugiura & Suzuki, 1982). The second site, shown underlined, was termed the secondary (2°) site which at many sites bears no resemblance to the reported selectivity for Fe-BLM ss-cleavage. While these studies have provided qualitative

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[1] Abbreviations: ds, double-stranded; ss, single-stranded; BLM, bleomycin; PAGE, polyacrylamide gel electrophoresis; 1°, primary; 2°, secondary; TBE, 89 mM Tris-HCl (pH 8.0), 89 mM boric acid, and 2 mM EDTA.

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information concerning the location of BLM-induced ds-breaks, they do not allow a basis on which to compare the relative frequencies of ds-cleavage occurring at individual sites. Information concerning the ss/ds-cleavage ratios for specific sequences might have importance in determining the relative contribution of ss- and ds-breaks to the genotoxicity of BLM (Bennett et al., 1993; Povirk, 1987; Povirk & Austin, 1991; Steighner & Povirk, 1990).

We now report a new method using hairpin oligonucleotides containing an "internal" ^{32}P-phosphodiester bond [i-^{32}P] which allows quantitative and simultaneous assessment of ss- and ds-cleavage products in a sequence-specific fashion. This approach takes advantage of the low backgrounds achievable using radiolabeled oligonucleotides and high-resolution PAGE under denaturing conditions and the ease of separating oligonucleotides differing by a single nucleotide in length. This method has allowed evaluation for the first time of ratios of ss- to ds-cleavage at specific sites over a range of Fe-BLM concentrations that vary from 5- to 70-fold. The results obtained using this method of analysis have been interpreted to indicate that a single Fe-BLM molecule, as originally proposed by Povirk (1983), can effect ds-cleavage. In the following paper the results using these hairpin-oligonucleotides to investigate the mechanism of site-specific ds-cleavage are reported (Absalon et al., 1995).

EXPERIMENTAL PROCEDURES

Materials. Bleomycin (Blenoxane) was obtained from Bristol-Meyers. Dilute solutions (448 μM in 5 mM HEPES, pH 7.5) were often stored at 4 °C for a period of a few months with no noticeable loss of activity. *Eco*RI and T4 polynucleotide kinase were obtained from New England Biolabs. The Klenow fragment of DNA polymerase I was isolated as described by Joyce and Grindley (1983). Deoxy-NTPs and NICK-50 columns were purchased from Pharmacia, and salmon testes DNA was purchased from Sigma. The ^{32}P-labeled nucleotides [α-^{32}P]dGTP (specific activity 3000 Ci mmol^{-1}) and [γ-^{32}P]ATP (specific activity 6000 Ci mmol^{-1}) were obtained from New England Nuclear/DuPont. Co(III)-BLM A_2, green and brown, were prepared as previously described (Chang & Meares, 1982) and purified to homogeneity by HPLC (Wu and Stubbe, unpublished results).

General. Electrophoresis was performed in 89 mM Tris-HCl, pH 8, 89 mM boric acid, and 2 mM EDTA (TBE buffer). Formamide gel loading buffer consisted of 90% deionized formamide in TBE buffer containing 0.025% (w/v) bromophenol blue and 0.025% (w/v) xylene cylanol. Maxam–Gilbert sequencing reactions were carried out by standard procedures (Maxam & Gilbert, 1980). Polyacrylamide gel electrophoresis (PAGE) under native and denaturing conditions was carried out as described in Sambrook et al. (1989).

Oligonucleotide Hairpin Template-Primers. The self-priming oligonucleotides prepared by the phosphoramidite method were purchased with the protecting groups removed from the Biopolymer Lab of M.I.T. or from Oligo's Etc. of Willsonville, OR. The self-priming hairpin oligonucleotides used for the preparation of GT-1, GT-2, GA-1, and GC-1 (Figure 1) listed in respective order are as follows: **1**, 5′-CGA-ATT-CTG-CGG-TAC-CCT-TTC-CCA-AAA-AGG-GAA-AGG; **2**, 5′-CGA-ATT-CTG-CTG-TAC-ACT-TTC-CCA-AAA-AGG-GAA-AGT; **3**, 5′-CGA-ATT-CCT-TTC-CCA-ACC-CAA-AAA-GGG-TTG-G; **4**, 5′-CGA-ATT-CTG-CGG-CGT-TTC-CCA-AAA-AGG-GAA-AC. Oligonucleotides **1**–**4** were purified by denaturing PAGE prior to further manipulation according to standard procedures (Sambrook et al., 1989) and stored in 5 mM HEPES (pH 7.5) with 5 mM NaCl at a typical concentration of 100 μM (oligonucleotide) as determined by its A_{260} nm using ϵ_{260} = 8.6 mM^{-1} (nucleotide) cm^{-1}. ^{32}P-Labeling of **1**–**4** on the 5′ hydroxyl group was accomplished using T4 polynucleotide kinase and [γ-^{32}P]ATP (Sambrook et al., 1989).

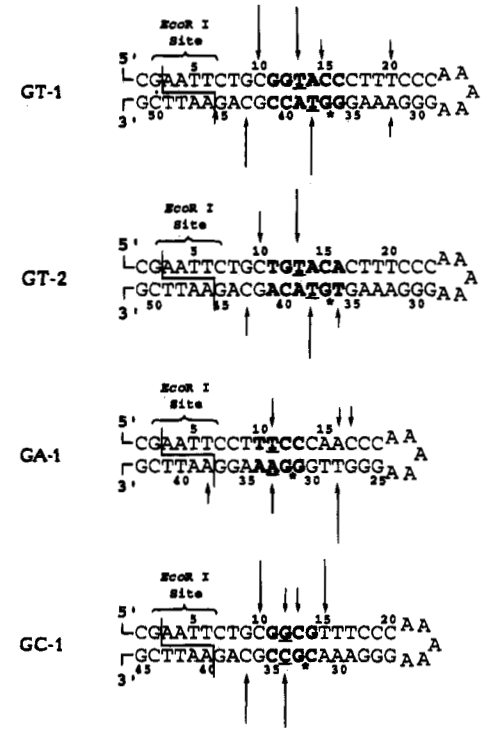

FIGURE 1: Oligonucleotides GT-1, GT-2, GA-1, and GC-1. The putative ds-cleavage sites are shown in bold type with the specific nucleotides degraded to form a ds-break shown underlined. The location of the internal ^{32}P-phosphodiester bond is shown with an asterisk. The arrows indicate the sites of cleavage observed using 5′-^{32}P oligonucleotides.

Preparation of Full-Length [5′-^{32}P]Hairpin Oligonucleotides: GT-1, GT-2, GA-1, and GC-1. [5′-^{32}P]Oligonucleotides **1**–**4** (100–200 pmol) were dissolved in a 40 μL solution of 50 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, and 30 mM NaCl, heated to 95 °C for 2 min in a heat block, and allowed to cool slowly in the block (~1 h) to room temperature. To the annealed oligonucleotide were added dGTP, dCTP, dATP, and TTP followed by ~10 units of the Klenow fragment of DNA polymerase I to give a final volume of 50 μL and final concentration of 400 μM in each dNTP. The polymerization reaction was allowed to proceed at room temperature for 20 min. Reactions were quenched with 100 μL of formamide gel loading buffer and the oligonucleotides purified by denaturing PAGE.

Preparation of [i-^{32}P]Hairpin Oligonucleotides: GT-1, GT-2, GA-1, and GC-1. Oligonucleotides **1**–**4** (250 pmol) in a 30 μL solution of 67 mM Tris-HCl (pH 7.5), 33 mM $MgCl_2$, and 41.7 mM NaCl were annealed as described above. [α-^{32}P]dGTP (125 μCi) and ~5 units Klenow were then added to this oligonucleotide to give a final volume of 50 μL, and the reactions were incubated at room temperature

for 15 min. Nonlabeled dGTP, dCTP, dATP, and TTP (400 μM final concentrations) and another ~5 units of Klenow were then added, bringing the final volume to 75 μL, and the reaction was incubated for another 20 min at room temperature. Unincorporated [α-^{32}P]dGTP and dNTPs were removed using a NICK column. The [i-^{32}P]hairpin oligonucleotides were isolated by ethanol precipitation, dissolved in 50 μL of formamide gel-loading buffer, and purified by denaturing PAGE. As an alternative to this one-pot method of labeling, the ^{32}P-labeled product produced during the initial incubation was separated from unincorporated [α-^{32}P]dGTP using a NICK column and ethanol precipitation prior to the enzymatic extension to the full length hairpin using 400 μM each unlabeled dNTP with ~5 units of Klenow in 50 μL of 50 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, and 30 mM NaCl.

Nondenaturing Gel Analysis of [5′-^{32}P] GT-1, GT-2, GA-1, and GC-1. Less than 1 pmol (20 000 Cerenkov cpm) of each of the [5′-^{32}P]oligonucleotides, GT-1, GT-2, GC-1, and GA-1, was heated to 95 °C for 2 min in a 40 μL solution containing 100 mM HEPES (pH 7.5), 100 mM NaCl, and 0.4 μg μL^{-1} salmon testes DNA. Each solution was allowed to slowly cool to room temperature in a heat block (~1 h) before receiving 10 μL of 40% sucrose gel-loading buffer. Each sample (25 μL) was subjected to electrophoresis through a 20% acrylamide gel lacking urea at constant power (10 W) for 20 h in a 4 °C room. The electrophoretic mobilities were compared to that of a [5′-^{32}P]oligonucleotide 25-bp standard prepared by annealing [5′-^{32}P]-TGC-CAT-CGA-TGG-TAG-CGG-CCG-CAA-G (~20 000 Cerenkov cpm) with ~10 nmol of its complementary strand in 20 μL of 100 mM HEPES (pH 7.5) and 100 mM NaCl.

EcoRI Truncation of Full-Length [i-^{32}P]Hairpin Oligonucleotides. Each full-length [i-^{32}P]hairpin oligonucleotide encodes an *Eco*RI recognition site and can be digested with *Eco*RI to yield a [i-^{32}P]hairpin with a four base 5′-staggered stem. Typical digestions contained 1−20 pmol of the preannealed [i-^{32}P]oligonucleotide (approximately 5 × 10^5 Cerenkov cpm) in a total volume of 50 μL, the manufacturer's buffer, and 24 units of *Eco*RI. Reactions were incubated at 37 °C overnight, terminated by the addition of two volumes of formamide gel loading buffer, and purified by denaturing PAGE.

Degradation of [^{32}P]Hairpin Oligonucleotides by BLM. Prior to all of the degradation reactions, each ^{32}P-oligonucleotide (~1 pmol) was added to a 40 μL solution containing 100 mM HEPES (pH 7.5), 100 mM NaCl, and 0.4 μg μL^{-1} salmon testes DNA (~1.2 mM nucleotide), heated to 95 °C for 2 min, and cooled to room temperature over ~1 h. "Activated BLM" was prepared *ex situ* by mixing equal volumes of 450 μM BLM in 5 mM HEPES (pH 7.5) and 450 μM $(Fe^{2+})(NH_4)_2(SO_4)_2$ at 4 °C for 60 ± 2 s, and an appropriately sized aliquot and H_2O were added to each individual reaction mixture to achieve the desired final concentration of Fe-BLM (14−112 μM) in a final volume of 80 μL. For degradation reactions using 0.78−14 μM of Fe-BLM, equal aliquots of 62.5 μM BLM and 62.5 μM $(Fe^{2+})(NH_4)_2(SO_4)_2$ were used in the *ex situ* formation of "activated BLM". Reactions were incubated at 4 °C for 10 min prior to the addition of 80 μL of 0.6 M NaOAc (pH 5.5) and 900 μL of ethanol. The samples were then chilled on dry ice (10 min), the precipitated DNA was collected and dissolved in 20 μL of formamide gel-loading buffer, and the radioactivity was quantified by the Cerenkov radiation prior to product separation by high-resolution PAGE (20% acrylamide containing 5% bis(acrylamide), TBE, 7 M urea).

DNA degradation reactions to be analyzed by nondenaturing PAGE were performed as described above with several modifications. Final reaction volumes were 20 μL, and the reactions were terminated by addition of 5 μL of gel-loading buffer (40% sucrose−EDTA, pH 7.4). The mixture was then immediately loaded onto a 20% native polyacrylamide gel.

Cleavage of [i-^{32}P]GT-1 by Co(III)-BLM. Reactions to investigate ss- and ds-cleavage by photoactivated Co(III)-BLM A_2 contained ^{32}P-labeled GT-1 (100 000 Cerenkov cpm), 0.2 μg μL^{-1} salmon testes DNA, 50 mM HEPES (pH 7.5), 50 mM NaCl, and 0−14 μM Co(III)-BLM A_2-"green" in 80 μL of final volume. The samples, in septum-stoppered borosilicate glass culture tubes (6 mm diameter), were irradiated with black light (broad band: 360 nm max output) for 15 min using a Rayonett Photo-Reactor in a 4 °C room. Under these conditions the temperature in the photolysis chamber was maintained between 15 and 20 °C. The DNA was then isolated by ethanol precipitation, redissolved in 100 μL of 1 M piperidine, and heated at 95 °C for 15 min (Saito et al., 1989; Sugiyama et al., 1990). The DNA was then again precipitated with ethanol and redissolved in 20 μL of formamide gel-loading buffer, and the products were separated by high-resolution PAGE.

Fe-BLM Cleavage of [i-^{32}P] GT-1 in the Presence of Co(III)-BLM. A 40 μL solution containing 100 000 Cerenkov cpm of [i-^{32}P]GT-1, 100 mM HEPES (pH 7.5), 100 mM NaCl, and 16 μg of salmon testes DNA was heated to 95 °C and cooled to room temperature as previously described. H_2O (15 μL) was added to this solution which was cooled to 4 °C. "Activated BLM" was prepared by incubating 5 μL of BLM (240 μM in 5 mM HEPES, pH 7.5) with 5 μL of 240 μM $(Fe^{2+})(NH_4)_2(SO_4)_2$ at 4 °C for 45 s. This mixture was then combined with 15 μL of 240 μM Co(III)-BLM A_2-"brown" for 15 s, and the entire BLM mixture was added to the DNA solution (final volume to 80 μL) to give 15 μM Fe-BLM and 45 μM Co(III)-BLM. Reactions were incubated at 4 °C for 10 min prior to the addition of 80 μL of 0.6 M NaOAc and 900 μL of ethanol. The DNA was precipitated as previously described, and the products were separated by 20% PAGE under denaturing conditions. Control reactions were identical to those described above except that Co(III)-BLM or Fe-BLM was omitted.

Quantitation of ^{32}P-Products of BLM Degradation. Quantitation of the individual ^{32}P-fragments separated by PAGE was accomplished using a Molecular Dynamics PhosphorImager (Johnston et al., 1990), equipped with Image-Quant (version 3). The software allows the data to be visualized in histogram form and for manual definition of the peak boundaries. The procedure used for this process is illustrated in Figure 2. A rectangle was defined through a portion of the lane containing the DNA fragments of interest such that the bands were evenly represented within the boundaries of the rectangle (I, Figure 2). A histogram was derived from the average of each pixel value spanning the width of the rectangle, and a base line was then drawn from valley-to-valley about each peak (II, Figure 2). Where two or more peaks were not clearly separated, or appeared in areas of high background, the base line was drawn valley-to-valley around the peak bunch and the peaks were separated by a vertical drop line reaching from the valley down to the previously drawn base line (III, Figure 2). The areas defined

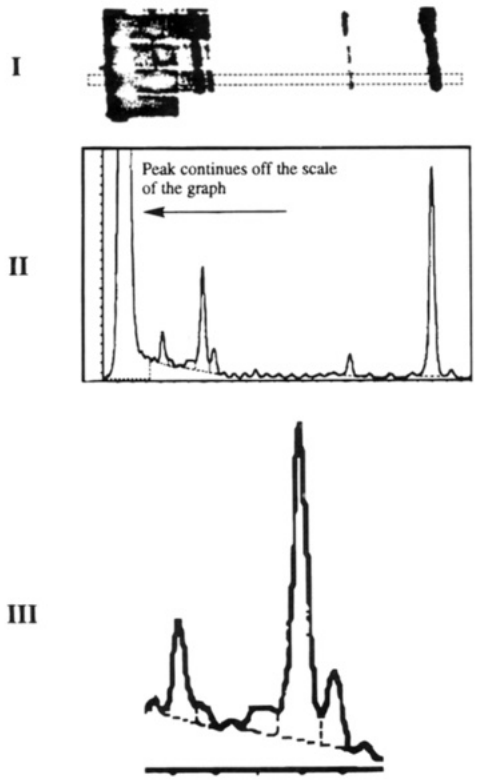

FIGURE 2: Method used for quantitation of ^{32}P-fragments separated by PAGE (described in detail in the Experimental Procedures).

within the histogram, vertical drop lines, and/or base lines were then computed and reported as relative values.

RESULTS AND DISCUSSION

Design of Hairpin Oligonucleotides To Examine Site-Specific ds-Cleavage. The four hairpins chosen for this study are shown in Figure 1. The rational for their design is briefly outlined. First, in the middle of the stem of each hairpin resides a putative ds-cleavage site. The site in GC-1 has previously been identified by Povirk and co-workers (Povirk et al., 1989; Steighner & Povirk, 1990) while the sites in GT-1, GT-2, and GA-1 have previously been identified by us using a similar sequencing method. Each oligonucleotide has been designed to have a stable duplex structure with a bulged loop of 5 nucleotides. Investigation by NMR spectroscopy of DNA hairpins containing loops of 3−5 nucleotides reveals that although the loop region is dynamic, the stem portion appears to be unperturbed beyond 3 bp from the loop (Baxter et al., 1993; Blommers et al., 1991; German et al., 1990; Gupta et al., 1993, 1987; Hare & Reid, 1986; Williamson & Boxer, 1989a,b). Thus our putative ds-cleavage sites in the center of the stems should be unperturbed by the loop structure. Likewise, these cleavage sites should be unperturbed by fraying at the end of each oligonucleotide.

The hairpin has also been designed so that BLM mediated ss- and ds-cleavage will result in ^{32}P-fragments sufficiently different in size so that they will be separated by denaturing PAGE. Additionally, an *Eco*RI restriction site has been built into the end of each hairpin. The reason for this latter addition, as outlined below, is that the *Eco*RI truncated hairpin can be used to provide further support for the identity of the fragment resulting from ds-cleavage. Finally, additional BLM ss-cleavage sites have been incorporated to produce a standard reference for quantitation of both ss- and ds-cleavage processes. This standard is particularly important in the quantitation of isotope effects discussed in the second paper in this series (Absalon et al., 1995).

Scheme 1: Preparation of [i-^{32}P]GT-1

Preparation of [5-^{32}P]- and [i-^{32}P]Hairpins. The strategy for synthesizing the internally labeled [i-^{32}P]hairpins is shown in Scheme 1 for GT-1. A self-priming 36-mer **1** is heated, annealed, and enzymatically extended using a pulse−chase method in which [α-^{32}P]dGTP is introduced in the pulse and the sequence completed with unlabeled dNTPs in the chase. All of the hairpins were labeled with [α-^{32}P]dGTP at the phosphodiester bonds indicated by ^{32}P in Figure 1. The unlabeled dGTP in the chase (400 μM) was sufficient to dilute out any remaining [α-^{32}P]dGTP (<1 μM) from the pulse so that no interference with sequencing analysis was observed. 5′-^{32}P-End labeling of the self-priming oligonucleotides **1**−**4** was accomplished using polynucleotide kinase and the full-length hairpins prepared analogously to the [i-^{32}P]hairpins. These complementary labeling methods ensured detection of almost all ss-and ds-cleavage products.

Evidence That the Structure of GT-1 Is the Predicted Hairpin. A detailed analysis in support of the hairpin structure for GT-1 (Figure 1) will be provided. Similar experiments have been carried out using the other three oligonucleotides.

Oligonucleotides capable of forming a hairpin structure are also capable of forming an oligomeric duplex containing a 5-bp bulge. The equilibrium between the two structures favors the monomeric or hairpin structure under low concentrations of oligonucleotide. Therefore, immediately prior to each degradation study, dilute solutions (nM) of oligonucleotide were heated to 95 °C to melt any secondary structure and slowly cooled to room temperature. To verify that a monomeric structure was indeed favored under the reaction conditions, [^{32}P]GT-1, subjected to this treatment, was analyzed by nondenaturing PAGE. The electrophoretic mobility of GT-1 (51 nucleotides long) was compared to that of a 25-bp duplex. Figure 3 shows that GT-1 migrates

FIGURE 3: Nondenaturing PAGE analysis of [5′-^{32}P]GT-1. The phosphorimage shows the migration of [5′-^{32}P]GT-1 relative to a 25-bp standard using a 20% polyacrylamide gel run under native conditions. Lane 1 contains [5′-^{32}P]GT-1. Lane 2 contains the 5′-^{32}P-labeled 25-bp oligonucleotide standard.

similarly to the 25-bp standard, supporting the conclusion that its structure is monomeric.

Fe-BLM Degradation of Hairpins. "Activated BLM" required for DNA degradation can be generated by incubation of Fe^{2+} and BLM in oxygenated buffers or from Fe^{3+} and BLM in the presence of mercaptoethanol and oxygenated buffers (Burger et al., 1981). To avoid problems associated with the reproducibility of the extent of DNA degradation, BLM was activated "*ex situ*" by the method of Burger et al. (1979, 1981). "Activated BLM" was prepared by combining equal volumes of BLM (450 μM) and ferrous ammonium sulfate (450 μM) in air saturated buffer at 4 °C for 60 s, and this mixture was then added directly to the reaction mixture containing the hairpin being investigated. The concentrations of Fe-BLM used varied from 0.8 to 120 μM, and the concentration of carrier DNA was 600 μM (in nucleotides). The products generated after incubation for 10 min at 4 °C were analyzed by denaturing PAGE. Typical results using [5′-^{32}P]GT-1 and [i-^{32}P]GT-1 are shown in Figure 4, panels a and b, respectively. The ^{32}P-fragments resulting from [5′-^{32}P]GT-1 were assigned on the basis of their electrophoretic mobilities relative to sequencing standards (Maxam & Gilbert, 1980). The sites of cleavage are summarized in Figure 1. The predominant sites of cleavage (C_{10}, T_{13}, A_{32}, T_{38}, C_{43}) are consistent with the previously reported selectivity for ss-cleavage by BLM (D'Andrea & Haseltine, 1978; Fox, 1990; Kuwahara & Sugiura, 1988; Sugiura & Suzuki, 1982). Minor extents of cleavage were also observed at C_{15} and T_{20}, neither of which follows the normal ss-cleavage selectivity for Fe-BLM. The significance of the cleavage occurring at these latter two sites has not been experimentally evaluated, but they may represent "secondary" sites involved in ds-cleavage at locations in GT-1 other than at the designed 5′-G*T*AC site.

The fragmentation patterns of [i-^{32}P]GT-1 are shown in Figure 4b. Tentative assignments are made on the basis of size of the fragments relative to Maxam−Gilbert sequencing markers and expectations based on the results of the experiments with the 5′-^{32}P-labeled material (Table 1). The ^{32}P-fragments generated from [i-^{32}P]GT-1 differ slightly in their electrophoretic mobilities compared to the Maxam−Gilbert fragments due to the differences in their 5′- and 3′-ends (Table 1).

A consequence of the perturbation of the electrophoretic mobility due to the ends of a [i-^{32}P]fragment, is that the two ^{32}P-fragments due to ss-cleavage at C_{10} and C_{43} are not resolved on the sequencing gel shown in Figure 4b. The expected length of the ^{32}P-fragment due to ss-cleavage at C_{10} is 41 nucleotides while the length of the fragment due to ss-cleavage at C_{43} is 42 nucleotides. The electrophoretic mobility of the 42-nucleotide fragment, however, is enhanced by its 3′-phosphoglycolate end which diminishes its separation from the 41-nucleotide fragment. In contrast, the two ^{32}P-fragments resulting from ss-cleavage at T_{13} and T_{38} (which also differ in length by 1 nucleotide) are satisfactorily resolved on the sequencing gel shown in Figure 4b. Because T_{13} and T_{38} are staggered in the 5′-direction (while C_{10} and C_{43} are staggered in the 3′-direction), the migration of the shorter ^{32}P-fragment (the product of ss-cleavage at T_{38}) is enhanced by the 3′-phosphoglycolate end, and therefore the separation of the two fragments is also enhanced.

Cleavage involving A_{32} of [i-^{32}P]GT-1 leads to the fastest migrating species on the gel shown in Figure 4b. Intriguingly, the ^{32}P-fragment migrating with the mobility expected for ss-cleavage at T_{20} is not seen on the gel, indicating that ss-cleavage at T_{20} does not occur to a appreciable extent. The implication from the absence of a ^{32}P-fragment from [i-^{32}P]GT-1 corresponding to ss-cleavage at T_{20}, combined with the presence of the corresponding ^{32}P-fragment from [5′-^{32}P]GT-1, is that cleavage at T_{20} is predominately, if not exclusively, associated with a ds-cleavage event probably involving cleavage at A_{32}. The implication of ds-cleavage at this site was the impetus for the design of hairpin, GA-1, to be discussed subsequently.

The most interesting cleavage product is the ^{32}P-fragment comigrating with the 24-mer Maxam−Gilbert standard. This fragment cannot be explained by any single lesion observed from the degradation of [5′-^{32}P]GT-1. The size of this fragment, however, is consistent with a ds-cleavage event involving T_{13} and T_{38}. Two additional lines of evidence support the assignment of this fragment as a ds-cleavage product. The first involves repetition of the above experiment on *Eco*RI-truncated [i-^{32}P]GT-1 and comparison of the resulting cleavage pattern to that obtained with the full length hairpin. The Fe-BLM-induced fragmentation pattern of the *Eco*RI-truncated [i-^{32}P]GT-1 resulted in a predictable change in the migration of all ^{32}P-fragments (compare lane 1 and lanes 2−6, Figure 5). Since only the electrophoretic migration of ^{32}P-fragments resulting from ds-cleavage are expected to be insensitive to the *Eco*RI truncation, the presence of a 24-nucleotide fragment in lanes 2−6 supports its assignment to that of ds-cleavage involving T_{13} and T_{38}.

Additional evidence supporting the assignment of the 24-nucleotide fragment is obtained by analysis of the Fe-BLM degradation of [i-^{32}P]GT-1 by native PAGE. Analysis under nondenaturing conditions is expected to resolve ds-cleavage fragments from GT-1 containing ss-breaks which migrate similarly to undamaged oligonucleotide. One major fast-migrating product (A, Figure 6a) is apparent on the nondenaturing gel. Isolation of this fragment from the gel with subsequent electrophoresis on a denaturing-gel showed that it comigrated with the 24-nucleotide fragment (compare lanes 1 and 3, Figure 6b). Furthermore, isolation of the material not resolved from the full-length hairpin on the native gel (B, Figure 6a) and subsequent electrophoresis on the denaturing gel showed that none of the 24-nucleotide

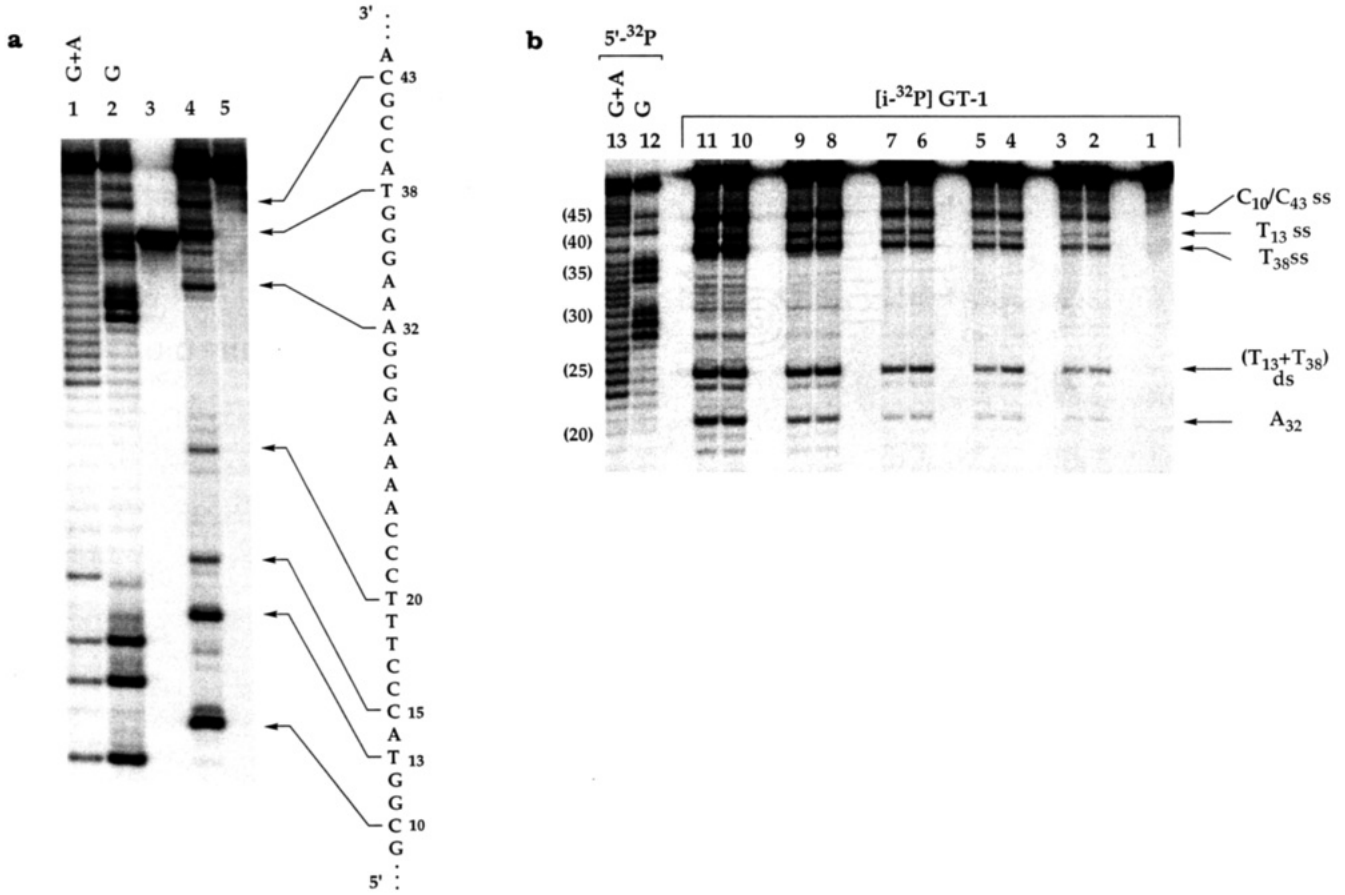

FIGURE 4: Fe-BLM degradation of [5′-^{32}P]GT-1. The phosphorimage shows the 20% polyacrylamide sequencing gel used to evaluate Fe-BLM cleavage of [5′-^{32}P]GT-1. Lanes 1 and 2, G+A and G Maxam−Gilbert markers, respectively. Lane 3, [5′-^{32}P] labeled **1**, a 36 oligonucleotide standard. Lane 4, [5′-^{32}P]GT-1 treated with 8 μM Fe-BLM. Lane 5, [5′-^{32}P]GT-1 without iron or BLM. (b)Fe-BLM degradation of [i-^{32}P]GT-1. The phosphorimage shows the 20% polyacrylamide sequencing gel used to evaluate Fe-BLM cleavage of [i-^{32}P]GT-1. Lanes 1−11 contain [i-^{32}P]GT-1 treated with the concentrations of Fe-BLM indicated (lane, concentration): 1, no drug; 2 and 3, 0.8 μM; 4 and 5, 1.6 μM; 6 and 7, 3.1 μM; 8 and 9, 6.2 μM; 10 and 11, 12.5 μM. Lanes 12 and 13 contain G and G+A Maxam−Gilbert markers using [5′-^{32}P]GT-1. The numbers in parentheses indicate the number of nucleotides of the Maxam−Gilbert marker beside it.

Table 1: Cleavage Products from Fe-BLM Degradation of [i-^{32}P] GT-1[a]

cleavage site	expected length (nuc)	expected 5′-end	expected 3′-end	migration rel to Maxam−Gilbert stds (nuc)	% cleavag of [i-^{32}P]-GT-1 [b]
C_{10} ss	41	P	OH	43	*c*
T_{13} ss	38	P	OH	40	1.2
T_{20} ss	31	P	OH	n.o.	
A_{32}[d]	19	P	OH	20−21	0.2
T_{38} ss	37	OH	PG	37	2.1
C_{43} ss	42	OH	PG	43	*c*
(G_9 + C_{43}) ds	33	P	PG	n.o.	
(C_{10} + G_{42}) ds	31	P	PG	n.o.	
(T_{13} + T_{38}) ds	24	P	PG	24	0.94

[a] Abbreviations: PG, phosphoglycolate; P, phosphate; OH, hydroxyl; n.o., not observed. [b] The total extent of degradation of [i-^{32}P]GT-1 for this set of data is 6.7%. [c] Fragments resulting from ss-cleavage at C_{10} and C_{43} of [i-^{32}P]GT-1 comigrate in Figure 4b. The sum of the ss-cleavage products at these sites represents 2.0% degradation. Quantitation of the products from Fe-BLM treatment of [5′-^{32}P]GT-1 indicates that the ratio of ss-cleavage at C_{10} to C_{43} is 3:1. [d] Ds- and ss-cleavage involving this site cannot be distinguished.

fragment was present while all other fragments were present (lane 2, Figure 6b). These data strongly support the assignment of the 24-nucleotide fragment from the Fe-BLM degradation of [i-^{32}P]GT-1 as the ds-cleavage product involving T_{13} and T_{38}.

For this ds-cleavage to be of mechanistic interest, the 24-nucleotide fragment must not be the result of two ss-breaks coincidentally occurring at T_{13} and T_{38} on the same [i-^{32}P]-GT-1. Quantitation of the amount of products produced from degradation of [i-^{32}P]GT-1 due to 12.5 μM Fe-BLM reveals that the observed cleavage occurring at T_{13} (ss and ds) represents 2.14% degradation while the cleavage observed at T_{38} represents 3.04% degradation (Table 1).[2] The multiplicative product of these extents of degradation (0.065%) represents the amount of ds-cleavage predicted by a coincident occurrence of ss-cleavage events. The amount of the 24-nucleotide fragment from 12.5 μM Fe-BLM represents 0.94% degradation, or 14 times the amount predicted by two coincident ss-breaks. The same calculation using the data obtained from the degradation of [i-^{32}P]GT-1 using 0.8 μM Fe-BLM (0.56% total degradation) predicts 0.00024% ds-cleavage while the actual observed amount (0.062%) is 258 times higher. The ds-cleavage observed in GT-1 is mechanistically significant.

The assay for ds-cleavage using [i-^{32}P]GT-1 might have also detected ds-cleavage involving 5′-d(G_9C_{10}/$G_{42}C_{43}$).

[2] The percentages for total cleavage at T_{13} and T_{38} do not include a correction for the extent of each fragment lost due to other cleavage events such as ss-cleavage at C_{10} and C_{43}. Since the extent of degradation at these sites is small (~2%), the contribution of these factors to the percent cleavage at T_{13} and T_{38} is negligible.

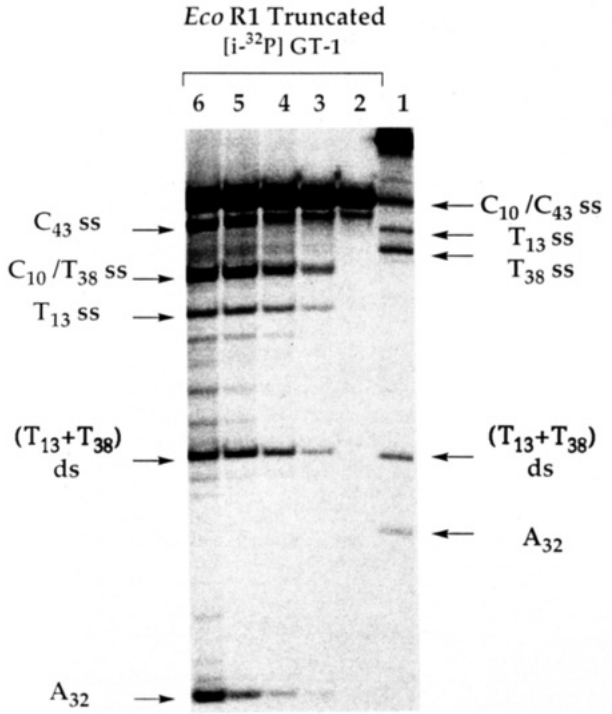

FIGURE 5: Fe-BLM degradation of *Eco*RI truncated [i-^{32}P]GT-1. Lane 1, full-length [i-^{32}P]GT-1 treated with 14 μM Fe-BLM. Lanes 2−6 contain truncated [i^{32}P] GT-1 treated with the concentrations of Fe-BLM indicated (lane, concentration): 2, no drug; 3, 7 μM; 4, 14 μM; 5, 28 μM; 6, 56 μM.

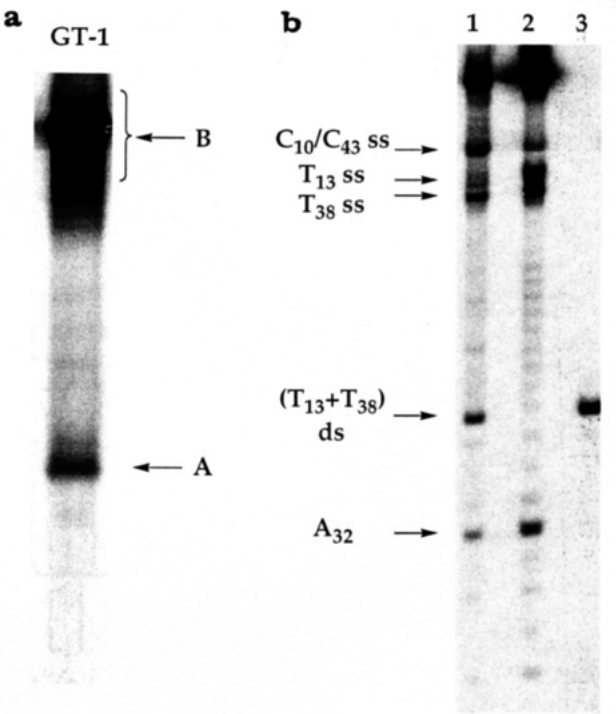

FIGURE 6: Nondenaturing PAGE analysis of Fe-BLM degradation of [i-^{32}P]GT-1. [i-^{32}P]GT-1 was treated with 30 μM Fe-BLM and then subject to electrophoresis using a nondenaturing 20% polyacrylamide gel. Fragment A and the broad region indicated as B were isolated and reanalyzed under denaturing conditions shown in panel b. (b) Reanalysis of fragments separated using native conditions. Fragments A and B from the gel shown in panel a were reanalyzed using a 20% polyacrylamide sequencing gel containing 7 M urea. Lane 1 contains [i-^{32}P]GT-1 treated with 30 μM Fe-BLM without previous separation of the fragments by nondenaturing PAGE. Lanes 2 and 3 contain A and B, respectively.

Although the surrounding sequence to this dinucleotide does not conform to a previously observed site for Fe-BLM-induced ds-cleavage, the pattern for sequence selectivity described by Povirk et al. (1989) predicts that if ds-cleavage were to occur it would involve cleavage at G_9 plus C_{43}, and/or C_{10} plus G_{42}, producing blunt ended DNA fragments. Either of these ds-cleavage events would have produced ^{32}P-fragments that would have easily been resolved from the other cleavage products during PAGE analysis (Table 1). These fragments, however, were not observed, indicating that significant ds-cleavage involving this 5′-GC fragment does not occur. Single-strand cleavage occurs at both C_{10} and C_{43} to about the same extent that ss-cleavage occurs at T_{13} and T_{38}. The lower limit for the detection of ds-cleavage at this dinucleotide by this assay has not yet been established; however, it is likely that if ds-cleavage had occurred to greater than 10% of the extent of ss-cleavage observed at C_{10} and C_{43}, it would have been detected. The paucity of ds-cleavage observed at these sites indicates that the efficiency of ds-cleavage can vary dramatically between sites where ss-cleavage by Fe-BLM occurs at approximately equivalent levels.

Degradation of [i-^{32}P]GT-1 by Co(III)-BLM. As a control experiment to demonstrate an apparent ds-cleavage resulting from two coincident ss-breaks, the degradation of [i-^{32}P]GT-1 mediated by Co(III)-BLM A_2-"green" irradiated with near-UV light was examined. Previous studies have shown that the Co(III)-BLM (green) cleaves DNA with similar sequence specificity to that observed with Fe-BLM, but does not result in ds-cleavage as analyzed by the conversion of supercoiled plasmid DNA to linear DNA (Chang & Meares, 1982, 1984; Saito et al., 1989). The predominate lesion produced by photoactivated Co(III)-BLM is the 4′-ketone abasic site (Saito et al., 1989; Sugiyama et al., 1990). This lesion requires base or piperidine treatment to effect strand scission (Sugiyama et al., 1986). Figure 7 shows the degradation of GT-1 studied as a function of concentration of Co(III)-BLM A_2 (green). Analogous to the observations made with Fe-BLM, ss-cleavage occurs at both T_{13} and T_{38} as well as at C_{43} and C_{10}. Cleavage occurs to a lesser extent at A_{32}. The 24-nucleotide fragment corresponding to ds-cleavage involving T_{13} and T_{38} is also observed, although only in the reactions for which a high extent of degradation has occurred. The 24-nucleotide fragment is first visible in the reaction containing 7.25 μM Co(III)-BLM in which the oligonucleotide has already suffered 42% degradation. The percent of degradation occurring at T_{13} is 8.4% while the cleavage occurring at T_{38} is 13%. A purely coincident mechanism for ds-cleavage, then, predicts that "ds-cleavage" should account for 1.1% of the ^{32}P-material. The actual observed amount of the 24-nucleotide fragment is 0.91%. "Double-strand cleavage" by Co(III)-BLM, then, can be adequately explained by a coincident mechanism. Interestingly, a ~27-nucleotide fragment is also observed in the reactions undergoing a large extent of degradation. The corresponding cleavage site for this fragment has not been unambiguously identified, but the size and extent of cleavage are consistent with a coincident amount of ss-breaks involving both T_{38} and C_{10} and not ds-cleavage due to a mechanistically significant process.

Examination of the Degradation of Additional Hairpins. Experiments similar to those described for GT-1 have been carried out with GT-2, GA-1, and GC-1. The results of the Fe-BLM-induced cleavage observed using 5′-^{32}P-oligonucle-

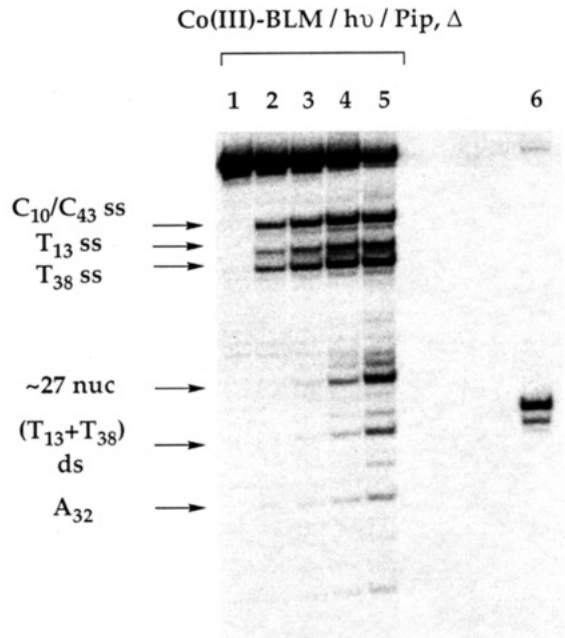

FIGURE 7: [i-^{32}P]GT-1 degradation by Co(III)-BLM A_2-"green". [i-^{32}P]GT-1 was irradiated with Co(III)-BLM A_2-"green" and the products were separated on a 20% polyacrylamide sequencing gel subsequent to incubation with 1 M piperidine at 95 °C. The concentration of Co-BLM used in each reaction is as follows: lane 1, 0; lane 2, 1.8 μM; lane 3, 3.6 μM; lane 4, 7.2 μM; lane 5, 14.5 μM. Lane 6 contains [i-^{32}P]GT-1 treated with *Rsa*I to produce a 25-base oligonucleotide size marker.

otides is presented in Figure 1. In general, cleavage was observed at all sites conforming to the reported sequence selectivity for ss-cleavage by Fe-BLM . Suprisingly, for reasons that are not clear, the extent of cleavage observed at T_{36} of GT-2 and C_{13} of GC-1 was only about 10−15% the extent of cleavage observed at other G-Py ss-cleavage sites. Minor sites of cleavage were observed in GA-1 at T_{11}, A_{16}, and C_{17}. Cleavage observed at T_{11} is consistent with ds-cleavage involving A_{33}. Cleavage at A_{16} and C_{17} may be involved in ds-cleavage in conjunction with T_{28}; however, this possibility was not explored further. The minor extent of cleavage observed at G_{12} in GC-1 is consistent with ds-cleavage involving C_{34}.

To directly observe ds-cleavage in these oligonucleotides, [i-^{32}P]GT-2, -GA-1, and GC-1 were treated with "activated BLM", and the electrophoretic migration of the ^{32}P-fragments was compared to Maxam−Gilbert standards. The results are summarized in Tables 2−4. In analogy to the results obtained with GT-1, a prominent amount of 24-nucleotide fragment due to ds-cleavage involving T_{13} and T_{38} was observed using [i-^{32}P]GT-2 (Table 2). Since the sequence of GT-2 differs from GT-1 only in the base pairs which immediately surround the GTAC site, the prominent ds-cleavage observed in GT-2 suggests that the recognition site for Fe-BLM mediated ds-cleavage may be adequately described by a 4-bp sequence. A 21-nucleotide fragment was observed in the degradation reactions using [i-^{32}P]GA-1 and GC-1 consistent with ds-cleavage occurring at A_{33} and T_{11} of GA-1, and G_{12} and C_{34} of GC-1. Double-strand cleavage in these latter two oligonucleotides, in contrast to GT-1 and GT-2, involves cleavage at a good BLM cleavage site (1° site) as well as cleavage at a 2° site that does not normally function as a good ss-cleavage site. In both cases blunt ended fragments are observed. In each of the three cases, the amount of ds-cleavage product was shown to be above the amount predicted by a coincident occurrence of ss-breaks.[3]

Table 2: Cleavage Products from Fe-BLM Degradation of [i-^{32}P]GT-2[a]

cleavage site	expected length (nuc)	expected 5′-end	expected 3′-end	migration rel. to Maxam−Gilbert stds (nuc)	% cleavage of [i-^{32}P]-GT-1[b]
C_{10} ss	41	P	OH	42−43	[c]
T_{13} ss	38	P	OH	39	1.6
T_{36}[d]	15	P	OH	15−16	0.26
T_{38} ss	37	OH	PG	37−38	2.5
C_{43} ss	42	OH	PG	43	[c]
(G_9 + C_{43}) ds	33	P	PG	n.o.	
(C_{10} + G_{42}) ds	31	P	PG	n.o.	
(T_{13} + T_{38}) ds	24	P	PG	24	1.2

[a] Abbreviations: PG, phosphoglycolate; P, phosphate; OH, hydroxyl; n.o., not observed. [b] The total extent of degradation of [i-^{32}P]GT-2 for this set of data is 7.0%. [c] Fragments resulting from ss-cleavage at C_{10} and C_{43} of [i-^{32}P]GT-2 comigrate. The sum of the ss-cleavage at these sites represents 1.2% degradation. Quantitation of these products from Fe-BLM treatment of [5′-^{32}P]GT-1 indicates that the ratio of ss-cleavage at C_{10} to C_{43} is ~14:1. [d] Ds- and ss-cleavage involving this site cannot be distinguished.

Table 3: Cleavage Products from Fe-BLM Degradation of [i-^{32}P]GA-1[a]

cleavage site	expected length (nuc)	expected 5′-end	expected 3′-end	migration rel. to Maxam−Gilbert stds (nuc)	% cleavage of [i-^{32}P]-GT-1[b]
T_{11} ss	32	P	OH	32	[c]
A_{16} ss	27	P	OH	n.o.	
C_{17} ss	26	P	OH	n.o.	
T_{28} ss[d]	15	P	OH	15−16	3.9
A_{33} ss	32	OH	PG	32	[c]
A_{38} ss	37	OH	PG	37	0.27
(A_{33} + T_{11}) ds	21	P	PG	21	0.31

[a] Abbreviations: PG, phosphoglycolate; P, phosphate; OH, hydroxyl; n.o., not observed. [b] The total extent of degradation of [i-^{32}P]GA-1 for this set of data is 5.9%. [c] Fragments resulting from ss-cleavage at A_{33} and T_{11} of [i-^{32}P]GA-1 are expected to comigrate. The sum of the ss-cleavage at these sites represents 1.4% degradation. Quantitation of these products from Fe-BLM treatment of [5′-^{32}P]GA-1 indicates that the ratio of cleavage at A_{33} to T_{11} is 4:1. [d] Ds- and ss-cleavage involving this site cannot be distinguished.

Table 4: Cleavage Products from Fe-BLM Degradation of [i-^{32}P]GC-1[a]

cleavage site	expected length (nuc)	expected 5′-end	expected 3′-end	migration rel. to Maxam−Gilbert stds (nuc)	% cleavage of [i-^{32}P]-GT-1[b]
C_{10} ss	35	P	OH	36	1.7
G_{12} ss	33	P	OH	33−34	[c]
C_{13} ss	32	P	OH	33	0.29
T_{15} ss	30	P	OH	31	1.5
C_{34} ss	33	OH	PG	33−34	[c]
C_{37} ss	36	OH	PG	37	2.1
(G_{12}+C_{34}) ds	21	P	PG	37	0.36
(G_9 + C_{37}) ds	27	P	PG	n.o.	
(C_{10} + G_{36}) ds	25	P	PG	n.o.	
(C_{13} + G_{33}) ds	19	P	PG	n.o.	

[a] Abbreviations: PG, phosphoglycolate; P, phosphate; OH, hydroxyl; n.o., not observed. [b] The total extent of degradation of [i-^{32}P]GC-1 for this set of data is 8.6%. [c] Fragments resulting from ss-cleavage at $G_{12}$and C_{34} of [i-^{32}P]GC-1 are expected to comigrate. The sum of the ss-cleavage at these sites represents 2.1% degradation. Quantitation of these products from Fe-BLM treatment of [5′-^{32}P]GC-1 indicates that the ratio of cleavage at C_{34} to G_{12} is ~5.5:1.

Table 5: Site-Specific Ss- to Ds-Cleavage Ratios

[i-^{32}P]-oligonucleotide	cleavage site	total oligonucleotide degradation (%)	ss- to ds-cleavage ratio
GT-1	5'-GG**T**ACC CCA**T**GG-5'	6.7	3.3
GT-2	5'-TG**T**ACA ACA**T**GT-5'	7.0	3.4
GA-1	5'-TT**T**CCC AA**A**GGG-5'	5.9	4.5
GC-1	5'-CG**C**GT GC**C**GCA-5'	8.6	5.8

Comparison of the ss- to ds-Cleavage Ratios Observed in GT-1, GT-2, GA-1, and GC-1. The hairpin-oligonucleotide assay provides a case study approach for comparison of the efficiencies of ds-cleavage by Fe-BLM at defined positions within specific DNA sequences. The ss- to ds-cleavage ratios obtained with the four [i-^{32}P]oligonucleotides used in this study are summarized in Table 5 for similar extents of oligonucleotide degradation. Double-strand cleavage occurring at the G*T*AC sites of GT-1 and GT-2 demonstrated the highest efficiency of ds-cleavage with ss/ds-cleavage ratios of 3.3 and 3.4, respectively. Both *T*s involved in ds-cleavage in these hairpins are good ss-cleavage, sites, and the absolute amount of ds-cleavage is extensive. Indeed, the extent of ds-cleavage at the G*T*AC in GT-1 is approximately equal to the extent of ss-cleavage occurring at C_{10} in the same hairpin (Table 1). In contrast to the ds-cleavage efficiency noted in GT-1 and GT-2, the CG*C*C site in GC-1 displayed a higher ss/ds ratio (~5.8). Barring differences due to exact sequence context, the ss/ds ratio is to some extent surprising given the attention that analogous sites in λc1 DNA have received as "mutational hot spots" for Fe-BLM (Povirk, 1987; Steighner & Povirk, 1990). Not included in Table 5 are the other 5′-G_9C_{10} sites in GT-1, GT-2, and GC-1 (Figure 1) in which significant ss-cleavage was observed, but for which no ds-cleavage could be detected. While it is possible that ds-cleavage may still occur at these sites, the efficiency of ds-cleavage must be very low compared to the other sites evaluated in this study. These results clearly indicate that the efficiency of ds-cleavage varies depending upon sequence context and, therefore, affirm that the "global" ss/ds-cleavage ratios reported using vector and plasmid DNA represent an average ratio for all Fe-BLM cleavage sites (Povirk et al., 1977; Lloyd et al., 1978; Mirabelli et al., 1980; Boger et al., 1994).

Ratios of ss- to ds-Cleavage as a Function of Concentration of Fe-BLM. Contradictory reports have appeared in the literature regarding the dependence of ds-cleavage on the order of the concentration of FeBLM. Povirk et al. (1977), studying the conversion of Col E1 supercoiled DNA to closed circular and linear DNA, reported that the ratio of ds-breaks to ss-breaks did not change over a factor of ~6.7 in extent of degradation. While these experiments in 1977 did not have exogenous iron added to the reaction mixtures, leaving ambiguous the identity of the metallo-BLM species responsible for the damage, the conclusion was drawn that the number of ds-breaks was "roughly" proportional to the BLM concentration. In a more recent paper Gonikberg and Odintsova (1987) reported that the occurrence of ds-breaks in phage PM2 DNA cross-linked with psoralen was proportional to the square of the Fe-BLM concentration over a concentration range of 0.3−3 μM. The authors thus arrived at diametrically opposed conclusions. Povirk cites his paper as indicating a single BLM molecule is responsible for one ds-cleavage event (Povirk, 1983), while Gonikberg and Odintsova (1987) suggest two molecules are responsible for each ds-break. We have therefore reexamined ds-cleavage occurring at specific sites in the hairpin oligonucleotides as a function of Fe-BLM concentration and extent of damage. The most dramatic results are shown in Figure 8 using [i-^{32}P]-GT-1. In this case the ss- to ds-cleavage ratio remains constant over a 70-fold range in Fe-BLM concentration and extent of degradation. Furthermore, in contrast to the observations made by Gonikberg and Odintsova (1987), the relative amount of ds-cleavage is linear with the concentration of Fe-BLM used to effect the cleavage. Similarly, the ss/ds cleavage ratio in [i-^{32}P]GA-1 remained constant over a 4.5-fold range in Fe-BLM concentration and extent of DNA degradation (data not shown).[4]

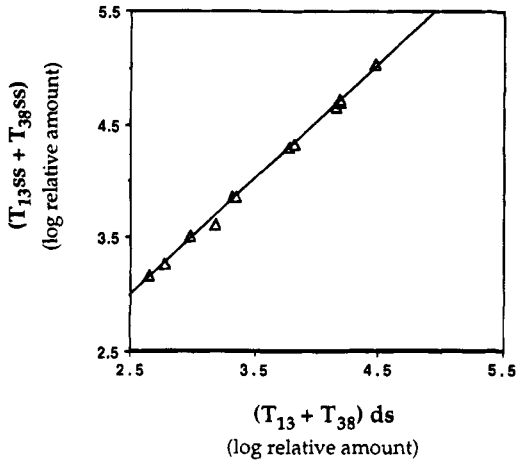

FIGURE 8: Fe-BLM-mediated ss- and ds-cleavage in [i-^{32}P]GT. The log of the relative extents of ss- and ds-cleavage occurring at T_{13} and T_{38} in [i-^{32}P]GT-1 was plotted using data from experiments employing from 0.78 to 56 μM Fe-BLM. The data corresponding to experiments using 0.78−12.5 μM was obtained from the gel shown in Figure 4b.

The simplest explanation of the independence of the ss/ds ratio upon Fe-BLM concentration and extent of DNA damage is that one molecule of BLM effects ds-cleavage. However, a second interpretation of these results, first suggested by Lloyd et al. (1978b) and recently reiterated by Keller and Oppenheimer (1987) is that a second molecule of Fe-BLM can bind in a highly cooperative fashion once the first molecule of BLM has been appropriately positioned on the DNA. To test this hypothesis involving cooperativity of binding, the ratio of ss- to ds-cleavage was redetermined

[3] A 21-nucleotide fragment in GC-1 can also be formed by two coincident cleavages occurring at T_{15} and C_{37}. Experiments with this hairpin were therefore limited to conditions in which less than ~10% degradation occurred to avoid a significant contribution to the 21-nucleotide fragments by this statistical event.

[4] Due to the inefficiency of ds-cleavage in GA-1, the ss- to ds-cleavage ratio could not be evaluated over a larger range of Fe-BLM concentration since greater extents of degradation result in putative "ds-cleavage" arising from coincident ss-cleavage events.

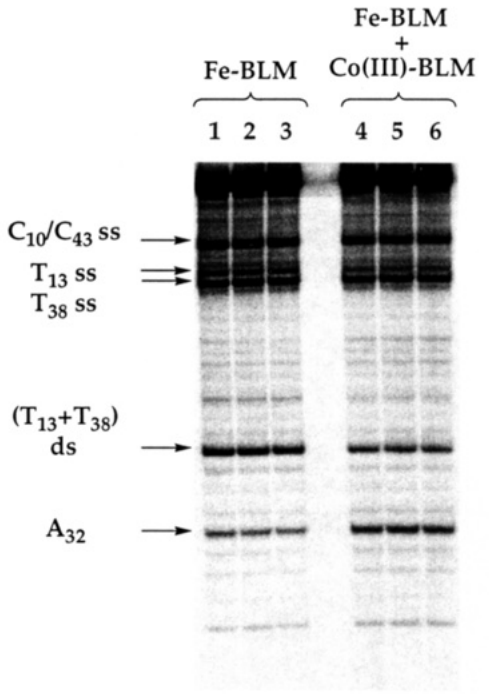

FIGURE 9: Degradation of [i-^{32}P]GT-1 in the presence of Co(III)-BLM. Lanes 1−3 contain [i-^{32}P]GT-1 treated with 15 μM Fe-BLM. Lanes 4−6 contain [i-^{32}P]GT-1 treated with a mixture of 15 μm Fe-BLM and 45 μM Co-BLM A_2 "brown".

in the presence of the "brown" form of Co(III)-BLM A_2. We hypothesized that CoBLM, a molecule that is chemically inert in the absence of light, possessing similar sequence selectivity to Fe-BLM (Chang & Meares, 1984; McLean et al., 1989), might interfere with the cooperative binding of a second molecule of Fe-BLM and thus decrease the amount of ds-cleavage. Furthermore, since "activated BLM" is prepared *ex situ* resulting in equal amounts of "activated-BLM" and nonactive Fe(III)-BLM (Burger et al., 1981), the inclusion of three additional equivalents of Co(III)-BLM to the reaction mixture results in a ratio of "active" BLM to "inactive" BLM of ~1:7. If Co(III)-BLM does indeed compete with "activated BLM" for DNA binding, then ds-cleavage might be expected to decrease by a factor of 16.0 [$(1/2)^2$ vs $(1/8)^2$]. The results shown in Figure 9 reveal a change in the ss- to ds-cleavage ratio from 3.1 in the reaction lacking Co-BLM, to 5.0 in the reaction in which it is included. Similarly, the ss/ds ratio failed to change significantly when [i-^{32}P]GA-1 and [i-^{32}P]GT-2 were treated with Fe-BLM in the presence of 2.5 equivs of "brown" Co(III)-BLM A_2, and 4 equivs of "green" Co(III)-BLM A_2, respectively (data not shown). The small increase in ss/ds cleavage ratio, in contrast with that predicted for a cooperative mechanism, might be expected as the high concentrations of inactive BLMs might nonspecifically bind, preventing the single BLM from the first site of cleavage to reorganize to the second site required for ds-cleavage. In conjunction with the Fe-BLM concentration dependence for ds-cleavage described above, these results allow us to favor the hypothesis proposed by Povirk and co-workers (Povirk et al., 1989; Steighner & Povirk, 1990) that a single BLM can be reactivated after an initial cleavage event to allow the occurrence of a second strand break. Further insight into this issue may benefit from a detailed kinetic analysis of BLM-mediated cleavage under one-turnover conditions.

SUMMARY

A new method has been developed to examine ds-cleavage events using hairpin oligonucleotides. This method has allowed determination for the first time of the ratio of ss- to ds-cleavage occurring at specific sites in a specific sequence context. The independence of this ratio from the BLM concentration is consistent with a mechanism in which a single molecule of Fe-BLM can mediate ds-cleavage. These hairpins, as outlined in the following paper, have provided a means to examine in more chemical detail the ds-cleavage event mediated by Fe-BLM.

REFERENCES

Absalon, M. J., Vanderwall, D. E., Wu, W., Kozarich, J. W., & Stubbe, J. S. (1995) *Biochemistry 34,* 2076−2086.
Baxter, S. M., Greizerstein, M. B., Kushlan, D. M., & Ashley, G. W. (1993) *Biochemistry 32,* 8702−8711.
Bennett, R. A., Swerdlow, P. S., & Povirk, L. F. (1993) *Biochemistry 32,* 3188−3195.
Blommers, M. J. J., van de Ven, F. J. M., van der Marel, G. A., & van Boom, J. H. (1991) *Eur. J. Biochem. 201,* 33−51.
Boger, D. L., Honda, T., Menezes, R. F., & Colletti, S. L. (1994) *J. Am. Chem. Soc. 116,* 5631−5646.
Burger, R. M., Horwitz, S. B., Peisach, J., & Wittenberg, J. B. (1979) *J. Biol. Chem. 254,* 12299−12302.
Burger, R. M., Peisach, J., & Horwitz, S. B. (1981) *J. Biol. Chem. 256,* 11636−11644.
Carter, B. J., de Vroom, E., Long, E. C., van der Marel, G. A., van Boom, J. H., & Hecht, S. M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 9373−9377.
Carter, B. J., Holmes, C. E., van Atta, R. B., Dange, V., & Hecht, S. M. (1991a) *Nucleosides & Nucleotides 10,* 215−227.
Carter, B. J., Reddy, K. S., & Hecht, S. M. (1991b) *Tetrahedron 47,* 2463−2474.
Chang, C.-H., & Meares, C. F. (1982) *Biochemistry 21,* 6332−6334.
Chang, C.-H., & Meares, C. F. (1984) *Biochemistry 23,* 2268−2274.
D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 3608−3612.
Fox, K. R. (1990) *Anti-Cancer Drug Des. 5,* 99−104.
German, M. W., Kalisch, B. W., Lundberg, P., Vogel, H. J., & van de Sande, J. H. (1990) *Nucleic Acids Res. 18,* 1489−1497.
Gonikberg, E. M., & Odintsova, S. P. (1987) *Mol. Biol. (Moscow) 20,* 1048−1052.
Grimwade, J. E., Cason, E. B., & Beerman, T. A. (1987) *Nucleic Acids Res. 15,* 6315−6329.
Gupta, G., Sarma, M. H., Sarma, R. H., Bald, R., Engelke, U., Oei, S. L., Gessner, R., & Erdmann, V. A. (1987) *Biochemistry 26,* 7715−7723.
Gupta, G., Garcia, A. E., & Hiriyanna, H. T. (1993) *Biochemistry 32,* 948−960.
Hare, D. R., & Reid, B. R. (1986) *Biochemistry 25,* 5341−5350.
Holmes, C. E., Carter, B. J., & Hecht, S. M. (1993) *Biochemistry 32,* 4293−4307.
Hüttenhofer, A., Hudson, S., Noller, H. F., & Mascharak, P. K. (1992) *J. Biol. Chem. 267,* 24471−24475.
Johnston, R. F., Pickett, S. C., & Barker, D. L. (1990) *Electrophoresis 11,* 355−360.
Joyce, C. M., & Grindley, N. D. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80,* 1830−1834.
Keller, T. J., & Oppenheimer, N. J. (1987) *J. Biol. Chem. 262,* 15144−15150.
Kuwahara, J., & Sugiura, Y. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85,* 2459−2463.
Lloyd, R. S., Haidle, C. W., & Hewitt, R. R. (1978a) *Cancer Res. 38,* 3191−3196.
Lloyd, R. S., Haidle, C. W., & Robberson, D. L. (1978b) *Biochemistry 17,* 1890−1896.
Magliozzo, R. S., Peisach, J., & Ciriolo, M. R. (1989) *Mol. Pharmacol. 35,* 428−432.

Maxam, A. M., & Gilbert, W. A. (1980) *Methods Enzymol. 65*, 499−560.
McLean, M. J., Dar, A., & Waring, M. J. (1989) *J. Mol. Recognit. 1*, 184−192.
Mirabelli, C. K., Huang, C.-H., & Crooke, S. T. (1980) *Cancer Res. 40*, 4173−4177.
Mirabelli, C. K., Huang, C.-H., Fenwick, R. G., & Crooke, S. T. (1985) *Antimicrob. Agents Chemother. 27*, 460−467.
Povirk, L. F. (1983) Bleomycin, in *Molecular Aspects of Anti-Cancer Drug Action* (Neidle, S., & Waring, M., Eds.) Vol. 3, pp 157−181, Macmillan, London.
Povirk, L. F. (1987) *Mutat. Res. 180*, 1−9.
Povirk, L. F., & Houlgrave, C. W. (1988) *Biochemistry 27*, 3850−3857.
Povirk, L. F., & Austin, M. J. F. (1991) *Mutat. Res. 257*, 127−143.
Povirk, L. F., Wübker, W., Köhnlein, W., & Hutchinson, F. (1977) *Nucleic. Acids Res.* 4, 3573−3580.
Povirk, L. F., Han, Y.-H., & Steighner, R. J. (1989) *Biochemistry 28*, 5808−5814.
Saito, I., Morii, T., Sugiyama, H., Matsura, T., Meares, C. F., & Hecht, S. M. (1989) *J. Am. Chem. Soc. 111*, 2307−2308.
Sambrook, J., Fritsch, E. F., & Mantiatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Steighner, R. J., & Povirk, L. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8350−8354.
Sugiura, Y., & Suzuki, T. (1982) *J. Biol. Chem. 257*, 10544−10546.
Sugiyama, H., Kilkuskie, R. E., Chang, L.-H., Ma, L.-T., Hecht, S. M., van der Marel, G., & van Boom, J. H. (1986) *J. Am. Chem. Soc. 108*, 3852−3854.
Sugiyama, H., Kawabata, H., Fujiwara, D., Y., & Saito, I. (1990) *J. Am. Chem. Soc. 112*, 5252−5257.
Umezawa, H. (1980) *Recent Progress in Bleomycin Studies*, in *Anticancer Agents Based on Natural Product Models*, pp 147−166, Academic Press, London.
Umezawa, H., Maeda, K., Takeuchi, T., & Okami, Y. (1966) *J. Antibiot. 19*, 200−209.
Williamson, J. R., & Boxer, S. G. (1989a) *Biochemistry 28*, 2819−2831.
Williamson, J. R., & Boxer, S. G. (1989b) *Biochemistry 28*, 2831−2836.

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