Influence of Chromatin Structure on Bleomycin−DNA Interactions at Base Pair Resolution in the Human β-Globin Gene Cluster[†]

Murray J. Cairns and Vincent Murray*

School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney, Australia 2052

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ABSTRACT: The DNA sequence specificity of bleomycin was examined in human cells and in purified genomic DNA. In each case, DNA damage sites were determined at nucleotide resolution in the human single-copy β-globin promoter and the locus control region (LCR) hypersensitive site 2 (HS-2). Exponential amplification of gene-specific genomic fragments was achieved by ligation-mediated PCR, and labeled reaction products were analyzed directly by sequencing gel electrophoresis. Bleomycin was found to cleave DNA preferentially at GC, GT, and GA dinucleotides. This study represents the first occasion that the sequence specificity of bleomycin has been determined in intact human cells at the single-copy gene level. The intensity of bleomycin damage sites in the LCR HS-2 was found to differ substantially between intact cells and purified DNA at putative transcription factor binding sites. Bleomycin activity was greatly reduced in cells at a tandem NF-E2/AP1 DNA sequence element. This footprint was strongest in K562 cells where the nuclear factor-erythroid 2 (NF-E2) is thought to bind. Protection and enhancement were also observed at other sequence elements in the HS-2 that associate with erythroid-specific and ubiquitous transcription factors. These results suggest that the activity of bleomycin is significantly reduced at the site of protein−DNA interactions in intact cells. This property of bleomycin is extremely useful in genomic "footprinting", where it has significant advantages over other commonly used agents.

The antibiotic bleomycin (BLM),[1] derived from *Streptomyces verticillus*, has anti-tumor activity and is used clinically to treat a range of neoplasias, such as squamous cell carcinoma, testicular carcinoma and malignant lymphoma (Krakoff, 1989). BLM induces single- and double-strand DNA cleavage. Double-strand breaks in the DNA are probably more resistant to repair and are therefore considered to be the biologically important lesion (Absalon et al., 1995). The affinity of BLM for nucleic acids is attributed to the positively charged flat planar bithiazole moiety, which is thought to intercalate between bases via the minor groove (Povirk et al., 1979). The DNA binding component of BLM is attached to a metal chelate that forms a coordination complex with ferrous ion (Fe^{2+}) in the activated molecule. Spontaneous oxidation of the Fe^{2+} to the ferric (Fe^{3+}) ion releases an electron in the presence of oxygen that produces a superoxide or hydroxyl radical capable of inducing extensive damage to the localized DNA (Ishida & Takahashi, 1975; Sausville et al., 1978; Burger et al., 1979).

The sequence specificity of DNA damage caused by BLM has been studied using a variety of oligonucleotide and plasmid DNA systems (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Kross et al., 1982; Murray & Martin, 1985b), as well as repetitive alphoid DNA sequences in intact human cells (Murray & Martin, 1985a). In these systems BLM was found to have a strong tendency to damage GC, GT, and GA dinucleotide and to a lesser extent AT and AC dinucleotides. BLM has been shown to preferentially damage DNA in the linker region of the nucleosome and at active genes, as compared to nontranscribed genes, in isolated nuclei (Kuo & Hsu, 1978; Kuo 1981). More recently BLM activity has been shown to be inhibited by the core protein in reconstituted nucleosomes (Smith et al., 1994). In the experiments described in this paper, the DNA sequence specificity of BLM was investigated in intact cells and compared to that in purified human DNA. The target DNA sequences were situated in the single-copy human β-globin gene cluster at the locus control region (LCR) hypersensitive site 2 (HS-2) and at the β-globin gene promoter. Analysis at the single copy level in intact cells permits the effects of DNA−protein interactions and chromatin microstructure to be investigated. Alphoid DNA is a very useful model for the study of DNA-damaging agents in intact cells; however, its tandemly repeating sequence is found in heterochromatin, and it does not allow the effects of transcription factors and chromatin microstructure to be investigated (Murray & Martin, 1985a).

The 5′ HS-2 is one of four erythroid-specific DNase I hypersensitive sites (DHS) that are found in the 15 kb section of the β-globin gene cluster comprising the LCR (Tuan et al., 1985; Forrester et al., 1987). The HS-2 extends for approximately 550 bp and contains a number of well-defined sequence elements that bind erythroid specific and constitutive transcription factors (Figure 1) (Bresnick & Felsenfeld, 1993). Experiments have shown that the LCR HS-2 region has classical enhancer activity for ϵ-globin and γ-globin gene expression (Moi & Kan, 1990; Ney et al., 1990). The β-globin gene promoter also presents an erythroid-specific DHS which contains motifs necessary for an active transcrip-

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* To whom correspondence should be addressed. Tel: (02) 385 2028. FAX: (02) 313 6271.

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[1] Abbreviations: AP-1, activator protein-1; BLM, bleomycin; DHS, DNase I hypersensitive site; DMS, dimethyl sulfate; HS-2, hypersensitive site 2; LCR, locus control region; NF-E2, nuclear factor-erythroid 2; PBS, phosphate-buffered saline.

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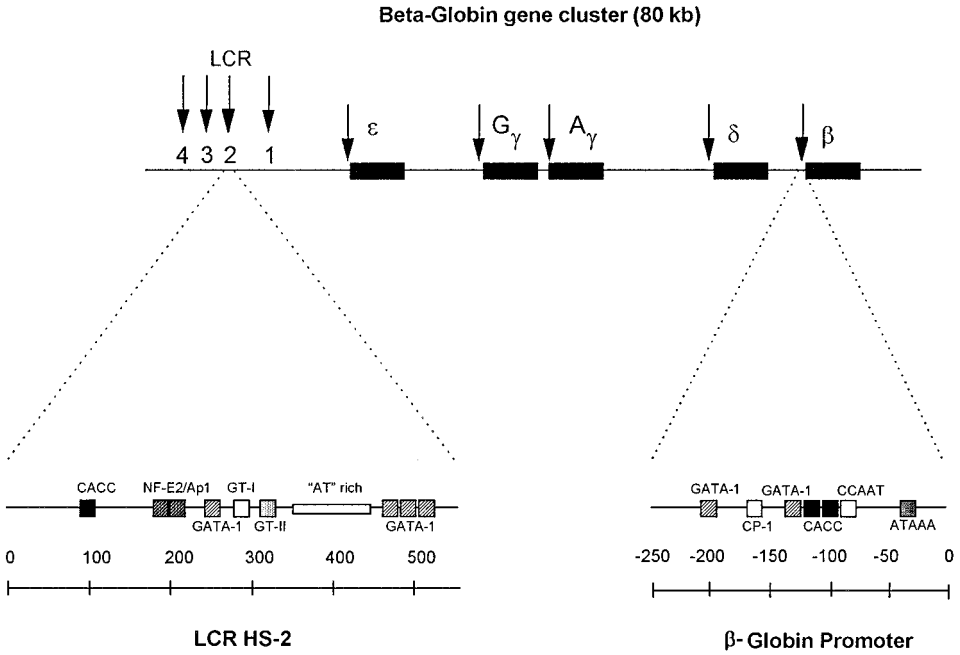

FIGURE 1: Map of the β-globin cluster showing the position of erythroid-specific DHSs (arrows) in relation to the β-like globin genes (large rectangles) and the LCR. The expanded view of the LCR HS-2 and the β-globin gene promoter shows the arrangement of putative erythroid specific and ubiquitous nuclear factor binding motifs (small rectangles).

tion assembly in the adult erythroblast (Figure 1) (de Boer et al., 1988; Mantovani et al., 1988).

In this study the human tissue culture cell line K562 was used because of its erythroid character (Rutherford et al., 1979; Benz et al., 1980). K562 cells express the ϵ-globin and γ-globin genes at a level that can be enhanced 2−5-fold by the addition of hemin (Charnay & Maniatis, 1983). K562 cells have a similar chromatin structure to that observed in foetal liver cells except that they do not display an erythroid-specific β-globin promoter DHS (Groudine et al., 1983; Tuan et al., 1985). In non-erythroid HeLa cells, the β-like globin genes are not expressed and the gene cluster does not contain a DHS. Comparison of DNA damage in these two human cell lines with purified human genomic DNA enabled the effect of protein−DNA interactions on BLM activity to be determined in intact cells. The analysis of BLM damage down to nucleotide resolution in a single-copy human gene from samples of total genomic DNA was achieved with the ligation-mediated PCR technique (Mueller & Wold, 1989; Pfeifer et al., 1989). Terminal 5′-phosphates generated by BLM cleavage enabled the DNA fragments to be ligated to a double-stranded oligonucleotide by T4 DNA ligase. The amplified damage fragments were positioned in the sequence by comparison to Maxam−Gilbert sequencing fragments amplified in the same system and electrophoresed on the same gel. Using this approach, DNA alkylation lesions induced by the anti-tumor drug analogues aldozelesin, bizelesin, and azirdinylbenzoquinones have already been mapped to nucleotide resolution in single-copy human genes (Lee et al., 1994a,b).

MATERIALS AND METHODS

Cell Culture. K562 cells were grown as a suspension culture with RPMI 1640 (0.01% penicillin/streptomycin/fungizome) supplemented with 10% foetal calf serum. Globin gene induction was initiated during logarithmic phase growth by the introduction of 50 μM hemin (Rutherford & Weatherall, 1979). The cells were then harvested by centrifugation 4 days after induction and washed with phosphate-buffered saline (PBS). HeLa cells were grown as a monolayer in RPMI 1640 supplemented with 2.5% newborn calf serum and 2.5% foetal calf serum. Cell cultures approaching 90% confluence were washed twice with PBS and detached by a 5 min pronase digestion. Detached HeLa cells were harvested as described for the K562 cells.

Bleomycin Damage. Centrifuged tissue culture cell pellets were resuspended in PBS containing 0.1−9 mM BLM of the clinical preparation blenoxane (Bristol-Meyers). After a 1 h incubation at 37 °C the cells were washed twice in PBS and resuspended in 50 mM Tris-HCl, pH 7.5, 20 mM EDTA. The DNA was then extracted and purified as described by Murray and Martin (1985a). Purified human DNA was incubated with 20−180 μM BLM and an equivalent concentration of $Fe^{2+}SO_4$ at 37 °C for 1 h and ethanol precipitated with 0.1 volumes of 3 M sodium acetate and 2 volumes of ethanol. Damaged and control DNA were redissolved in 10 mM Tris-HCl, pH 8.8, 0.1 mM EDTA.

Oligonucleotide Primer Sequences. Oligonucleotide sequences for the human β-globin gene cluster were derived from the Genbank sequence humhbb that covers 73 326 bp of the human β-globin region on chromosome 11. The LCR HS-2 specific primers for analyzing the coding strand were 5′-ATATGTCACATTCTGTCTCAGGCATCC-3′ (first-strand synthesis) and 5′-GTCTCAGGCATCCATTTTCTTTATG-3′ (PCR); the HS-2 noncoding strand 5′-TTCCTGTTA-CATTTCTGTGTGTCTC-3′ (first-strand synthesis) and 5′-CATTAGTGACCTCCCATAGTCCAAGCA-3′ (PCR); the coding strand of the β-globin promoter 5′-CAAATGTAAG-CAATAGATGGCTCTG-3′ (first-strand synthesis) and 5′-TCCTGCCCTCCCTGCTCCTGGGAGTAG-3′ (PCR); the noncoding strand of the β-globin promoter 5′-CTTCCACTTT-TAGTGCATCAATTTC-3′ (first-strand synthesis) and 5′-TCAATATGCTTACCAAGCTGTGATTCC-3′ (PCR).

Genomic Sequencing and LMPCR. Purified human DNA (20−40 μg) was incubated with formic acid (G+A reaction) or hydrazine (C+T) at 20 °C for 1−3 min, washed by ethanol precipitation, and then redissolved in 1 M piperidine (Maxam & Gilbert, 1980). After an incubation at 90 °C for 30 min the DNA was ethanol precipitated again before extensive lyophilization in a vacuum-centrifuge to remove the last trace of piperidine. The sample was then redissolved in 10 mM Tris-HCl, pH 8.8, 0.1 mM EDTA.

The relative sequence positions of BLM-induced lesions were determined with reference to the chemical sequencing fragments in a two-primer ligation-mediated PCR. The first-strand synthesis was performed using 0.5−2.0 μg of previously denatured (95 °C for 5 min) genomic DNA in a 5 μL reaction volume consisting of 40 mM NaCl, 10 mM Tris-HCl, pH 8.8, 5 mM $MgSO_4$, 0.01% gelatin, 300 μM each of dATP, dGTP, dTTP, and dCTP, 0.17 units of *Vent* DNA polymerase (New England Biolabs) and 10 pmol of the first gene-specific primer [this method was adapted from Garrity and Wold (1992)]. The reaction mixture was covered with oil and incubated at 95 °C for 30 s, 60 °C for 10 min, and 76 °C for 10 min. After being cooled on ice, 2.4 μL of the reaction product was transferred from beneath the oil to a new tube containing 3.6 μL of the ligation mixture. The ligation mixture consisted of 20 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 0.2 units of T4 DNA ligase (Pharmacia), and 10 pmol of double-stranded unidirectional linker which was prepared by annealing two oligonucleotides with the sequences 5′-GAAGAGAAGGT-3′ and 5′-CCAAACGCCATTTCCACCTTCTCTTC-3′. The ligation was incubated for approximately 16 h at 17 °C. The PCR phase of the procedure was performed in the same tubes by the addition of 4 μL containing 166 nmol of $(NH_4)_2SO_4$, 670 nmol of Tris-HCl, pH 8.8, 67 nmol of $MgCl_2$, 3 nmol each of dATP, dGTP, dTTP, and dCTP, 0.74 units of *Taq* DNA polymerase (Perkin Elmer Cetus), and 1 pmol of 5′ ^{32}P-labeled second gene-specific primer. After being covered with mineral oil, the reaction was incubated through 19 cycles at 95 °C for 30 s, 60 °C for 60 s, and 72 °C for 90 s. At completion, 2 μL of each sample was combined with 2 μL of loading dye consisting of 80% formamide, 50 mM NaOH, 1 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol and electrophoresed on a 6% (w/v) polyacrylamide sequencing gel. The gel was vacuum dried on 3MM paper.

Densitometry. Sequencing gels were exposed for a minimum of 16 h to a phosphor storage screen and scanned using a Molecular Dynamics PhosphorImager. Image analysis was performed using ImageQuant software. At each band position, the intensity was integrated (using areas) and assigned a sequence location with reference to the Maxam and Gilbert sequencing ladder. The background was subtracted and intensity values determined. Each intensity value was then normalized by dividing by the sum of band intensities for the whole lane. The relative damage intensity at each band position in cells compared to purified DNA was then calculated as a ratio. The Log_{10} of this ratio (cell damage/purified DNA) was then plotted against the nucleotide sequence location. In this type of graph, relative damage protection is observed as negative values whereas relative damage enhancement is observed at positive values.

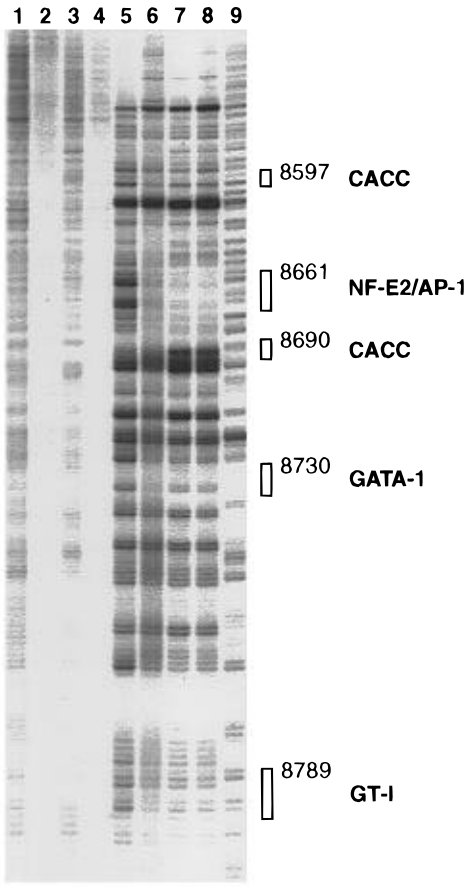

FIGURE 2: Phosphorimage of a DNA sequencing gel comparing BLM damage on the coding strand of the LCR HS-2 in various environments and amplified by LMPCR. Lanes 1−4 were undamaged controls for purified DNA, HeLa cells, uninduced K562 cells, and hemin-induced K562 cells respectively. Lanes 5−8 were derived from BLM damage to purified DNA (15 μM), HeLa cells (1 mM), uninduced K562 cells (1 mM), and hemin-induced K562 cells (3 mM), respectively. Lane 9 was a Maxam−Gilbert G+A DNA sequencing reaction amplified by LMPCR. The position of transcription factor motifs are indicated by the rectangles on the right side of the image.

RESULTS

Modified LMPCR Protocol. The LMPCR procedure used in these experiments is a simplified version of the method of Garrity and Wold (1992). The first-strand synthesis and linker ligation phases are similar to those in the published technique. After the linker ligation each sample was heat denatured and used directly in the PCR without prior ethanol precipitation. The PCR reaction phase was performed using *Taq* DNA polymerase and a ^{32}P-labeled gene-specific primer. This enabled LMPCR products to be labeled directly, avoiding the need for a labeled third gene-specific primer in a linear amplification reaction or membrane transfer and hybridization. This is a substantial time-saving measure and reduces the overall sample manipulation during an experiment.

DNA Sequence Specificity of BLM in the β-Globin LCR HS-2. Genomic DNA fragments generated by BLM cleavage were amplified by LMPCR with LCR HS-2 specific primers and resolved on a DNA sequencing gel. The sequence specificity was determined by direct alignment with LMPCR-amplified Maxam and Gilbert sequencing fragments. Figures 2 and 3 are phosphorimages derived from sequencing gels that display the effects of BLM cleavage in

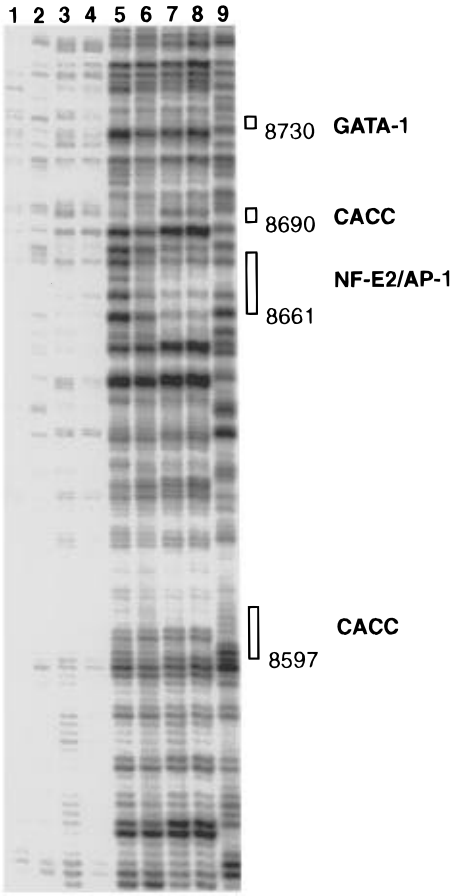

FIGURE 3: Phosphorimage of a DNA sequencing gel comparing BLM damage on the noncoding strand of the LCR HS-2 in various environments and amplified by LMPCR. The arrangement of samples was the same as in Figure 2.

the coding and noncoding strands, respectively. In each sequencing gel, BLM damage in purified (control) DNA was compared to BLM damage in HeLa cells, uninduced K562 cells, and hemin-induced K562 cells. Undamaged DNA from each of these environments was also included to indicate the level of background noise in the DNA samples.

The sequence specificity of BLM observed in the purified DNA sample was similar to that previously reported for purified plasmid DNA with sites occurring preferentially at GC, GT, and GA dinucleotides. The strongest damage sites occurred at dinucleotides that are part of an alternating purine−pyrimidine sequence (Table 1). A similar pattern of BLM cleavage was found in the DNA samples damaged in intact cells with the exception of discrete regions along the sequence where damage intensity was reduced or enhanced with respect to the purified control DNA.

Densitometric analysis of BLM damage intensity was used to determine the level of relative protection or enhancement at each site in the HS-2. A graph showing the relative intensity ratios (cells/purified DNA) was drawn in Figure 4 to chart the position and amplitude of these fluctuations. The most prominent feature of this data is a large protection area (indicated by negative values) covering a 27 bp region at sequence positions 8656−8683. This segment is also conspicuous in the sequencing gels where a number of bands in both the coding and noncoding strands are substantially reduced in cells leaving a clearly defined protein-footprint (Figures 2 and 3). The position of this section of damage protection corresponds to the tandem NF-E2/AP-1 motifs

Table 1: DNA Sequence Specificity of BLM Damage in the β-Globin LCR HS-2[a]

	Sequence	Purified DNA % total	HeLa Cells % total	Induced K562 cells % total	transcription factor binding site
Coding Strand	tgtGTaac	5.1	6.4	2.5	
	tgtGTgcc	5.0	4.7	1.6	
	tcaGCcta	4.3	3.5	1.1	
	tgaGTcat	4.1	1.4	0.1	*
	tgaGTcat	3.9	1.3	0.1	*
	agaGTgat	3.7	3.5	0.9	
	ggtGTgtg	3.6	4.6	1.3	*
	gatGActc	3.0	1.9	0.4	
Non-Coding Strand	ccaGAagg	4.5	3.9	5.0	
	tcaGCatt	3.3	1.6	0.5	*
	ggaGTcat	3.2	1.8	2.5	
	taaGCctc	3.1	2.1	4.4	
	tcaGCatg	2.9	1.9	0.9	*
	gggGCctg	2.9	3.4	3.9	
	gggGCact	2.8	2.3	2.7	
	catGActc	2.7	1.2	0.9	*

[a] The eight most intense BLM damage sites were arranged in decreasing order for each strand with purified DNA. The percentages of total damage for each damage site are shown for purified DNA, HeLa cells, and induced K562 cells. An asterisk indicates that a transcription factor binding site is present at this site and has affected the level of damage. The sequences are written from 5′ to 3′, with the capital letters representing the site of damage.

which are thought to bind the nuclear factor erythroid-2 (NF-E2) in erythroid cells. Footprint analysis of this sequence in plasmid DNA has shown that the ubiquitous activator protein-1 (AP-1) will also bind to the consensus sequences within this motif. BLM-damaged DNA derived from non-erythroid HeLa cells is also protected (to a lesser extent) at this sequence element (Figures 2 and 3).

There are a number of other positions in the HS-2 sequence that show relative protection or enhancement of BLM cleavage (Figures 2−4). There is evidence of protection at a CACC motif (bp 8596−8601) in the coding and noncoding strands in induced K562 cells and in the noncoding strand in HeLa cells. However, at the other CACC motif (bp 8690−8694), there is enhancement visible in both strands of K652 cells and possibly in HeLa cells. Further downstream at positions 8726 and 8733 in the coding strand and possibly also in the noncoding strand there are sites of BLM damage protection near and within the erythroid GATA-1 motif (bp 8730−8735) in the two cell lines.

A densitometric comparison was also made between BLM damage in hemin-induced and non-induced K562 cells. No significant differences were apparent between these two cellular environments.

DNA Sequence Specificity of BLM in the β-Globin Promoter. BLM fragments from both strands of the β-globin promoter were amplified by LMPCR and resolved on DNA sequencing gels (Figures 5 and 6). The DNA sequence specificity of BLM in the β-globin promoter was very similar in each environment and consistent with observations in the LCR HS-2. The intensity of BLM damage at various positions was compared between cells and purified DNA using densitometry. The results of this analysis was presented in a plot of the sequence position against the intensity ratio (cells/purified DNA) (Figure 7).

BLM damage in induced K562 cells compared to purified DNA did not demonstrate any outstanding feature similar

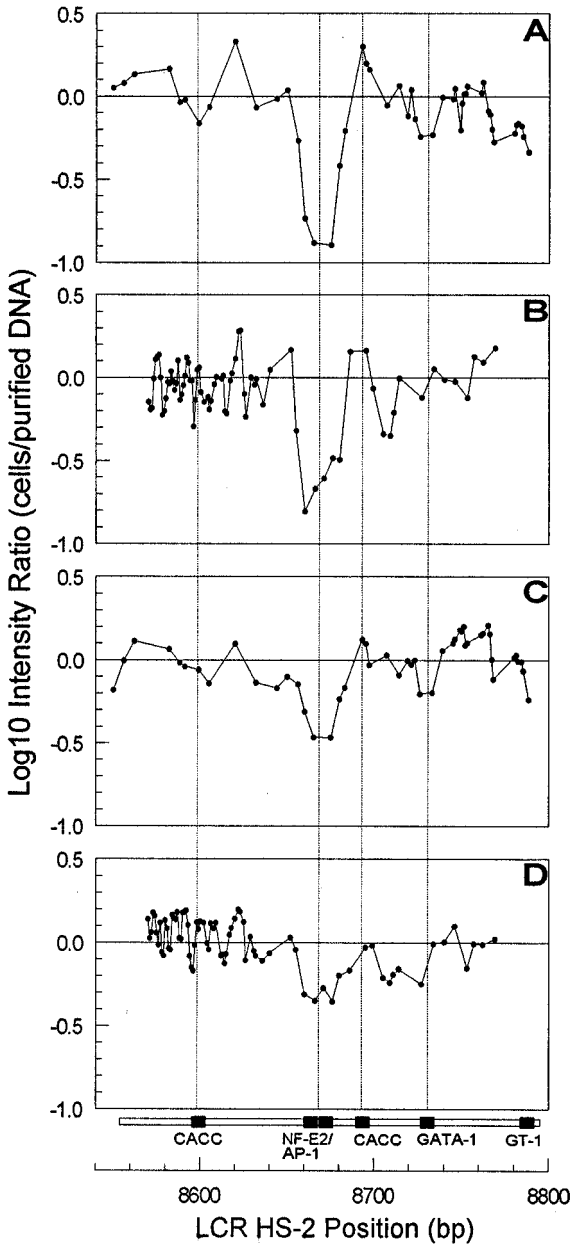

FIGURE 4: Graphs of band intensity ratios with respect to BLM damage positions in the LCR HS-2. The band intensity at damage positions was quantitated, and Log_{10} of the ratio of cell damage/purified DNA damage was calculated and plotted with respect to the sequence position. Relative protection of BLM damage in cells is visible at positions where the ratio is negative. Enhancement of damage in cells is denoted by positive values. Panel A represents the coding strand in induced K562 cells; panel B represents the noncoding strand in induced K562 cells; panel C represents the coding strand in HeLa cells; panel D represents the noncoding in HeLa cells.

to the protection seen in the NF-E2/AP-1 motif of the LCR HS-2. However, there was an area of protection between positions 61 990−62 010 in both the coding and noncoding strands in cells compared to purified DNA (Figure 7). A consensus binding motif for the transcription factor CP-1 is contained within this area. Further downstream in the vicinity of a GATA-1 element (62 036−62 041) there are also protected sites. The distal CACC motif at bp 62 050−62 055 has a protected site in the coding strand and a slightly enhanced damage site in the noncoding strand. The proximal CACC element at bp 62 065−62 069 was not subject to any

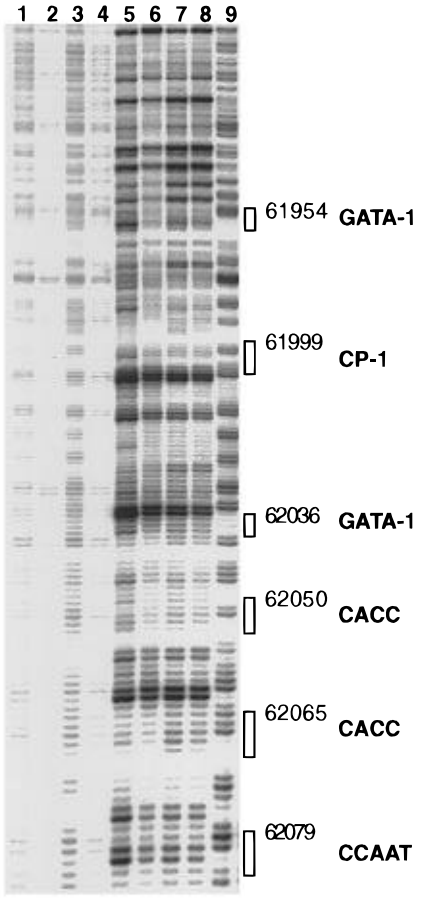

FIGURE 5: Phosphorimage of a DNA sequencing gel comparing BLM damage on the coding strand of the β-globin promoter in various environments and amplified by LMPCR. The arrangement of samples was the same as in Figure 2.

protection or enhancement of BLM damage. The CCAAT box was protected at a number of sites in both strands.

HeLa cell damage by BLM in the region just described followed a similar pattern of intensity difference compared to purified DNA as that seen in the K562 cells. However, upstream of position 61 990 there was an extended area of protection in the coding strand corresponding to approximately 150 bp that was not observed in K562 cells (lane 6, Figure 5). This effect could not be fully explored in the noncoding strand as it was partially beyond the window defined by the primer system used.

No significant differences in BLM activity were observed between the hemin-induced K562 cells and uninduced K562 cells. This is not surprising, as β-globin gene is not expressed in K562 cells and therefore its promoter is probably not responsive to changes in induction status.

DISCUSSION

The DNA sequence specificity of BLM was determined in two regions of the human single-copy β-globin cluster: the erythroid specific DNase I hypersensitive site 2 of the locus control region and at the β-globin gene promoter. Each of these target sequences was damaged in four different environments: induced and non-induced erythroid K562 cells, non-erythroid HeLa cells, and purified human DNA. The DNA sequence selectivity of BLM cleavage in these environments was found to be the same as previously reported (D'Andrea & Haseltine, 1978; Takeshita et al., 1978;

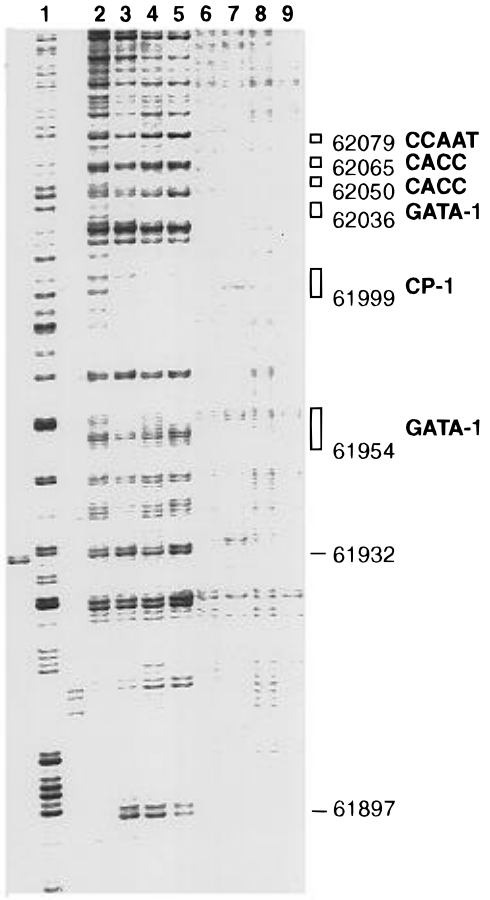

FIGURE 6: Phosphorimage of a DNA sequencing gel comparing BLM damage on the noncoding strand of the β-globin promoter in various environments and amplified by LMPCR. Lane 1 was a Maxam−Gilbert G+A DNA sequencing lane amplified by LMPCR. Lanes 2−5 were LMPCR products derived from BLM damage to purified DNA (15 μM), HeLa cells (500 μM), K562 cells (100 μM), and hemin-induced K562 cells (500 μM), respectively. Lanes 6−9 were undamaged controls for purified DNA, HeLa cells, uninduced K562 cells, and hemin-induced K562 cells, respectively.

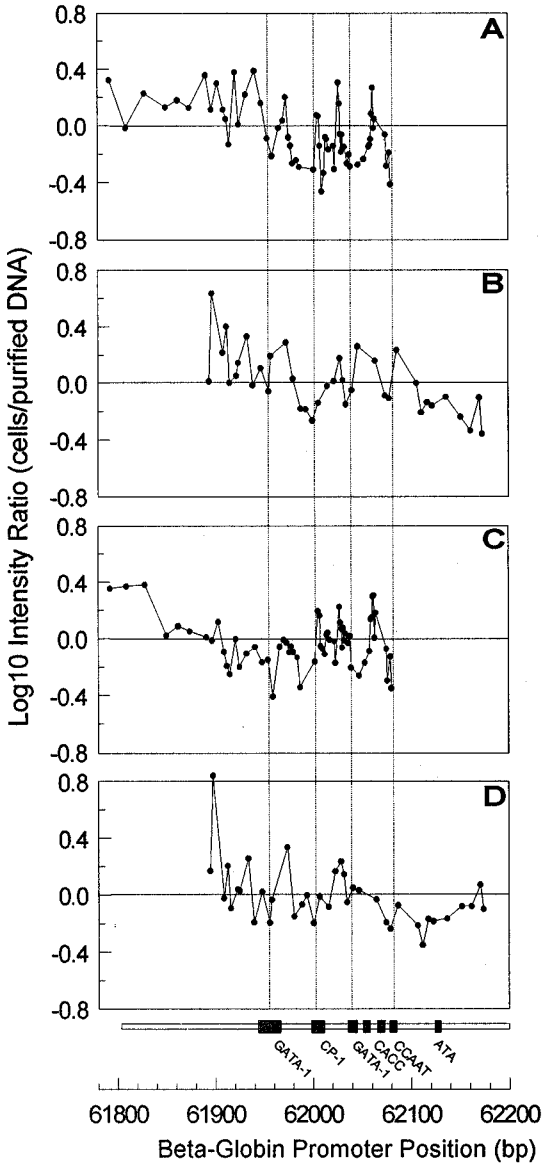

FIGURE 7: Graphs of band intensity ratios with respect to BLM damage positions in the β-globin promoter. See Figure 4 for more detailed explanation.

Kross et al., 1982; Murray & Martin, 1985a,b; Murray et al., 1988). The sequences GC, GT, and GA were preferentially damaged (Table 1). The sites of most intense damage occurred at runs of alternating purine−pyrimidine bases as found previously (Murray & Martin, 1985b). To our knowledge, this study represents the first occasion that the sequence specificity of BLM has been determined in intact cells at the single-copy gene level. Previously the sequence specificity of BLM damage had been investigated in the tandemly repeated alphoid DNA sequence (Murray & Martin, 1985a).

The intensity of BLM damage was found to be modulated in the vicinity of certain DNA sequence elements in intact cells compared to the purified DNA control. These sites of relative protection and enhancement of DNA damage are due to the more complex environment in intact cells with DNA binding proteins likely to be the major component affecting damage intensity. DNA damage protection analysis (footprinting) of these sequences in purified DNA (Bresnick & Felsenfeld, 1993; de Boer et al., 1988; Mantovani et al., 1988; Moi & Kan, 1990; Ney et al., 1990) and in intact cells (Reddy & Shen, 1991; Ikuta & Kan, 1991; Reddy et al., 1994) has established a wealth of data concerning nuclear factor binding status in expressing and non-expressing systems. The globin gene chromatin microstructure revealed by these studies provides an ideal basis for exploring the effect of BLM in this environment.

BLM activity in the β-globin LCR HS-2 was affected at a number of putative nuclear factor binding sites in intact cells. The most substantial protection was observed in K562 cells at the tandem NF-E2/AP-1 motif with a 10-fold reduction in relative damage intensity, measured at two damage sites in the coding strand. This indicates that BLM activity is severely inhibited by protein−DNA interactions at this element in K562 cells. The NF-E2 protein is probably responsible for shielding the DNA in this region, although the AP-1 factor can also bind to this sequence. The AP-1 factor is possibly responsible for the weaker footprint seen in HeLa cells. Footprints were also seen at other transcription factor binding sites in the HS-2 such as the GATA-1, GT-I, and the distal CACC element (bp 8596−8601). GATA-1 is also an erythroid-specific transcription factor. BLM damage protection at this motif and at the ubiquitous GT-I and CACC (distal) was much weaker in comparison to that observed at the NF-E2/AP-1 site. Interestingly the proximal CACC element (bp 8690−8694) was found to be

slightly hypersensitive to BLM in both strands compared to the purified control sample. Reddy et al. (1994) did not observe any effect on DMS activity at this sequence in K562 cells although footprints were found in adult erythroblasts. The effect on BLM activity at this position may have been influenced by neighboring interactions at the NF-E2/AP-1 binding sites. Alternatively some species of CACC binding factors associated with this site may enhance BLM damage. In K562 cells it may have been caused by an erythroid-specific CACC binding factor which has been observed to interact with this particular CACC motif (Mantovani et al., 1988).

Very little difference was observed between hemin-induced K562 cells and the uninduced K562 cells in the LCR HS-2 sequence. It is possible that globin gene induction in these cells does not lead to changes in transcription factor binding in the HS-2. This, however, is not consistent with DMS protection patterns in intact cells, which did show changes at some positions in response to hemin induction of K562 cells (Ikuta & Kan, 1991). These differences in DMS activity in response to hemin induction (not seen with BLM) are perhaps due to fundamental differences in reaction mechanism and specificity, that make it more sensitive in this instance than BLM. It is also possible that differences in chromatin structure between induced and uninduced genes are abolished by the introduction of BLM-induced nicks into an active loop of chromatin (Villeponteau & Martinson, 1987; Luchniq et al., 1988). However, nicks in chromatin are not expected to be produced by DMS-induced DNA methylation.

An unusual aspect of these studies is the footprints observed with the non-erythroid HeLa cells. This phenomenon has been observed by other workers (Reddy & Shen, 1991; Ikuta & Kan, 1991; Reddy et al., 1994). Despite the lack of a DNase I hypersensitive site, it appears that DNA binding proteins are able to interact with DNA in HeLa cells. It is possible that the chromatin structure has been modified in the highly transformed and subcultured HeLa cells, leading to a more open chromatin conformation that allows ubiquitous transcription factors such as the AP-1 protein and other factors to bind to DNA.

The β-globin promoter sequence was also used as a target for BLM in cells and purified DNA. The intensity of damage varied to some extent between purified DNA and intact cells, indicating that chromatin structure could have been influencing BLM activity at this sequence. BLM damage protection was apparent in cells at a number of transcription factor binding sites, including the minimal promoter CCAAT, the erythroid-restricted GATA-1, and a CP-1 motif (Chodosh et al., 1988). This result was quite unexpected as the β-globin gene is not expressed in K562 cells or the non-erythroid HeLa cell line (Charnay & Maniatis, 1983). Furthermore in contrast to adult erythroblasts, no transcription factor binding was observed in genomic DMS footprint analysis of K562 cells (Reddy et al., 1994). It is, however, possible that BLM is more sensitive to low levels of nuclear factors that may be present at the β-globin promoter in this non-expressing system. In this event the chromatin structure existing in K562 cells at this position might allow *trans*-acting factors (where available) to access the sequence despite the absence of a β-globin promoter DHS.

BLM is an ideal agent for genomic footprinting because it enters intact cells and has been shown to damage purified DNA in preference to DNA associated with protein. This endows it with considerable advantage over nucleases such as DNase I and micrococcal nuclease, as they cannot penetrate intact cells and will only enter permeabilized cells or isolated nuclei. In this latter system there is the possibility that a factor or factors are lost during the isolation process which gives a false picture of chromatin microstructure. Dimethyl sulfate (DMS) damages DNA in intact cells and is a popular agent for analysis of protein−DNA interactions in intact cells. DMS has been used effectively in this capacity to analyze nuclear factor contacts in the β-globin cluster (Reddy & Shen, 1991; Ikuta & Kan, 1991; Reddy et al., 1994). BLM compared favorably with DMS as a genomic footprinting agent in the LCR HS-2, with well-defined protection sites in both the coding and noncoding strands of the NF-E2/AP-1 motif. DMS protection sites are much weaker in this element compared to those observed with BLM, especially in the coding strand. The superior footprinting achieved by BLM at this site compared to DMS can be attributed to a number of possible factors. BLM activity is probably impaired at the site of protein−DNA interactions as a consequence of steric hindrance. The large bulky structure of BLM could be excluded from binding to DNA associated with various transcription factors, whereas the much smaller DMS molecule is perhaps less likely to be inhibited from methylating the target DNA in the same circumstances. Another possibility is that BLM is more sensitive to protein contacts in the minor groove and along the sugar−phosphate backbone, as it associates with DNA via the minor groove and induces damage to deoxyribose. In contrast, DMS binds mainly to the N7 of guanine and hence is likely to be sensitive to protein DNA interactions in the major groove, particularly those involving the N7 of guanine. BLM is thought to bind to its DNA target through an intercalation mode. This may be disturbed at the site of protein−DNA contact by the perturbation of base stacking interactions. Subtle influences on DNA microstructure of this nature would not be reflected by DMS binding patterns.

The influence of transcription factors on the activity of BLM has not previously been described. However, investigations in bulk chromatin and in precisely phased reconstituted nucleosomes have shown that BLM activity is modulated by protein−DNA interactions occurring in the nucleosome (Kuo & Hsu, 1978; Smith et al., 1994). In this study there is some suggestion that a phased nucleosome was exerting a protective influence across an area of the β-globin promoter sequence (approximately 150 bp of the coding strand) in HeLa cells (Figures 5 and 7). This effect observed in the coding strand was not verified in the noncoding strand although this may be in part due to the limits imposed by the primer system employed for its analysis.

The activity of BLM appears to be substantially inhibited at DNA sequences in contact with transcription factors. This novel finding could have important implications for the understanding of the mechanism of action of bleomycin at the molecular/cellular level and in the design of analogues based on BLM.

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