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DNA Methylation Diminishes Bleomycin-Mediated Strand Scission

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ABSTRACT: Three DNA duplexes differing substantially in sequence were derived from pBR322 plasmid DNA and supercoiled SV40 DNA by digestion with appropriate restriction endonucleases. Following treatment with the restriction methylase *Hha*I (recognition sequence: GCGC) or *Hha*I and *Hpa*II (CCGG), the unmethylated and methylated DNAs were compared as substrates for the antitumor agent bleomycin. Bleomycin-mediated strand scission was shown to diminish substantially at a number of sites in proximity to the methylated cytidine moieties, especially where multiple sites had been methylated within a DNA segment of limited size. Detailed analysis of the DNA substrates revealed that both strands of DNA within a methylated region became more refractory to cleavage by bleomycin and that the protective effect could extend as many as 14 base pairs in proximity to the 5-methylcytidine moieties. Among the methylated DNA segments that became more resistant to bleomycin cleavage was a *Hpa*II site of SV40 DNA, methylation of which has previously been shown to diminish the synthesis of the major late viral capsid protein following microinjection into *Xenopus laevis* oocytes. Study of the cleavage reaction at varying salt levels suggested that diminished bleomycin strand scission may be due, at least in part, to local conformational changes of the DNA to Z form (or other non-B-form structures). The results are generally consistent with the hypothesis that one mechanism for the expression of selective therapeutic action by certain DNA damaging agents could involve the recognition of specific methylation patterns.

DNA methylation at specific sites has been shown to affect regulation of gene expression in several eukaryotic systems. In many instances, the methylation of critical sites in genetic sequences leads to their inactivation [for reviews, see Razin & Riggs (1980) and Doerfler (1984)]. The use of restriction methylases and restriction endonucleases has led to a greater understanding of the presence and location of 5-methylcytosine within eukaryotic genes and its relationship to gene expression. Critical methylation sites leading to gene inactivation have been localized to the 5′ end and in the promoter region of several genes (Langner et al., 1984, and references cited therein; Stein et al., 1983; Ott et al., 1982).

The involvement of DNA methylation in gene regulation in normal cells has led to the suggestion that aberrant gene expression observed in neoplastic cells is related to alterations in DNA methylation patterns (Wilson & Jones, 1983; Drahovsky & Boehm, 1980; Ehrlich & Wang, 1981). Several lines of evidence support this hypothesis. Total genomic DNA methylation is often abnormal in tumor cells (Diala et al., 1983; Gama-Sosa et al., 1983). Chemical carcinogens have been shown to inhibit DNA methylation in vitro (Wilson & Jones, 1983; Wojciechowski & Meehan, 1984) and to induce hypomethylation in vivo (Boehm & Drahovsky, 1983). Azacytidine, a drug that induces decreased methylation of DNA in vivo, has been shown to induce tumorigenesis concomitant with chromosome changes and specific demethylation in preproinsulin, Ha-*ras*, and Ki-*ras* genes (Harrison et al., 1983). In addition, a site-specific DNA methylation defect within the human c-*myc* oncogene has been demonstrated in cultured tumor cell lines (Cheah et al., 1984).

This evidence suggests that one mechanism for selective chemotherapeutic action by DNA damaging antitumor agents could involve recognition of specific methylation patterns. We tested this hypothesis using the antitumor antibiotic bleomycin, which is thought to mediate its chemotherapeutic effect by degrading cellular DNA (Suzuki et al., 1968; Umezawa, 1977). Bleomycin (BLM)[1] cleaves DNA in vitro in the

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[1] Abbreviations: BLM, bleomycin; DTT, dithiothreitol; bp, base pair(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide.

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presence of metal ions, predominantly at 5′-GC-3′ and 5′-GT-3′ sequences (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1982). We now report that the relative efficiency of bleomycin cleavage in vitro is often reduced at sites that have been methylated by restriction methylases. By the use of three different DNA oligomers we examine the generality of this phenomenon as well as the effect of ionic strength on bleomycin-mediated strand scission of methylated DNA. We also demonstrate that the cleavage sites reduced by methylation can extend as far as 14 base pairs in proximity to 5-methylcytidine residues.

Experimental Procedures

Materials. Blenoxane was obtained from the National Cancer Institute and was fractionated as described (Chen et al., 1977; Oppenheimer et al., 1979). Supercoiled SV40 DNA and DNA polymerase I (Klenow fragment) were purchased from Bethesda Research Labs. Supercoiled pBR322 plasmid DNA was isolated from *Escherichia coli* strain Ja221 according to the procedure of Clewell (1972). Calf thymus DNA was sonicated, deproteinized with phenol, and dialyzed extensively against 10 mM Tris, pH 7.4, containing 50 mM NaCl. Restriction methylases *Hha*I and *Hpa*II were purchased from New England Biolabs. Solutions of Fe(II) and dithiothreitol (DTT) were prepared just before use.

Preparation of DNA Restriction Fragments. Supercoiled pBR322 plasmid DNA was digested with *Bam*HI and labeled at the 3′ end with [α-^{32}P]dATP and the Klenow fragment of DNA polymerase I (Sanger & Coulson, 1975). Enzymatic digestion with *Sal*I and *Eco*RI yielded two singly end labeled DNA fragments, 381 and 280 base pairs in length, which were isolated from a 5% polyacrylamide gel as described (Maxam & Gilbert, 1980). Similarly, cleavage of supercoiled SV40 DNA with *Ban*I, followed by 3′ end labeling, digestion with *Alu*I, and polyacrylamide gel isolation yielded a 113 base pair long singly end labeled DNA fragment. The 113-bp DNA fragment was also 5′ end labeled at the *Ban*I site as described (Maxam & Gilbert, 1980), followed by *Alu*I digestion.

In Vitro Methylation of DNA Fragments. End-labeled DNA fragments (0.1–1.0 μg) were methylated at 37 °C for 2 h in 100 μL of 50 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, 5 mM 2-mercaptoethanol, and 300 μM *S*-adenosylmethionine with 48 units of *Hha*I methylase and/or 10 units of *Hpa*II methylase. The reaction was terminated by ethanol precipitation. In order to verify that the methylase reaction was complete, these DNA fragments along with nonmethylated DNA substrates were subjected to *Hha*I and/or *Hpa*II restriction enzyme digestion by using at least 10 units/μg of DNA. In all cases, methylated DNA fragments were fully protected from restriction enzyme cleavage while the nonmethylated substrates were fully cleaved.

DNA Cleavage by Fe(II)·BLM. The standard reaction mixture (20-μL total volume) contained 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1000–2000 cpm of ^{32}P-end-labeled DNA made up to 50 μM (bp) with sonicated calf thymus DNA, 1 mM DTT (when present), and Fe(II)·BLM A_2 at concentrations noted in the figure legends. Some reactions contained higher NaCl concentrations as noted. After incubation at 25 °C for 60 min, the DNA was isolated by ethanol precipitation, washed, dried, and suspended in 4 μL of a pH 8.3, 100 mM Tris–borate/50% formamide loading buffer. The sample was heat denatured at 90 °C for 2 min, loaded onto a 0.4 mm thick, 40 cm long, 8% polyacrylamide (1:20 cross-linked)/50% urea gel, and electrophoresed at 1500–1800 V for 2–4 h. Autoradiography was carried out with an intensifying screen at −70 °C on Kodak X-Omat AR film.

Analysis of DNA Cleavage Gels. The autoradiograms from the gels were scanned with an LKB 2202 laser densitometer interfaced to a Hewlett-Packard 3390 integrator. The background absorbance due to the film density was subtracted from the scans. The data are reported as histograms in the form of fractional cleavage $f = A_i/A_t$, where A_i is the area of a peak corresponding to cleavage efficiency at band i and A_t is the sum of the areas of all of the cleavage band peaks within the gel lane.

Results and Discussion

Two end-labeled DNA fragments from plasmid pBR322 were chosen because they contained recognition sites for two different restriction methylases, *Hha*I (GCGC) and *Hpa*II (CCGG). These restriction methylases introduce a methyl group at the 5-position of the central two cytidines (on opposite strands) of their respective recognition sequences. The fragments were methylated with either *Hha*I only or both *Hha*I and *Hpa*II in order to introduce different levels of methylation. These fragments, along with the corresponding nonmethylated pBR322 DNA fragments, were subjected to cleavage by Fe(II)·BLM A_2 or Fe(II)·BLM A_2 + DTT. The ^{32}P-end-labeled products were analyzed by denaturing polyacrylamide gel electrophoresis in order to determine the relative efficiencies of cleavage at specific sites.

Figure 1 shows an autoradiogram of a sequencing gel for an experiment using the 381-bp fragment. Lanes 1–3 show the cleavage pattern induced by Fe(II)·BLM A_2 in the presence of DTT for the unmethylated and two methylated DNAs, respectively, while lanes 4–6 illustrate the pattern in the absence of DTT to permit the assessment of any possible effect of a reducing agent on strand scission of methylated DNA by bleomycin. Both in the absence and in the presence of DTT, the patterns indicated the characteristic bleomycin sequence specificity at 5′-GC-3′ and 5′-GT-3′ sites demonstrated previously (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1982). However, it was evident that one of the 5′-GC-3′ bleomycin cleavage bands present on the nonmethylated DNA substrate (lanes 1 and 4) was extensively diminished on the methylated substrates (lane 2, 3, 5, and 6). This band corresponded to the central 5-methylcytidine of the *Hha*I recognition sequence.

Three other methylation sites were examined on this DNA fragment, another *Hha*I site and two *Hpa*II sites. There were no differences in bleomycin cleavage efficiencies at the other *Hha*I site. There was, however, decreased cleavage in lanes 3 and 6 in the vicinity of the *Hpa*II sites near the top of the gel. Lanes 2 and 5, which were not methylated by *Hpa*II, were not affected in this region.

The autoradiogram shown in Figure 1 was converted to a histogram (Figure 2) by densitometric measurements in order to permit quantitation of the cleavage efficiencies at each site. Cleavage at the inner C (118) of the *Hha*I methylated site (116–119) was clearly diminished for those DNA substrates in which this cytidine was methylated. The outer C of the methylation recognition sequence at site 116 was relatively unaffected. DNA cleavage at the other *Hha*I methylation site (144–147) occurred primarily at the outer C (144) and was unaffected by the state of methylation.

Densitometric analysis of the *Hpa*II methylation region in Figure 1 was not possible due to the position of the relevant bands on the gel. However, visual inspection of Figure 1 indicated that cleavage bands within this region were decreased in lanes 3 and 6. Other *Hpa*II sites were examined in more detail on a 280-bp DNA fragment from pBR322, shown in Figure 3. In this histogram (Figure 3a) it can be seen that

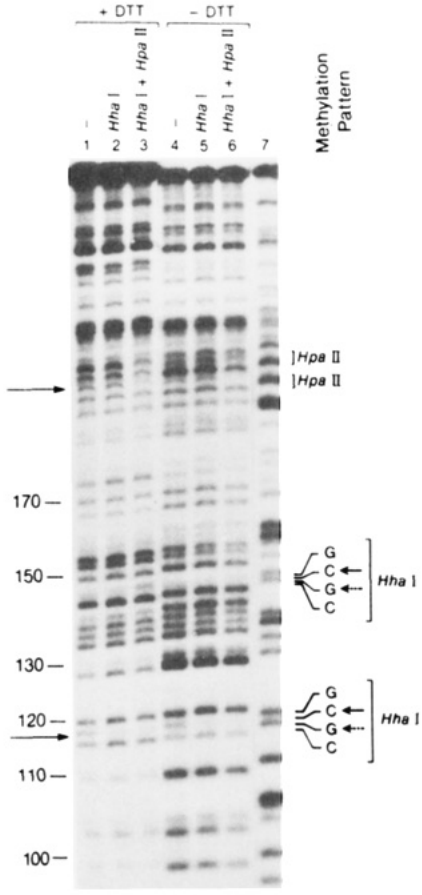

FIGURE 1: The 381-bp DNA fragment was methylated with *Hha*I (lanes 2 and 5) or *Hha*I and *Hpa*II (lanes 3 and 6). These substrates, along with the nonmethylated DNA (lanes 1 and 4), were subjected to Fe(II)·BLM A_2 digestion. Lanes 1–3, 0.5 μM Fe(II)·BLM A_2 + 1 mM DTT; lanes 4–6, 5 μM Fe(II)·BLM A_2; lane 7, G lane (Maxam & Gilbert, 1980). Numerals in the margin of the autoradiogram refer to oligonucleotide size. Solid arrows in the right margin refer to cytidines that are methylated by the indicated enzyme; dotted arrows refer to guanosines that are base paired to methylated cytidines. Solid arrows in the left margin refer to sites whose cleavage is diminished by methylation. The darker band in lane 3 at position 146 was an artifact not reproduced in other experiments.

many bleomycin cleavage sites are substantially diminished within one 14 base pair long DNA sequence (sites 26, 30, 32, 33, 35, and 39). Two CCGG sequences occur within this region. The effect was observed only for the DNA duplex containing 5-methylcytidines at these CCGG sequences; methylation of cytidines at the *Hha*I site had no effect on DNA strand scission.

The affected bleomycin cleavage sites for this DNA fragment occurred at bases that were not directly methylated but were either near or between the methylated CCGG sequences. If the observed diminution of bleomycin-mediated DNA strand scission were simply due to an unfavorable steric interaction between the 5-methyl group of cytidine and some portion of the bleomycin molecule, then the effect would be expected to be most pronounced at the site of methylation or at least in close proximity to this site. That diminution of strand scission was observed at six positions over a 14-bp region (cf. Figure 3, bases 26–39) suggests that this entire DNA segment could have undergone a conformational change unfavorable to subsequent drug interaction. Interestingly, methylation of an isolated *Hha*I site further upstream (60–63) had no effect on bleomycin cleavage. This implied that flanking sequences as well as the number of methyl groups within a DNA region could be important determining factors for the expression of bleomycin selectivity.

Since cytidine methylation is known to reduce the concentration of salt required to induce a B → Z conformational change in DNA (Behe & Felsenfeld, 1981), the effect of higher NaCl concentrations on Fe(II)·BLM A_2 mediated strand scission of the 280-bp fragment was studied. As shown in parts b and c of Figure 3 (containing 0.5 and 1.0 M NaCl, respectively) higher salt concentrations increased the difference in extent of BLM cleavage of the methylated and nonmethylated DNAs.

Although the methylation of every cytidine in the alternating poly(dG-dC)·poly(dG-dC) has been shown to stabilize the Z conformation of DNA (Behe & Felsenfeld, 1981), it is not known what effect a limited number of methyl groups introduced at specific sites has on DNA conformation. The putative altered conformation within these methylated DNA fragments that is unreceptive to bleomycin may be Z-DNA or some other Z-like or non-B DNA conformation. It has been hypothesized that the methylation of eukaryotic DNA in vivo at 5′-CG-3′ sites can induce a similar conformational change, resulting in altered chromatin structure and differential affinities for DNA binding proteins (Ehrlich & Wang, 1981). Bleomycin can

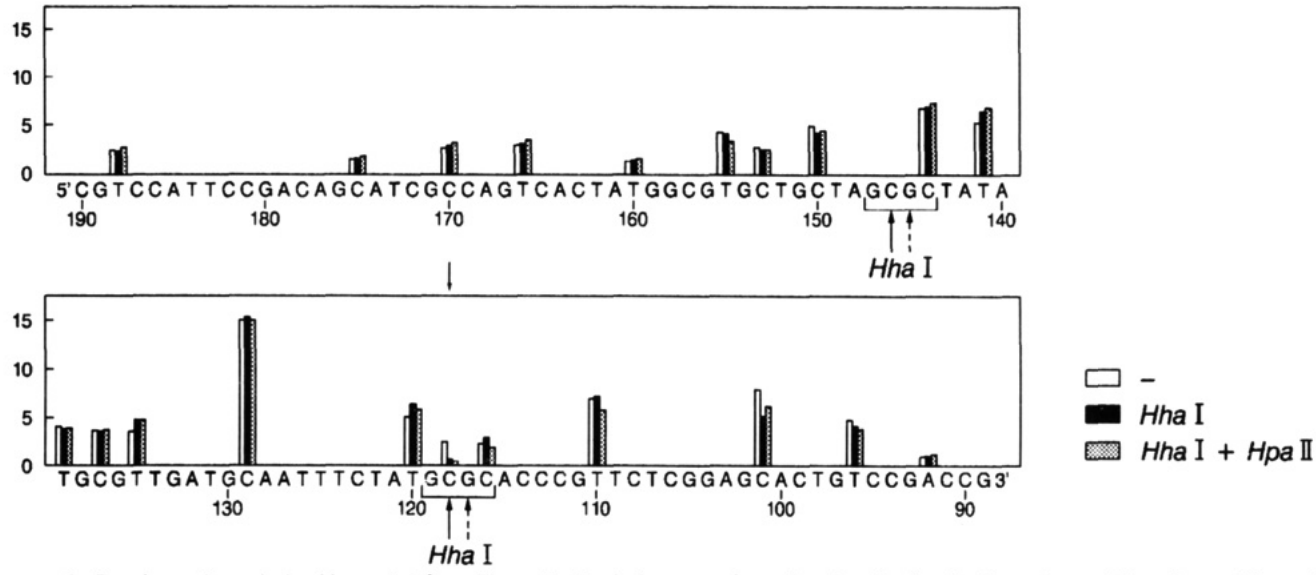

FIGURE 2: Densitomeric analysis of lanes 4–6 from Figure 1. Analysis was performed as described under Experimental Procedures. Numerals and arrows refer to the same sites as in Figure 1.

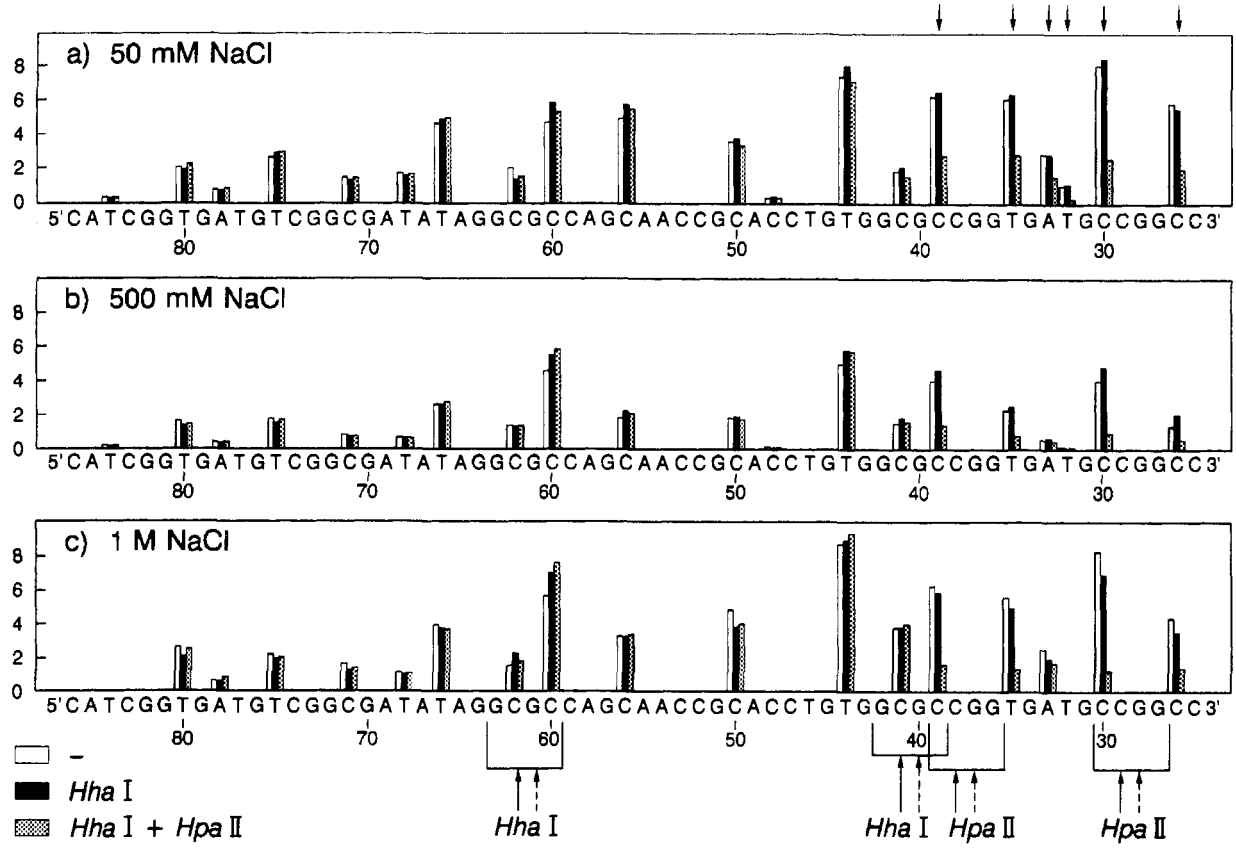

FIGURE 3: Densitometric analysis of cleavage of unmethylated and methylated DNA (280-bp segment) by BLM A_2. DNA cleavage was carried out with 1 μM Fe(II)·BLM A_2 + 1 mM DTT in the presence of (a) 50 mM NaCl, (b) 500 mM NaCl, or (c) 1 M NaCl. Arrows at the top of the figure indicate positions of diminished DNA cleavage.

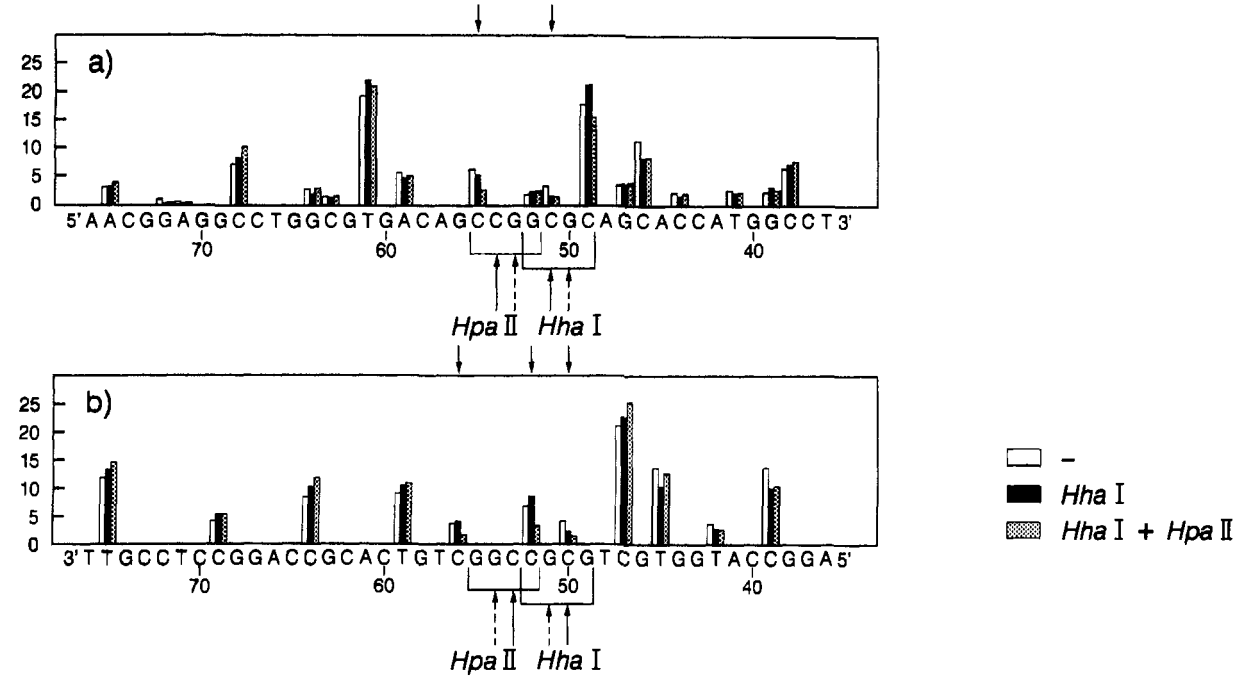

FIGURE 4: Analysis of effects of DNA methylation on BLM A_2 mediated cleavage of both strands of a 113 bp long DNA duplex. DNA digestion was carried out with 1 μM Fe(II)·BLM A_2 + 1 mM DTT in the presence of 50 mM NaCl with (a) 3′-end-labeled DNA or (b) 5′-end-labeled DNA.

recognize this change and may be useful as a probe to study small segments of methylated DNA embedded in nonuniform sequences. Although the observations outlined here seem most likely to be due to an altered DNA conformation, it is clear that additional experimentation will be required to establish definitively the molecular basis for the observed diminution of DNA strand scission. Of particular concern in this context is the apparent ability of a single pair of methylated cytidines (base opposite 117 and base 118, Figures 1 and 2) to alter DNA conformation.

A DNA fragment from SV40 was also investigated for methylation-induced changes in bleomycin cleavage. This fragment (113 bp long) was separately labeled at the 5′ and 3′ ends, methylated with *Hha*I or *Hha*I/*Hpa*II, and subjected

to treatment with Fe(II)·BLM A_2. As shown in Figure 4, cytidine methylation resulted in diminished BLM cleavage on both strands of the double helix (bases 55a, 51a, 56b, 52b, and 50b). DNA cleavage at the cytidines within the *Hha*I methylation site (51a and 50b) was affected on both methylated substrates (*Hha*I and *Hha*I/*Hpa*II), while those in the vicinity of the *Hpa*II site (55a, 56b, and 52b) were affected only on the *Hha*I/*Hpa*II-methylated DNA substrate. Thus, as had been seen for the 280-bp DNA fragment, these data illustrated that nonmethylated cytidines in proximity to methylated cytidines may not be good substrates for BLM-induced cleavage. For the sequences studied, *Hha*I methylation seemed to affect cleavage only at the C in the middle of the GCGC recognition sequence, while the (additional) *Hpa*II methylation at CCGG sequences appeared to affect cleavage at bases peripheral to the recognition sequence. In this context, it should be noted that the *Hha*I recognition sequence is also a good BLM recognition sequence (D'Andrea & Haseltine, 1978; Takeshita et al., 1978; Mirabelli et al., 1982).

While the results obtained here are consistent with the hypothesis that certain DNA damaging antitumor agents might mediate selective damage to transformed cells by exhibiting greater activity toward undermethylated promoter regions of active genes in such cells, it must be emphasized that the present results were carried out exclusively with isolated DNA fragments. Nonetheless, two intriguing observations have been made that may be pertinent to the hypothesis under investigation. The first is that methylation of the CCGG *Hpa*II site of SV40 DNA (corresponding to base pairs 52–55 in Figure 4) has been studied for its effect on gene expression in *Xenopus laevis* oocytes (Fradin et al., 1982). When this sequence was methylated in vitro and microinjected into oocytes, there was a marked reduction in the synthesis of the major late viral capsid protein, relative to the synthesis by an unmethylated control. Thus, a DNA sequence whose methylation has been shown to influence susceptibility to bleomycin in vitro is also known to regulate gene expression in vivo. The second observation involves the antineoplastic agent *m*-AMSA, whose mechanism of action is believed to involve topoisomerase II mediated DNA interaction (Nelson et al., 1984). In a recent report, Zwelling et al. (1984) have provided evidence that undermethylated DNA may also be a better substrate for this agent in vivo.

Registry No. Bleomycin, 11056-06-7.

REFERENCES

Behe, M., & Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1619–1623.

Boehm, T. L. J., & Drahovsky, D. (1983) *Recent Results Cancer Res. 84*, 212–225.

Cheah, M. S. C., Wallace, C. D., & Hoffman, R. M. (1984) *J. Natl. Cancer Inst. 73*, 1057–1065.

Chen, D. M., Hawkins, B. L., & Glickson, J. D. (1977) *Biochemistry 16*, 2731–2738.

Clewell, D. B. (1972) *J. Bacteriol. 110*, 667–676.

D'Andrea, A. D., & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3608–3612.

Diala, E. S., Cheah, M. S., Rowitch, D., & Hoffman, R. M. (1983) *J. Natl. Cancer Inst. 71*, 755–764.

Doerfler, W. (1984) *Adv. Viral Oncol. 4*, 217–246.

Drahovsky, D., & Boehm, T. L. J. (1980) *Int. J. Biochem. 12*, 523–528.

Ehrlich, M., & Wang, R. Y.-H. (1981) *Science (Washington, D.C.) 212*, 1350–1357.

Fradin, A., Manley, J. L., & Prives, C. L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5142–5146.

Gama-Sosa, M. A., Slagel, V. A., Trewyn, R. W., Oxenhandler, R., Kuo, K. C., Gehrke, C. W., & Ehrlich, M. (1983) *Nucleic Acids Res. 11*, 6883–6894.

Harrison, J. J., Anisowicz, A., Gadi, I. K., Raffeld, M., & Sager, R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6606–6610.

Langner, K.-D., Vardimon, L., Renz, D., & Doerfler, W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2950–2954.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499–560.

Mirabelli, C. K., Ting, A., Huang, C.-H., Mong, S., & Crooke, S. T. (1982) *Cancer Res. 42*, 2779–2785.

Nelson, E. M., Tewey, K. M., & Liu, L. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1361–1365.

Oppenheimer, N. J., Rodriguez, L. O., & Hecht, S. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5616–5620.

Ott, M.-O., Sperling, L., Cassio, D., Levilliers, J., Sala-Trepat, J., & Weiss, M. C. (1982) *Cell (Cambridge, Mass.) 30*, 825–833.

Razin, A., & Riggs, A. D. (1980) *Science (Washington, D.C.) 210*, 604–610.

Sanger, F., & Coulson, A. R. (1975) *J. Mol. Biol. 94*, 441–448.

Stein, R., Sciaky-Gallili, N., Razin, A., & Cedar, H. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2422–2426.

Suzuki, H., Nagai, K., Yamaki, H., Tanaka, N., & Umezawa, H. (1968) *J. Antibiot. 21*, 379–386.

Takeshita, M., Grollman, A. P., Ohtsubo, E., & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5983–5987.

Umezawa, H. (1977) *Lloydia 40*, 67–81.

Wilson, V. L., & Jones, P. A. (1983) *Cell (Cambridge, Mass.) 32*, 239–246.

Wojciechowski, M. F., & Meehan, T. (1984) *J. Biol. Chem. 259*, 9711–9716.

Zwelling, L. A., Minford, J., Nichols, M., Glazier, R., & Shackney, S. (1984) *Biochem. Pharmacol. 33*, 3903–3906.