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Methylation-Related Chromatin Structure Is Associated with Exclusion of Transcription Factors from and Suppressed Expression of the *O*-6-Methylguanine DNA Methyltransferase Gene in Human Glioma Cell Lines

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There is considerable interest in identifying factors responsible for expression of the *O*-6-methylguanine DNA methyltransferase (MGMT) gene, as MGMT is a major determinant in the response of glioma cells to the chemotherapeutic agent 1,3 bis(2-chloroethyl)-1-nitrosourea. Recently we have shown that MGMT expression is correlated in a direct, graded fashion with methylation in the body of the MGMT gene and in an inverse, graded fashion with promoter methylation in human glioma cell lines. To determine if promoter methylation is an important component of MGMT expression, this study addressed the complex interactions between methylation, chromatin structure, and in vivo transcription factor occupancy in the MGMT promoter of glioma cell lines with different levels of MGMT expression. Our results show that the basal promoter in MGMT-expressing glioma cell lines, which is 100% unmethylated, was very accessible to restriction enzymes at all sites tested, suggesting that this region may be nucleosome free. The basal promoter in glioma cells with minimal MGMT expression, however, which is 75% unmethylated, was much less accessible, and the basal promoter in nonexpressing cells, which is 50% unmethylated, was entirely inaccessible to restriction enzymes. Despite the presence of the relevant transcription factors in all cell lines examined, in vivo footprinting showed DNA-protein interactions at six Sp1 binding sites and one novel binding site in MGMT-expressing cell lines but no such interactions in nonexpressors. We conclude that in contrast to findings of previous in vitro studies, Sp1 is an important component of MGMT transcription. These correlations also strongly suggest that methylation and chromatin structure, by determining whether Sp1 and other transcription factors can access the MGMT promoter, set the transcriptional state of the MGMT gene.

Expression of the *O*-6-methylguanine DNA methyltransferase (MGMT) gene is a major determinant in the response of glioma cells to the chemotherapeutic agent 1,3 bis(2-chloroethyl)-1-nitrosourea (BCNU) (8). Since the majority of glioma cells express the MGMT gene (8), understanding factors that regulate MGMT expression is important for the design of therapeutic strategies to inhibit MGMT expression and thereby overcome BCNU resistance. Recent studies have shown that cytosine methylation may be one factor that influences MGMT gene expression (5, 7, 23, 30, 31).

The mechanism by which cytosine methylation influences gene expression is unclear. Methylation of GC-rich promoters, through normal or abnormal processes, is clearly associated with loss of gene expression (16), but studies addressing the molecular mechanisms that suppress transcription have not been definitive. One proposed mechanism suggests that methylation of cytosines within transcription factor binding sites interferes directly with DNA-protein interactions (25). This mechanism, however, is obviously limited to CpG-containing binding sites and is irrelevant to transcription factors such as Sp1, whose binding is methylation independent (12). A second proposed indirect mechanism is thought to involve protein mediators, such as MeCP1, that bind in a non-sequence-specific manner to methylated DNA, prevent transcription factor access, and thereby maintain the chromatin in a transcriptionally inactive state (2). The interrelationship of MeCPs with other chromosomal proteins (e.g., histones) in the formation or maintenance of inactive chromatin is not understood. Methylation-related chromatin structures could explain why many genes are not expressed in cells that contain all the relevant transcription factors (2, 16). As the MGMT promoter is very GC rich and lacks a TATA box (14), and as MGMT-expressing and -nonexpressing cells contain all of the transcription factors necessary to support the activity of a transfected MGMT promoter (13, 20), methylation and chromatin structure of the MGMT promoter may be involved in MGMT transcription. We have recently shown through high-resolution methylation analysis that the methylation status of CpGs throughout the basal MGMT promoter correlated in an inverse, graded fashion with MGMT expression (7).

This study was designed to address the relative contribution of methylation and chromatin structure in MGMT gene expression and to dissect the complexity of protein-promoter interactions. Using glioma cell lines with a wide range of MGMT expression and differential promoter methylation, we examined both the chromatin structure and in vivo transcription factor-promoter interactions in the basal MGMT promoter. The results of our in vivo studies, which contrast with those of previous in vitro studies (13, 14), provide compelling evidence for the involvement of methylation and chromatin structure in MGMT gene transcription.

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MATERIALS AND METHODS

Cell culture. The glioma cell lines used in this study were established from grade III to IV human astrocytomas and glioblastomas. The glioma cell lines used were A1235, Cla (L. Erickson, Loyola Medical Center, Maywood, Ill.) SF767 (Brain Tumor Research Center, University of California, San Francisco), and Hs683, T98, and U138 (American Type Culture Collection, Rockville, Md.). Normal human T lymphocytes were supplied by P. McAllister (Loyola Medical Center).

Analysis of MGMT mRNA and MGMT activity. The relative amount of MGMT mRNA and MGMT activity in each glioma cell line was determined by Northern (RNA) blot analysis and by a restriction endonuclease assay, respectively, both as previously described (10, 32). The MGMT activity assay measured the extent to which glioma cell sonic extracts (10 to 50 μg of total cellular protein) repair methyl group adducts at O^6-guanine within a radiolabeled 18-bp DNA substrate.

Analysis of *Msp*I accessibility to the MGMT promoter within nuclei. Cells were washed twice with cold 1× phosphate-buffered saline (PBS) and harvested by scraping into 8 ml of fresh 1× PBS. The cells were centrifuged (5 min, 3,500 rpm) and then resuspended in 1.0 ml of cold reticulocyte standard buffer (10 mM Tris [pH 8.0], 10 mM NaCl, 3 mM $MgCl_2$) plus 0.05% Nonidet P-40 to lyse the cells. Nuclei were pelleted by centrifugation (12,000 rpm, 4 s), washed twice in 1× *Msp*I buffer (NEBL no. 2; New England Biolabs, Beverly, Mass.), and resuspended in 350 μl of fresh 1× *Msp*I buffer. Nuclei equivalent to 30 μg of DNA were incubated with 20 to 400 U of *Msp*I (10 min, 37°C). DNA was then isolated from the nuclei (24), precipitated, and resuspended in double-distilled H_2O (1.0 μg of DNA per μl). Five micrograms of DNA from the nuclei digests was analyzed by linker-mediated PCR (LMPCR) as described below except that autoradiograph exposures were for 2 to 5 h, with intensifying screens.

In Vivo DMS footprint analysis of the MGMT promoter. Glioma cells were treated with 0.1% dimethylsulfate (DMS) in fresh medium (37°C, 2 min) and then washed three times with 1× PBS. DNA was then isolated (24), resuspended in 1 M piperidine, and heated for 30 min at 95°C. Following precipitation, the DNA was washed twice with 80% ethanol and lyophilized overnight. The DNA was resuspended in double-distilled H_2O, and 5 μg was analyzed by LMPCR.

The LMPCR protocol was based on the method described by Pfeifer et al. (21) and consisted of extension, ligation, and amplification steps. All DNA primers for LMPCR except an 11-nucleotide (nt) linker primer were gel purified. For extension reactions, a 15-μl reaction mixture containing 5.0 μg of cleaved genomic DNA, 0.5 pmol of the extension primer (for promoter region 1, 5′-CGGGCCATTTGGCAAACTAAG-3′, corresponding to MGMT promoter nt 655 to 675; for promoter region 2, 5′-AGGCACAGAGCCTCAGGCGGAAG CT-3′, corresponding to nt 805 to 823), and 1× Sequenase buffer (United States Biochemical, Cleveland, Ohio) was incubated at 95°C for 3 min and then at 60°C for 30 min. The reaction mixture was cooled on ice, and 7.5 μl of deoxynucleoside triphosphate (dNTP) mix (final concentrations in mix were 0.062 mM dGTP, 0.188 mM 7-deaza-dGTP, and 0.2 mM each dCTP, dATP, and dTTP [Pharmacia, Piscataway, N.J.]), 0.5 μl of 0.5 M $MgCl_2$, 0.95 μl of 1 M dithiothreitol, and 1.5 μl of a 1:4 dilution (in Tris-EDTA [pH 8.0]) of Sequenase version 2.0 (United States Biochemical) were added. Following primer extension (48°C, 15 min), the reaction mixtures were cooled on ice, 6 μl of cold 300 mM Tris (pH 7.7) was added, and the Sequenase was heat inactivated (67°C, 15 min). The reaction mixture was cooled on ice. In ligation steps, a double-stranded DNA linker (21) was ligated to the extension products by addition of 45 μl of a ligation mix (13.33 mM $MgCl_2$, 30 mM dithiothreitol, 1.66 mM ATP, 83.3 μg of bovine serum albumin, 100 pmol of linker DNA, 3 U of T4 DNA ligase [Promega] to each reaction mixture. After ligation (18°C, 12 to 16 h), the reaction mixture was heated (70°C, 10 min) and then cooled on ice. The DNA was precipitated (along with 10 μg of yeast tRNA), washed with 70% ethanol, lyophilized, and resuspended in 67 μl of double-distilled H_2O. The ligated DNA was then incubated in a 100-μl reaction mixture containing 10 μl of dNTP mix (0.067 mM dGTP, 0.133 mM 7-deaza dGTP, 0.2 mM each dATP, dCTP, and dTTP), 1× Stoffel fragment buffer, 2.5 mM $MgCl_2$, 10 U of the Stoffel fragment of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 10 pmol each of the longer (25-mer) linker primer and a nested gene-specific primer (for promoter region 1, 5′-AGGCACA GAGCCTCAGGCGGAAGCT-3′, nt 674 to 698; for promoter region 2, 5′-TGGGCATGCGCCGACCCGGTC-3′, nt 841 to 861) (13) and amplified by PCR (5 min at 95°C followed by 18 cycles of 95°C for 1 min, 66°C for 2 min, and 76°C for 3 min, with a 5-s extension of the 76°C step after each cycle and 10 min at 76°C after cycle (18). ^{32}P-labeled PCR products were generated through two additional PCR cycles with a second nested end-labeled primer (promoter region 1, 5′-AGGCA CAGAGCCTCAGGCGGAAGCTGGGA-3′, nt 674 to 702; promoter region 2, 5′-TGGGCATGCGCCGACCCGGTCG GG-3′, nt 841 to 864). Seven microliters of a mix containing 1× Stoffel buffer, 2.5 mM $MgCl_2$, 0.1 U of Stoffel fragment per μl, and 4.0 pmol of the ^{32}P-labeled primer was added to the amplification reaction mixture. Following two cycles of PCR (same parameters as specified above except that annealing was at 67°C and extension was at 77°C), the DNA was extracted, precipitated, and resuspended in 10 μl of LMPCR dye (80% formamide, 45 mM Tris base, 45 mM boric acid, 1 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). Three to 5 μl of the sample was electrophoresed (55 W, 2 to 3 h) through a 6% denaturing polyacrylamide gel and then detected by autoradiography (6 to 18 h of exposure).

Gel mobility shift assay. Cells (5×10^6 to 10×10^6) from each cell line were harvested, centrifuged, and flash frozen. The frozen cell pellet was resuspended in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 25% (vol/vol) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol and centrifuged at 100,000 × *g* for 5 min at 4°C. The supernatants were assessed for basal Sp1 binding activity. Binding reactions were performed by addition of 10 μg of whole cell extract protein to a mixture containing 0.1 ng of a ^{32}P-labeled double-stranded Sp1 oligonucleotide (5′-GCTCGCCCCGCCCCGATCGAAT-3′ [27]) in 25 μl of binding buffer [10 mM Tris (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 10 μg of bovine serum albumin, 0.5 μg of poly(dI-dC) · poly(dI-dC)]. Following incubation at 25°C for 20 min, the protein-bound and unbound (free) oligonucleotides were electrophoretically (40 mA, 4°C) separated in a nondenaturing 4% polyacrylamide gel in 6.7 mM Tris (pH 7.5)–1 mM EDTA–3.3 mM sodium acetate. Gels were dried and exposed to X-ray film. For the competition experiments, the binding reaction mixtures contained either a 100-fold molar excess of an unlabeled Sp1 oligonucleotide (self competition) or a 100-fold molar excess of an unlabeled consensus heat shock element oligonucleotide (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′ [1]) (non-self competition).

TABLE 1. MGMT expression in glioma cells

Glioma cell line	MGMT mRNA level[a] (% of T98 level)	MGMT activity[b] (% of T98 level)
U138	76.8	96 ± 12
SF767	61.6	110 ± 19
Hs683	33.3	38 ± 9
A1235	0	<1
Cla	0	<1
Cro	0	<1

[a] Values represent means of two experiments.
[b] Values represent means ± standard deviations of three experiments.

RESULTS

MGMT expression. The levels of MGMT mRNA and MGMT activity of various glioma cell lines relative to the T98 cell line are presented in Table 1. Normal human T cells, which were also used in this study, had a two- to threefold-greater level of MGMT expression than the T98 cell line. The similarity in relative levels of MGMT mRNA and MGMT activity within each cell line suggests that the MGMT gene is regulated at the transcriptional level. The transcription rate, however, is apparently too slow to measure by nuclear run-on analysis (not shown).

***Msp*I accessibility to the MGMT promoter within nuclei.** Because the methylation status of the MGMT promoter was associated with MGMT expression in a graded, inverse fashion (7), we investigated the possibility that methylation influences the chromatin structure of the MGMT promoter and thus MGMT transcription. Chromatin structure of the MGMT promoter was analyzed by incubation of nuclei from MGMT-expressing and -nonexpressing cells with *Msp*I followed by LMPCR analysis of the DNA.

Incubation of the SF767 nuclei (high expression) with 20 U of *Msp*I (Fig. 1, lanes 1 to 3) resulted in a much greater cleavage at all promoter *Msp*I sites tested compared with that in Hs683 (minimal MGMT expression) and Cla (no MGMT expression) nuclei. Similar results were seen at an additional *Msp*I site (nt 884; not shown) and three *Ava*II sites (nt 773, 953, and 1084 [7]). Together, these sites encompass 372 nt (712 to 1084), a region of accessible DNA much larger than would be expected if normal nucleosomal phasing was present (18). Incubation of nuclei (from the same isolation) with an excess of *Msp*I (Fig. 1, lanes 4 to 9) resulted in measurable cleavage at all sites, indicating that the absence of LMPCR products is likely not a result of contaminants from the nuclei isolation that completely inhibit *Msp*I digestion or enzymes used in LMPCR. The *Msp*I sites at 712, 722, and 738 (lanes 1 to 3) appear to have graded degrees of accessibility across the cell lines that parallel, in an inverse fashion, the graded promoter methylation, suggesting that methylation and chromatin structure are closely linked in the MGMT promoter. These results raise the possibility that the restriction enzyme-inaccessible promoter in cells with little or no MGMT expression may also be inaccessible to endogenous transcription factors.

Association between methylation, chromatin structure, and DNA-protein interactions in the MGMT promoter. Specific in vivo protein-DNA interactions in the MGMT promoter were examined by LMPCR of DNA from glioma cells that were exposed to DMS. The LMPCR products generated from this DNA represent the guanines that are accessible to DMS within intact cells. As DMS is not blocked by nucleosomes or MeCPs but is blocked by many transcription factors (21), the DMS-footprinted protein-DNA interactions are qualitatively different from those assessed by *Msp*I accessibility.

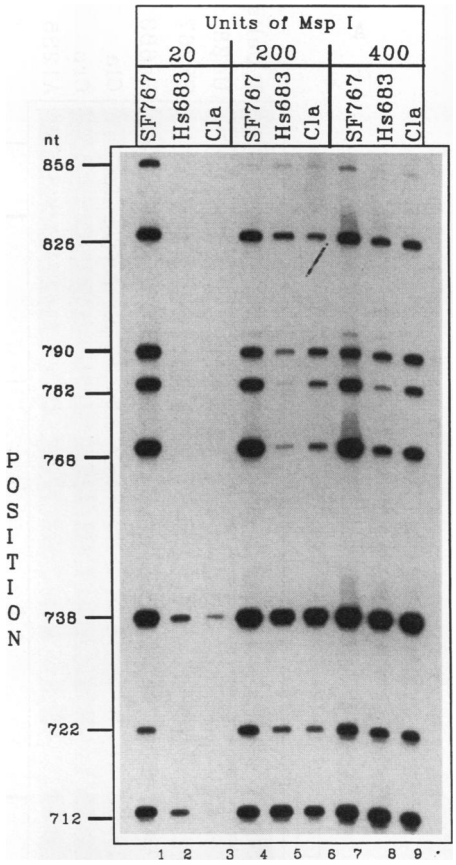

FIG. 1. LMPCR analysis of *Msp*I accessibility to the MGMT promoter within intact nuclei from cells with high levels of MGMT expression (SF767), low levels of MGMT expression (Hs683), and no MGMT expression (Cla). Nuclei were incubated with 20 to 400 U of *Msp*I for 10 min, the DNA was isolated, and 5 μg was analyzed by LMPCR. One-fifth of the reaction mixture was separated on a 6% denaturing polyacrylamide gel and autoradiographed for 2 to 5 h.

Figure 2 shows the in vivo footprint analysis of the MGMT promoter, in MGMT-expressing and -nonexpressing cell lines, from nt 700 to 865 (Fig. 2A) and 865 to 1050 (Fig. 2B), which together include the transcription start site (nt 955) and the basal promoter elements (nt 883 to 974) (14). There are no detectable footprints in any of the cells in the region spanning nt 700 to 845 (Fig. 2A), which includes a putative Sp1 recognition site (nt 708 to 713). The guanines at positions 849, 851, and 852, however, are protected from DMS in the expressing cells (lanes 1 to 3) but not in the poorly expressing (lane 4) and nonexpressing cells (lanes 5 to 7), indicating the presence of an MGMT expression-associated DNA-protein interaction. The protected sequence does not match any known consensus sequence for transcription factor binding and may thus represent a novel protein binding site. In the promoter region spanning nt 865 to 1050, there are six footprints in cells with high levels of MGMT expression but none in the low expressors and nonexpressors. All six of these footprints correspond to Sp1 recognition sequences and have 5′ hypersensitive guanines followed by at least five protected guanines. There are no footprints, however, at the two overlapping Sp1-like sequences spanning nt 875 to 885 or at the putative CCAAT box (nt 870 to 876) (14) in any of the cells tested (Fig. 2B). Normal human T cells, which have high levels

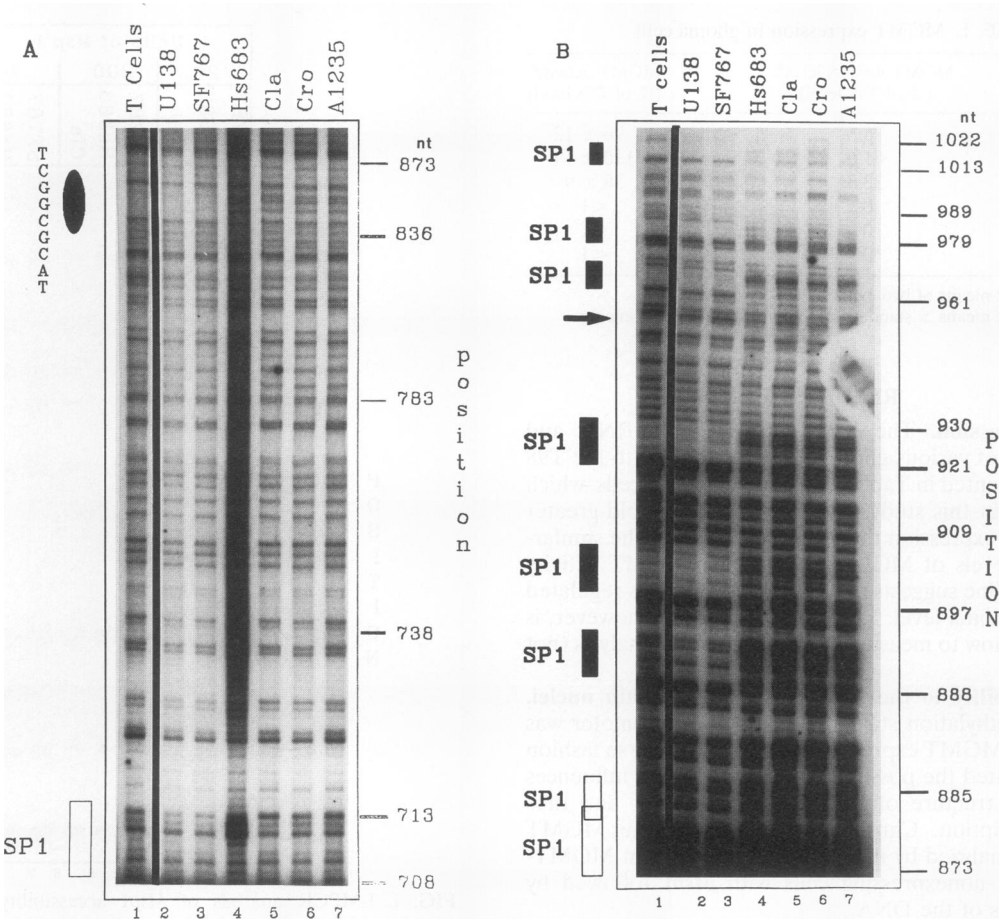

FIG. 2. In vivo footprint analysis of DNA-protein interactions in the MGMT promoter. Normal human T lymphocytes (lane 1) and two glioma cell lines all with high levels of MGMT expression (lanes 2 and 3), one glioma cell lines all with low levels of MGMT expression (lane 4), and three nonexpressing glioma cell lines (lanes 5 to 7) were incubated with 0.1% DMS (2 min, 37°C). DNA was isolated from the cells and cleaved with piperidine, and 5 μg was analyzed by LMPCR. One-half of the reaction mixture was separated on a 6% denaturing polyacrylamide gel and autoradiographed for 6 to 18 h. (A) Analysis of promoter nt 700 to 865; (B) analysis of promoter nt 865 to 1050. □, Sp1 sites unoccupied in all cells tested; ■, Sp1 site that is footprinted in lanes 1 to 3 but not in lanes 4 to 7; ⬮, potentially novel site occupied in lanes 1 to 3 only (protected guanines shown vertically). → in panel B indicates the transcription start site.

of MGMT expression, have a pattern of footprints identical to that of the glioma cell lines with high levels of MGMT expression, indicating that these DNA-protein interactions are not confined to cell lines. Despite the lack of Sp1 footprints in the nonexpressing cells, Sp1 protein was present in all the cell lines, as assessed by gel shift analysis with a radiolabeled oligomer containing a single Sp1 site (Fig. 3). These correlative data strongly suggest that the chromatin structure of the MGMT promoter influences transcription factor access to the promoter in vivo and likely influences the expression state of the MGMT gene.

DISCUSSION

The indirect model by which methylation is associated with gene expression predicts that transcription factors interact with unmethylated, accessible promoters but not with methylated, inaccessible promoters (3). Studies of the CpG island-containing promoters of the X-linked genes *HPRT* (15) and *PGK1* (22) support this model. The majority of CpG islands associated with autosomal genes, however, are unmethylated in all normal cells, regardless of the expression state of the gene (16), and are therefore not regulated by promoter methylation in normal cells. During tumorigenesis and viral transformation however, many of the normally unmethylated CpG islands become methylated, coincident with loss of expression of the associated gene (16). It is possible that the relationship between methylation and loss of gene expression during these abnormal processes is mediated in an indirect fashion similarly to X-linked genes, but this has not been directly tested. As MGMT expression is suppressed in nearly one-half of simian virus 40-transformed cell lines tested (11) and MGMT-deficient tumors have been identified (6), the MGMT gene may be one example in which this methylation-related gene inactivation occurs. The present study analyzed the role of methylation, chromatin structure, and in vivo transcription factor-promoter interactions in the expression of the MGMT gene in glioma cell lines and clearly demonstrates that a methylation-related chromatin structure is an important component of MGMT expression.

Analysis of *Msp*I accessibility in this study demonstrated that chromatin structure and methylation in the MGMT promoter are closely linked. The MGMT promoter was much more accessible to *Msp*I in cells with a completely unmethylated promoter than in cells with promoters that are, on average, only 74% unmethylated (Hs683) or 50% unmethylated (Cla).

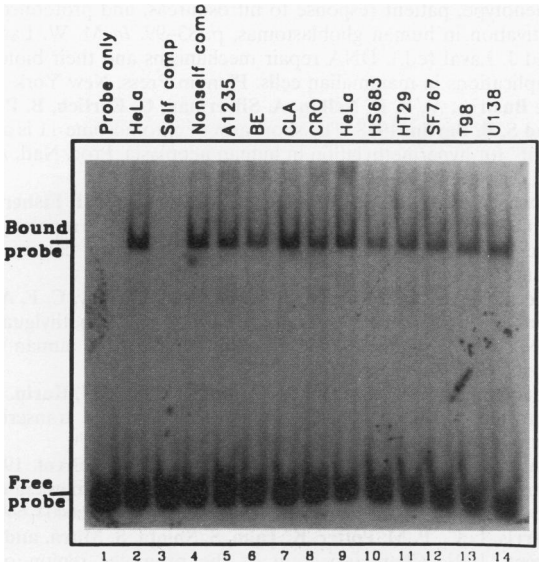

FIG. 3. Gel mobility shift analysis of basal Sp1 binding activity. Ten micrograms of protein extract from each cell line was incubated with a double-stranded, ^{32}P-labeled oligonucleotide containing Sp1 consensus sequences. The protein-bound and unbound (free) oligonucleotides were separated by electrophoresis through a 4% nondenaturing polyacrylamide gel and then detected by autoradiography. Lanes: 1, no protein; 2, 10 μg of HeLa protein extract; 3, HeLa extract preincubated with excess cold Sp1 oligonucleotide (specific competitor); 4, HeLa extract preincubated with excess cold heat shock element oligonucleotide (nonspecific competitor); 5 to 14; 10 μg of protein extract from the indicated cell lines.

The close association between methylation and chromatin structure was demonstrated by the graded fashion in which accessibility of at least three *Msp*I sites correlated with methylation. The accessibility of all sites tested over 372 nt (712 to 1084) of the basal promoter in the nuclei from SF767 cells (Fig. 4) indicates that this may be a nucleosome-free region, as DNA wrapped in nucleosomes would be protected from restriction enzyme digestion (29). Conversely, the inaccessibility of the promoter at all sites over 372 nt in the Cla cells (Fig. 4) suggests that nucleosomes and/or methylated DNA-binding proteins are present and possibly involved in setting the transcriptionally inactive state of the promoter in these cells. The results of the chromatin structure analysis of the MGMT promoter are consistent with the idea that chromatin structure is an important part of MGMT transcription. According to the indirect model, the methylation-related chromatin structure in the promoter of the gene may influence MGMT expression by allowing or excluding transcription factor access to relevant promoter sequences.

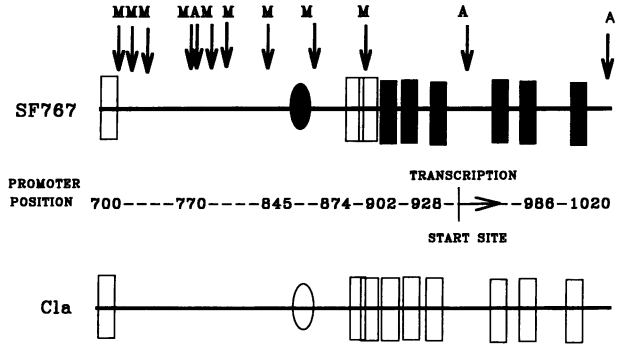

FIG. 4. Summary diagram of in vivo footprints in and restriction enzyme accessibility to the MGMT promoter in cells with high levels of MGMT expression (SF767) and cells with no MGMT expression (Cla). Sp1 sites occupied (■) and unoccupied (□) and potentially novel footprinted sites occupied (⬮) and unoccupied (○) are indicated. ↓ indicates restriction enzyme-accessible site. M, *Msp*I; A, *Ava*II.

The methylation-related chromatin structure in the MGMT promoter is closely associated with in vivo transcription factor occupancy. In addition to the general association between accessible chromatin and transcription factor occupancy, several footprints that are positioned closely to restriction enzyme accessible sites reveal specific examples of this association. For example, the protected guanine at nt 852 was only 3 nt away from an accessible *Msp*I site, and the presumably Sp1 protected guanine at 965 was only 7 nt away from an accessible *Ava*II site (Fig. 4). Since Sp1 is the only detectable factor interacting with the basal promoter elements (nt 883 to 974), we conclude that Sp1 may be sufficient, in conjunction with protein-protein interactions, for basal-level MGMT transcription. DNA-protein interactions were not detected on the opposite strand (nontranscribed strand) in the basal promoter (data not shown), which includes an imperfect Sp1 consensus sequence. We cannot, however, exclude the possibility that other transcription factor-promoter interactions relevant to basal expression occur at nonguanine residues, although there are no other known transcription factor sites within the minimal promoter elements. It is not unexpected that there are no discernible footprints in the promoter of Hs683 cells, which express very low levels of MGMT, because the Hs683 promoter is inaccessible relative to the SF767 promoter. It is probable that Sp1 does nevertheless interact with the Hs683 promoter but only too infrequently, as dictated by accessibility, to detect with DMS footprinting. The footprint at nt 845 to 852, while not within the proposed basal promoter, is associated with MGMT transcription, as it is present in the expressing cell lines and T cells and absent from all three nonexpressing glioma cell lines. This presumably expression-related protein-promoter interaction is of note for two reasons. First, previous in vitro studies showed that addition of promoter nt 807 to 883, which includes the footprinted site, to the basal promoter did not increase MGMT promoter-driven chloramphenicol acetyltransferase expression (14), suggesting that these sequences are inconsequential to MGMT promoter activity. As the effect of deletion of only these sequences from the full promoter was not tested, it remains possible that these sequences are important for promoter activity but that their contribution is influenced by interactions with other promoter regions. Such interactions have been noted for synergistically functioning yet distant Sp1 sites (28). Second, the DNA-protein interaction is novel, as it involves nucleotides that do not correspond to any known transcription factor binding site. Although the footprinted sequence has minimal homology to an Sp1 site (7 of 10 nt), and several sequences that deviate from the canonical Sp1 site still bind Sp1 (4), the pattern of the protected sequence is distinct from the canonical Sp1 protection, as seen at the other six footprints in the MGMT promoter (Fig. 2B). Three additional sequences previously described as potential Sp1 sites on the basis of sequence analysis (14) are not protected in any of the cells tested, suggesting that these sites do not function as Sp1 sites in vivo. Since these sites have only marginal similarity to Sp1 sites (7 of 10 nt), it is possible that these sequences are incapable of binding Sp1. These in vivo results further support the idea that methylation-related

differences in chromatin structure of the MGMT promoter determine whether Sp1, and possibly other factors necessary for MGMT expression, can interact with the MGMT promoter. This mechanism for gene inactivation may be relevant to many other genes that are inactivated by abnormal methylation (3, 9). It of course remains unclear whether the methylation changes cause changes in gene expression or whether methylation plays a more passive role, such as maintenance of the expression state of the gene. Additionally, methylation analysis of the MGMT promoter in tumor samples from various stages of glioma progression will determine if the changes that we observed in cultured cells are related to the tumorigenic state. Because aberrant CpG island methylation is often associated with genetic loci thought to harbor tumor suppressor genes (9), the indirect mechanism may ultimately be involved in the relationship between methylation and tumor formation or progression. Our findings that the promoter in the MGMT-expressing cell lines is unmethylated, accessible, and occupied by transcription factors suggest that MGMT expression is dependent on these factors. Since MGMT expression is a major determinant in sensitivity versus resistance to BCNU (8, 19, 26), methylation, chromatin structure, and transcription factor occupancy of the promoter could be viewed as critical elements of the molecular mechanisms that determine the chemosensitivity of these glioma cell lines. In support of this view, MGMT-expressing SF767 cells are much more resistant to BCNU than are the nonexpressing Cla cells (19, 26). The fact that gliomas of oligodendrocytic origin are especially sensitive to nitrosourea-based chemotherapy (17) implies that MGMT gene inactivation may be a frequent event, possibly mediated by changes in methylation, chromatin structure, and transcription factor access, in these tumors.

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