Graded Methylation in the Promoter and Body of the O\textsuperscript{6}-Methylguanine DNA Methyltransferase (MGMT) Gene Correlates with MGMT Expression in Human Glioma Cells*

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Expression of the O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT) gene in human glioma cell lines is strongly associated with resistance to the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (1–5). Although all normal cells and the majority of normal human tumor cells express the MGMT gene, 20–30% of glioma cell lines are devoid of MGMT mRNA and MGMT protein (1–5). This lack of MGMT expression is presumed, but not proven, to be associated with a defect in transcription and results in an increased sensitivity to the cytotoxicity induced by BCNU (1, 3, 4, 5). Because some malignant gliomas are responsive to BCNU chemotherapy (2, 6, 7) and gliomas lacking MGMT activity have been reported (8), the level of MGMT expression may be relevant in the clinical response of gliomas to BCNU.

Several studies suggest that MGMT transcription is associated with changes in both the promoter and body of the MGMT gene (9–12). The promoter for the MGMT gene has CpG island characteristics and lacks TATA and CAAT boxes, similar to many housekeeping genes (13). MGMT promoter elements required for basal promoter activity (41% of full activity) are located between nucleotides 886 and 1157 of the 1157-bp promoter and contain the transcription start site (nt 955) and six putative Sp1 recognition sites (13). In human cells transfected with chloramphenicol acetyltransferase constructs containing the MGMT promoter, both MGMT expressing and nonexpressing cell lines contained the factors necessary for transcription initiation from the MGMT promoter (14, 15), implying that differences between the transfected and endogenous MGMT promoters such as methylation status and/or chromatin structure may be important. Changes in the body of the gene may also, however, be relevant in MGMT expression as several studies have shown that the cytosine methylation patterns in MGMT exons are altered in nonexpressing tumor cells relative to expressing cells (9–11).

While methylation clearly can be involved in setting the state of gene activation/inactivation (16–23), a limited amount of studies suggest that graded methylation, both in promoter and body regions of genes, may be associated with more graded levels of gene expression (24). We have recently demonstrated that the translated exons of the MGMT gene, which are located 50 kb through 120 kb 3' of the promoter, are methylated in expressing cell lines but proportionately, rather than variably demethylated in cell lines with reduced MGMT expression (9). These studies raise the possibility that methylation, in addition to being a mechanism associated with regulation of gene expression in an all or none fashion, may also be associated with subtle changes in the level of gene expression.

To further understand the role of methylation in the regulation of MGMT expression, and to further investigate the possibility that methylation can be associated with gene expression in a graded fashion, we addressed the relationship between methylation and expression, both in the promoter and body of the MGMT gene, in glioma cell lines exhibiting a wide range of MGMT expression. To determine if the differential methylation is confined to exons or if the body of gene is uniformly methylated, and to determine whether this methylation correlates with MGMT expression, we analyzed the methylation status of several intron regions, 4–10 kb distant from the nearest exons, across the body of the gene, in a number of glioma cell lines expressing different levels of MGMT. In the same cell lines, we also determined the methylation status of...
25 Cpgs in the MGMT promoter, including Cpgs in the basal promoter elements, using linker-mediated PCR (LMPCR) technology. Methylation was quantitated in the promoter and body of the MGMT gene to determine how closely the level of methylation is linked with MGMT expression. Additionally, to examine the possibility that the differences in methylation of the MGMT promoter might influence the local chromatin structure, and thus transcription, we examined the accessibility of the MGMT promoter in intact nuclei to the restriction enzyme AvaII. The results of these studies provide evidence that methylation may influence or maintain MGMT expression through alterations in chromatin structure. The results also provide an example of an endogenous human gene in which graded methylation is associated with graded changes in gene expression.

Materials and Methods

Cell Culture—The 10 glioma cell lines used in this study were established from grade III-IV human astrocytomas and glioblastomas. The glioma cell lines used were A1235, CLA, CRO, NAT (L. Erickson, Loyola University Medical Center, Maywood, IL), SF763, SF767 (Brain Tumor Research Center, University of California, San Francisco, CA), HS883, T98, U138, and U373 (purchased from American Type Culture Collection, Rockville, MD). The cell lines were all grown in α-minimal essential medium (αMEM, Flow Laboratories, Rockville, MD), supplemented with 10% fetal bovine serum (Hyclone Corp., Logan, UT) and 1% penicillin-streptomycin (GIBCO). The cell lines were maintained in log phase growth at 37 °C in a 95% air, 5% CO₂ atmosphere.

Genomic Library Screening and Restriction Enzyme Mapping—A genomic library was constructed from the DNA of a human fibroblast cell line (WI38) inserted in the phage vector Lambda FIX I1 (Stratagene, La Jolla, CA). The library was restricted with BamHI, HindIII, or EcoRI with NotI (Stratagene). The DNA products of the NotI digest were then gene fragments were cut from phage vector DNA by complete digestion (BRL) and analyzed by Southern blot with a 32P-end-labeled oligonucleotide primer method (25) with [γ-32P]CTP (specific activity 3000 Ci/mmol, Amersham Corp.).

Analysis of MGMT mRNA Levels—Total cellular RNA was isolated from 10–20 x 10⁶ cells of each glioma cell line by a guanidinium isothiocyanate lysis procedure (26). The levels of MGMT mRNA within each cell line were determined by Northern blot analysis as described previously (9). Relative hybridization of the MGMT cDNA probe to glioma MGMT mRNA was quantitated with a Betascope 603 blot analyzer (Betagen, Inc., Waltham, MA). MGMT mRNA levels are expressed as percent of T98 and represent the average of two independent Northern blots.

MGMT Activity Assay—The relative amount of MGMT activity in each glioma cell line was determined by a restriction endonuclease assay (27). Restriction enzyme digestion (HpaII and MspI, respectively) of the 3'-most exon were identified in cloned genomic DNA by PCR amplification (as described above) using primers complementary to the 3'-end of the MGMT cDNA (primer 1, complementary to nucleotides 590–610, primer 2, complementary to nucleotides 776–795). This region was designated exon 5.

Analysis of MGMT Exons—Four exons were identified in the genomic clones by Southern blot analysis with radiolabeled MGMT cDNA fragments (listed above). To facilitate sequencing of two MGMT exons, the genomic DNA fragments containing the exons were subcloned into plasmid vectors. A 4.8-kb BamHI/HindIII fragment of genomic clone 1c containing the 5'-most exon (designated exon 2) of the CDNA was used, subcloned into the plasmid pGEM-3Zf (Promega, Madison, WI), and a 2.8-kb NotI/NaeI fragment (from genomic clone 7c) containing a second exon was subcloned into pBluescriptII SK+ (Stratagene). The resultant subclones were designated p5'BH and p7cNN, respectively. The exon regions of these subclones were subcloned with MGMT cDNA primers using the dideoxy sequencing method (Then Track Sequencing System, Promega). Hybridization of a 32P-labeled HindIII/HhaI fragment of the MGMT cDNA (see above) to Southern blots of genomic clones 5b identified a fourth translated exon, designated exon 4. Nucleotide complementarity of the 3'-most exon were identified in cloned genomic DNA by PCR amplification (as described above) using primers complementary to the 3'-end of the MGMT cDNA (primer 1, complementary to nucleotides 590–610, primer 2, complementary to nucleotides 776–795). This region was designated exon 5.

Restriction enzyme (BamHI, HindIII, and EcoRI) maps of the 28 cloned MGMT gene fragments were determined as follows. The MGMT gene fragments were cut from phage vector DNA by complete digestion with NotI (Stratagene). The DNA products of the NotI digest were then partially digested in separate reactions with BamHI, HindIII, or EcoRI (BRL) for 10 min. DNA fragments were separated by electrophoresis in 0.8% agarose for 15 min and Southern blotting was performed following the procedures of Dr. Mark Philipsen (29). DNA was isolated from the nuclei according to Saluz and Philipsen (29). DNA was isolated from the nuclei according to Saluz and Philipsen (29).

Linker-mediated PCR Analysis of MGMT Promoter Methylation—For methylation analysis by LMPCR, nuclei were isolated from the glioma cell lines according to a method described by Wijnholds and Philipse (28). DNA was isolated from the nuclei according to Saluz and Yost (30) and cut with EcoRI (5 U) to reduce viscosity. Following extraction and precipitation, the DNA was dialyzed in dH₂O and then cleaved with genomic DNA sequence-specific DNA primers as described by Maxam and Gilbert (31).

The LMPCR protocol was based on the method described by Pfifer et al. (32). It consisted of denaturation, ligation, and amplification steps. All DNA primers for LMPCR were gel purified on an agarose gel and eluted except an 11-mer primer for EcoRI. For extension reactions, a 15-min reaction containing 5.0 μg of cleaved genomic DNA, 0.5 pmol of the extension primer (for promoter region I), 5'-CGGGCCATTCTGGGCAAACTAAG, corresponding to nucleotides 576–575, for region II, 5'-GAGCCAGAGGCTTGCAGGCGGAAAGCCT-3', corresponding to nt 868–873, and a 2× PCR buffer (United States Biochemical Corp. (USB), Cleveland, OH) was

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In a 3ZP-labelled 18 bp DNA substrate, the mean between duplicate experiments was 42%. Values for MGMT activity, determined by an in vitro assay that measured the ability of glioma cell protein extracts, were similar to the levels of MGMT mRNA in each cell line (Table I), consistent with transcriptional control of MGMT expression. Differences in the rank order of cell lines according to MGMT mRNA versus MGMT activity may reflect small differences in post-transcriptional events such as mRNA stability, translational efficiency, or protein half-life.

**Analysis of the MGMT Gene Coding Sequences**—To develop probes for methylation analysis across the body of the MGMT gene, a genomic library was screened with an MGMT cDNA probe. Twenty-two positive clones were analyzed for BamH I (B), HindIII (H), and EcoR I (E) restriction sites, aligned, and partially sequenced. Eight of these clones with minimal overlap were used to generate a preliminary map of the MGMT gene consisting of four translated exons and spanning >80 kb (exon map).

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**RESULTS**

**MGMT Expression in Glioma Cell Lines**—The level of MGMT expression in the glioma cell lines was determined at the mRNA and protein levels. MGMT mRNA levels from duplicate Northern blots hybridized with the MGMT cDNA are expressed as percent of T98 MGMT mRNA in Table I. The relative levels of MGMT activity, quantitated from an in vitro assay of glioma protein extracts, were similar to the levels of MGMT mRNA in each cell line (Table I), consistent with transcriptional control of MGMT expression.

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**Table I**

| Glioma cell line | MGMT mRNA levels | MGMT Activity (% of T98) |
|------------------|------------------|--------------------------|
| T-98             | 100              | 100                      |
| SF-763           | 78.8             | 121 ± 15                 |
| U-138            | 76.8             | 96 ± 12                  |
| SF-767           | 61.6             | 110 ± 19                 |
| Nat              | 48.5             | 73 ± 12                  |
| U-733            | 38.4             | 38 ± 2                   |
| HS-683           | 38.3             | 38 ± 9                   |
| A1235            | 0                | <1                       |
| Cla              | 0                | <1                       |
| Cro              | 0                | <1                       |

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**Fig. 1. Restriction enzyme map of the coding sequences of the human MGMT gene.** A genomic library derived from human fibroblast cell line DNA was screened with an [3P]-labelled MGMT cDNA that spanned nucleotides 70-777 (33). Eight positive clones analyzed by restriction enzyme mapping with BamH I (B), EcoRI (E), and HindIII (H) were aligned to form a discontinuous map of the gene. Exons (•) within two genomic subclones, p5 BH and p7CNN, were sequenced and compared with the corresponding cDNA nucleotides (13). Nucleotides complementary to cDNA sequences 580-776 were identified within genomic clone 11b (exon 5) by PCR amplification with primers defining this cDNA region. Gaps in the map represent intron regions of >20 kb (15). The * denotes the EcoRI site analyzed for methylation in Fig. 2. The positions and lengths of the intron probes used for methylation analysis are indicated by solid or dashed lines located below the subclones.
cDNA-specific primers, spans >185 bp and extends into the 3' nontranslated region. These genomic clones likely include all translated exons and span the body of the MGMT gene (15).

**Methylation at an Intron 1 Site Correlates in a Direct, Graded Fashion with MGMT Expression**—In order to examine the relationship between methylation and MGMT expression in a quantitative fashion, the percent of MGMT gene alleles that were methylated at an MGMT intron I site, 5 kb upstream of the translation start site (' in Fig. 1), within each glioma cell line was determined by Southern blot analysis with EcoRI-digested glioma DNA (Fig. 2A) and then compared with MGMT mRNA levels by linear regression analysis (Fig. 2B). As a result of the methylation sensitivity of EcoRI (EcoRI will not cleave at its recognition site, GAATTC, if the cytosine is followed by a guanine and the cytosine of this CG dinucleotide is methylated) (34), when the 3' EcoRI site flanking the 3.8-kb EcoRI intron 1 segment of the MGMT gene (see Fig. 1) is methylated in glioma DNA, the DNA is cut at the next available EcoRI site, approximately 0.7 kb downstream, generating a larger, 4.5-kb fragment. The proportion of 4.5- to 4.5 + 3.8-kb fragments in the DNA of each cell line is therefore a measure of the percent of methylated (at this site) MGMT gene alleles within a population of glioma cells. In Fig. 2A, the cell lines are arranged from left to right in order of decreasing MGMT expression. The results of this analysis demonstrate that methylation is graded across the cell lines at the intron 1 EcoRI site and increases with increasing MGMT expression (Fig. 2). The methylation status of this intron 1 site correlates (r = 0.857, Fig. 2B) with MGMT mRNA levels in a similar, positive fashion as previously seen in MGMT exons (9–11). The percent methylation at this site within each cell line varied on average less than 20% between two experiments (which were done 4 months apart) indicating that the partial methylation status of this site within each cell line may be stable over time.

**Uniform Methylation across the Body of the MGMT Gene Correlates Directly with MGMT Expression**—In order to determine if the direct correlation between methylation status of MGMT exons (9–11) (and intron 1) and MGMT gene expression is uniform across the body the MGMT gene, Southern blots of MspI- (methylation insensitive) and HpaII- (methylation sensitive) digested glioma DNA were hybridized with restriction fragments of genomic clones from 5', middle, and 3' regions of the body of the MGMT gene.

Analysis of the methylation status of the body of the gene is shown in Fig. 3. A 3.8-kb EcoRI intron 3 fragment, located 10 kb 3' of exon 3 (see Fig. 1) was hybridized to Southern blots of MspI (Fig. 3A) and HpaII (Fig. 3B) cut glioma DNA. The cell lines are arranged from left to right in order of decreasing MGMT expression. Fig. 3A shows that the pattern of hybridization to MspI-digested DNA is the same in all lanes indicating that, by Southern blot analysis, the number and position of MspI sites is the same in all cell lines. The results of the HpaII digest, shown in Fig. 3B, demonstrate that this region is heavily methylated in the four high expressing cell lines (lanes 1–3) and relatively unmethylated in the low (lanes 5 and 6) and non-expressors (lanes 7–9). The strong hybridization to high molecular weight HpaII fragments from cell lines with high MGMT expression (lanes 1–4) indicates that in a significant proportion of these cells all HpaII sites are methylated over 7–12 kb of intron 3. The presence of a 1.1-kb fragment in all lanes (Fig. 3B), which corresponds to a MspI fragment (Fig. 3A), indicates that the two HpaII sites flanking this fragment are unmethylated to an extent, estimated by hybridization intensity, in all cell lines, with a slightly greater number of unmethylated alleles in the nonexpressing cell lines. The presence of both the methylated and unmethylated fragments in HpaII-
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Fig. 3. Southern blot analysis of MspI/HpaII site (CCGG) methylation in MGMT intron 3. DNA from MGMT expressing (MGMT +) and nonexpressing (MGMT -) glioma cell lines was digested with either MspI (7–10 μg DNA) (A) or HpaII (7–10 μg DNA) (B). Complete digestion of each sample was demonstrated by analysis of the digestion products of a uniformly 32P-labeled MGMT cDNA incubated with aliquots (20 μl) of the primary digestion reactions (data not shown). The DNA was analyzed by Southern blot with a 32P-labeled 3.8-kb MGMT intron 3 fragment derived from genomic clone 7C and located 10 kb 3' of exon 3 (see Fig. 1).

digested DNA from the high MGMT expressing glioma cell lines suggests the existence of allelic differences (within a single cell) and/or population differences (within each cell line) in MGMT gene methylation patterns. The variable hybridization intensities within each lane and across lanes likely reflect the degree of heterogeneity in methylation at various MGMT intron sites, i.e., a greater degree of heterogeneity in MGMT gene methylation within a cell line results in fewer copies of a given HpaII fragment and thus weaker hybridization signal for that fragment.

In other regions across the body of the MGMT gene (see intron probes, Fig. 1) methylation analysis (not shown) yielded results very similar to those in Fig. 3, i.e., the MGMT gene was methylated in MGMT expressing cell lines and relatively unmethylated in nonexpressing cell lines. These results suggest that, within each cell line, the methylation status of the MGMT gene is uniform over the >80 kb defined by the probes used and correlates in a positive, graded fashion with MGMT expression across the cell lines.

MGMT Promoter Methylation Correlates in an Inverse, Graded Fashion with MGMT Expression—The methylation status of the MGMT promoter was determined by LMPCR analysis of hydrazine-treated glioma DNA. As hydrazine, in the presence of 1.5 μ NaCl, reacts preferentially with cytosine but not 5-methylcytosine (35), methylated cytosines decrease the intensity, or cause the disappearance of cytosine bands in sequenced DNA. We analyzed methylation in two regions of the MGMT promoter that together likely contain all the basal promoter elements and approximately 60% of full promoter activity (13). Region I includes nucleotides 703–800 and region II, which spans the transcription start site (nt 955), includes the basal promoter elements (nucleotides 865–1020) (13). The full nucleotide sequence of these regions in the cloned promoter as well as the positions of guanines in the glioma DNA were also confirmed using genomic sequencing chemicals and LMPCR.

Fig. 4, A and B, represent autoradiographs of the promoter methylation analysis in region I and region II, respectively. In both promoter regions, the relative intensity of most bands representing potentially methylated cytosines (numbered arrows) decreases, indicating fewer unmethylated cytosines, from SF767 (high expressing) to Hs683 (low expressing) and Cla- (nonexpressing) amplified DNA. Methylation in these MGMT promoter regions appears uniform within each cell line and correlates in an inverse, rather than direct, fashion with MGMT expression.

The radioactive signal from 25 of the cytosine bands (of a CpG) was quantitated directly from the polyacrylamide gels and compared with a nearby non-CpG cytosine to obtain a
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FIG. 4. Methylation analysis of the MGMT promoter in high MGMT expressing (SF767), low MGMT expressing (Hs683), and nonexpressing (Cla) glioma cell lines by LMPCR. DNA from three glioma cell lines and plasmid DNA containing a 1.2-kb BamHI/StfI fragment of the MGMT promoter was reacted with genomic sequencing chemicals. All nucleotides (G, guanine; A, adenine; T, thymine; and C, cytosine) in the cloned DNA and guanines and cytosines in the glioma DNA spanning promoter nt 703–800 (A, region I) (13) and 865–1020 (B, region II) were analyzed by LMPCR. A MGMT promoter-specific primer (region I, nt 655–675 and region II, nt 805–823) (13) was annealed to the cleaved DNA and extended (48 °C, 15 min) with Sequenase. Extension products were ligated to a double-stranded linker and then amplified by PCR (5 min, 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) with the longer (25-mer) linker primer and a nested gene-specific primer (for promoter region 1, nt 674–698, for promoter region 2, nt 841–861). 32P-Labeled PCR products were generated through two additional PCR cycles with a second nested end-labeled primer (for promoter region I, nt 674–702, for promoter region 2, nt 841–864). Following two cycles of PCR (same parameters as above except annealing was at 67 °C and extension at 77 °C), the DNA was extracted, precipitated, and resuspended in 10 μl. Three to 5 μl of the sample was electrophoresed through a 6% denaturing polyacrylamide gel and then detected by autoradiography (6–18 h exposure). ▲ indicates cytosines within a CpG.

Relative ratio (CpG cytosine/non-CpG cytosine) that reflects the level of methylation at that site. The relative ratios from glioma cell DNAs were compared with plasmid DNA containing the MGMT promoter (100% unmethylated at all sites) (region I, Fig. 5A) or to the SF767 cell line DNA (region II, Fig. 5B). The results are expressed as percent of MGMT promoter alleles within each cell line that are unmethylated at a particular site, as the signal being measured is a result of unmethylated cytosines. Methylation appears uniform within each cell line and graded across the cell lines at 8 of 10 sites (exceptions are sites 3 and 6) in region I and 13 of 15 sites (exceptions are sites 11 and 13) in region II, and correlates in an inverse, rather than direct fashion with MGMT expression. Of the four non-graded sites, three (sites 3, 6, and 11) are still methylated to a greater extent in the nonexpressing cell lines compared with expressing cell lines, and one (site 13) is unmethylated in all cell lines. The graded methylation patterns at the majority of sites across the cell lines implies that there is a close, inverse relationship between methylation of the promoter and MGMT expression.

Restriction Enzyme Accessibility to the MGMT Promoter within Nuclei Is Associated with MGMT Expression—The uniformity of promoter methylation within each cell line suggested to us that the overall level of promoter methylation might influence MGMT transcription through an indirect mechanism, involving methylation-related changes in chromatin structure. As a measure of chromatin structure, we analyzed the accessi-
Fig. 5. Quantitation of promoter methylation in high MGMT expressing (SF767, ■), low expressing (Hs683, □), and nonexpressing (C1a, ▶) glioma cell lines. Promoter methylation was quantitated by measuring radioactive signal from the LMPCR products in polyacrylamide gels using a Betascope 603 blot analyzer. Methylation values of each CpG were calculated as the ratio of unmethylated cytosine (in a CpG) to a
Fig. 6. LMPCR analysis of AvaII accessibility to the MGMT promoter within intact nuclei. Nuclei isolated from high MGMT expressing (SF767), low expressing (Hs683), and nonexpressing (Cla) glioma cells were incubated with 16 U of AvaII for 10 min at 37 °C. DNA was isolated from the nuclei, and 5 μg was analyzed by LMPCR as described in Fig. 4, except only one-fifth of the final reaction was analyzed, and autoradiograph exposures were 2–5 h with intensifying screens. The LMPCR products of 127 nt (arrow in A, promoter region I) and 140 nt (arrow in B, promoter region II) are derived from glial DNA cut at the AvaII site at promoter nt 776 or 956 (13), respectively.

DISCUSSION

Changes in the methylation status of a particular gene or gene region may occur as a result of one or several normal (X chromosome inactivation, imprinting, tissue-specific gene expression) (36) or abnormal (tumorigenesis, oncogene-induced transformation) (37, 38) processes. During X chromosome inactivation, methylation of gene-associated CpG islands and variable methylation of the body of genes are associated with complete transcriptional silencing of gene expression (36). Similarly, the widespread hypomethylation and CpG island hypermethylation frequently observed in tumor cells are associated with complete suppression of transcription from affected genes (37, 39, 40). These observations raise the possibility that during the progression of methylation changes, whether through normal or abnormal events, intermediate levels of cytosine methylation may exist, which in turn may be associated with diminishing, graded gene expression. Identification of intermediate levels of methylation associated with diminished, but not silenced, gene expression would provide a closer link between methylation and gene expression, and would also be useful in studying potential mechanisms by which methylation influences gene expression.

In the present study, the graded methylation and correlations between methylation and MGMT expression suggest that in both the promoter and body of the MGMT gene methylation may influence the level of MGMT expression. Several previous studies have attempted to analyze methylation in the MGMT promoter in cells with different levels of MGMT expression. One study examining the methylation status of HpaII sites in the MGMT promoter failed to show a clear association between promoter methylation and MGMT gene expression, although the close proximity of the 14 HpaII sites in the promoter region precluded resolution of the methylation status of these sites (12). Analysis of the methylation status of a single site 70 bp upstream of the transcription start site demonstrated a negative, but not absolute association between methylation and MGMT expression (12), although MGMT promoter activity studies demonstrated that deletion of 76 bp 5' from, and including, this site did not alter promoter strength (13). Another study examining methylation at HpaII sites in the MGMT promoter concluded that the promoter was methylated to a greater extent in MGMT expressing cell lines compared with nonexpressors, although this study also used Southern blot analysis and thus did not resolve the relevant HpaII fragments (15). It is unclear from these studies whether the methylation status of the MGMT promoter is associated with MGMT expression. In the present study, the graded methylation across the cell lines at 21 of 25 CpGs tested in the MGMT promoter indicates that the methylation status is uniform within each cell line and that there is a close, inverse association between overall promoter methylation and MGMT expression. The overall promoter methylation, expressed as the average “percent unmethylated” of all CpG sites, was similar in region I and II (for region I, 93% in SF767, 73% in Hs683, and 45% in Cla, and for region II (relative to SF767), 100% in SF767, 74% in Hs683, and 58% in Cla). The region II values were expressed relative to SF767 because the intensity of cytosine bands corresponding to the more 3'-cytosines in the plasmid DNA was somewhat diminished and thus difficult to accurately quantitate. As the three 5'-most cytosines of CpGs in the plasmid region II were similar in degree of unmethylation to those in the SF767 cell line, however, and as most sites in SF767 region I were completely unmethylated (given the 8.6% average experimental error), it seems reasonable to assume region II cytosines in SF767 DNA are nearly 100% unmethylated. It should be noted that in cells that do not express the MGMT gene, methylation of the promoter, while less than in expressing cells, was still only 50%, rather than 100% methylation noted in X-linked inactive genes (17). These data suggest that while inactive genes on the X chromosome may be 100% methylated, complete methylation may not be necessary for processes involved in promoter inactivity. In contrast to promoter methylation, the relative contri-
bution of methylation in the body of genes to the control of gene expression is less understood, although the conserved methylation status in the body of many genes implies that these sites are important, at least in genes where the body methylation correlates with gene expression. While methylation in the MGMT promoter correlates inversely with MGMT expression, the uniform methylation over the body of the MGMT gene and graded methylation at the intron EcoRI site correlated directly with MGMT expression, suggesting that methylation in the body may also influence MGMT expression in a graded fashion. Although the intron I EcoRI site analysis is consistent with the methylation analysis at HpaII sites in the same region (not shown), the possibility that the inhibition of EcoRI digestion at the internal EcoRI site in MGMT intron 1 is a result of a point mutation or small deletion that destroys the EcoRI site cannot be excluded. We would not consider this possibility likely, however, as the probability of this specific mutation occurring independently in all 10 of the unrelated glioma cell lines tested is exceptionally low. Because the body of the MGMT gene is methylated in MGMT expressing normal human brain cells and T lymphocytes (not shown), the close association between hypomethylation of the MGMT gene and lack of MGMT expression in glioma cell lines suggests that maintenance of appropriate levels of methylation may be important for MGMT expression. This hypothesis is supported by experiments demonstrating that treatment of MGMT expressing tumor cell lines with 5-azacytidine decreased methylation in the body of the gene to a level comparable to a nonexpressing cell line and significantly decreased MGMT mRNA levels (9, 41). Additionally, two reports (42, 43) have demonstrated that treatment of MGMT nonexpressing human cell lines with 5-azacytidine did not restore the DNA repair phenotype associated with MGMT expression, suggesting that the absence of MGMT expression is not solely due to a reversible hypermethylation, as has been demonstrated in the promoter region of other genes (44), but may also involve methylation in the body. The hypomethylated body of the MGMT gene is, however, minimally permissive to transcription, as low levels of MGMT mRNA are detected in the two glioma cell lines (Hs683 and U373) and a 5-azacytidine-treated cell line (HT29) (9) in which the body of the MGMT gene is relatively unmethylated. In contrast, an induction in MGMT expression was observed following exposure to 5-azacytidine in two MGMT nonexpressing cell lines, although these studies did not assess methylation in the body of the gene (41). The direct correlation between methylation and MGMT expression in the body and indirect correlation in the promoter, demonstrated in the present study, suggest that methylation in both the promoter and body may influence MGMT expression, although probably through separate mechanisms.

One mechanism by which methylation may influence MGMT expression involves alterations in chromatin structure. The significantly greater restriction enzyme accessibility of the MGMT promoter in nuclei from glioma cells with high levels of MGMT expression relative to glioma cells with low or no MGMT expression, demonstrated in this study, suggests that the promoter is in a more open, transcriptionally competent state in the glioma cells with high levels of MGMT expression. The absence or significantly decreased amount of LMPCR product from the nuclei digests of low (Hs683) and nonexpressers (Clia) is likely not a result of contaminating material in the nuclei isolation or LMPCR reactions as parallel digests of nuclei (from the same isolation) with vast excess amounts of a second restriction enzyme, followed by LMPCR, generated equal amounts of LMPCR products from all three cell lines (data not shown). We therefore conclude that the differences in the nuclei digests reflect significant differences in the chromatin structure of the MGMT promoter in cells with different levels of MGMT expression. The more accessible chromatin structure would appear better suited for interaction with transcription factors and thus may directly facilitate expression of the MGMT gene. The relatively inaccessible, yet only partially methylated, promoter in the cells with little or no MGMT expression suggests that small, rather than complete, changes in methylation are sufficient to significantly affect chromatin structure and possibly transcription. It is possible that there is a threshold of methylation, probably less than 50% average methylation in promoters, that is sufficient for rendering the chromatin inaccessible. The inaccessible promoter in cells with low levels of MGMT expression, however, suggests that either the closed chromatin structure is not sufficient for complete suppression of MGMT transcription, or that the inaccessibility in these cells is lesser than in the nonexpressing cells and thus permissive to low levels of MGMT expression. Although there are clear differences in promoter accessibility between the cells with high levels of MGMT expression and those with little or no MGMT expression, we cannot determine from these experiments if there are subtle differences in promoter accessibility between the low and nonexpressors. Potential differences in promoter accessibility between the low and nonexpressors may become apparent through extensive analysis with different concentrations of AcaII, or with other restriction enzymes that cleave at nearby sites. Ultimately, however, the identification of subtle differences in chromatin structure that are relevant to MGMT transcription relies on in situ footprinting analysis. These experiments are currently underway. In the MGMT promoter, there are numerous consensus sequences for transcription factor binding including 11 SP-1 sites, AP-1 and AP-2 sites, and glucocorticoid-responsive and heat shock elements (13). Since there are six SP-1 sites in the MGMT minimal promoter, including several flanking the differentially accessible AcaII site (nt 956), and SP-1 is an important component of transcription regulation from other GC-rich, TATA-less promoters (44), the accessibility of the SP-1 sites may be relevant in transcriptional regulation of MGMT expression.

In summary, the present study strengthens the link between cytosine methylation and gene expression by identifying a gene with high, low, and intermediate levels of methylation that correlate in a graded fashion with gene expression. The demonstration that MGMT gene expression correlates directly with methylation in the body of the gene and inversely in the promoter suggests that methylation both in the promoter and body of the gene may influence MGMT expression in a graded fashion. Since such intermediate levels of methylation exist in the MGMT gene, and the mechanisms of transcriptional regulation of genes with GC-rich, TATA-less promoters are poorly defined, the MGMT gene may be a suitable model for studying the relative contribution and interrelatedness of methylation, chromatin structure, and transcription factor/promoter interactions.

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