Epigenetic regulation of O^6-methylguanine-DNA methyltransferase gene expression by histone acetylation and methyl-CpG binding proteins

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Abstract

Transcriptional silencing of the DNA repair gene, O^6-methylguanine-DNA methyltransferase (*MGMT*) in a proportion of transformed cell lines is associated with methylated CpG hotspots in the *MGMT* 5′ flank. The goal of the study was to evaluate the mechanism by which CpG methylation of the *MGMT* promoter region influenced silencing of the gene. Analysis of histone acetylation status in two regions of the promoter using chromatin immunoprecipitation assay showed that a higher level of histone acetylation was associated with expression in three MGMT-expressing cell lines (HeLa CCL2, HT29, and Raji) compared with three MGMT-silenced cell lines (HeLa S3, BE, and TK6). To determine how the modulation of CpG methylation and histone acetylation influenced MGMT expression, we exposed the cells to 5-aza-2′ deoxycytidine (5-Aza-dC), inhibitor of DNA methylation, which strongly up-regulated MGMT expression in three MGMT-silenced cell lines whereas trichostatin A, inhibitor of histone deacetylase, weakly induced MGMT. However, combined treatment with 5-Aza-dC and trichostatin A significantly up-regulated *MGMT* RNA expression to a greater extent than in cells treated with either agent alone suggesting that histone deacetylation plays a role in MGMT silencing but that CpG methylation has a dominant effect. Consistent with enhanced MGMT expression, 5-Aza-dC increased the association of acetylated histone H3 and H4 bound to the *MGMT* promoter. Chromatin immunoprecipitation analysis of methyl-CpG binding domain containing proteins detected a greater amount of MeCP2, MBD1, and CAF-1 bound to the *MGMT* promoter in MGMT-silenced cells. Our findings implicate specific MBD proteins in methylation-mediated transcriptional silencing of *MGMT*. [Mol Cancer Ther 2005;4(1):61–9]

Introduction

The DNA repair protein, O^6-methylguanine-DNA methyltransferase (MGMT), removes alkyl adducts from the O^6-position of guanine in DNA and protects cells against the mutagenic, carcinogenic and cytotoxic effects of alkylating agents. MGMT is expressed in all human cells, tissues, and most tumors, although its expression varies widely (1). Furthermore, MGMT is silenced in a significant proportion (~20-30%) of malignant and transformed cell lines and a fraction of primary tumors. Such silenced cells are hypersensitive to cancer therapeutic and carcinogenic alkylating agents (2).

MGMT gene has a CpG-rich promoter containing a CpG island with no TATA or CAAT boxes. There are six putative Sp1 recognition sites, along with two glucocorticoid receptor binding elements, two activator protein-1 (AP-1), and two putative AP-2 binding sequences (3). MGMT-silenced cells lack detectable levels of *MGMT* RNA and protein. However, no gross rearrangements nor deletions of the gene have been reported suggesting that the absence of RNA is due to regulation at the transcriptional level. Previous reports from our lab and others have shown that *MGMT* suppression is strongly linked to CpG methylation in the 5′ flanking region of the gene (4–7). We also reported that in cell lines and xenografts, there are two hotspot regions in the 5′ region of the *MGMT* gene where CpG methylation is almost always associated with MGMT silencing (8). Whereas methylation is clearly involved in MGMT silencing, the precise mechanism of methylation-mediated transcriptional inactivation of the *MGMT* gene is not understood.

DNA methylation can repress gene transcription either by interfering with the sequence-specific binding and/or function of transcription factors due to cytosine methylation of the recognition sites (9, 10) or by recruiting methyl CpG binding domain (MBD) containing family of proteins. MBD proteins mediate transcriptional repression via corepressors and by altering the chromatin structure, or by blocking access to transcription machinery (11, 12). Based on studies demonstrating the connection between DNA methylation and histone deacetylation (13, 14), a model has been proposed for methylation-mediated transcriptional silencing. According to this model, MBD proteins bind to methylated CpGs and recruit histone deacetylase (HDAC) either directly or via a corepressor complex. The HDAC removes acetyl groups from the

lysines on the NH_2-terminal histone tails, which restores the positive charge on the lysine residues and promotes the stabilization of histone-DNA interaction. This results in chromatin condensation, inaccessibility of transcription factors, and transcriptional inactivation. Conversely, histone acetylation reverses the process leading to a more open chromatin, accessibility of transcription factors, and transcriptional activation.

Five methyl-CpG binding proteins, MeCP2, MBD1, MBD2, MBD3, and MBD4 have been identified to date in mammalian systems. Four of these proteins, MeCP2, MBD1, MBD2, and MBD4 bind to methylated CpG through the conserved protein motif called the MBD (15). MeCP2, MBD1, and MBD2 have been shown to recruit HDAC and function as transcriptional repressors. MBD3 is a non-DNA binding component of the Mi-2/NuRD corepressor complex, and MBD4 has uracil DNA glycosylase activity and has been implicated in DNA repair (16). Another protein, Kaiso, binds methylated DNA through a zinc finger motif and behaves as a transcriptional repressor (17).

The present study was designed to first assess whether histone acetylation is associated with CpG methylation in the *MGMT* promoter region and evaluate the relative contribution of histone acetylation to MGMT expression. Second, the goal was to identify the MBD proteins associated with *MGMT* promoter in MGMT expressing and silenced cell lines and thereby understand the role of the MBD proteins in mediating *MGMT* transcriptional repression.

Materials and Methods

Cell Culture

The human cervical tumor cell lines HeLa S3 and CCL2 were grown in Eagle's minimum essential medium supplemented with 10% FCS. The human colon adenocarcinoma cell lines BE and HT29, were grown in McCoy's 5A medium supplemented with 10% FCS. The human B lymphoblast cell lines, TK6, and Raji were cultured in RPMI 1640 containing 10% FCS. All cells were grown at 37°C in 95% humidified air and 5% CO2. All cells were obtained from American Type Culture Collection (Manassas, VA).

5-Aza-2′ Deoxycytidine and Trichostatin A Treatment of Cells

Cells were seeded 24 hours before treatment. Cells were then given one of the following treatments. (a) 5-Aza-2′ deoxycytidine (5-Aza-dC; 1 μmol/L; Sigma, St. Louis, MO) treatment for 1 to 4 days. Medium containing 5-Aza-dC was changed every 24 hours. (b) Trichostatin A (TSA, 330 nmol/L, Sigma) treatment was for 24 hours. (c) In cells treated with both the drugs, 5-Aza-dC (1 μmol/L) was present in culture for either 1, 2, 3, or 4 days and the addition of TSA (330 nmol/L) was for the last 24 hours before harvesting the cells.

Immunoblotting

Cell lysates were prepared with mammalian protein extraction reagent (Pierce, Rockford, IL) containing protease inhibitors. Equal amounts of proteins were size fractionated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. Membranes were probed with antibodies against MeCP2 (Upstate Biotechnology, Lake Placid, NY), MBD1 (Santa Cruz Biotechnology, Santa Cruz, CA), MGMT (Chemicon International, Temecula, CA), and β-tubulin (Sigma). The proteins were detected with the enhanced chemilumiscence Western blotting detection system (Amersham Biosciences, Piscataway, NY).

Western Blot Analysis of Isolated Histones

Cells were cultured as described above. After 24 hours of culture without or with TSA (330 nmol/L), cells were harvested and washed in ice-cold PBS. Nuclei were isolated by dounce homogenization of cells in 1 mL of lysis buffer [10 mmol/L Tris-HCl (pH 6.5), 50 mmol/L sodium bisulfite, 8.6% sucrose, 1% Triton X-100, and 10 mmol/L $MgCl_2$] and centrifuged at 1,000 rpm for 5 minutes, washed twice in lysis buffer and later with Tris-EDTA solution [10 mmol/L Tris-HCl (pH 7.4) and 13 mmol/L EDTA]. Histones were isolated from the nuclear fraction by acid extraction as described (18). Histones (25 μg) were separated on 15% SDS-PAGE minigels, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed with different antibodies. The following antibodies were used: antiacetylated histone H3, anti-histone H3, anti-acetylated histone H4, anti-histone H4, (Upstate Biotechnology). The proteins were detected with the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences).

Chromatin Immunoprecipitation Assay

We used the chromatin immunoprecipitation (ChIP) assay kit from Upstate Biotechnology and followed the manufacturer's protocol with some modifications. The proteins were cross-linked to the DNA, by the addition of formaldehyde to the culture medium to a final concentration of 1%, and were incubated at 37°C for 10 minutes. The cross-linking reaction was quenched by adding 125 mmol/L glycine (in PBS) for 5 minutes at room temperature. The medium was removed and the cells were suspended in 1 mL of ice-cold PBS containing protease inhibitors. Cells were pelleted, resuspended in 0.2 mL of SDS lysis buffer per 10^6 cells, and incubated on ice for 10 minutes. Sonicated lysates were centrifuged at 14,000 rpm at 4°C for 15 minutes. Supernatants were diluted 5-fold in ChIP dilution buffer (provided in the kit). An aliquot (100 μL) of the chromatin preparation was set aside and designated as Input fraction. To reduce the nonspecific background, the chromatin solution was precleared with 50 to 80 μL of salmon sperm DNA/Protein A agarose slurry (50% slurry) rocking for 1 hour at 4°C. The cleared chromatin was immunoprecipitated with 5 μg of either anti-acetylated histone H3, anti-acetylated histone H4, anti-MeCP2, anti-MBD1, or anti-CAF-1 (p150; Santa Cruz Biotechnology) antibody and incubated overnight at 4°C with rotation. Later, salmon sperm DNA/Protein A agarose slurry was added to these samples and rocked for 1 hour at 4°C. Protein A immune complexes were collected by centrifugation and washed with the recommended

buffers for 5 minutes each. Immune complexes were eluted twice with 250 μL of elution buffer for 15 minutes at room temperature. Twenty microliters of 5 mol/L NaCl were added to the combined eluates, and the samples were incubated at 65°C for 4 hours; 10 mmol/L EDTA, 40 mmol/L Tris-HCl (pH 6.5), and 20 μg proteinase K were then added to the samples and incubated at 45°C for 1 hour. DNA (both from immunoprecipitation samples and Input) was recovered by phenol extraction, ethanol precipitation, and analyzed by PCR.

PCR Amplification

Two regions of the *MGMT* promoter designated hotspot 1 region and AP-1 region (Fig. 1), and a region of the γ-actin promoter were analyzed by PCR. The primer pairs used for the hotspot region of the *MGMT* promoter for ChIP analysis were 5′-GACAGGAAAAGGTACGGGCCATT (forward) and 5′-GAGCCGAGGACCTGAGAAAAGCAA-GAGAG (reverse). These primers amplify the hotspot 1 and the minimal promoter region located upstream of the transcription start site. A second set of primers used to amplify the AP-1 region of the *MGMT* promoter were 5′-GCTCCAGGGAAGAGTGTCCTCTGCTCCCT (forward) and 5′-GGCCTGTGGTGGGCGATGCCGTCCAG (reverse). This region includes two AP-1 binding sites. The primer pairs used for γ-actin promoter ChIP analysis were 5′-GCCAGCTCTCGCACTCTGTT (forward) and 5′-AGATCGCAACCGCCTGGAAC (reverse; ref. 18). The optimal PCR conditions were determined with buffers from Epicentre (Madison, WI) for each PCR pair. The annealing temperatures for hotspot region, AP-1 region, and γ-actin were 60C, 66C, and 62C, respectively. PCR products were resolved on 1.2% agarose gels, stained with ethidium-bromide, and quantitated using AlphaImager 2000 documentation and analysis software v.4.0.3. (Alpha Innotech Co., San Leandro, CA).

Reverse Transcription-PCR

Total RNA was isolated using the RNAqueous kit (Ambion, Austin, TX). RNA (2 μg) was reverse transcribed using avian myeblastosis virus reverse transcriptase, deoxynucleotide triphosphates, and oligo-dT primers (Invitrogen, Carlsbad, CA). Amplification of cDNA was done using primers specific for *MGMT* and *β-actin*. Primers specific for *MGMT* cDNA amplification were 5′-CGACTTGG-TACTTGGAAAAATG (forward) and 5′-CGCATCCGATG-CAGTGTTAC (reverse). The primer pair specific for *β-actin*

cDNA was 5′-GTGGGGCGCCCCAGGCACCA (forward) and 5′-CTCCTTAATGTCACGCACGATTTC (reverse). Amplification of *β-actin* cDNA served as an internal control. PCR amplification for the *MGMT* and *β-actin* mRNA was done at a 60C annealing temperature to yield 702 and 540 bp products, respectively. The PCR products were resolved on 1.2% agarose gel, stained with ethidium bromide, and quantitated as described above.

Results

Differences in the Histone Acetylation Status in the MGMT Expressing and Silenced Cell Lines

To determine whether histone acetylation is associated with MGMT expression, we compared the levels of acetylated histones H3 and H4 (AcH3 and AcH4) bound to the 5′ flank of the *MGMT* promoter in the immuno-precipitated chromatin from three MGMT-expressing cell lines (HeLa CCL2, HT29, and Raji) and three MGMT-silenced cell lines (HeLa S3, BE, and TK6) using the ChIP assay. Two different regions of the *MGMT* promoter were amplified, designated as the hotspot 1 and AP-1 regions to yield PCR products of 324 and 394 bp, respectively (Fig. 1). The PCR fragment with hotspot 1 extends from −314 to +10 nucleotides relative to the transcription start site which also includes the minimal promoter and has 41 CpGs. Previously, we have shown that these CpGs are almost always methylated in MGMT-silenced cells (8). The PCR fragment of the AP-1 region extends from −541 to −934 nucleotides and includes two AP-1 sites. This sequence was chosen for comparison because it contains only eight CpGs and the methylation of CpGs upstream of −245 bp is not associated with MGMT silencing (19).

Figure 2 shows that the amount of acetylated histones H3 and H4 associated with the hotspot region in the *MGMT* promoter was lower in MGMT-silenced (TK6, BE, and S3) cells when compared with MGMT expressing (Raji, HT29, and CCL2) cells. Similarly, the amount of acetylated histones H3 and H4 associated with the AP-1 region in the *MGMT* promoter was also reduced in MGMT-silenced cells when compared with those of MGMT expressing cells. Because methylation of the AP-1 region has not been previously shown to be associated with MGMT silencing, a reduction in the amount of acetylated histones in this region is likely mediated by the closed chromatin
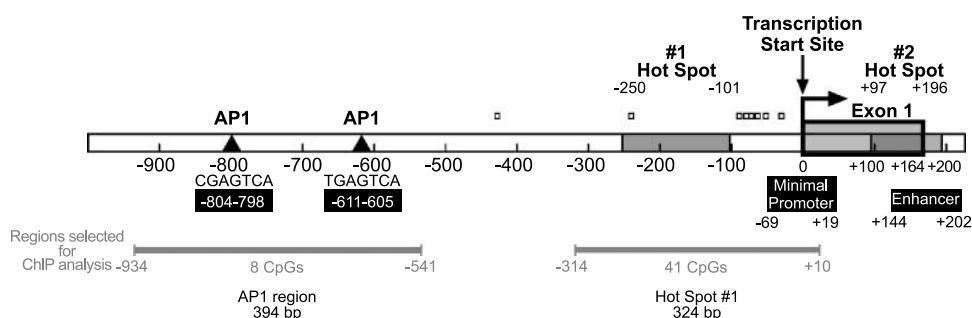

Figure 1. Schematic representation of the Hot Spot and AP1 regions in the *MGMT* promoter used for ChIP analysis. The CpGs in the two Hot Spot regions are almost always methylated in MGMT-silenced cells (8). □, SP1 sites.
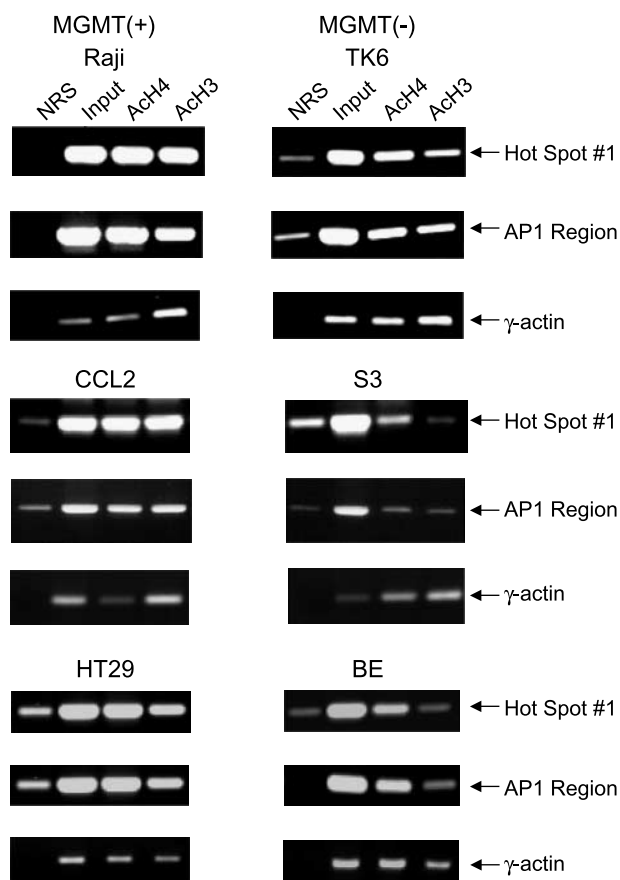
Figure 2. Comparison of histone acetylation status between MGMT-expressing and MGMT-silenced cell lines in the Hot Spot #1 and AP1 regions of the *MGMT* promoter. ChIP assay for Raji, TK6, CCL2, S3, HT29, and BE cell lines after ChIP with anti-acetyl-H4 or H3 antibodies. *NRS,* ChIP with normal rabbit serum; *Input,* DNA isolated from the lysate before immunoprecipitation; *AcH4,* DNA immunoprecipitated with anti-acetyl-H4 antibody; *AcH3,* DNA immunoprecipitated with anti-acetyl-H3 antibody. Amplification of γ-actin promoter served as a positive control.

throughout the *MGMT* promoter region. Consequently, these results suggest that the enrichment of acetylated histones H3 and H4 in both the hotspot and AP-1 promoter regions is associated with open chromatin and transcriptional activation of MGMT in expressing cell lines.

To determine whether the differences in the histone acetylation status were specific to *MGMT*, we examined the level of acetylation of histones H3 and H4 in *γ-actin* promoter which is constitutively expressed in all actively proliferating cells (Fig. 2). No consistent differences were observed in the abundance of acetylated histones H3 and H4 bound to the *γ-actin* promoter when comparing the MGMT-expressing and MGMT-silenced cell lines. Normal rabbit serum immunoprecipitations were done as a negative control, and although no PCR product was detected for *γ-actin*, a nonspecific product was observed in some of the *MGMT* promoter analysis samples. Nevertheless, if this signal is subtracted from the experimental samples, the interpretation of the results remains the same.

Cellular Level of Histones

Next we asked if inhibition of histone deacetylases with TSA resulted in a global increase in cellular histone acetylation. We determined the level of histone acetylation in BE and TK6 cells after treatment with TSA for 2, 4, 8, 12, 16, and 24 hours. Western blot analysis showed that in untreated TK6 cells, the level of acetylated histone H4 was very low (Fig. 3). Treatment with 330 nmol/L TSA resulted in the accumulation of AcH4 up to 4 hours and remained unchanged during the 24-hour period, only a slight increase in the levels of AcH3 was observed. For BE cells, an increase in both AcH3 and AcH4 was observed following TSA treatment (data not shown).

Up-Regulation of MGMT RNA Expression by 5-Aza-dC and TSA

To test whether demethylation of DNA could reverse MGMT silencing, 5-Aza-dC, an inhibitor of DNA methyltransferase was used as demethylating agent. Treatment of all three MGMT-silenced cell lines with 1 μmol/L 5Aza-dC alone up-regulated *MGMT* mRNA expression as determined by reverse transcription-PCR (Fig. 4). The TK6 cells seem to respond faster than the other two cell lines with a significant increase in MGMT expression measured at 2 days compared with 3 days for BE and S3 cells. To examine the potential role of histone hyperacetylation in the control of MGMT expression, the cells were also treated with the inhibitor of histone deacetylases, TSA. When cells were treated with 330 nmol/L TSA alone for 24 hours, only a slight up-regulation of MGMT was observed in all three cell lines suggesting that TSA alone cannot activate MGMT expression, and that TSA-sensitive HDAC activity is not critical for methylation mediated silencing of MGMT. However, when TSA was added to the cells for the final 24 hours of the 5-Aza-dC exposure, MGMT expression was enhanced above the level induced by 5-Aza-dC alone for all three of the MGMT silenced cell lines. Although the data for HeLa S3 do not seem statistically significant, there is clearly a trend towards TSA enhancing MGMT expression above that generated by 5-Aza-dC alone. The most significant up-regulation was observed in BE cells following 2 days of exposure to 5-Aza-dC. When TSA
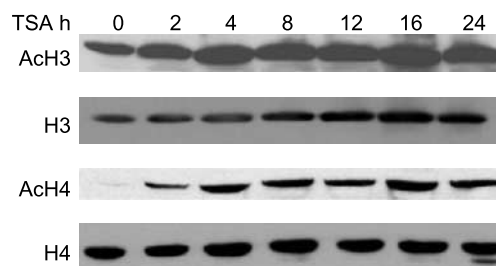

Figure 3. Time course of TSA treatment in TK6 cells. Western blot analysis of nuclear histones. *AcH3,* acetylated histone H3; *H3,* total histone H3; *AcH4,* acetylated histone H4; *H4,* total histone H4.
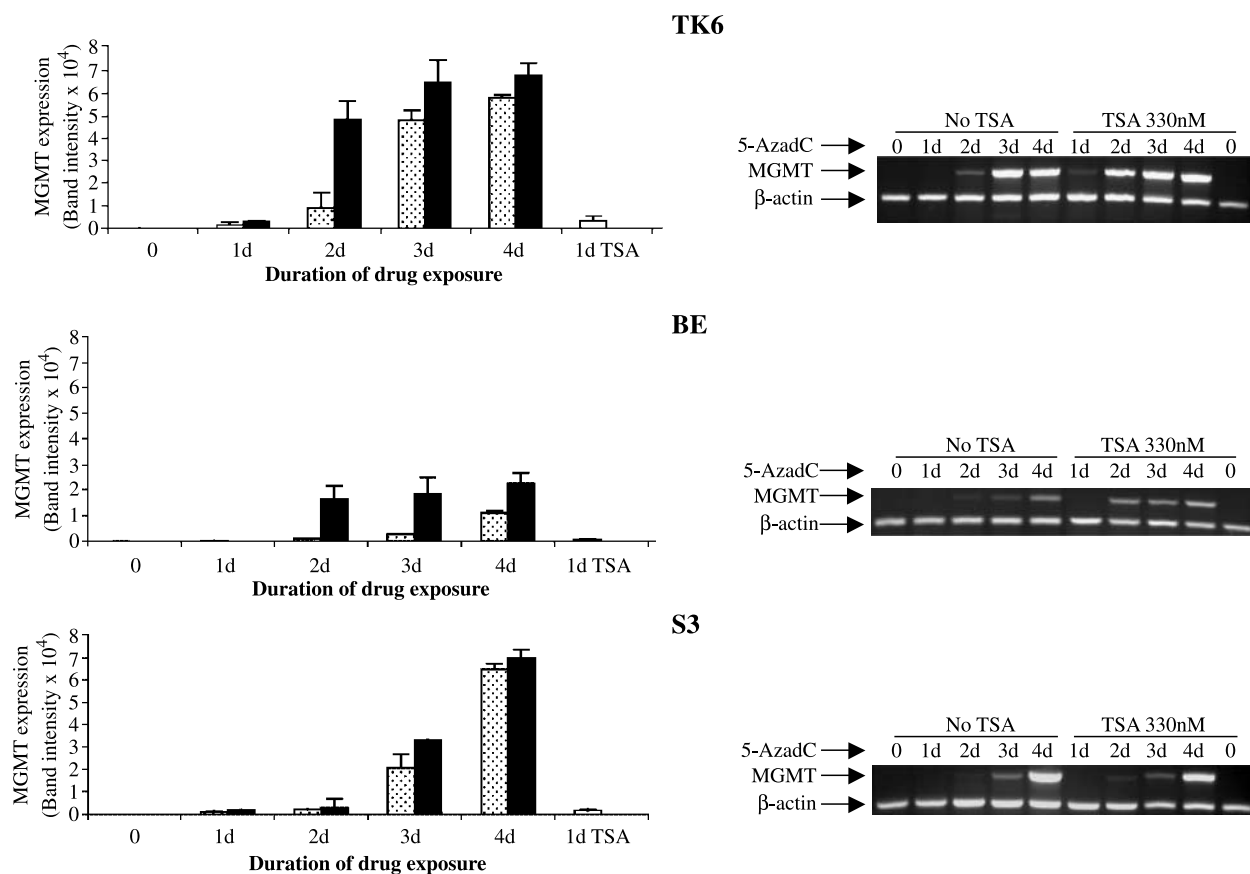
Figure 4. *MGMT* RNA expression following exposure to 5-Aza-dC and TSA in different cell lines measured by reverse transcription-PCR analysis. Data for TK6, BE, and S3 cells after treatment with either 5-Aza-dC or TSA, or with combination of these agents. Representative multiplex reverse transcription-PCR gels together with a histogram depicting the mean band intensity (normalized to the β-actin signal) from at least three independent experiments. Expression of β-actin served as an internal control. The intensity of the PCR bands was quantitated by Alphalmager 2000 v4.0.3 system. *Open columns,* TSA alone – treated samples; *shaded columns,* 5-Aza-dC – treated samples; *solid columns,* 5-Aza-dC + TSA – treated samples.

was added to BE cells for the final 24 hours of the 2-day 5-Aza-dC exposure, MGMT expression was induced 13-fold above the level induced by 5-Aza-dC or TSA alone.

These data show an apparent synergistic effect on up-regulating MGMT expression when TK6 and BE cells were first treated with a DNA methyltransferase inhibitor and followed by an HDAC inhibitor. Treatment of cells with TSA for longer than 24 hours resulted in cell death, and therefore MGMT expression could not be measured for additional time points of TSA exposure. However, as shown in Fig. 3, the effect of TSA on global histone acetylation was maximal at 4 hours showing that exposure time of >24 hours probably would not have resulted in enhanced effects on MGMT expression.

Effect of 5-Aza-dC on the Association of Acetylated Histones with the *MGMT* Promoter

We investigated the effect of 5-Aza-dC on the acetylation status of histones H3 and H4 in the *MGMT* promoter using ChIP assay in MGMT-silenced HeLa S3 cells. As shown in Fig. 5, the proportion of acetylated histones H3

and H4 bound to the hotspot region was enhanced 3.5- and 7.5-fold respectively, after treatment of cells with 5-Aza-dC for 4 days. In contrast, the association of these acetylated histones to the AP-1 region increased only 1.5-fold following 5-Aza-dC exposure.

Methyl-CpG Binding Proteins and Regulation of MGMT Expression

Methyl-CpG binding proteins are interpreters of the DNA methylation signal (20). MBD proteins, (MeCP2, MBD1, and MBD2) and Kaiso have been shown to function as transcriptional repressors through interaction with HDAC complexes (12, 17). Chromatin assembly factor 1 (CAF-1) is a multiprotein complex composed of three subunits, p150, p60, and p48. CAF-1 initiates nucleosome assembly by recruiting acetylated histones onto newly replicating DNA (21) and is one of the key players in the determination and inheritance of chromatin states (22). To assess whether MBD proteins were involved in MGMT silencing, we examined expression of different MBD proteins in the cells and their association with the *MGMT* promoter using the ChIP assay. Figure 6 depicts the total
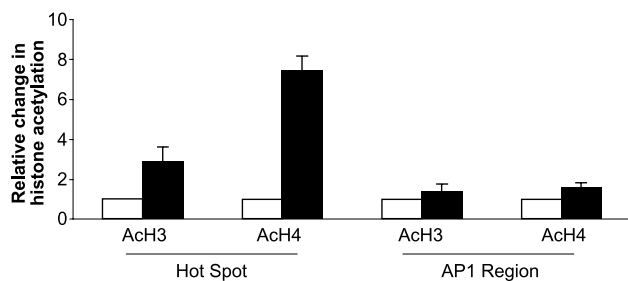
Figure 5. ChIP assay in HeLa S3 cells. Effect of 5-Aza-dC on the binding of acetylated histones to the Hot Spot and AP1 regions of the MGMT promoter. *Open columns,* untreated samples; *solid columns,* 5-Aza-dC – treated samples. *Columns,* mean of three independent experiments; *bars,* SD.

amount of MeCP2, MBD1, CAF-1 proteins in a panel of MGMT expressing and silenced cell lines determined by Western blot analysis. MBD1 was expressed in all cell lines at approximately equal levels. However, MeCP2 and CAF-1 expression was lower in HT29 and Raji cells respectively.

In vivo Binding of MBD Proteins to MGMT Promoter

The ChIP assay (Fig. 7A) showed that the relative amount of MeCP2 and MBD1 associated with the hotspot region in the *MGMT* promoter was 3.3- to 3.7-fold higher in MGMT-silenced HeLa S3 cell line than in MGMT expressing HeLa CCL2 cell line. Because MBD1 has been shown to interact with the COOH terminus of CAF-1, we also investigated the binding of the CAF-1 to the *MGMT* promoter. The abundance of CAF-1 at the hotspot region was almost 2-fold greater in S3 than in CCL2 cell lines. In the AP-1 region, the proportion of bound MeCP2 was 1.75-fold higher in S3 cells compared with the CCL2 cells, but there was no difference in the binding of MBD1 and CAF-1 between these two cell lines (Fig. 7B). These results show differences in the binding of MBD proteins to the hotspot region compared with the AP-1 region of the *MGMT* promoter and suggest that MeCP2 and MBD1 are involved in methylation mediated MGMT silencing. As a control, we evaluated MeCP2, MBD1, and CAF-1 binding to the *γ-actin* promoter, a constitutively expressed gene. As expected, we did not detect binding of any of these proteins to the *γ-actin* promoter (Fig. 7C), demonstrating the specificity of MBD protein binding to the *MGMT* promoter.

Discussion

Previous studies from our lab and others have shown that MGMT silencing strongly correlates with promoter hypermethylation. In this study, we tested the hypothesis that silencing of the *MGMT* promoter is mediated by DNA methylation and histone deacetylation. To examine whether there is any association between histone acetylation and MGMT expression, we first determined the differences between histone acetylation status in MGMT-expressing

and MGMT-silenced cell lines using the ChIP assay (Fig. 2). In three MGMT expressing cell lines, higher levels of acetylated histones H3 and H4 were associated with the *MGMT* promoter when compared with MGMT-silenced cell lines which supports the model that gene expression is associated with histone acetylation. Similar observations were reported by Bhakat and Mitra (23) who showed a reduced level of acetylated histone H4 bound to methylated promoter DNA compared with the unmethylated promoter.

Several lines of evidence suggest that histone acetylation plays a role in transcriptional regulation, probably by altering the chromatin structure. HDACs and histone acetyltransferases act together to modulate chromatin structure and transcriptional activity by changing the pattern of acetylation status of histones (24–26). Because of the known dynamic linkage between DNA methylation and histone deacetylation, we examined whether modulating methylation and histone acetylation could affect MGMT expression. Exposure of cells to the DNA methyltransferase inhibitor, 5-Aza-dC reduced DNA methylation and up-regulated *MGMT* RNA expression (Fig. 4). Although we observed that the accumulation of total cellular acetylated histones increased in cells cultured with TSA (Fig. 3), only weak expression of *MGMT* RNA was detected (Fig. 4). The activation of MGMT by 5-Aza-dC and not TSA alone supports the dominant role that methylation plays in the silencing of MGMT which is further illustrated by the observation that inhibition of methylation by 5-Aza-dC followed by inhibition of histone deacetylation by TSA, synergistically enhanced *MGMT* transcription in all three cell lines (Fig. 4). Furthermore, we observed an increase in binding of acetylated histones to both regions of the *MGMT* promoter following treatment with 5-Aza-dC (Fig. 5). Other laboratories have also reported robust re-expression of *p16 INK4a* and *estrogen receptor-α* genes by combined DNA methyltransferase and HDAC inhibition (27–29). The mechanism for this synergistic effect is thought to be due to the formation of condensed chromatin induced by extensive methylation concomitant with binding of MBD proteins and their associated corepressor complexes. Reactivation of *MGMT* gene would require at least partial demethylation to unfold the chromatin with the consequent loss of repressor complexes. Inhibition of
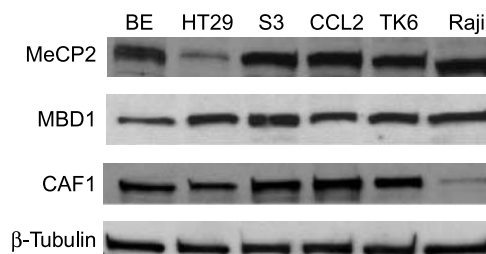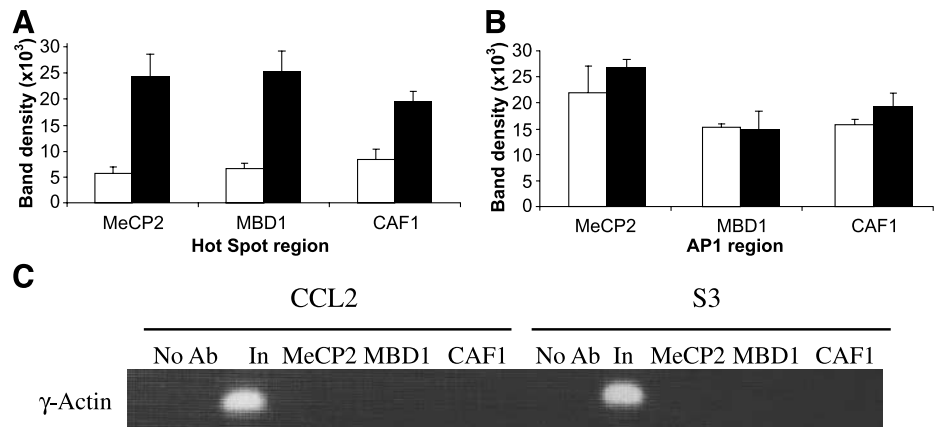

Figure 6. Western blot analysis of MBD proteins in different cell lines. β-Tubulin was used as a loading control.

Figure 7. Comparison of binding of MBD proteins to the Hot Spot #1 (**A**) and AP1 region (**B**) of the *MGMT* promoter in MGMT expressing (*CCL2, open columns*) and silenced (*S3, solid columns*) cell lines by ChIP assay using anti-MeCP2, MBD1, or CAF-1 antibodies. Amplification of *γ-actin* promoter served as a negative control (**C**). Highly expressed *γ-actin* has no MeCP2, MBD1, nor CAF-1 associated with it. *No Ab,* ChIP with no antibody; *In,* DNA isolated from the lysate before immunoprecipitation; *MeCP2,* DNA immunoprecipitated with anti-MeCP2 antibody; *MBD1,* DNA immunoprecipitated with anti-MBD1 antibody; *CAF1,* DNA immunoprecipitated with anti-CAF-1 antibody. *Columns,* mean of three independent experiments; *bars,* SD.

HDACs would then become advantageous and these events would enable the accessibility of transcription factors and RNA polymerase to the transcription start site leading to activation of *MGMT* gene.

To identify proteins responsible for interpretation of the methylation signal within the *MGMT* gene sequence, we chose to evaluate MeCP2, MBD1, and CAF-1 binding to the *MGMT* promoter. MeCP2 and MBD1 can act as methylation-dependent transcriptional repressors by binding to methylated CpGs and mediating repression through the association with HDACs (30–32). CAF-1 is involved in nucleosome assembly in chromatin and has been shown to colocalize with MBD1 to heavily methylated pericentromeric heterochromatin in mouse cells (33). In general, expression of these three proteins in cell extracts was comparable in the panel of cell lines we examined (Fig. 6). ChIP assay data showed differences in the binding of MeCP2, MBD1, and CAF-1 at the hotspot and AP-1 regions between the MGMT-expressing (CCL2) and MGMT-silenced (S3) cells (Fig. 7). In the hotspot region comprising of 41 CpGs, binding of all the three proteins was 2- to 4-fold higher in MGMT-silenced cells when compared with MGMT-expressing cells. The binding of CAF-1 to the *MGMT* promoter (Fig. 7) along with MBD1 is corroborated by the report that MBD1 partners with the COOH terminus of the p150 subunit of CAF-1 (33). However, the lack of differences in the proportion of binding of MBD1 and CAF-1 in AP-1 region may be due to the low density of CpGs. Moreover, the association of methylation status of the CpGs in the AP-1 region with MGMT silencing is unknown. Our data suggest that the hotspot region with a high density of methylated CpGs in the MGMT-silenced cells, has higher occupancy of MeCP2 and MBD1 with their associated corepressor complexes resulting in a very compact chromatin. The reason for the low but detectable levels of MeCP2 and MBD1 bound to the hotspot region in MGMT expressing cells, which lack CpG methylation is not clear. It is possible that these data reflect the presence of a subset of MGMT-silenced cells within the MGMT-expressing population.

Our results are consistent with the recent reports that MeCP2 and histone H3 methylated at K9 are bound preferentially to the *MGMT* CpG island in the MGMT-negative cell lines (34, 35). The report by Kondo et al. (35) evaluated the binding of H3 methylated or acetylated at Lys-9 to three areas of the *MGMT* promoter which also span the hotspot region. They observed that histone H3 Lys-9 methylation directly correlated and histone H3 Lys-9 acetylation inversely correlated with *MGMT* promoter methylation. Fuks et al. (36) reported that MeCP2 mediates methylation of H3K9 by recruiting histone methyltransferase *in vivo*. CAF-1 also interacts with heterochromatin proteins (HP1; ref. 37). HP1α, which normally associates with heterochromatin, recognizes and binds to H3 methylated at K9 sites (38). The heterochromatinization probably results in the exclusion of transcription factor binding to this region.

There are 6 SP1 sites within 100 bp upstream and two others further upstream of the transcription start site of *MGMT* (Fig. 1). However, the lack of MGMT expression in MGMT-silenced cells probably reflects the alteration of nucleosomal structure modulated by methylation excluding SP1 from binding to the MGMT promoter (39, 40).

In summary, methylation-mediated MGMT silencing seems to be associated with a reduction in acetylated histones H3 and H4 bound to the promoter region. MeCP2 binds to the methylated DNA and recruits HDACs and histone methyltransferases leading to changes in the pattern of histone modifications and alterations in chromatin structure. Similarly, MBD1 bound to methylated DNA associates with CAF-1, which in turn may be linked to HP1α anchored to histone H3K9me sites. Therefore, in MGMT-silenced cells, we hypothesize that a multiprotein complex containing MBD1/CAF-1/HP1α together with MeCP2, HDACs, and histone H3K9 methyltransferase forms at the hotspot region of the *MGMT* promoter resulting in a condensed, closed chromatin, and transcriptional inactivation of MGMT. This is the first report that implicates the MBD1/CAF-1/HP1 complex in methylation-mediated transcriptional silencing of *MGMT* gene.

Reactivation of MGMT can be achieved by demethylation and inhibition of histone deacetylation. Future studies using coimmunoprecipitation, ChIP assay, and RNA interference to elucidate the precise role of individual components of the MGMT promoter transcription repressive complex will be pursued.

Tumors with a hypermethylated *MGMT* promoter that do not express MGMT are more sensitive to alkylating drugs than those that express MGMT (41), and therefore *MGMT* hypermethylation could potentially be used as a clinical predictive marker for therapeutic response of tumors. However, tumors with silenced MGMT accumulate more point mutations (e.g., in K-*ras*, p53) than other tumors resulting in genomic instability (42), and colon tumors with both *hMLH1* and *MGMT* hypermethylation confer resistance to methylating agent induced cell death. Pharmacologic intervention against DNMT and HDAC to control the expression of tumor suppressor genes has been proposed as a novel anticancer therapeutic modality but a better understanding of the effects of these drugs on specific genes, and how drug resistance might be affected is warranted.

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