CpG methylation-dependent repression of the human $O^6$-methylguanine-DNA methyltransferase gene linked to chromatin structure alteration

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The mechanism of inactivation of the $O^6$-methylguanine-DNA methyltransferase (MGMT), responsible for repair of mutagenic and cytotoxic $O^6$-alkylguanine, in Mex$^{-}$ tumor cells, is not completely understood. We have examined the role of CpG methylation in the human MGMT promoter in a luciferase (luc) reporter plasmid and associated alteration in chromatin structure. Methylation of 16% CpG sequences in promoter and flanking sequences in the plasmid with $Hpa$II methylase reduced luciferase activity by 10–12-fold, while methylation of all CpG sites, including those in the luc coding sequence, as well as the promoter sequence blocked expression completely. Repression of luc expression due to partial but not complete CpG methylation could be reversed by histone deacetylase inhibitor trichostatin A (TSA). However, 5-azacytidine, which reverses CpG methylation, but not TSA, could reactivate silent MGMT gene in Mex$^{-}$ HeLa MR cells. Furthermore, chromatin immunoprecipitation (ChIP) assay showed reduced level of acetylation of H4 histone bound to the methylated promoter compared with the non-methylated promoter. These results suggest that complete repression of the MGMT gene in Mex$^{-}$ cells requires methylation of CpG sequences in both promoter and neighboring regions of the gene, resulting in inactive, condensed chromatin state of the gene.

Introduction

$O^6$-Alkylguanine, a critical mutagenic and carcinogenic lesion, is induced in DNA by alkylating carcinogens and N-chlorehetyl-N-nitrosourea-(CNU) based drugs such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (1–3). BCNU, effective in therapy of many tumors, induces the formation of $O^6$-alkylguanine in DNA and in vitro lesion, is induced in DNA by alkylating carcinogens and N-chlorehetyl-N-nitrosourea-(CNU) based drugs such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (1–3). BCNU, effective in therapy of many tumors, induces the formation of $O^6$-alkylguanine in DNA and

Abbreviations: AcH4, acetylated histone H4; ChIP, chromatin immunoprecipitation; 5-azaC, 5-azacytidine; McCP, methyl-CpG binding protein; MGMT, $O^6$-methylguanine-DNA methyltransferase; ML, MGMT-luciferase; RT–PCR, reversed transcription–polymerase chain reaction; TSA, trichostatin A.
Methylated CpG-dependent repression may occur due to alterations in the chromatin structure that are mediated by interaction with methyl-CpG-binding proteins (MeCP) (32). Two such proteins have been identified (MeCP1 and MeCP2), both of which bind specifically to methylated DNA regardless of the sequence context (33,34). It has been suggested that both MeCP2 and MeCP1 repress transcription by recruiting histone deacetylase complexes to the methylated DNA sequences, which cause compaction of the chromatin by deacetylating histones (32).

While methylation is clearly involved in setting the inactive versus active state of the MGMT gene, several studies indicated that graded methylation, both in the promoter region and the body of the gene, may regulate the level of gene expression (23,31). The present studies were designed to assess the relative contribution of methylation of the promoter and neighboring sequences and chromatin structure in MGMT gene expression, and thereby to dissect the complex relationship between the extent of methylation and chromatin structure. Many questions related to the dynamics of CpG methylation are difficult to address due to the extreme complexity of the genome structure and unusually large size of the MGMT gene (35). We have, therefore, utilized the approach of plasmid reporter expression driven by the MGMT promoter in order to test how CpG methylation density affects promoter activity via alterations in chromatin structure. Our results support the conclusion that the density of MGMT promoter methylation alone does not play a determining role in MGMT activity, but rather that methylation of both the promoter and neighboring sequences and associated alteration of chromatin structure are critical for complete silencing of the gene.

Materials and methods

Cell lines and reagents

MIA PaCa-2 (ATCC CRL-1420) cells were maintained in DMEM high glucose medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 2.5% horse serum (Life Technologies), 1 mM sodium pyruvate (Sigma Chemical Co., Saint Louis, MO) and 100 μg/ml each of penicillin and streptomycin. HeLa S3 (ATCC CCL2.2) and HeLa MR, a gift from Robert B. Varmus, were maintained in DMEM/F12 supplemented with 10% FBS, and antibiotics. All cells were grown at 37°C in 5% CO₂. Trichostatin A and 5-azaC were purchased from BIOMOL (Plymouth Meeting, PA) and Sigma, respectively.

Plasmids

The MGMT promoter-luciferase reporter constructs p-954/23ML (containing the MGMT promoter sequence from -954 to +23 bp), and p-72/23ML (containing the region from -72 to +23 bp) were described earlier (17). Partially methylated constructs were generated by ligating methylated and unmethylated portions of the plasmid (30–50 μg) using 3 U of T4 DNA ligase (USB). Appropriate DNA fragments were purified with a Prep-Gene kit (Bio-Rad, Hercules, CA) for methylation as described below. Ligation was carried out after complete methylation of fragments, and the ligated products were eluted from an agarose gel. The DNA was extracted with phenol–chloroform (1:1 v/v), and precipitated with ethanol and then dissolved in TE (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA); the DNA concentration was determined spectrophotometrically, and its quality by agarose gel electrophoresis.

In vitro DNA methylation

Plasmid DNA (15 μg) was methylated with SsoI or HpaII or HhaI methylase (14–18 U) for 6 h at 37°C according to the manufacturer’s instructions. The DNA was then extracted with phenol–chloroform and precipitated with ethanol. The completeness of DNA methylation was confirmed by digestion with the methylation-sensitive restriction endonuclease HpaII; only the plasmid completely resistant to digestion was used in the transfection experiments. Double methylation of the plasmid used sequential methylation with HpaII and HhaI methylase.

Reporter expression

MIA PaCa-2 cells were transfected using LipofectAMINE (Life Technologies) according to the manufacturer’s instructions. Twenty-four hours after the cells were seeded on 60 mm plates, MGMT promoter-reporter constructs (5 μg/dish) were used for transfection, along with 1 μg of the β-galactosidase expression plasmid, pCMV β (Clontech, Palo Alto, CA) as an internal control, so that co-expressed β-gal activity could be used to correct for variation in transfection efficiency. The cells were harvested 48 h after transfection and the luciferase activity in the cell lysate was measured in a luminometer using the luciferase assay kit (Promeza, Madison, WI). All transfections were carried out in duplicate for each experiment, and three to four independent experiments were performed to determine standard deviation. For better transfection efficiency, HeLa S3 and MIA PaCa-2 cells were transfected in a few experiments by electroporation (36).

Chromatin immunoprecipitation (ChIP) assay

Immunoprecipitation of chromatin containing nuclear and episomal DNA with anti-acetylated histone H4 (AcH4) antibody was performed as described earlier (36). PCR amplification of immunoprecipitated DNA was carried out with diluted aliquots, using the oligonucleotides 5’-GCCCTCCAGGGAAAGGTTGGTCTGCCCCT and 5’-GGCCTGGTGGGGGATGCCGCCTCAG as 5’ and 3’ primers, respectively, which encompass the 395 bp promoter region of MGMT. The PCR products were separated by agarose gel electrophoresis and their sequences confirmed directly. To ensure that the PCR was carried out in the linear range, we quantified the PCR products generated from different amounts of input DNA. PCR amplification from immunoprecipitated DNA for β-actin sequence (990 bp) was carried out using 5’-GGCACAGTGAATGCTGAAACA and 5’-AGTACTTGGCCTCAGGAGA as 5’ and 3’ primers, respectively. In some experiments, MIA PaCa-2 cells were transfected with SsoI- or HpaII-methylated or mock-methylated MGMT promoter-reporter (p-954/23ML) and the ChIP assay was performed as before. In these cases the 3’ primer for PCR (5’-GGCCTGGTGGGGGATGCCCGTCCAG) corresponded to the 3’ end of the MGMT promoter, and the 5’ primer (5’-TGATCTCATGGTACTGTAACTG) to a sequence in the pGLO2 basic vector.

5-AzaCytidine treatment

HeLa MR cells were repeatedly exposed to 5-azaC (2 μM), with a total of eight treatments over a 16 day period. The cells were seeded in 100 mm plates and incubated for 12 h before treatment with 5-azaC, and then grown for another 36 h. Afterwards, either fresh medium including 5-azaC was added or the cells were subcultured for the next treatment. After completion of treatment, one aliquot of the cells was treated with trichostatin A (TSA) (100 ng/ml) for 24 h before harvesting for analysis of MGMT RNA or protein.

RNA isolation and RT–PCR

RNA was extracted by using RNA-Zol reagent (Tel-Test, Friendswood, TX) and 1 μg RNA was reverse-transcribed with oligo (dT) primer and MuLV reverse transcriptase (Perkin Elmer, Boston, MA) according to the manufacturers’ protocol. The resulting cDNA was amplified under the following PCR conditions; 2 min 95°C for the first cycle; 95°C for 55 s, 60°C for 55 s, and 72°C for 2 min for the next 34 cycles; and a final elongation step at 72°C for 10 min. The sense 5’-ATGGCAACGATTGTGTTGAAAT and antisense 5’-GAAACCGGGATGTGGTGAAGC primers corresponded to sequences in MGMT exons 2 and 3, respectively. This inter-exonic amplification of 264 bp sequence in MGMT cDNA avoided contamination with PCR product generated from the genomic DNA. The RT–PCR products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and visualized by UV light.

Immunoblot analysis

SDS–PAGE (12.5% polyacrylamide) was carried out with 50 μg protein/lane for western analysis with polyclonal anti-MGMT antibody (17).

Southern blot analysis

Genomic DNA (15 μg), isolated from HeLa MR cells (37), was digested with 30 U HpaI or MspI at 37°C for 4–6 h, then re-digested overnight with fresh enzyme before blot analysis using 32P-labeled MGMT cDNA as the probe (37,38).

Results

Effect of in vitro CpG methylation on MGMT promoter activity

We investigated the effect of CpG methylation density on MGMT promoter activity by measuring reporter gene
expression in transiently transfected cells. MGMT promoter-driven luciferase expression was analyzed after transfecting MIA PaCa-2 cells with plasmids having methylation of 0, 16, 36 or 100% of the CpG sites in the 1 kb MGMT promoter region (Figure 1), and also methylation of remaining sequence of the plasmid, including the luciferase coding sequence. To achieve these different CpG methylation densities sequences, we methylated the p-954/+24ML MGMT promoter-reporter construct, with HpaII or HhaI plus HpaII or SssI methylase. Luciferase gene expression from a completely unmethylated plasmid was found to be 10–12-fold higher than that from a 16% methylated promoter, and was 300–400-fold higher than that from a 100% methylated promoter (Figure 2). The data indicate that repression of MGMT promoter activity was a graded function of CpG methylation density, such that even a low level of methylation inhibited gene expression significantly, whereas extensive methylation prevented expression almost completely. An SV40 promoter-dependent luciferase expression plasmid, pGL2-control, from which p-954/+24 ML plasmid was generated by substitution of the SV40 promoter with the MGMT promoter, was used as the control. As shown in Figure 2, partial methylation of the pGL2-control vector by HpaII or HhaI–HpaII methylase decreased luciferase gene expression by 2–4-fold, and complete methylation by SssI methylase decreased expression by some 20-fold. Because the pGL2-control vector and MGMT promoter-luciferase construct are identical except for the promoter region, this result indicates that a high density of CpG methylation in the MGMT promoter was responsible for the complete inhibition of luciferase expression.

**Methylation of MGMT promoter alone is not sufficient for its complete repression**

To investigate whether methylation of all CpG sites in only the MGMT promoter region can completely suppress transcription, an MGMT promoter-reporter plasmid (pGL2-/-/MGMTme) was generated by cassette methylation of only the promoter region. The MGMT promoter fragment, containing –954 to +24 bp, was methylated with SssI methylase or mock-methylated, and then ligated to an unmethylated pGL2 basic vector using the KpnI–HindIII sites. To examine the effect of methylation, unmethylated plasmid (pGL2-/-/MGMTme), and a plasmid methylated only in the promoter region (pGL2-/-/MGMTme) were used for transient transfection of MIA PaCa-2 cells, along with plasmids which were methylated at all CpG sites (pGL2me/-/MGMTme). Luciferase expression from the plasmid methylated only in the MGMT promoter was 2.5–4-fold lower than that from the unmethylated plasmid (Figure 3). However, luciferase expression from partially methylated plasmid was 90–100-fold higher than that from the fully methylated plasmid. We conclude from these results that high-density methylation in the MGMT promoter region (Figure 1) significantly inhibited transcription when the rest of the plasmid was unmethylated; however, dense methylation of the promoter region alone was necessary but not sufficient for completely inhibiting transcription. Thus, the dramatic inhibition of MGMT promoter activity observed in the completely methylated plasmid must result from methylation of plasmid sequences outside the promoter region.

**Repression of activity of methylation-free promoter by methylation of neighboring sequences**

To examine the effect of flanking sequence methylation on MGMT promoter activity, we constructed, pGL2me/-/MGMTme, in which the MGMT promoter region was unmethylated but the rest of the plasmid was methylated. A 10–15-fold lower level of luciferase expression from this plasmid relative to the fully unmethylated plasmid was observed (Figure 3). Luciferase expression from the reporter with the unmethylated MGMT promoter (pGL2me/-/MGMTme) was 20–30-fold higher than that of the fully methylated pGL2me/-/MGMTme plasmid.

**MGMT promoter activity is dictated by global methylation**

A 72 bp MGMT promoter sequence containing 12 CpG sites was shown previously to be the minimal promoter sequence required for luciferase expression (17). We methylated this minimal promoter-reporter construct (p-72/+24ML) with HpaII or SssI methylase. Luciferase expression from the unmethylated minimal promoter was 5–8-fold higher than that from the HpaII-methylated reporter, and 300-fold higher

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**Fig. 1.** Physical map of the MGMT promoter region showing the locations of CpG sequences (vertical bars), and HpaII- (square) and HhaI- (triangle) sensitive subsets in these sequences. The nucleotides are numbered 5’ (–) and 3’ (+) from the transcription start site shown by the arrow (+1).

**Fig. 2.** Inhibition of the MGMT promoter by CpG methylation. The MGMT promoter-luciferase (p-954/+24ML) or SV40-promoter luciferase plasmid was methylated for transfection of MIA PaCa-2 cells, as described in the Materials and methods. The data represent the average ± SD of three independent experiments performed in duplicate; AU, arbitrary units.
than from the SssI-methylated reporter in which all CpG sites were methylated (Figure 4).

**Association of methylated MGMT promoter with reduced acetylation of H4 histone**

Recent studies indicate that methylation of CpG represses transcription via recruitment of histone deacetylase complexes to the methylated DNA by Me-CPs (39,40). Histone deacetylation induces chromatin condensation, which in turn leads to repression. To determine if the complete silencing of fully methylated MGMT promoter-reporter, observed in transient transfection, is consistent with a model in which methylation is coupled to acetylation of histones bound to the MGMT promoter, we used the ChIP assay to immunoprecipitate chromatinized plasmid DNA from Mia PaCa-2 cells transfected with HpaII- or SssI-methylated MGMT promoter-reporter plasmid (p-954/+24ML). DNA, immunoprecipitated with anti-AcH4 antibody, was analyzed by PCR with primers specific for the MGMT promoter region. Figure 5 shows that the amount of MGMT promoter sequence was significantly decreased (10–13-fold) in the immunocomplex containing AcH4 from fully methylated MGMT promoter-reporter (lane 6) relative to that from unmethylated or partially methylated reporter (lanes 2 and 4, respectively). The data and the appropriate controls further support our conclusion of decreased association of methylated MGMT promoter with acetylated H4 histone in chromatinized plasmid. Thus, for example, little or no MGMT promoter sequence was detected by PCR assay in the absence of anti-AcH4 antibody (Figure 5, lanes, 1, 3 and 5). Furthermore, no PCR product was detected in the absence of formaldehyde cross-linking (data not shown).

**TSA increases transcription from both methylated and unmethylated MGMT promoters**

In view of reduced level of acetylation in H4 histone associated with the methylated MGMT promoter relative to the unmethylated promoter, we tested the effect of TSA, a specific inhibitor of histone deacetylase (41), which increases the level
of histone acetylation, and activates transcription and reporter expression (36). TSA has been shown to partially relieve transcriptional repression mediated by Me-CPs (39,40). We have shown previously that TSA activates the MGMT promoter via enhanced histone acetylation and chromatin modification (36). To test whether histone acetylation could overcome inhibition of the methylated MGMT promoter, MIA PaCa-2 cells were transfected with unmethylated, partially methylated or fully methylated MGMT promoter-reporter constructs by electroporation. After 24 h, the cells were treated with various amounts of TSA for another 24 h. Compared with the untreated cells, the MGMT promoter activity was increased by some 50-fold for unmethylated plasmid after TSA treatment, 150–200-fold for partially methylated MGMT plasmid and 10–20-fold for fully methylated plasmid (Figure 6). Expression of luciferase from the partially methylated MGMT promoter after TSA treatment was comparable with that observed with the unmethylated promoter reporter. The data suggest that histone hyperacetylation induced by TSA can overcome transcriptional repression caused by partial methylation of the MGMT promoter. However, TSA cannot restore full promoter activity when the expression plasmid is fully methylated.

Abundance of acetylated H4 histone bound to the endogenous MGMT promoter in MGMT-expressing and non-expressing cell lines

We used ChIP assay to investigate whether the endogenous MGMT promoters in MGMT-expressing HeLa S3 and non-expressing HeLa MR cells are associated with different amounts of acetylated H4 histone. It is evident that the MGMT promoter sequence was significantly enriched (3–4-fold) in the immunocomplex of AcH4 from HeLa S3 cells (Figure 7A, lane 2), compared with that from HeLa MR cells (Figure 7A, lane 4). As expected for the control, in the absence of anti-AcH4 antibody no MGMT promoter sequence was detected by the PCR assay (lanes 1 and 3). In another control, no significant difference was found in the abundance of AcH4 bound to the β-actin gene sequence in HeLa S3 versus HeLa MR cells (Figure 7A, lower panel, lanes 2 and 4). These results strongly suggest significant difference in the chromatin structure associated with histone acetylation of the MGMT gene in Mex+ HeLa S3 versus Mex- HeLa MR cells.

Reactivation of MGMT in HeLa MR cells following 5-azacytidine treatment

The absence of an RT–PCR product of MGMT mRNA confirmed the lack of MGMT expression HeLa MR (Mex-) cells (Figure 7B, lane 2). Because the MGMT promoter region in these cells contains a smaller amount of bound AcH4 relative to S3 cells (Figure 7A), we asked whether chromatin alteration due to histone deacetylation is associated with the Mex- phenotype of HeLa MR cells. Treatment of these cells with 100 ng/ml TSA for 24 h could not reactivate MGMT expression (Figure 7B, lane 3). We then investigated whether MGMT expression in HeLa MR could be activated by depleting the level of 5-meC in the DNA. Repeated 5-azaC treatment, as described in the Materials and methods, reactivated MGMT expression as indicated by western analysis (Figure 7C, lane 4). MGMT was undetectable in control cells as expected (lane 2). While treatment with TSA alone could not reactivate MGMT expression (lane 3), TSA post-treatment of 5-azaC-treated cells increased MGMT level by 5–6-fold (lane 5).

The CpG methylation status of the MGMT gene downstream of the promoter was examined by Southern analysis using the entire cDNA as the probe. Genomic DNA was isolated from 5-azaC-treated or parental HeLa MR cell lines, and digested with the methylation-sensitive enzyme HpaII. A control experiment was carried out using MspI, an HpaII isoschizomer that is insensitive to CpG methylation. Digestion of DNA with MspI produced three major fragments that hybridized with MGMT cDNA probe (Figure 8, lane 2). Analysis of HpaII-digested DNA revealed substantial methylation in the body of the MGMT gene in parental Mex- HeLa MR cells (lane 3), and also showed that significant demethylation occurred after...
5-azaC treatment (lane 4), with concomitant reactivation of MGMT expression. Taken together, the data indicate that demethylation of MGMT gene by 5-azaC treatment could restore MGMT expression in Mex⁻ HeLa MR cells, and that the complete lack of MGMT expression could be correlated with methylation of both the body and promoter of the gene.

Discussion

MGMT expression ranges from a high level in some tumor cells to an undetectable level in Mex⁻ tumor cells (9–11). Elucidating the molecular basis for this variation of expression is extremely important from both basic and clinical perspectives. Although several studies showed that downregulation of the human MGMT gene was linked to the presence of methylated CpGs in the promoter region (23–26), other studies indicated that regulation of MGMT expression was complex, and that over-expression of MGMT was in fact observed in some cells which contain hypermethylated CpG sequences in its gene (31). Furthermore, while the observed phenotypic reversal from Mex⁻ to Mex⁺ by 5-azaC treatment indicates involvement of CpG demethylation in this process, it is not clear which CpG sites are critical for the Mex⁻ phenotype.

We have used a reporter expression assay to re-examine the role of CpG methylation in downregulation of the MGMT promoter. Because the degree of inhibition of gene expression appears to be correlated with the density of CpG methylation (21,22), we investigated MGMT promoter activity as a function of three levels of CpG methylation density. Our initial results suggested that nearly complete suppression of the MGMT promoter occurred as a result of methylation of promoter CpG sites, which are present at a much higher density in the promoter of MGMT than of SV40 (Figure 2). However, subsequent experiments suggested that promoter methylation by itself plays only a modest role in MGMT repression (Figure 3). Although a previous study showed that in vitro methylation of a 1.2 kb MGMT promoter-reporter with HpaII or HhaI methylase suppressed MGMT promoter activity (28), the relative contribution of methylation of the promoter versus the neighboring sequences, including the luc coding region and rest of the plasmid, had not been addressed. By using a cassette methylation strategy, we examined reporter
expression as a function of selective methylation of the MGMT promoter region, or the neighboring sequence in the expression plasmid. Our data clearly indicate that a significant level of transcription occurs even when the MGMT promoter alone is highly methylated. Thus, promoter methylation by itself may not account for the MGMT-negative phenotype in Mex cells. Previous studies showing that MGMT silencing in Mex glioma cells was independent of the methylation status of the CpG island in its promoter support this conclusion (42). Furthermore, the mapping of methylated CpG sites revealed that MGMT expression was not correlated with methylation of the core MGMT promoter in variants of Mex Raji cells (43). Both Mex and Mex variants showed similar pattern of high-density methylation in the MGMT promoter indicating that MGMT could be expressed from a methylated promoter (43).

The significant inhibition of luciferase expression from the pGL2me/MGMT plasmid (which has an unmethylated MGMT promoter but methylated non-promoter region including the luc coding sequence), indicates that either: (i) methylation of the coding region can affect transcriptional elongation or (ii) the global chromatin structure induced by methylation of the region inhibits transcription initiation from a methylation-free MGMT promoter. The latter possibility is supported by an earlier observation that methylation of the 5′-MGMT CpG island, which includes binding sites for relevant transcription factors, is not required for silencing of the gene in T98G glioma cells (42). Rather, a condensed chromatin structure of the gene induced by methylation of distal sequences prevents binding of transcription factors to the promoter, and thus inactivates it (42). In comparing luciferase expression from two fully methylated MGMT promoter-reporter plasmids (Figures 2 and 4), differing in the length of the promoter, our study shows that the global methylation density, rather than promoter size, impacts the transcriptional activity.

Although the MGMT promoter was present in an episome in our studies, our results may be qualitatively relevant to regulation of the endogenous MGMT gene, including its extinction in Mex cells. In principle, it should be possible to carry out complete analysis of CpG methylation sites in the promoter and body of the MGMT gene in several Mex and Mex cells by bisulfite sequencing. While such a study will elucidate the role of methylation and histone acetylation in MGMT gene regulation, it requires prohibitive efforts for the 150 kb gene. It may also be worthwhile to analyze the changes of methylation induced chromatin structure by restriction enzyme accessibility to the MGMT promoter and body of the gene. Our results showing activation of MGMT in Mex HeLa MR cells by 5-azaC concomitant with demethylation of its coding sequences, as indicated by Southern analysis, suggests that both promoter and body of the MGMT gene is hypermethylated in Mex cells. Furthermore, the Mex to Mex conversion of a naturally occurring HeLa subline indicates that the lack of MGMT expression is not due to gross rearrangement or deletion of the gene. However, these sublines including HeLa MR or HeLa S3 are not isogenic. Cairns-Smith and Karran described a cell line that could switch between Mex and Mex phenotypes in different culture conditions without 5-azaC treatment (24). In that case, the MGMT gene in the non-expressing state was found to be hypermethylated, and underwent demethylation at CpG sequences, coincident with reappearance of the MGMT mRNA.

Although these results support our conclusion, there are additional complications that many Mex cells were found to be hypomethylated in the MGMT gene sequence, and that 5-azaC treatment could not reactivate Mex BE and IMR-90-890 lines (30,44). It is possible that demethylation occurs at different rates in different cell lines, or that demethylation of some critical CpG sequences is essential for MGMT gene activation. Moreover, direct correlation of the MGMT expression level with methylation in the body of the gene in some glioma cell lines suggests that maintenance of appropriate levels of methylation may be important for MGMT expression (42).

One mechanism by which methylation could influence MGMT expression involves binding of MeCP to CpG sites (33,34). Several recent studies have indicated that MeCPs recruit histone deacetylase complexes to methylated DNA, leading to histone deacetylation and chromatin condensation (39,40). Our observation that inhibition of histone deacetylation by TSA partially relieved transcriptional repression of the methylated reporter plasmid is consistent with this model. It is also supported by our ChIP studies showing that fully or partially methylated MGMT promoters in reporter plasmids were associated with smaller amounts of hyperAcH4 than the unmethylated promoter. That TSA treatment could overcome transcriptional repression due to partial methylation further strengthens the likelihood that histone deacetylation is involved in methylation-mediated MGMT gene suppression. We showed earlier that TSA enhances MGMT promoter activity 30–50-fold in an unmethylated reporter plasmid (36). The 150-fold enhancement of the same promoter after TSA treatment in a partially methylated plasmid could simply reflect the difference in the basal level of promoter activity in the two
cases. Partial methylation decreased the basal level expression of the reporter.

We made the intriguing observation that TSA could not reactivate the MGMT promoter in a fully methylated plasmid. One possible explanation is that an unusual chromatin structure leading to extinction of the gene is induced by extensive methylation, and may be mediated by binding to MeCPs. Reactivation of the gene would require at least partial demethylation, together with histone acetylation to unfold the chromatin structure. Activation of the silent MGMT gene by 5-azaC but not by TSA in HeLa MR cells, whose promoter region contains a low level of acetylated histone, supports this possibility. It is interesting to note that our results are similar to those which showed that the methylated and silent fragile X mental retardation gene (FMR1) could not be reactivated by TSA alone, but only after 5-azaC treatment (45).

Finally, the present study provides additional evidence for a causal relationship between methylation-dependent chromatin alteration and transcriptional regulation, and suggest how methylation, histone acetylation and chromatin structure work together in modulating MGMT expression. Elucidating the biochemical basis of how graded methylation affects chromatin remodeling and can completely repress the MGMT gene may provide important clues about regulation of other large mammalian genes.

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