Methyl-CpG Binding Domain Protein 2 Represses Transcription from Hypermethylated \( \pi \)-Class Glutathione \( S \)-Transferase Gene Promoters in Hepatocellular Carcinoma Cells*

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During the pathogenesis of human hepatocellular carcinoma (HCC), the CpG island encompassing the \( \pi \)-class glutathione \( S \)-transferase gene (GSTP1) becomes hypermethylated. Repression of transcription accompanying CpG island hypermethylation has been proposed to be mediated by methyl-CpG binding domain (MBD) proteins. We report here that inhibition of transcription from hypermethylated GSTP1 promoters in Hep3B HCC cells, which fail to express GSTP1 mRNA or GSTP1 polypeptides, appears to be mediated by MBD2. Treatment of Hep3B cells with 5-aza-deoxycytidine (5-aza-dC), a methyltransferase inhibitor, activated GSTP1 expression, whereas treatment with trichostatin A, a histone deacetylase inhibitor, had little effect. To more precisely assess the contribution of the pattern of GSTP1 CpG island methylation on GSTP1 mRNA expression, Hep3B cells were treated for 72 h with 5-aza-dC and then subjected to limiting dilution cloning. Bisulfite sequencing was used to map the methylation patterns of the GSTP1 promoter region in GSTP1-expressing and -non-expressing clones. In the clone that expressed GSTP1 mRNA determined by Northern blot analysis and quantitative reverse transcriptase (RT)-PCR, widespread demethylation of at least one GSTP1 allele was evident. Chromatin immunoprecipitation experiments revealed the presence of MBD2, but not Sp1, at the GSTP1 promoter in Hep3B cells. In contrast, Sp1 was detected at the GSTP1 promoter in a GSTP1-expressing Hep3B 5-aza-dC subclone. To test whether MBD2 might be responsible for the inhibition of GSTP1 transcription from hypermethylated GSTP1 promoters, siRNAs were used to reduce MBD2 polypeptide levels in Hep3B cells. SssI-catalyzed methylation of GSTP1 promoter sequences resulted in diminished luciferase reporter activity after transfection into Hep3B cells. However, when hypermethylated GSTP1 promoter sequences were transfected into Hep3B cells that had been treated with siRNA-targeting MBD2 mRNA, no repression of luciferase reporter expression was evident. These findings implicate MBD2 in the repression of GSTP1 expression associated with GSTP1 CpG island hypermethylation in HCC cells.

DNA methylation changes stereotypically accompany carcinogenesis. Although global DNA methylation levels decrease in cancer, CpG island sequences tend to be targets for hypermethylation (1, 2). Hypermethylation of CpG islands appears responsible for the transcriptional silencing of critical genes, including caretaker genes and tumor suppressor genes, that may be selected during the development of cancer and during cancer progression in a variety of human cancers (3). Normal CpG dinucleotide methylation patterns are thought to be established during embryonic development and maintained by DNMT1, a DNA methyltransferase targeted to DNA replication sites via interaction with PCNA‡ (4–6). In hepatocellular carcinoma (HCC), a number of genes are known to accumulate aberrant CpG island hypermethylation changes, including GSTP1, p16, and E-cadherin (7–12). The mechanism by which CpG island hypermethylation, amid global hypomethylation, appears in HCC or in other human cancers has not been established.

The mechanism by which hypermethylation at CpG islands acts to suppress the expression of genes is an area of active research. Methyl-CpG binding domain (MBD) family proteins have been identified as candidate mediators of this process. All MBD proteins contain a conserved methyl-CpG binding domain, first identified in MeCP2 (13–15). MeCP2 is capable of binding DNA containing a single \( 5\text{-mCpG} \). MeCP2 also contains a transcriptional repression domain that permits interaction with Sin3a and histone deacetylase (HDAC) to form one postulated \( 5\text{-mCpG} \)-dependent transcriptional repression complex (13, 16, 17). MDB2, which also binds DNA containing \( 5\text{-mCpG} \), has been shown to be a part of another transcriptional repression complex, containing HDACs, MBD3, and Mi-2-NuRD proteins (18). The Mi-2-NuRD complex appears capable of disrupting histone-DNA interactions to promote chromatin remodeling (19). For cancer genes inactivated by somatic CpG island hypermethylation, the role of HDACs in transcriptional silencing is unclear. For some genes, treatment with trichostatin A, an HDAC inhibitor, is sufficient to reverse the repression associated with CpG island hypermethylation, whereas for other genes, TSA treatment alone is unable to restore gene expression (17, 20, 21). Treatment with a combination of TSA and a DNMT inhibitor has been reported to trigger the reactivation of some cancer genes carrying somatic CpG island hypermethylation (20).

GSTP1, encoding the human \( \pi \)-class glutathione \( S \)-transferase, has been reported to be targeted for somatic CpG island hypermethylation changes by guest on July 18, 2018http://www.jbc.org/Downloaded from

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The abbreviations used are: PCNA, proliferating cell nuclear antigen; 5-aza-dC, 5-aza-deoxycytidine; TSA, trichostatin A; HDAC, histone deacetylase; siRNA, small interference RNA; HCC, hepatocellular carcinoma; MBD, methyl-CpG binding domain; DNMT, DNA methyltransferase 1; RT, reverse transcriptase; MES, 4-morpholineethanesulfonic acid.
hypermethylation in 85% of HCCs as well as in 30% of breast cancers and in >90% of prostate cancers (7, 22–24). Hep3B cells, a human HCC line, have been shown to contain densely hypermethylated GSTP1 CpG island sequences and to be devoid of GSTP1 mRNA (7). We report here that in Hep3B HCC cells, repression of GSTP1 associated with CpG island hypermethylation was reversed by treatment with 5-aza-deoxycytidine (5-aza-dC) but was not unaffected by treatment with TSA. Furthermore, when Hep3B cells were treated with 5-aza-dC for 72 h, subjected to limited dilution cloning, and then assessed by quantitative RT-PCR for GSTP1 mRNA and by bisulfite genomic sequencing for GSTP1 CpG island methylation, Hep3B-5-aza-dC clones that express genomic sequencing for cells, repression of GSTP1 was monitored by Northern blot analysis using GSTP1 cDNA as a probe (upper panel). Ethidium bromide staining of rRNA was used as a loading control (bottom panel).

**FIG. 1. Activation of GSTP1 expression in Hep3B cells by treatment with 5-aza-dC.** Hep3B cells were treated with 5-aza-dC (1 μM) or TSA (100 nM) for 24, 48, and 72 h. Expression of GSTP1 mRNA was monitored by Northern blot analysis using GSTP1 cDNA as a probe (upper panel). Ethidium bromide staining of rRNA was used as a loading control (bottom panel).

**EXPERIMENTAL PROCEDURES**

**Culture of Hep3B Cells and Treatment with 5-aza-dC and TSA—** Human Hep3B cells were propagated in vitro in minimal Eagle’s medium (Mediatech) supplemented with 1.0 mM sodium pyruvate and 10% fetal bovine serum (Invitrogen) (25). Treatment of Hep3B cells with 5-aza-dC (Sigma) and with TSA (Sigma) was accomplished by adding the drugs to complete growth medium at a concentration of 1 μM for 5-aza-dC and 100 ng/ml for TSA. Stock solutions of 5-aza-dC, 1 mM in MeSO, and TSA, 100 mg/ml in ethanol, were stored at −20 °C. To isolate individual Hep3B-5-aza-dC clones with reduced MeCP2 levels, cells with reduced MeCP2 levels, were incapable of repressing transcription from SeesI-methylated GSTP1 promoters. All of the data collected suggest that MD2, perhaps via an HDAC-independent pathway, acts to repress transcription from hypermethylated GSTP1 promoters in HCC cells.

**Detection of GSTP1 mRNA by Northern Blot Analysis and GSTP1 Polypeptides by Immunoblot—** Total RNA was isolated from Hep3B cells and Hep3B-5-aza-dC clones using an RNeasy® RNA isolation kit (Qiagen) and quantified using an orcinol assay (26). Purified RNAs (20 μg) were electrophoresed on 1.5% agarose gels in the presence of 2.2 M formaldehyde, transferred to Zeta-Probe® GT (Bio-Rad) filters, and hybridized with 0.1 ng of cDNA as a probe.

**Detection of GSTP1 mRNA Using Quantitative RT-PCR—** Total RNA from each of the Hep3B-5-aza-dC clones was subjected to quantitative RT-PCR for GSTP1 mRNA using a iCycler IQ™ multi-color real time PCR system (Bio-Rad). Before PCR, cDNA was synthesized from 1 μg of RNA using and Omniscript® RT kit (Qiagen). PCR reactions included cDNA from 125 ng of RNA, sense (5′-GGCCAGGCTGGCTTACAGATG-3′) and antisense (5′-gagactacacgtgac-3′) primers, and the Master Mix from a Quantitect™ SYBR® Green® PCR kit (Qiagen). PCR cycles were 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Cloned GSTP1 cDNA was used as a standard for quantification. As an internal control, TBP mRNA, encoding the TATA-binding protein, was also detected by quantitative RT-PCR using specific sense (5′-caagacaacctgcaagcat-3′) and antisense (5′-tcttctgcagccattgcac-3′) primers. PCR cycles for TBP cDNA detection were 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Each of the PCR assays was run in triplicate, and the GSTP1 and TBP copy numbers were estimated from the threshold amplification cycle numbers using software supplied with the iCycler IQ™ Thermal Cycler.

**Bisulfite Genomic Sequencing for Mapping GSTP1 CpG Island DNA Methylation Patterns in Genomic DNA—** Genomic DNA was isolated from Hep3B cells using the DNAeasy® kit (Qiagen). To map CpG dinucleotides in the GSTP1 CpG island region, a bisulfite genomic sequencing approach was undertaken (24, 29). Purified DNAs (500 μg) were treated with EcoRI, admixed with salmon sperm DNA (2.5 μg), and then treated with sodium bisulfite as previously described (27). The bisulfite-treated DNA was then subjected to two rounds of PCR to amplify GSTP1 CpG island alleles using primers that recognize the antisense strand of GSTP1 after bisulfite conversion. First-round PCR primers were 5′-AAGCAACAATTCTAAACCTACTA-3′ and 5′-GTTCTCGAGATCCGATTTTGATGTTG-3′; second-round PCR primers were 5′-AACCTTAAAACACAAGTAAACACAT-3′ and 5′-TGGGTATTGTGGAGAATTTT-3′. PCR reaction conditions have been described previously. To permit DNA sequencing of individual GSTP1 CpG island alleles, PCR products were purified by electrophoresis on 6% agarose gels using the Qiagen® gel extraction kit (Qiagen), ligated into pCR1.1TOP™ cloning vectors (Invitrogen), and then introduced into TOP 10® One Shot competent bacteria (Invitrogen). Plasmid DNA, isolated using Qiaprep Spin Miniprep kit, was subjected to DNA sequence analysis using M13 sequencing primers.

**Chromatin Immunoprecipitation—** 8–10 × 10^6 growing Hep3B cells or Hep3B-5-aza-dC clone 5 cells were fixed with 1% formaldehyde for 10 min at 37 °C, washed twice in ice-cold PBS containing protease inhibitor III (Calbiochem), and then recovered by scraping and centrifugation at 325 × g for 5 min (30). Cell pellets were resuspended in 200 μl of chromatin lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), incubated for 10 min at 4 °C, and then sonicated for 40 s using a Vers sonic micropipette sonicator to reduce DNA fragment size to 400–600 bp. The sonicated chromatin lysates were clarified by centrifugation at 14,000 × g for 10 min at 4 °C, and the supernatants were added to 10 ml of precipitation buffer (0.01% SDS, 1.1% Triton X-100, 167 mM NaCl), and 1.2 mM EDTA in 16.7 mM Tris-HCl, pH 8.1). After preclearing with 400 μl of salmon sperm DNA/protein A-agarose (Upstate Biotech) by incubation at 4 °C for 30 min with gentle agitation and then centrifugation at 325 × g for 1 min, nucleoprotein complexes were separated into 1 ml aliquots for immunoprecipitation using specific antibodies to MBD2, MeCP2, Sp1, and acetylated histone H4 (all from Upstate Biotechnology). 5–10 μg of antibody solution was added to 1 ml of nucleoprotein complexes. Antibody-nucleoprotein complex mixtures were incubated at 4 °C overnight with gentle agitation. Immunocomplexes were collected by the addition of 60 μl of salmon sperm DNA/protein A-agarose (Upstate Biotech), incubated for 1 h at 4 °C with rotation, and then centrifugation at 325 × g for 1 min. Pelleted immunocomplexes were washed in TE buffer (10 mM Tris, 1 mM EDTA) and then recovered by scraping and centrifugation at 325 × g for 1 min. The immunoprecipitated DNA was treated with 1 ml of proteinase K solution, digested with 10 μg/ml of DNase I, and then recovered by ethanol precipitation. DNA aliquots were then resuspended in 20 μl of TE buffer (10 mM Tris, 1 mM EDTA) and then analyzed by Southern blot hybridization using the GSTP1 probe.
eluted from the final washed immunoprecipitates in 250 μl of 1% SDS and 0.1 M NaHCO₃ by incubation at room temperature for 15 min. To reverse the cross-linking of protein to DNA, 20 μl of 5 N HCl was added to the eluted immunoprecipitates and incubated at 65 °C overnight. Proteins were digested by adding 2 μl of proteinase K (10 mg/ml), 10 μl of 0.5% EDTA, and 20 μl of Tris-HCl, pH 6.5, and incubating the mixture for 1 h at 45 °C. DNA was recovered by phenol/chloroform extraction and EtOH precipitation. To detect GSTP1 expression in Hep3B cells, the drug-treated cells were subjected to limiting dilution cloning, and eight individual clones were isolated. GSTP1 expression was monitored by Northern blot analysis as described for Fig. 1.

FIG. 3. GSTP1 expression by Hep3B clones isolated after 72 h of 5-aza-dC exposure. Hep3B cells were treated with 5-aza-dC (1 μM) for 72 h, then maintained in complete growth medium without drugs thereafter. The drug-treated cells were subjected to limiting dilution cloning, and eight individual clones were isolated. GSTP1 expression was monitored by Northern blot analysis as described for Fig. 1.

CUGAGGGUGCCUGUGCUG-3', siRNAs were transfected into Hep3B cells using Oligofectamine™ (Invitrogen). An additional siRNA transfection was undertaken 48 h later to increase the efficiency of target protein knock-down. The effectiveness of the target protein reduction was monitored by immunoblot analysis. Total protein extracts, prepared by lysing cells in 2% SDS, were electrophoresed on 10% polyacrylamide gels (Novex) in MES running buffer (Novex), transferred to nitrocellulose membranes (Invitrogen), and then probed with antibodies to MBD, MeCP2, acetylated histone H4, and lamin A/C (Upstate Biotechnology, Calbiochem) using horseradish peroxidase-conjugated anti-IgG (Amersham Biosciences) as previously described.

Transient Transfection Analysis of the Effects of Cpg Island Hypermethylation on GSTP1 Promoter Activity—GSTP1 promoter-luciferase reporter constructs (pGL3 vector, Promega) containing sequences from −408 to 5' of the GSTP1 transcription start site to +36 were treated with Sss1, a Cpg methylase, or left untreated and then transfected into Hep3B cells using LipofectAMINE™ (Invitrogen) (24). After 48 h, the transfected cells were lysed using passive lysis buffer (Promega). Luciferase reporter activity was assayed using a Dual-Luciferase® reporter assay system (Promega) and a 1450 MicroBeta® JET luminometer (Wallac). A cytomegalovirus promoter-β-galactosidase reporter construct was used to monitor transfection efficiency.

RESULTS

Reactivation GSTP1 Expression from Hep3B Cells Containing Hypermethylated GSTP1 Cpg Islands Using a DNA Meth-
Although \( \pi \)-class glutathione \( S \)-transferases appear to be up-regulated in rat models of HCC, the human \( \pi \)-class glutathione \( S \)-transferase is not expressed in human HCCs or by the human HCC cell line, Hep3B (5). In normal human liver tissue, the \( GSTP1 \) CpG island is unmethylated, even though \( GSTP1 \) is usually not expressed (7, 32). However, in Hep3B HCC cells, the \( GSTP1 \) promoter has been previously shown to be heavily methylated (7). When we subjected Hep3B cells to treatment with 5-aza-dC, a DNMT inhibitor, or with TSA, an HDAC inhibitor, \( GSTP1 \) expression was evident only in cells treated with 5-aza-dC within 72 h (Fig. 1). To ascertain whether combinations of 5-aza-dC and TSA might be more effective at restoring \( GSTP1 \) expression than 5-aza-dC alone, Hep3B cells were treated sequentially for 48 h with 5-aza-dC and/or TSA to a total 96 h of drug treatment (Fig. 2). Prior exposure of Hep3B cells to TSA did not potentiate the effect of 5-aza-dC on increasing \( GSTP1 \) mRNA levels, nor did exposure to TSA after 5-aza-dC treatment have any synergistic effect on restoring \( GSTP1 \) expression. These findings are consistent with a role for \( GSTP1 \) CpG island hypermethylation in the silencing of \( GSTP1 \) transcriptional in Hep3B cells and further suggest that the mechanism of methylation-associated inhibition of \( GSTP1 \) transcription may not require HDACs.

**Bisulfite Genomic Sequencing Analysis of Individual Hep3B Clones Isolated after 5-aza-dC Treatment**—To better characterize the effect of CpG island hypermethylation on \( GSTP1 \) expression in Hep3B cells, we treated the cells for 72 h with 5-aza-dC and then isolated individual Hep3B-5-aza-dC subclones by limiting dilution cloning. Eight Hep3B-5-aza-dC clones were recovered, and three of the clones expressed significant levels of \( GSTP1 \) mRNA by Northern blot (Fig. 3) and quantitative RT-PCR analyses (Fig. 4). DNMT inhibitors have been reported to restore the expression of many genes repressed by CpG island hypermethylation in cancer cells; however, a reduction in gene expression and a remethylation of CpG island sequences after prolonged passage in cell culture have been described for some such genes (33). In T24 bladder cancer cells, restoration of \( p16 \) mRNA expression by treatment...
with 5-aza-dC was completely reversed after 21 population doublings in the absence of the inhibitor (33). Remarkably, in Hep3B-5-aza-dC clones 2, 5, and 7, \textit{GSTP1} mRNA expression remained stable for at least 8 months during continuous cell culture in the absence of 5-aza-dC (not shown). Whether the apparent differences in propensity for CpG island remethylation between \textit{p16} in T24 cells and \textit{GSTP1} in Hep3B cells can be attributed to differences in selection for loss of \textit{p16} versus \textit{GSTP1} expression or to some mechanism has not been established (24). When genomic DNA from each of the clones was subjected to bisulfite genomic sequencing, capable of mapping 5-mCpG dinucleotides at the \textit{GSTP1} transcriptional regulatory region, a reduction in \textit{GSTP1} CpG island hypermethylation was evident only in Hep3B-5-aza-dC clones that expressed \textit{GSTP1} mRNA (Fig. 5). Of interest, the PCR primers for bisulfite genomic sequence analysis flanked a polymorphic (ATA-AAA) repeat located 506 bp 5’ of the \textit{GSTP1} transcription start site, permitting discrimination of CpG dinucleotide methylation patterns on individual \textit{GSTP1} alleles. Hep3B cells and four of the Hep3B-5-aza-dC clones were found to contain three different (ATAAAA), repeat lengths, consistent with an instability of this polymorphic repeat at some point during the development, isolation, and propagation of Hep3B HCC cell line. Four of the Hep3B-5-aza-dC clones appeared to have lost \textit{GSTP1} allele 3 after 5-aza-dC treatment and limiting dilution cloning. For the three Hep3B-5-aza-dC clones with a reduction in \textit{GSTP1} CpG island methylation, the reduction was restricted to one \textit{GSTP1} allele. Furthermore, each of the three Hep3B-5-aza-dC subclones displayed reversal of \textit{GSTP1} CpG island hypermethylation at different \textit{GSTP1} alleles, suggesting no bias of 5-aza-dC action toward any specific \textit{GSTP1} allele. Hep3B-5-aza-dC clones 2 and 5 appeared to have completely reversed \textit{GSTP1} hypermethylation at a \textit{GSTP1} allele; Hep3B-5-aza-dC clone 7 only partially reversed the \textit{GSTP1} CpG island hypermethylation. These experiments further support a direct correlation between \textit{GSTP1} CpG island hypermethylation and \textit{GSTP1} repression.

Chromatin Immunoprecipitation Analyses of Active and Inactive \textit{GSTP1} Promoters—To ascertain whether MBD family proteins formed transcriptional repression complexes at hypermethylated \textit{GSTP1} CpG islands, we performed chromatin immunoprecipitation analyses of Hep3B cells, which contain only hypermethylated \textit{GSTP1} CpG islands and fail to express \textit{GSTP1} mRNA, and of Hep3B-5-aza-dC clone 5 cells, which contain one unmethylated \textit{GSTP1} CpG island allele and express high levels of \textit{GSTP1} mRNA (Fig. 6). Antibodies to Sp1 and acetylated histone H4 were used to detect active transcription complexes, whereas antibodies to MBD2 and MeCP2 were
used to detect repressive transcription complexes. For Hep3B cells, MBD2 and perhaps a small amount of MeCP2, but not Sp1 nor acetylated H4, were detected at the GSTP1 promoter. In contrast, for Hep3B-5-aza-dC clone 5 cells, Sp1 and a small amount of acetylated histone H4 were detected at the GSTP1 promoter on at least some GSTP1 alleles, whereas reduced levels of MBD2 and MeCP2 were present. Differences in levels of GSTP1-MBD2 and GSTP1-MeCP2 nucleoprotein complexes in Hep3B cells versus Hep3B-5-aza-dC clone 5 cells were not attributable to differences in MBD2 or MeCP2 polypeptide levels, because both proteins were readily detected in protein extracts from both cell lines. Thus, the presence of at least one unmethylated GSTP1 promoter allele permitted the assembly of GSTP1-protein complexes containing the transcriptional trans-activator Sp1 and histone H4, whereas the exclusive presence of hypermethylated GSTP1 promoter alleles only allowed the assembly of GSTP1-protein complexes containing MBD family proteins.

SssI-catalyzed CpG Methylation of GSTP1 Promoter Sequences Reduces GSTP1 Promoter Activity in Both Hep3B Cells and Hep3B-5-aza-dC Clone 5 Cells—Although the stable high level GSTP1 expression induced by brief treatment of Hep3B-5-aza-dC clone 5 cells with 5-aza-dC was correlated with reversal of GSTP1 CpG island hypermethylation and with the assembly of an Sp1-containing complex at the GSTP1 promoter, in principle, DNA methylation-independent increases in trans-activation activity might still contribute to the high level of GSTP1 expression in the Hep3B-5-aza-dC clone 5 cells. Also, nucleoside DNMT inhibitors have been reported to increase the expression of some genes in the absence of alterations in DNA methylation (34–36). Nonetheless, when unmethylated GSTP1 promoter sequences were transfected into Hep3B and Hep3B-5-aza-dC clone 5 cells, similar luciferase reporter expression levels, normalized to cytomegalovirus promoter-driven β-galactosidase reporter expression levels, were seen (Fig. 4). Furthermore, 5-aza-dC treatment did not appear to increase the activity of unmethylated GSTP1 promoters (Fig. 7). However, when GSTP1 promoter sequences were treated with the CpG methylase SssI before transfection, a marked reduction in luciferase reporter expression in both Hep3B and Hep3B-5-aza-dC clone 5 cells was observed (Fig. 4).

siRNA Knock-down of MBD2 and MeCP2 in Hep3B Cells Implicates MBD2 in Hypermethylation-dependent GSTP1 Repression—To test whether MBD2, MeCP2, or both MBD2 and MeCP2 were responsible for repression of transcription from hypermethylated GSTP1 promoter alleles, the levels of the MBD family proteins were reduced in Hep3B cells by treatment with specific siRNAs capable of degrading mRNA transcripts in a target specific manner (Fig. 8). The effectiveness of siRNA knock-down of MBD family proteins was monitored by immunoblot analysis. Remarkably, when an SssI-methylated GSTP1 promoter was transfected into Hep3B cells treated with siRNA-targeting MBD2 mRNA, the reduction in MBD2 protein levels appeared to render the Hep3B cells incapable of repressing GSTP1 transcription. Finally, a combined knock-down of MBD2 levels and MeCP2 levels in Hep3B cells was no better at reversing alleviating repression from hypermethylated GSTP1 promoters as a knock-down of MBD2 alone. Considered along with the finding that MBD2 is located at hypermethylated GSTP1 promoters in Hep3B cells, the lack of repression activity for hypermethylated GSTP1 promoters in Hep3B cells with reduced MBD2 levels strongly suggest that MBD2 likely medi
MBD2 and DNA Methylation-associated GSTP1 Repression

Fig. 8. Alleviation of repression from hypermethylated GSTP1 promoters after targeted reduction of MBD2 using siRNA. Hep3B cells were repeatedly transfected with siRNA-targeting mRNA encoding lamin A, MBD2, and MeCP2. Reductions in the levels of targeted proteins were monitored by immunoblot analysis. After two siRNA treatments, 5-aza-dC methylated (meth) GSTP-P1 promoter activity was assessed via transient transfection as described for Fig. 7.

MeCP2

MD2

MBD2

H4

MeCP2

DISCUSSION

All of the data collected suggest that CpG island hypermethylation is responsible for transcriptional silencing of GSTP1 in Hep3B cells. GSTP1 repression was reversed by treatment with 5-aza-dC, a DNMT inhibitor, but not with TSA, an HDAC inhibitor. For certain genes silenced by CpG island hypermethylation, treatment with TSA can activate gene expression, indicating the participation of HDACs in transcriptional repression, whereas for other genes, TSA alone is incapable of restoring gene function (17, 20, 21). In our chromatin immunoprecipitation experiments, we detected more acetylated histones in association with active GSTP1 promoters (in Hep3B-5-aza-dC clone 5 cells) than in association with inactive GSTP1 promoters (in parent Hep3B cells), suggesting that histone acetylation likely accompanies GSTP1 transcription. However, the absence of TSA stimulation of GSTP1 expression from hypermethylated GSTP1 promoters in Hep3B cells suggests that HDACs do not play a critical role in CpG island hypermethylation-associated GSTP1 repression.

The mechanism by which aberrant methylation patterns develop in cancer cells has not been determined. Several cytosine methyltransferase genes have been identified and characterized. Dnmt1, Dnmt3a, and Dnmt3b are each essential for mouse development (37). DNMT1, thought to function as a maintenance methyltransferase in normal cells, is present at replication foci during the S phase of the cell cycle (6). Under certain circumstances, DNMT1 may also promote de novo CpG dinucleotide methylation (38, 39). In cancer cells, DNMT3a and DNMT3b may contribute to both de novo and to maintenance DNA methylation in some way. HCT116 colorectal carcinoma cells carrying disrupted DNMT1 alleles display only a ~20% reduction in 5-mCpG (40). Furthermore, although DNMT3a and DNMT3b seem to be expressed at high levels during embryonic development and at low levels in normal adult tissues, increased expression of DNMT3a and DNMT3b mRNA has been reported in human cancers (41–43). Nonetheless, DNMT1 has been more prominently implicated in the earliest stages of cancer development than other DNMTs. Apcmin/+ mice develop fewer intestinal polyps when crossed to a Dnmt1– background (44). Dnmt1 also appears essential for fos transformation of rat fibroblasts in vitro, as forced Dnmt1 overexpression recapitulates the fos-transformed phenotype, and antisense Dnmt1 cDNA inhibits transformation by fos (38). Despite these observations, whether DNMT1 acts to facilitate cancer development through catalyzing de novo CpG island methylation has not been irrefutably established. DNMT1 has been reported to act as a transcriptional repressor, independent of DNA methyltransferase activity, by forming complexes with HDAC2 and DMAP1 (45).

The MBD proteins all contain sequences similar to a 60–80-amino acid motif shown in MeCP2 to be responsible for 5-mCpG binding. MeCP2, the first of these proteins to be identified, acts as a transcriptional repressor via interaction with Sin3A and HDACs. MECP2 mutations are responsible for Rett’s syndrome, a neurodegenerative disorder in females, and for severe mental retardation and death in males. Targeted disruption of Mecp2 leads to a similar phenotype in mice (46). In our studies, although we found a small amount of MeCP2 in association with hypermethylated GSTP1 promoters in Hep3B cells by chromatin immunoprecipitation, we were unable to increase GSTP1 promoter activity by treatment with siRNA targeting MECP2 mRNA in the setting of GSTP1 promoter hypermethylation. These data suggest that MeCP2 is not required for transcriptional repression from hypermethylated GSTP1 promoters in Hep3B cells. MeCP1, a multi-component transcriptional repression complex, contains MBD2, MBD3, and Mi-2/NuRD proteins (18). MBD2 most likely serves to recruit MeCP1 complex proteins to hypermethylated transcriptional promoters, because MBD3 does not bind 5-mCpG (15, 47). Perhaps for this reason, cells from Mbd2−/− mice are unable to prevent transcription from exogenous hypermethylated SV40 promoters, whereas Mbd3−/− cells remain capable of promoter hypermethylation-associated repression (47). We detected MBD2 bound to hypermethylated GSTP1 promoters in Hep3B cells by chromatin immunoprecipitation, and we showed that a reduction in MBD2 levels prevented repression of GSTP1 associated with hypermethylation. Confirming these findings, preliminary data collected using MCF-7 breast cancer cells suggests that siRNA knock-down of MBD2 triggers induction of GSTP1 mRNA expression despite the presence of hypermethylated GSTP1 promoters. The participation of MBD2 in the silencing of hypermethylated GSTP1 promoters in Hep3B cells may provide a partial explanation for the failure of TSA to reactivate GSTP1 expression. MeCP1 contains the SWI/SNF helicase Mi-2 as well as HDACs (18). Co-transfection of cDNA encoding a dominant-negative Mi-2 has been reported to alle-

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viate repression from a model hypermethylated transcriptional promoter (18). For \( \text{GSTP1} \) in Hep3B cells, CpG island hypermethylation appears to cause transcriptional silencing by an MBD2-dependent but HDAC-independent mechanism. Perhaps Mi-2 or some other MBD2-associated protein may help mediate repression from hypermethylated promoters. In all, our findings support a critical role for MBD2 in the silencing of genes targeted for somatic CpG island hypermethylation during cancer development.

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