Journal of Cellular Biochemistry 97:1300–1316 (2006)

Epigenetic Regulation of Metallothionein-I Gene Expression: Differential Regulation of Methylated and Unmethylated Promoters by DNA Methyltransferases and Methyl CpG Binding Proteins

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Abstract Metallothioneins (MTs) are a group of cysteine-rich stress response proteins that scavenge reactive oxygen species and heavy metals. Recently, we have shown that MT-I promoter is methylated and suppressed in some solid and liquid tumors and can be robustly activated following treatment with inhibitors of DNA methyltransferase (DNMT) and histone deacetylase (HDAC). Here, we have analyzed MT-I chromatin structure in active, unmethylated (Hepa cells) and in repressed, methylated state (lymphosarcoma cells). Restriction enzyme accessibility assay showed that the MT-I promoter has an open conformation in unmethylated state as opposed to refractory chromatin structure in methylated state. Positioning of nucleosomal arrays on the methylated promoter further confirmed the closed chromatin structure of the methylated promoter. Chromatin immunoprecipitation (ChIP) assay demonstrated that the unmethylated promoter is associated with K9-acetyl, K4-methyl, and S10-phospho histone H3 whereas the methylated promoter is predominantly associated with K9-methyl H3. HP1α that recognizes K9-methyl H3 inhibited methylated MT-1 promoter activity whereas closely related HP1γ repressed the promoter irrespective of its methylation status. Ubiquitously expressed DNA methyltransferase 1 (DNMT1) suppressed MT-I promoter activity irrespective of its methylation status that does not require its catalytic activity. The DNMT1-mediated repression of MT-I promoter was relieved by trichostatin A, an HDAC inhibitor. Among the methyl CpG binding proteins, MBD2 and MBD4 specifically associated with the methylated promoter and inhibited its activity. In contrast, MBD1 and MeCP2 interacted with both promoters and suppressed the promoter activity irrespective of its methylation status. These results demonstrate that the methylated and unmethylated MT-I promoter are differentially regulated by DNA methyltransferase and methyl-CpG binding proteins, and DNMT1 could suppress MT promoter by a transcriptional mechanism independent of its enzymatic function. These studies suggest that the components of epigenetic machinery differentially regulate methylated and unmethylated MT-I gene expression. J. Cell. Biochem. 97: 1300–1316, 2006.

Key words: metallothionein; DNA methylation; DNA methyltransferase 1; methyl CpG binding proteins; heterochromatin protein-1

Methylation of C-5 of CpG base pairs is the predominant epigenetic modification in the regulation of gene expression. DNA methylation is essential for mammalian development [Reik et al., 2001; Li, 2002; Jaenisch and Bird, 2003; Jacob and Motiwala, 2005]. Recent studies have shown that genome wide DNA methylation profile in cancer cells is profoundly altered, resulting in silencing of tumor suppressor genes and activation of oncogenes (for review, see Baylin and Bestor, 2002; Herman and Baylin, 2003; Egger et al., 2004; Szyf, 2005).

Sarmila Majumder and Kalpana Ghoshal contributed equally to this work.

Grant sponsor: National Institute of Health; Grant numbers: ES10874, CA81024.

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Received 31 October 2005; Accepted 1 November 2005
DOI 10.1002/jcb.20738

Methylation is initiated by de novo methyltransferases, DNMT3A and DNMT3B that transfer methyl group from S-adenosylmethionine (Ado-Met) to C-5 of CpG base pairs. The maintenance methyltransferase DNMT1 then methylates the newly synthesized strand complimentary to the hemimethylated DNA that occurs concurrently with DNA replication. Dnmt1 and Dnmt3b are essential for development in mice whereas Dnmt3a null mice die immediately after birth (for review, see Chen and Li, 2004; Goll and Bestor, 2005). Mutations in DNMT3B in humans cause ICF (Immunodeficiency, Centromeric instability, and Facial anomaly) syndrome [Hansen et al., 1999; Xu et al., 1999].

The major biological role of methylation is to silence genes such as proviral or tissue-specific genes permanently. Recent study demonstrated that methylation can also regulate inducible expression of a specific gene BDNF whose expression is dramatically induced after membrane depolarization in neuronal cells [Chen et al., 2003; Martinowich et al., 2003]. The epigenetic silencing of genes is mediated through recruitment of a group of proteins, called methyl CpG binding proteins (MBDs) that act as docking sites for co-repressor proteins such as Sin3a, histone deacetylases, histone methyltransferases, and heterochromatin protein 1α (HP1α) [Wade, 2001; Fuks et al., 2003; Geiman et al., 2004]. Mammalian cells contain five MBDs with highly homologous methyl CpG-binding domain. Kaiso, a β-catenin-interacting protein, with no signature MBD domain also binds to methylated DNA [Ng and Bird, 1999; Jorgensen and Bird, 2002]. The mechanism of targeting of MBDs to different regions of the genome is not known.

The regulation of gene expression in the chromatin context involves dynamic changes in post-translational modifications of nucleosomal histones (for review, see Jenuwein and Allis, 2001; Fischle et al., 2003; Margueron et al., 2005). Histones are the targets of numerous signal transduction pathways resulting in a variety of post-translational modifications of these proteins. Among these modifications, the role of acetylation, phosphorylation, and methylation in gene expression are extensively explored [Berger, 2002; Hake et al., 2004; Mahadevan et al., 2004; Margueron et al., 2005]. These modifications act on the same or different histones in synergistic or antagonistic manner to regulate gene expression [Jenuwein and Allis, 2001; Fischle et al., 2003; Wang et al., 2004a]. Recent studies from lower eukaryotes (Arabidopsis) and mammals have shown that DNA methylation and histone modifications, specifically hypoacetylation and K9 methylation, are interrelated [Freitag and Selker, 2005; Stancheva, 2005].

The present study was undertaken to explore the molecular mechanisms for the epigenetic regulation of metallothionein (MT) gene expression. MTs are a group of highly conserved heavy metal-binding, stress-inducible proteins that maintain metal homeostasis and scavenge free radicals [Ghoshal and Jacob, 2001; Sato and Kondoh, 2002; Palmiter, 2004; Feng et al., 2005]. We have previously shown that *MT-I* gene is silenced by methylation in mouse lymphosarcoma cells [Majumder et al., 1999; Ghoshal et al., 2002a] and in rat hepatoma [Ghoshal et al., 2000], which could be restored in synergistic manner by limited demethylation with 5-azacytidine and hyperacetylation of histones with HDAC inhibitor [Ghoshal et al., 2004]. In the present study, we show that different post-translationally modified core histones and methyl CpG-binding proteins are differentially associated with the methylated and unmethylated promoters, and that DNMT1 suppresses the MT-1 promoter by a transcriptional mechanism independent of its catalytic activity. We also demonstrate that some components of the repressor complex exhibit differential effects on the methylated and unmethylated MT-I promoters whereas some modulate the promoter activity irrespective of its methylation status.

MATERIALS AND METHODS

Construction of Plasmids

The construction of the expression vectors for MBD1, MBD2, and MeCP2 is described earlier [Ghoshal et al., 2004]. MBD4 cDNA was amplified from mouse lung cDNA library by RT-PCR and cloned into pcDNA3.1 to generate expression vectors. Construction of DNMT1-FlagX3 and DNMT1-(ΔCAT)-Flag3 are described earlier [Ghoshal et al., 2005].

Antibodies: Antibodies against MBD1, MeCP2, MBD4 were raised in our laboratory against recombinant proteins [Majumder et al., 2002; Ghoshal et al., 2002a, 2004; Datta et al., 2003; Bai et al., 2005]. Anti-DNMT1 antibody

was a generous gift from Shoji Tajima. Antibodies against post-translationally modified histones, MBD2 and HDAC1 were from Upstate Biotechnology.

Restriction Enzyme Accessibility Assay

Hepa and P1798 cells in log phase were harvested, washed with PBS, and nuclei were isolated by disruption of the plasma membrane with NP-40 lysis buffer as described [Ghoshal et al., 2002b]. Identical number of nuclei (1×10^5/reaction) isolated from Hepa and P1798 cells were partially digested with 50 U of restriction enzyme at 37°C for 10 min. The reaction was stopped and DNA purified. Genomic DNA (1 mg) was then completely digested with a second enzyme and an aliquot (250 ng) was subjected to LM-PCR with strand-specific primers for MT-I [Ghoshal et al., 2002b]. The ^{32}P-labeled reaction products were separated on a sequencing gel and the dried gel was subjected to autoradiography.

Micrococcal Nuclease Mapping by Indirect End-Labeling

MNase mapping was done as described [Weinmann et al., 1999]. Nuclei (1×10^7/digestion) from P1798 cells were resuspended in MNase digestion buffer and were digested with 30 and 60 U of MNase (Pharmacia) for 5 min at room temperature. The reaction was stopped and the DNA was purified. An aliquot of DNA was digested with Bgl I or Sac I, separated on agarose gel and subjected to Southern blot analysis with random primed, ^{32}P-labeled 180 bp Hae II fragment of mouse MT-I promoter as probe.

Cell Culture, Treatment With Inhibitors and Transient Transfection Assay

HepG2 (human hepatoma) and Hepa (mouse hepatoma) cells were grown in DMEM containing 5% fetal bovine serum (FBS) and P1798 (mouse lymphosarcoma) cells were grown in RPMI 1640 supplemented with 5% FBS as described [Ghoshal et al., 2002a]. HepG2 cells were transfected as described [Majumder et al., 2001, 2003] and treated with 300 nM trichostatin A for 12 h. After 24–48 h in the fresh medium, the luciferase activity was measured in cell lysate. The MT-I promoter (firefly luciferase) activity (RLU1) was normalized with plasmid copy number in transfected cells that was measured in the cell lysate by real-time PCR with primers specific for firefly luciferse coding region (F: 5′-GGATTCTAAAACGGATTACCAGGG-3′ and R: 5′-AGTTCTATGAGGCAGAGCGACACC-3’). Real-time RT-PCR reactions were carried out using total RNA as described [Bai et al., 2005; Ghoshal et al., 2005]. The copy number of pMT-I-Luc in the cell lysate was calculated from the standard curve containing 10-fold serial dilutions of the plasmid. PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C (for MT-I or luciferase), 60°C (for 18S rRNA) for 30 s, 72 °C for 30 s, and a dissociation cycle of 95°C for 60 s and 54.5°C for 30 s (to check the formation of primer dimers). Dissociation profile of the amplified products indicated that none of the primer pairs generated dimers. Histones were isolated by acid extraction of nuclei as described [Ghoshal et al., 2002a].

DNA Methylation In Vitro With Bacterial Methylases

Hind III-Bgl II fragment of pMTI-Luc [Majumder et al., 2001] was methylated with M. Sss I, or M. Hha I methylase (New England Biolabs) in the presence (methylated) or absence (mock-methylated) of S-adenosyl methionine (Ado-Met) as described earlier [Ghoshal et al., 2004; Datta et al., 2005]. The completion of the methylation reaction was determined by digestion of the fragment with methylation sensitive enzymes BstU I, Hpa II, or Hha I for M. Sss I, M. Hpa II, and M. Hha I methylases, respectively. The methylated promoter fragment was then ligated to the same sites of pGL2-basic vector (Promega). The ligated plasmid was separated on an agarose gel, purified using gel extraction kit (Qiagen) and the plasmid concentrations were measured at 260 nm. The copy number of the transfected plasmid was measured in the cell lyaste by real-time PCR using luciferase specific primers as described above.

Western Blot Analysis

Whole cell extracts prepared from HepG2 cells overexpressing different MBDs, Dnmts, and histone K-9 methyltransferase (SUV39H1) were separated by SDS–PAGE and subjected to immunoblot analysis with the respective antiserum [Majumder et al., 2002; Datta et al., 2003; Ghoshal et al., 2004]. The antigen-antibody complex was detected using ECL™ kit (Amersham) following the manufacturer's protocol.

ChIP-CHOP Assay

Formaldehyde cross-link chromatin with an average size of 600–1,000 bp was prepared as described [Weinmann and Farnham, 2002; Ghoshal et al., 2002a, 2004]. The antibodies used for the Western blot ChIP analyses were against different post-translationally modified histone H3 [S10-phospho (cat #05–817); K9-acetyl (cat #07–352), K4-trimethyl (cat #07–473); K9-trimethyl (cat 07–442)], and H4 [K5, K8, K12, and K16 acetyl H4 (cat #06–598)] and MBDs [Ghoshal et al., 2002a, 2004]. Immunoprecipitated and input DNAs were divided into three equal aliquots which were digested with Hpa II or Msp I or mock-digested followed by amplification using the ^{32}P-labeled primers specific for mouse MT-I promoter to determine the methylation status of the pulled down DNA: mMTI-F: 5′-GATAGGCCGTAATATCGGGGA-AAGCAC-3′ and mMTI-R: 5′-GAAGTAC TCAG-GACGTTGAAGTCGTGG-3′. The size of the PCR products for mMT-I promoters is 302 bp, which was separated on a polyacrylamide gels and subjected to autoradiography and phosphorimager analysis. ^{32}P-labeled PCR products were quantified using Imagequant software (Molecular Dynamics) and the results were depicted as the ratio of DNA precipitated with the antibodies to the input DNA.

ChIP assay with anti-MBD antibodies was performed as described earlier [Ghoshal et al., 2002a] with one modification. Immunoprecipitated and input DNAs were analyzed by real-time PCR with the MT-I promoter specific primers.

RESULTS

Chromatin Structures of Transcriptionally Active (Unmethylated) and Repressed (Methylated) MT-I Promoter Are Distinct

To elucidate the role of chromatin structure on MT-I gene expression we selected Hepa cells that actively express MT-1 and P1798 (lymphosarcoma) cells in which it is silenced due to promoter methylation. In Hepa cells, MT-I expression is induced 12–15-fold after zinc treatment [Daniels and Andrews, 2003] whereas in P1798 cells, it can be activated by zinc only after demethylation with 5-azacytidine [Ghoshal et al., 2002a]. Bisulfite genomic sequencing demonstrated that the promoter is methylation-free in Hepa cells (data not shown) and heavily methylated in P1798 cells [Ghoshal et al., 2002a]. These two cells are, therefore, ideal models to study the regulation of MT-I gene expression by epigenetic mechanisms.

We used restriction enzyme accessibility assay to compare chromatin structure of the MT-I promoter in these two cell lines. In this assay, the nuclei from Hepa cells were digested for a short time with methylation insensitive restriction enzymes Alu I or Msp I for limited cleavage of the MT-I promoter without disrupting the integrity of the nuclei (in vivo). We selected Alu I and Msp I as the MT-I promoter harbors sites of these enzymes (Fig. 1A). DNA isolated from these cells digested with the same enzyme was used as positive control (in vitro), and nuclei incubated without restriction enzymes (mock digested) as negative control. The purified DNA was further digested to completion with a second enzyme Taq I (for Alu I digested DNA) and with Alu I (for Msp I digested DNA). To analyze the alteration of restriction enzymes (Alu I or Msp I) accessibility to this site, identical amounts of DNA after complete digestion (with Taq I or Alu I, respectively) were subjected to LM-PCR (Fig. 1B,C). Amplification of LM-PCR products corresponding to all four Alu I sites indicates that these sites on MT-I promoter are accessible to the restriction enzyme in vivo (Fig. 1B, lanes 4 and 5). Similarly, Msp I was also accessible in Hepa cells (Fig. 1C, lanes 4 and 5). In contrast, MT-I promoter in P1798 cells was totally resistant to in vivo digestion with Alu I (Fig. 1B, lanes 6, 7) or Msp I (Fig. 1C, lanes 6, 7). These results indicate that the unmethylated MT-I promoter in Hepa cells has open chromatin structure while the methylated MT-I promoter in P1798 cells has a closed chromatin structure.

Regularly Spaced Nucleosomes Are Positioned Only on the Methylated MT-I Promoter

To get insight into the influence of chromatin structure on MT-I gene expression, we determined the nucleosome organization of the endogenous gene promoter in transcriptionally repressed and active state by micrococcal nuclease (MNase) digestion followed by indirect end labeling [Weinmann et al., 1999]. MNase preferentially digests DNA in the linker region between nucleosomes. Consequently, digestion with limiting amount of MNase will generate a DNA ladder that differs in size by multiples of

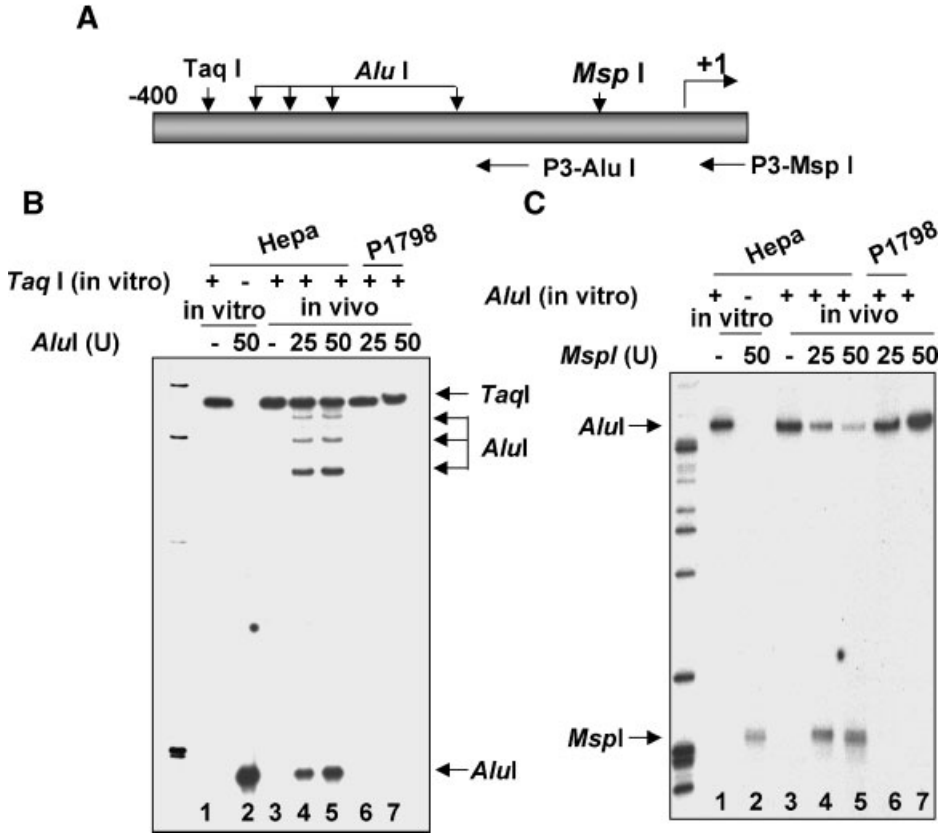


Fig. 1. Restriction enzyme accessibility assay of MT-I promoter in Hepa and lymphosarcoma (P1798) cells. **A**: Schematic diagram of MT-I promoter depicting restriction enzyme sites. **B** and **C**: LM-PCR profile of in vivo Alu I and Msp I-digested DNAs, respectively. B: Nuclei (5×10^6) from Hepa and P1798 cells were digested with Alu I at 37°C for 10 min (in vivo). Nuclei incubated in the buffer alone were used as control. DNA was purified from the nuclei and was digested with a second enzyme (Taq I) (in vitro). Identical amount (250 ng) of DNA from each group as well as naked DNA digested with respective restriction enzymes was subjected to LM-PCR with strand-specific primers (third primer P3 is labeled with ^{32}P). The reaction product was separated on a sequencing gel and subjected to autoradiography. C: Nuclei were digested with Msp I in vivo followed by digestion of the purified DNA with Alu I in vitro.

140 bp, the size of DNA wrapped around a nucleosome (Fig. 2A). If the DNA is nucleosome-free, MNase will digest DNA at all base pairs with equal probability generating a smear as observed when naked DNA is digested with MNase (Fig. 2B, lanes 2,6). Nuclei isolated from P1798 cells were digested with increasing amounts of MNase followed by digestion of the purified DNA with the restriction enzyme Bgl I or Sac I. Equal amount of DNA from each group was separated on an agarose gel and subjected to Southern blot analysis with ^{32}P-labeled Hae II (180 bp) fragment of MT-I promoter as probe (Fig. 2A). DNA digested with the Sac I or Bgl I alone gave specific product of 2.4 kb and 4.5 kb, respectively (Fig. 2B, lanes 1,2). When MNase-digested DNA was cleaved with Sac I or Bgl I in vitro, the probe detected bands ~140 bp apart, indicative of regularly spaced nucleosomes (lanes 4,7 and 5,8, respectively). Treatment of Hepa nuclei (where MT-I promoter is unmethylated) with increased concentrations of MNase resulted in disappearance of the Bgl I or Sac I fragment without forming nucleosomal ladder (data not shown) suggesting this region of the promoter is nucleosome-free in Hepa cells. These results indicate that inhibitory nucleosomes are positioned at regular intervals only on the methylated MT-I promoter.

Histone Code for Unmethylated and Methylated MT-I Promoter Is Distinct

Since post-translational modifications of core histones control nucleosome positioning and compaction of chromatin, we next determined the nature of post-translationally modified histones H3 and H4 associated with active and inactive MT-I promoters. The levels of different

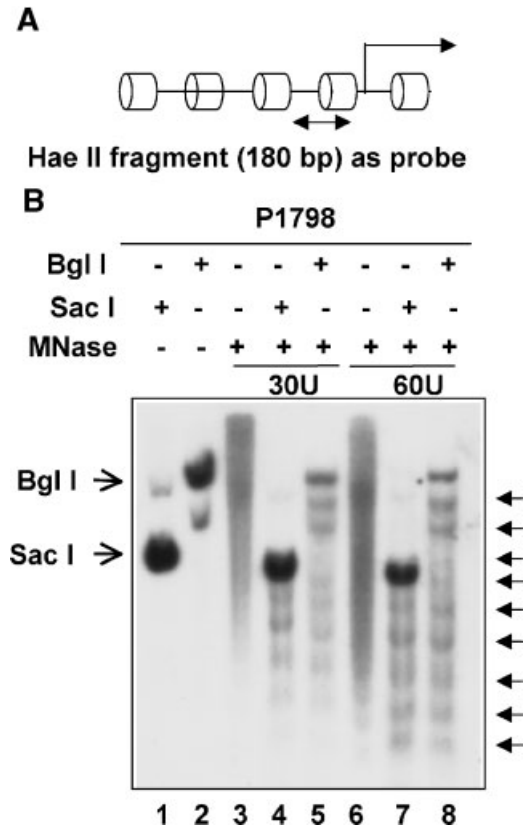


Fig. 2. Micrococcal nuclease mapping of the methylated MT-I promoter in P1798 and Hepa cells. **A**: Schematic diagram depicting regularly spaced nucleosomes positioned on MT-I promoter and the Hae II fragment used as probe for indirect end labeling. **B**: Nuclei (5×10^6) isolated from P1798 cells were digested with 30 and 60 U of micrococcal nuclease for 5 min at 37°C. Nuclei incubated without MNase were used as control. The reaction was stopped, DNA was purified, and an aliquot (40 μg) from each sample was digested with Bgl I or Sac I, separated on a 1.2% agarose gel and was subjected to Southern blot analysis with ^{32}P-labeled, random primed Hae II fragment (180 bp) of mouse MT-I promoter as probe. The blot was analyzed by autoradiography.

modified histones are comparable in Hepa and lymphosarcoma cells (Fig. 3A). However, differential association of the post-translationally modified histones H3 and H4 with the promoter was observed in two cell types (Fig. 3B,C). The cross-linked chromatin (same amount of DNA) from Hepa and lymphosarcoma cells was precipitated with antibodies specific for different post-translationally modified histones, and the immunoprecipitated and input DNAs were subjected to ChIP-CHOP assay (see Methods) followed by PCR with MT-I promoter specific primers. This region of the promoter harbors one Hpa II/Msp I site (Fig. 4B, upper panel). The lack of amplified product in Hpa II-digested DNA confirmed that the promoter is unmethylated in Hepa cells (Fig. 3B). Comparable level of PCR product in undigested and Hpa II-digested DNA from P1798 cells indicates that MT-I promoter is predominantly methylated in these cells (Fig. 3B). The quantification of the PCR products demonstrated predominant association of K9-acetyl H3 with the active MT-I promoter in Hepa cells (Fig. 3B,C) whereas multiply acetylated H4 was associated with both active (in Hepa sells) and inactive promoters (in P1798 cells). In contrast, K9-trimethyl H3 was exclusively associated with the methylated MT-I promoter (Fig. 3B,C), whereas K4 trimethyl H3 was solely associated with the unmethylated active promoter. Similarly, the level of S10-phospho H3 associated with the unmethylated promoter in Hepa cells was at least fivefold higher than that with the methylated promoter in P1798 cells (Fig. 3B,C). Therefore, the nature of modified histones associated with both promoters reflects their transcriptional competence.

Methylation Density Dependent Repression of Mouse MT-I Promoter

We next sought to investigate the mechanism by which DNA methylation influences MT-I chromatin structure and represses the promoter. As a first step towards this goal, we determined whether the decrease in mouse MT-I promoter activity depends on methylation density at the promoter. To generate the methylated plasmid, the promoter fragment was methylated with M. Sss I, M. Hha I, or M. Hpa II methylases followed by ligation into the pGL2-basic vector (Fig. 4A). Transient transfection of HepG2 cells with these plasmids showed that dense methylation with M.Sss I methylase (25 CpGs on the promoter) abolished the promoter activity whereas methylation of 4 CpGs (Hha I sites) resulted in 65% reduction of both the basal and Zn-induced activity of the promoter. Methylation of a single CpG base pair (Hpa II site) led to only 38% reduction of the basal and zinc-induced activity of the promoter compared to the mock-unmethylated controls (Fig. 4B). These data clearly demonstrated that the MT-I promoter activity is indeed inversely correlated with the extent of methylation.

Both HP1α and HP1γ Inhibit MT-I Promoter Activity

Nucleosomal K9 methyl histone H3 is recognized by heterochromatin 1 family of protein,

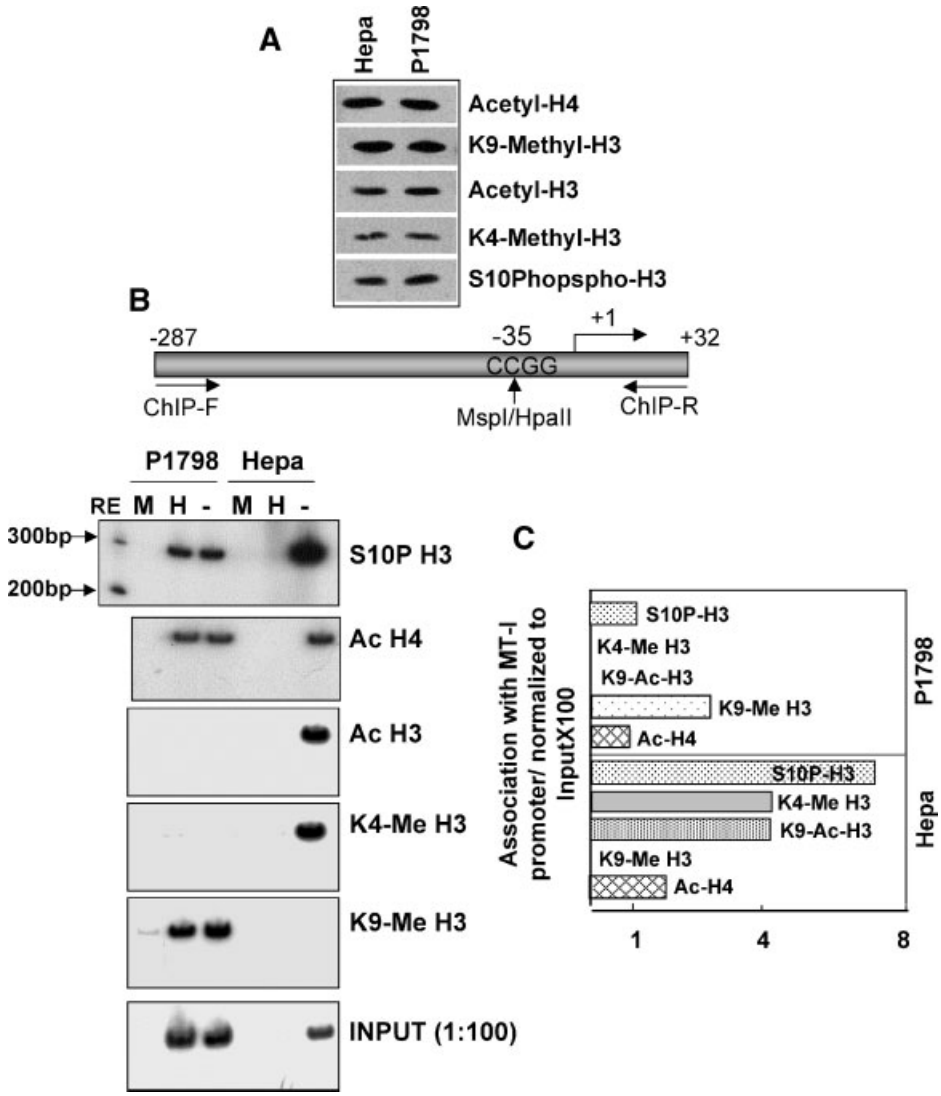


Fig. 3. Association of different post-translationally modified histones with the unmethylated and methylated MT-I promoters as measured by ChIP assay. **A**: The level of modified histones in Hepa and P1798 cells. Purified histones (10 μg) were separated by SDS-PAGE (15% acrylamide), transferred to nitrocellulose membrane and subjected to western blot analysis with antibodies specific for different post-translationally modified histones. **B**: Identical amount of formaldehyde cross-linked chromatin containing DNA from each cell line was subjected to immunoprecipitation with antibodies specific for each modified histone and the complex was pulled down with protein A beads. Immunoprecipitated DNA was purified and subjected to semi-quantitative PCR with ^{32}P labeled primers specific for MT-I promoter. Mock-immunoprecipitated (no antibody) was used as negative control. Input DNA used for PCR was 100 fold less that the amount used for ChIP. The PCR product was separated on a polyacrylamide (6%) gel and analyzed by autoradiography and phosphorImager analysis. **C**: Quantitative analysis of the association of different modified forms of histone H3 and acetylated histone H4 with the promoter normalized to the input. The phosphorImager signal in each band was quantified using ImageQuant software.

HP1α that propagates repressive chromatin structure by recruiting histone K9 methyltransferase [Lachner et al., 2001]. Association of K9 methyl H3 with the methylated promoter led us to explore the role of HP1α in MT-I promoter regulation. For this purpose, we co-transfected HepG2 cells with pMT-I-luc and HP1α or its closely related isoform HP1γ. The methylated MT-I promoter was more sensitive to HP1α-mediated inhibition than the mock-methylated promoter (45% vs. 28%) (Fig. 5A). In contrast, HP1γ inhibited activity (~50%) of both promoters (Fig. 5A). Western blot analysis demonstrated that the ectopic expression of these two proteins was comparable (Fig. 5B). To confirm that these proteins are indeed associated with MT-I promoter in the chromatin context, we performed ChIP assay. The results showed that

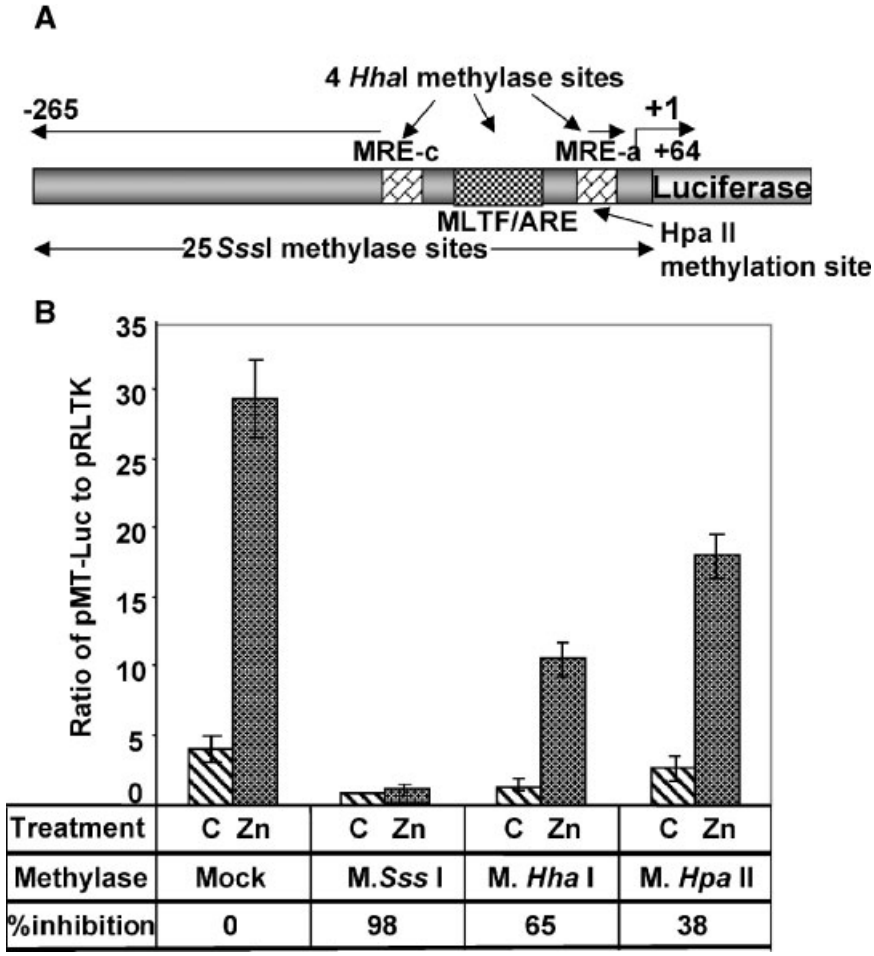


Fig. 4. The basal and zinc-induced activity of the MT-I promoter methylated at different CpG with bacterial methylases. **A**: Schematic diagram of the promoter depicting the Sss I, M. Hha I, and M. Hpa II sites. **B**: MT-I promoter-driven luciferase activity methylated at different density. Hind III/Bgl II fragment on MT-I promoter was methylated with specific methylases using Ado-Met as methyl donor. Mock-methylated (in absence of Ado-Met) promoter was used as control. The methylated DNA was then ligated to the pGL2-basic vector at the same site and the ligated plasmid DNA was purified from the agarose gel. The same amount of DNA (500 ng) was transfected into HepG2 cells using calcium phosphate precipitation method with pRLTK (50 ng) as an internal control. After 24 h, cells were equally divided into two and 12 h later one was treated with zinc sulfate (50 μM) for 6 h. Cellular extracts were analyzed for firefly luciferase (RLU1) and renilla luciferase (RLU2) activities. The results are mean of triplicate experiments ± SE.

both HP1α and HP1γ are associated with unmethylated (in Hepa cells) and methylated (in P1798 cells) MT-I promoter to similar extent (Fig. 5C). These data suggest that HP1α is recruited predominantly to the methylated MT-I promoter by K9 methyl-H3 and that HP1γ inhibits MT-I promoter activity by a distinct mechanism.

MBD2 and MBD4 specifically associate with the methylated MT-I promoter and repress its activity whereas MBD1 and MeCP2 impede MT-I promoter irrespective of its methylation status. Next, we examined the role of methyl CpG-binding proteins (MBDs) in the regulation of MT-I promoter. For this purpose, we first identified the MBDs associated with the MT-I promoter by chromatin immunoprecipitation (ChIP) assay. Western blot analysis demonstrated that both cells express comparable level of MBDs (Fig. 6A). The antibodies were raised against the C-terminal recombinant proteins lacking highly homologous N-terminal MBD domain and do not cross-react to one another [Majumder et al., 2002; Ghoshal et al., 2004]. ChIP assay demonstrated that MBD2 and MBD4 were exclusively associated with the methylated promoter whereas MBD1 and MeCP2 were associated with both unmethy-

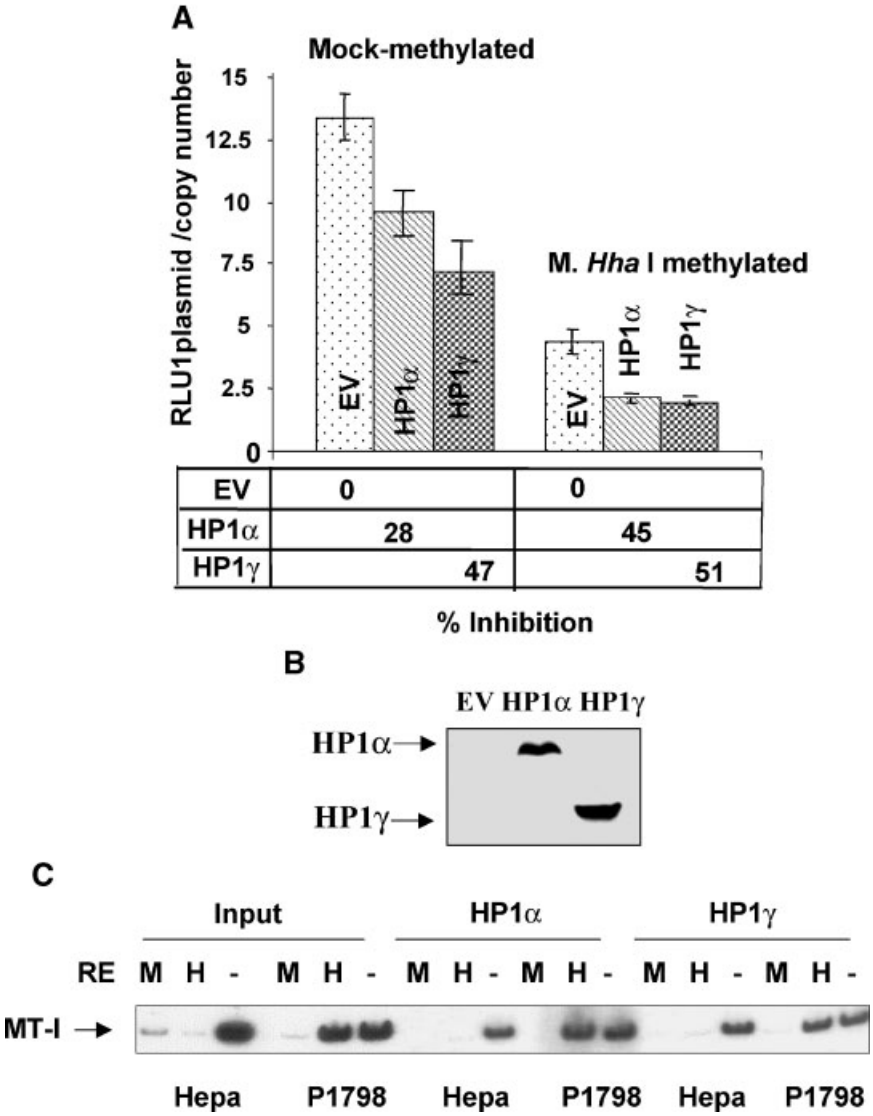


Fig. 5. Both HP1α and HP1γ inhibit MT-I promoter activity. **A**: Methylated MT-I promoter activity is more sensitive to inhibitory effect of HP1α. HepG2 cells were cotransfected with pMTI-Luc (500 ng) (mock-methylated or methylated) with 2 μg of the empty vector or Flag-tagged pcHP1α, or pcHP1γ. After 36 h, RLU1 was measured and normalized to the copy number of pMT-I-Luc in cell extract. Each experiment was done in triplicate and repeated three times. **B**: Comparable expression of ectopic HP1α and HP1γ in HepG2 cells. Western blot analysis (100 μg protein) of cell extracts with anti-Flag antibody. **C**: HP1α and HP1γ are associated with both unmethylated and methylated MT-I promoters. Formaldehyde cross-linked chromatin from Hepa (MT-I unmethylated) and P1798 (MT-I methylated) was immunoprecipitated with HP1α and HP1γ antibody and the DNA pulled down was subjected to PCR with ^{32}P-labeled primers specific for MT-I promoter and amplified products were analyzed in phosphorimager and quantified by volume analysis program.

lated (in Hepa cells) and methylated (in P1798 cells) promoters (Fig. 6B,C). The association of all four MBDs with the methylated promoter was higher compared to their association with the unmethylated promoter (Fig. 6B,C). Significantly less association of MBD2 with the methylated promoter in comparison to other MBDs is probably due to its very low abundance in lymphoid-derived cells. Preferential association of MBD4, a G:T mismatch repair enzyme, with the methylated promoter is likely due to frequent occurrence of CpG:TpG mismatches caused by deamination of methyl cytosines [Hendrich et al., 1999]. Robust association of MeCP2 with the unmethylated promoter occurs through its nonspecific DNA-binding domain located within the transcriptional repressor domain [Klose and Bird, 2004] whereas that of

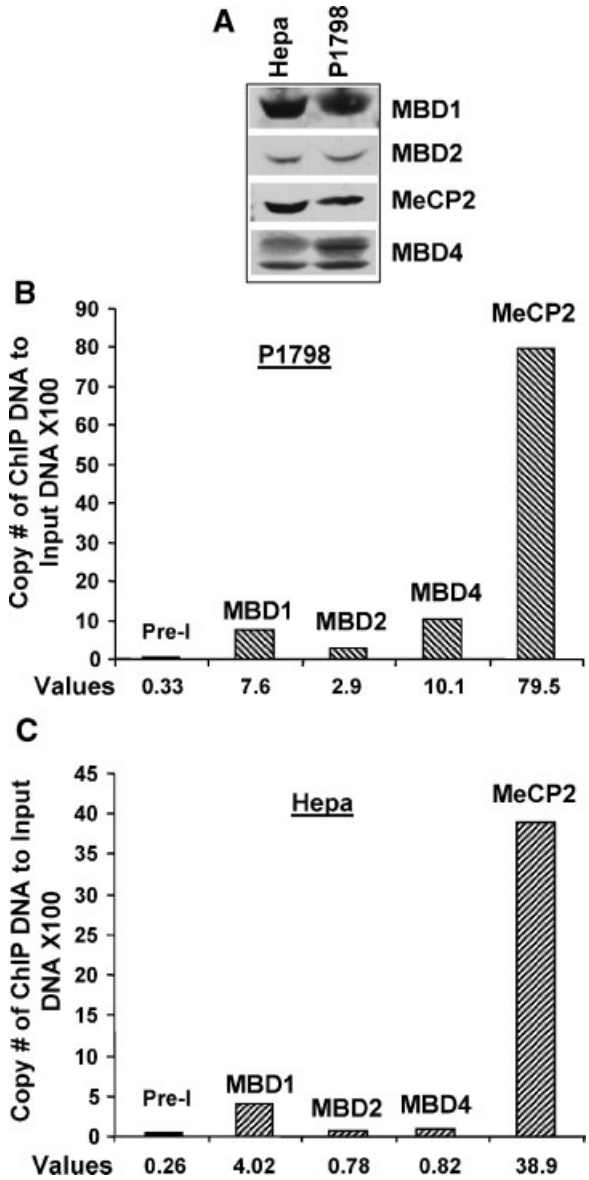


Fig. 6. Expression of different MBDs in Hepa and lymphosarcoma cells and their association with the unmethylated and methylated MT-I promoter. **A**: Levels of MBD proteins in Hepa and P1798 cells. Whole cell extracts (250 μg of protein) from each cell prepared in cell lysis buffer containing 1% SDS were subjected to immunoblot analysis with specific antibodies. Anti-rabbit IgG conjugated to HRP was used as secondary antibody except for MBD2 where anti-sheep IgG was used. The Signal was developed using ECL™ reagent (Amersham). **B**: Association of MBDs with methylated MT-I prompter (P1798). ChIP assay was performed as described for histones using antibodies specific for MBD1-3 and MeCP2 by real time PCR. The copy numbers of MT-I promoter in immunoprecipitated DNA were normalized to that of input DNA. **C**: Association of MBDs with unmethylated MT-I prompter (Hepa).

MBD1 is mediated through CXXC domain at the N-terminus. These results demonstrate differential association of MBDs with methylated and unmethylated MT-I promoter in chromatin context.

To determine the functional significance of the association of MBDs with MT-I promoter, we studied their role in regulating MT-I promoter activity by transient transfection assay. Methylated (M. Hha I) or mock-methylated MT-I promoter ligated to pGL2-basic vector (mock-pMT-Luc and meth-pMT-Luc) were co-transfected into HepG2 cells, along with MBD expression vectors. Expression of ectopic MBDs in HepG2 cells was comparable (Fig. 7A). The effect of MBDs on MT-I promoter activity was assessed 48 h post transfection. MBD2 specifically suppressed the methylated promoter (54% basal and 64% Zn-induced) whereas its effect on unmethylated promoter was relatively low (12% basal and 4% Zn-induced) (Fig. 7B, compare lanes 7, 8, 9, 10 with lanes 1, 2, 3, 4). Like MBD2, MBD4 specifically inhibited the methylated promoter activity, (43% basal and 49% Zn-induced) (Fig. 7B, compare lanes 7, 8, 11, 12 with lanes 1, 2, 5, 6) whereas the activity of the unmethylated promoter remained unaltered. On the other hand, ectopic expression of MeCP2 resulted in more than 50% inhibition of both basal and Zn-induced activity of the promoter irrespective of its methylation status (Fig. 7C, lanes 1, 2, 5, 6 and lanes 7, 8, 11, 12). Similarly, MBD1 exhibited a very strong inhibitory effect on both promoters (80–90%) (Fig. 7C, compare lanes 1–4 with lanes 7–10). Differential modulation of MT-I promoter activity by MBDs correlated well with their specific interactions with the endogenous promoter in the chromatin context in P1798 (methylated) and Hepa (unmethylated) cells (Fig. 7B,C).

DNA Methyltransferase 1 (DNMT1) Inhibits MT-I Promoter Activity That Does Not Require its Catalytic Domain

DNMT1 is a large polypeptide that harbors different motifs at its N-terminus in addition to its catalytic domain at the C-terminus (Fig. 8A). It can mediate gene silencing either by methylating CpG base pairs or independent of catalytic activity through recruitment of co-repressor complexes [Rountree et al., 2000; Chen and Li, 2004]. Therefore, we sought to examine the potential role of DNMT1 in regulating MT-I

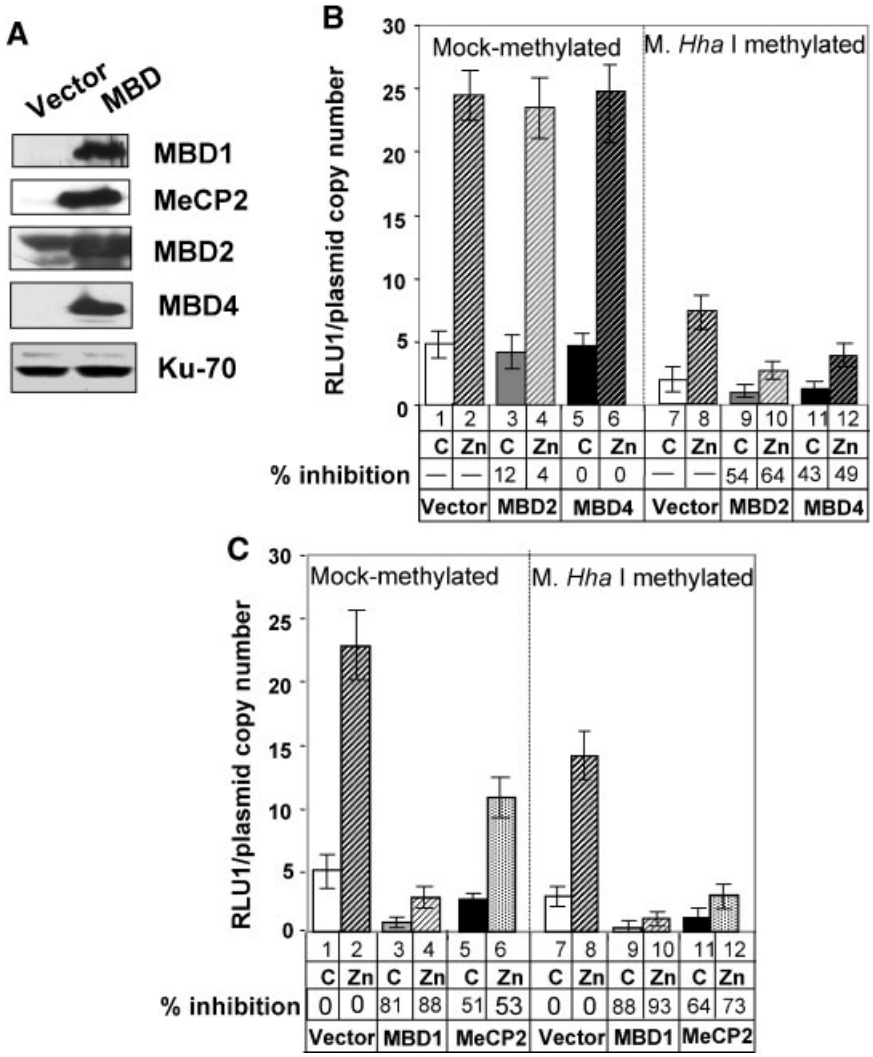


Fig. 7. Differential effects of MBDs on methylated and unmethylated MT-I promoter. **A**: Levels of endogenous and overexpressed-MBDs in HepG2 cells. Whole cell extracts (100 μg of protein) were subjected to Western blot analysis with antibodies specific for each MBDs. **B** and **C**: Effect of ectopic expression of different MBDs on the mock-methylated and Hha I methylated MT-I promoter. HepG2 cells were cotransfected with pMTI-Luc (500 ng) (mock-or methylated) with 4 μg of the empty vector or pcMBD1, pcMBD2, pcMBD4, or pcMeCP2 along with pRLTK (50 ng). After 24 h, cells were equally divided into two and 12 h later treated with zinc for 6 h. RLU1 values were normalized to the copy number of pMT-I-Luc. The results are mean of triplicate experiments ± SE.

promoter activity by overexpressing the wild type (WT) or catalytic domain deletion mutant (ΔCAT) (Fig. 8B). As expression of the full-length protein was significantly less than that of ΔCAT (Fig. 8B), we transfected threefold more of the wild-type DNMT1 to achieve comparable expression of the two proteins. The wild-type DNMT1 inhibited the activities of both methylated and mock-methylated MT-I promoter (~70%) (Fig. 8C,D). The Zn-induced activities of both promoters were similarly repressed by DNMT1 (Fig. 8C,D). To determine the role of the catalytic activity of DNMT1 in this process, we transfected ΔCAT to HepG2 cells to achieve comparable level of expression (1 μg of the ΔCAT vs. 4 μg of the wild type). ΔCAT inhibited both basal and Zn-induced activity of mock- and methylated-MT-I promoter to the same extent (~65%) (Fig. 8C,D). The methylation status of the transfected MT-I-Luc as measured by ChIP-CHOP assay was not significantly altered in cells expressing wild type DNMT1 (data not shown). These results reinforce the notion that de novo methylation of the unmethylated promoter and spreading of methylation across Hha I sites in the methylated promoter

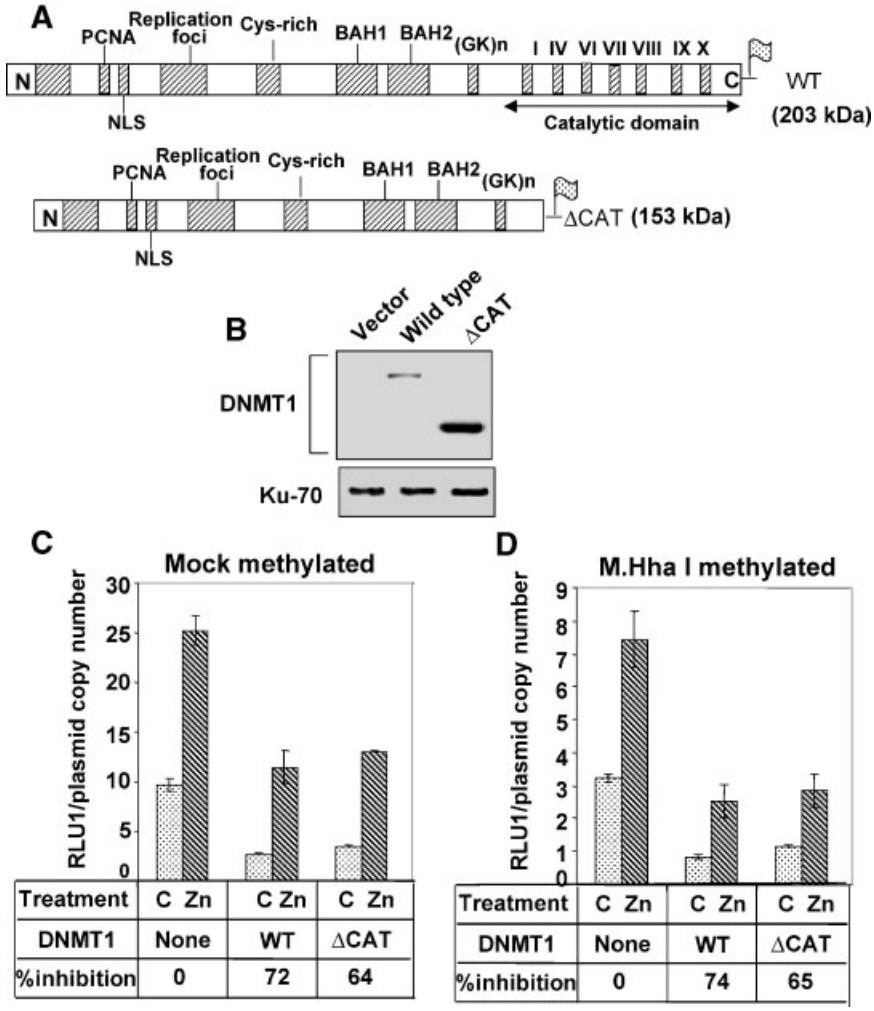
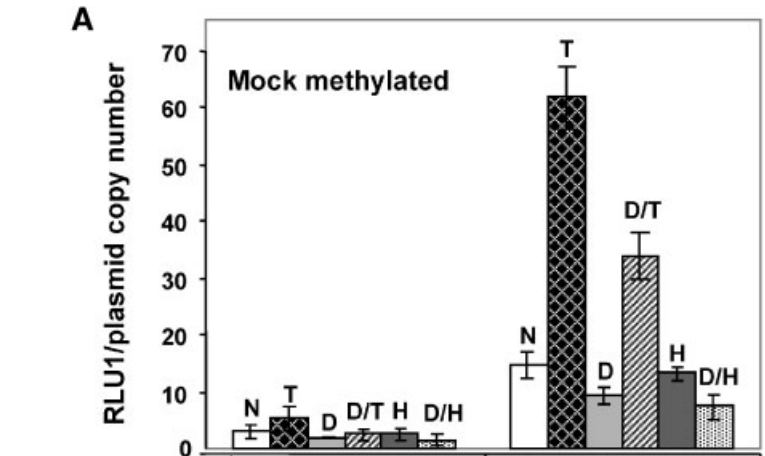


Fig. 8. DNMT1 inhibits both unmethylated and methylated MT-I promoter independent of its catalytic function. **A**: A schematic diagram depicting different domains of DNMT1. **B**: Western blot analysis of ectopic DNMT1 with anti-Flag (M2) antibody in HepG2 cells transfected with 4 μg of each expression vector. **C** and **D**: HepG2 cells were cotransfected with pMTI-Luc (500 ng) (unmethylated or methylated) along with 4 μg of the empty vector or pcDNMT1 or 1 μg of pcDNMT1/ΔCAT. After 24 h, cells were equally divided into two and 12 h later treated with zinc (50 μM). After 6 h, cells were harvested, RLU1 was measured and normalized to copy number of pMT-I-Luc. The results are mean of triplicate experiments ± SE.

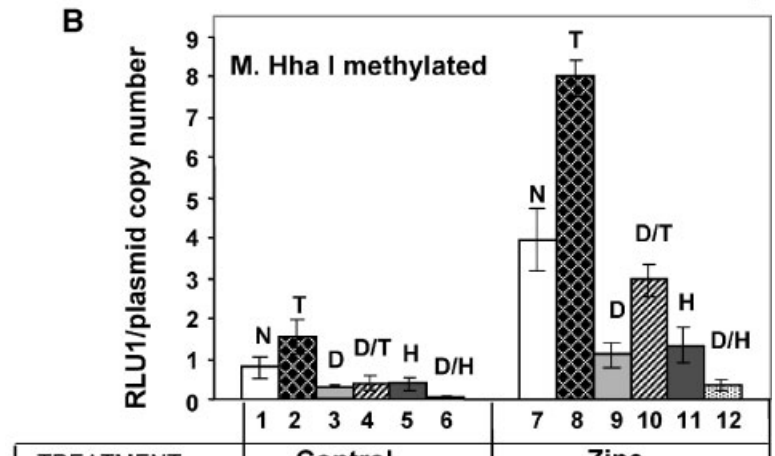
are not involved in DNMT1-mediated repression of MT-I promoter at least within first 48 h of transfection.

DNMT1 and HDAC1 Co-Operatively Inhibit Methylated MT-I Promoter Activity That Can Be Relieved by Trichostatin A

The N-terminal domains of DNMT1 recruit a variety of repressors such as DMAP1 and HDAC1/2 [Robertson et al., 2000; Rountree et al., 2000]. To determine the role of HDACs in MT-I promoter regulation, HepG2 cells were co-transfected with either mock-pMT-Luc or meth-pMT-Luc along with DNMT1 and HDAC1. The inhibitory effect of DNMT1 on the basal and Zn-induced activity of both promoters (mock- and methylated) was further enhanced upon co-expression of HDAC1. Overexpression of HDAC1 alone inhibited the basal (24%) but not Zn-induced activity of the mock-methylated promoter (Fig. 9A, compare lanes 1 with 5 and 7 with 11). However, both the basal and inducible activity of the methylated promoter was inhibited (50 and 66%, respectively) by HDAC1 alone (Fig. 9B, compare lanes 1 with 5 and 7 with 11). These results suggest that HDAC1 hypoacetylates the mock-methylated promoter in uninduced state to inhibit its activity. Upon Zn treatment, HDAC1 is displaced from the promoter due to binding of MTF-1, the key transcription factor activated by Zn [Wang et al., 2004b] to MREs (metal



TREATMENT	Control	Zinc
N: None	1	1
T: TSA	+1.65 fold	+4 fold
D: DNMT1	- 45%	- 35%
D/T:DNMT1/TSA	- 17%	+2.3 fold
H: HDAC1	-24%	no change
D/H: DNMT1/HDAC1	-56%	-50%



TREATMENT	Control	Zinc
N: None	1	1
T: TSA	+2 fold	+2-fold
D: Dnmt1	- 65%	- 62%
D/T: DNMT1/TSA	- 52%	- 25%
H: HDAC1	- 50%	- 66%
D/H: DNMT1/HDAC1	- 94%	- 92%

Fig. 9. DNMT1 and HDAC1 synergistically suppress MT-I promoter activity that could be relieved by trichostatin A (TSA), a HDAC inhibitor. **A** and **B**: Effect of DNMT1 and HDAC1 alone or in combination on unmethylated or methylated pMT-I-luc in HepG2 cells. HepG2 cells were co-transfected with pcDNMT1 (2 μg), HDAC1 (2 μg), or both. After 24 h, cells were equally divided and 12 h later treated with TSA (300 nM) or vehicle (ethanol) followed by Zn treatment 6 h later. RLU1 values were normalized to pMT-I-Luc copy number in the extract. The results are mean of three experiments performed in triplicate ± SE.

response elements). In contrast, the methylated promoter is probably occupied by a tightly associated MBD–co-repressor complex that could not be displaced by MTF-1 and its co-activators. Treatment of cells with trichostatin A (TSA), an HDAC inhibitor, abolished the inhibitory effect of DNMT1 on both the basal and Zn-induced activity of mock-methylated

MT-I promoter (Fig. 9A, lanes 1, 3, 4 and 7, 9, 10); whereas the activity of the methylated promoter was only partially recovered (Fig. 9B, lanes 1, 3, 4 and 7, 9, 10). These data reemphasize the involvement of HDAC1 in DNMT1-mediated repression of MT-I promoter. DNMT1 in combination with HDAC1 exhibited synergistic repression on the methylated promoter (90%) (Fig. 9B, lanes 1, 3, 6 and 7, 9, 12) compared to the unmethylated promoter (55%)(Fig. 9A, lanes 1, 3, 6 and 7, 9, 12). These results suggest that although both DNMT1 and HDAC1 are involved in the repression of methylated as well as unmethylated promoters, the nature of the repressor complex associated with these promoters is likely to be distinct.

DISCUSSION

MTs are stress response proteins involved in the detoxification of toxic metals and scavenging reactive oxygen species (ROS). In some cancer cells, the *MT* genes are silent due to promoter methylation [for review, see Ghoshal and Jacob, 2001]. Our earlier study showed that methylated MT-I promoter can be synergistically activated by treatment with inhibitors of DNA methyltransferase and HDACs [Ghoshal et al., 2002a]. The present study focused on detailed analysis of chromatin structure of MT-I promoter in expressing cells and in cells where it is silent due to promoter methylation. We also explored the mechanism of methylation-mediated silencing of the promoter. The results showed that the refractory chromatin structure of the methylated promoter is due to positioning of regularly spaced nucleosomes that are enriched in hypoacetylated, K9-trimethylated histone H3. In contrast, the association of K9 acetyl-, K4-trimethyl-, S10-phospho-histone H3 with the unmethylated promoter appears to be the signature of the active promoter. It is noteworthy that multiply acetylated H4 was associated with both promoters suggesting its minimal role in regulating MT-I expression. The association of acetyl-, S10-phospho-, and K4-methyl histone H3 with the unmethylated MT-I promoter in Hepa cells remained unaffected in cells treated with zinc (data not shown). There are two possible explanations for this result. First, unmethylated MT-I promoter has an open chromatin structure even in uninduced state and treatment of the cells with exogenous zinc or other inducers primarily causes activation of specific transactivators such as MTF-1 and glucocorticoid receptor (GR) that bind to cognate cis elements. The cleavage of MT-I immediate upstream promoter by restriction enzymes (Fig. 2B,C) and lack of nucleosomal array on the promoter (Fig. 3) in Hepa cells indicate that it is poised for transcriptional activation. Second, low-level zinc in the culture medium could induce open chromatin conformation of MT-I promoter by mobilizing nucleosomes positioned at specific regions of the unmethylated promoter. MNase mapping followed by indirect end labeling could not, however, identify such nucleosomes on the immediate upstream promoter in Hepa cells (data not shown).

Methylation suppresses gene expression either by directly inhibiting access of transactivators to cognate cis elements or indirectly by forming repressive chromatin structure. MT-I promoter harbors the cognate sites for myriad transcription factors. Binding of MTF-1 to MREs is differentially modulated by methylation. For example, the occupancy at MRE-d, the most potent among the MREs, is not affected by CpG methylation whereas binding at MRE-a is compromised [Radtke et al., 1996]. This probably explains low but significant (38%) inhibition of the promoter activity by methylation at a single site (Hpa II) located within MRE-a (Fig. 4). Since the DNA-binding activity of MTF-1 specifically at the most critical MRE-d is not inhibited by CpG methylation, it is likely that recruitment of MBDs as co-repressors plays a critical role in MT-I suppression in lymphosarcoma (P1798) cells. Methylation density dependent repression (Fig. 5) of MT-I promoter also suggests repressive function of MBDs. Indeed ChIP assay and overexpression studies confirmed differential role of MBDs in regulating MT-I gene expression. The specific association of MBD2 with the methylated promoter and its suppression confirms the selective effect of this MBD on the methylated promoter. We observed similar effect of MBD2 on human ribosomal RNA promoter that harbors a CpG island [Ghoshal et al., 2004]. MeCP2 probably interacts with the unmethylated MT-I promoter through its transcriptional repressor domain that harbors a DNA-binding motif in addition to the N-terminal methyl CpG-binding domain. In this context, it is noteworthy that MeCP2 represses an imprinted gene *DLX5* by binding at a single unmethylated CpG base

pair on the promoter. This gene is deregulated in MeCP2-null mice and in Rett syndrome patients leading to neurological abnormalities [Nan and Bird, 2001; Klose and Bird, 2004]. Among the MBDs, MBD1 associates with both methylated and unmethylated MT-I promoters and inhibits both promoters equally well. The interaction of MBD1 with the unmethylated promoter is probably mediated through its CXXC domain [Jorgensen et al., 2004] and with the methylated promoter through its N-terminal methyl CpG-binding domain.

Involvement of DNMT1 as a transcriptional repressor of *MT-I* gene independent of its methyltransferase activity deserves some comments. Mammalian DNMT1 has evolved from bacterial DNA methyltransferase that has minimal N-terminal region [Goll and Bestor, 2005]. The large N-terminal region of mammalian DNMT1 harbors a variety of motifs involved in interactions with proteins like PCNA [Chuang et al., 1997], DMAP1, HDAC1/2 [Robertson et al., 2000; Rountree et al., 2000], MeCP2 [Kimura and Shiota, 2003], and SUV39H1 [Fuks et al., 2003]. Recent studies have shown that knockdown of DNMT1 in human cancer cells induces certain gene expression by a mechanism that does not involve DNA methylation [Milutinovic et al., 2004]. Likewise, DNMT3A can be recruited by the transcription factor RP58 to repress its target genes by deacetylation of histone [Fuks et al., 2001]. Similarly, DNMT3B interacts with the chromatin remodeling factor SNF2 [Geiman et al., 2004] and recruits HDAC2 to its target genes and facilitates neurite outgrowth of PC12 in response to NGF [Bai et al., 2005]. It is likely that these mammalian enzymes have acquired the ability to function as either transient transcriptional repressors that involves reversible histone deacetylation, or to mediate permanent gene silencing by promoter methylation.

Finally, the significance of silencing of MT genes in some cancer cells merits discussion. MT-I and MT-II are ubiquitous proteins that act as reservoir for heavy metals and scavenge free radicals (for review, see Andrews, 1990; Ghoshal and Jacob, 2001). It has been shown that MT protects mice from hepatic hyperplasia induced by hepatitis B virus surface antigen [Quaife et al., 1999]. Agents such as alcohol and xenobiotics or viral infection may render these cells susceptible to genetic alteration predisposing them to carcinogenesis. A recent study showed that both MT-2A and MT-1 variants located in tandem spanning 75 kb region on chromosome 16 are co-ordinately deregulated in DNMT1 and DNMT3B null colon cancer cells [Zhang et al., 1997] suggesting the involvement of CpG island methylation in silencing these genes. It would be of interest to explore whether methylation or transcriptional repression is responsible for MT gene repression in human primary tumors, or whether transcriptional suppression is involved in initiation of tumor induction, specifically hepatocellular carcinomas, followed by permanent silencing due to promoter methylation at later stages of tumor growth. Studies along these lines are in progress.

ACKNOWLEDGMENTS

We thank Dr. David Schultz for HP1α and HP1γ expression vectors and antibodies, Dr. Ann Smith for Hepa cells, Dr. Stuart Schriber for HDAC1 expression vectors, and Dr. Shoji Tajima for anti-DNMT1 antibodies. This study was supported, in part, by grants ES10874 and CA81024 from National Institute of Health. We thank Shoumei Bai for critically reading the manuscript.

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