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**DNA METHYLATION-RELATED CHROMATIN MODIFICATION IN
THE REGULATION OF MOUSE DELTA-OPIOID RECEPTOR GENE**

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The abbreviations used are: mDOR, mouse δ -opioid receptor; MBD2, methyl-CpG-binding domain protein 2; MeCP2, methyl-CpG-binding protein 2; HAT, histone acetyltransferases; Adc, 5-aza-2'-deoxycytidine; ChIP, chromatin immunoprecipitation; LM-PCR, ligation-mediated polymerase chain reaction; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

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ABSTRACT

DNA methylation plays critical roles in gene silencing through chromatin modification. We previously reported that promoter region CpG methylation repressed mouse δ -opioid receptor (mDOR) gene expression. In the current study, we demonstrated that the methylation of mDOR gene promoter is correlated with a repressive chromatin structure that has less *HaeIII* and *MspI* nuclear accessibility and more deacetylated histone H3 and H4 than that of unmethylated mDOR promoter. Chromatin immunoprecipitation (ChIP) analysis showed the association of a methyl-CpG-binding domain protein 2 (MBD2) with methylated mDOR promoter. Transient expression of MBD2 enhanced the repression of partially methylated mDOR promoter activity and this repression was partially reversed by treatment of trichostatin A, a specific histone deacetylase inhibitor, indicating that MBD2 may mediate DNA methylation-related chromatin modification through recruiting histone deacetylases to mDOR promoter region. In addition, trichostatin A treatment increased both methylated mDOR promoter activity in a transient transfection assay and endogenous mDOR mRNA level in Neuro2A cells. Taken together, these results demonstrate that the mDOR gene expression is regulated by DNA methylation-related chromatin modification, especially histone acetylation and deacetylation.

INTRODUCTION

Three major types of opioid receptors referred to as μ , δ and κ , are the primary action sites for exogenous opioid alkaloids and endogenous opioid peptides. These receptors mediate a variety of pharmacological effects and have been implicated in many diverse physiological functions as well (Olson et al., 1993). They are members of the seven-transmembrane receptor superfamily and are coupled to their effectors by heterotrimeric GTP-binding proteins (G proteins) (Law and Loh, 1999). Although all three opioid receptors can initiate similar opioid-induced effects such as analgesia, each receptor type exhibits a distinct pharmacological profile, which correlates with the overlapping but unique anatomical distribution of the corresponding opioid receptor gene expression (George et al., 1994). Due to the parallels in opioid receptors distribution and the sites of opioids actions, it was hypothesized that regulation of opioid receptors' gene expression could provide opportunity to maximize the pharmacological benefits of opioids by manipulation of opioid receptors' expression level (Law and Loh, 1999).

The actions of opioids mediated via δ -opioid receptor include spinal analgesia, locomotion, limbic effects, and the δ -opioid receptor-mediated neuromodulation (Simonds, 1988). The δ -opioid receptor gene is expressed in a cell type-specific manner. The δ -opioid receptor mRNA is prominent in different regions of the brain, including cerebral cortex, olfactory tubercle, hippocampus, caudate putamen, and nucleus accumbens (George et al., 1994), although it can be found in periphery nervous system and some immune cells. In addition, the expression of δ -opioid receptor is tightly controlled during development and not detectable until postnatal stages (Zhu et al., 1998). Furthermore, levels of δ -opioid receptor mRNA can be regulated by various agents in some neuronal cells (Abood and Tao, 1995; Beczkowska et al., 1996; Jenab and Inturrisi, 1997). All of these indicate that the expression of δ -opioid receptor is

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under temporal and spatial control. Understanding the molecular mechanisms of the expression of δ -opioid receptor will gain insights of its functions corresponding to different development status and physiological condition.

Characterization of the δ -opioid receptor gene promoter from mouse (mDOR) has provided insights into regulatory mechanisms of mDOR gene transcription. Previous studies from our laboratory showed that the mDOR gene is TATA-less and contains multiple transcriptional initiation sites (Augustin et al., 1995). The minimal promoter resides -262 to -141-bp upstream from ATG (+1) site and contains several elements with binding activity for transcriptional factors Sp1/Sp3, USF, and Ets-1 (Liu et al., 1999; Sun and Loh, 2001). Furthermore, the promoter region of mDOR gene is rich in G+C content and contains a putative CpG island. Methylation of mDOR gene promoter has been shown to repress mDOR transcription through binding to a methyl-CpG binding domain protein (MBD), MBD2 (Wang et al., 2003).

Although methylation of DNA at cytosine residues of the CpG dinucleotides has long been implicated in transcriptional repression during embryonic development, genomic imprinting, and X-chromosome inactivation (Jaenisch and Bird, 2003), the mechanisms of the methylation-mediated inhibition of transcription remained elusive until the discovery of methyl-CpG binding proteins such as MeCP2 and MBD2. These proteins bind specifically to methylated DNA regardless of the sequence context (Nan et al., 1996), which may prevent the functional binding of transcription factors. Furthermore, it has been demonstrated that both MBD2 and MeCP2 are associated with other transcriptional corepressors and chromatin modifiers, such as histone deacetylase (Bird and Wolffe, 1999). The histone deacetylase removes acetyl group from histones which allows stronger interactions between the DNA backbone and histones, and

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induces a tight chromatin structure that is inaccessible to the transcription machinery. All of these suggest a model in which methyl-CpG-binding proteins act as adaptors between methylated DNA and repressive chromatin by recruiting accessory proteins that are able to modify chromatin structure and the transcriptional activity of the gene.

The present studies were designed to characterize the relationship between promoter region DNA methylation and chromatin modification, and their roles in the regulation of mDOR gene expression. We used nuclear accessibility assay, ligation-mediated polymerase chain reaction (LM-PCR), and chromatin immunoprecipitation (ChIP) analysis to characterize the chromatin structure and modifications of the mDOR gene in two cell lines, NS20Y and Neuro2A, each with different methylation levels and δ -opioid receptor expression levels (Wang et al., 2003). The relationship between DNA methylation and chromatin modification was further investigated using the approach of plasmid reporter driven by the mDOR promoter. We show here that DNA methylation-related chromatin modification is important for the regulation of mDOR gene expression.

MATERIALS AND METHODS

Cell Culture, Drug Treatments, RNA Isolation and Reverse Transcription-PCR (RT-PCR)

Mouse neuroblastoma NS20Y and Neuro2A cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. The cells were incubated at 37°C in an atmosphere of 10% CO₂. For RT-PCR analysis of mDOR mRNA level after trichostatin A treatment, Neuro2A cells were seeded in six-well plates overnight and treated the next day with trichostatin A (1, 5, 10, 50, 100 nM) for 24-h. Total RNA was isolated and RT-PCR was performed as described previously (Wang et al., 2003).

In vitro Methylation of Reporter Plasmid

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The mDOR promoter-luciferase reporter construct pD262 (containing the mDOR promoter sequence from -262 to ATG start site) was described earlier (Liu et al., 1999). Plasmid DNA was methylated with *SssI* or *HpaII* methylase overnight at 37°C according to the manufacture's instruction (New England Biolabs). The completeness of DNA methylation was confirmed by digestion with the methylation-sensitive restriction enzyme *HpaII* (New England Biolabs). Only the plasmid completely resistant to digestion was used.

Transient Transfection and Reporter Gene Assay

Neuro2A cells were plated 24-h prior to transfection at a density of 3×10^5 cells/well onto six-well culture plates. Transfection was carried out using the Effectene Transfection reagent (Qiagen) as described by the manufacture. Cells were washed and lysed with lysis buffer (Promega) 48-h after transfection. The trichostatin A treatment was carried out for 24-h before collection.

HaeIII and MspI Nuclear Accessibility Assays

Cells grown to confluence were washed once in ice-cold PBS and pelleted at 1500 rpm. The cell pellet was resuspended in ice-cold NP-40 lysis buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 0.15 mM spermine, and 0.5 mM spermidine) (Weinmann et al., 1999) and incubated on ice for 5 min. Nuclei were isolated at 1000 rpm followed by washing once in *MspI* buffer or *HaeIII* buffer (Roche). A series of increasing concentration of restriction enzyme *MspI* (0, 2, 4, 8 U/10µl) or *HaeIII* (0, 6, 12 U/10 µl) from Roche was used to digest the isolated nuclei at 37°C for 30 min or 20 min, respectively. The genomic DNA was isolated using QiaQuick PCR purification Kit (Qiagen) according to the manufacture's instructions and eluted in 10 mM Tris-HCl, pH 8.5. The DNA purified from the *MspI* digestion was then digested by *BglII* and the DNA purified from the *HaeIII* digestion was then digested by *StuI* to serve as

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internal controls. After phenol/chloroform extraction and ethanol precipitation, 1 µg of DNA was used in the Ligation-Mediated PCR as described below.

Ligation-Mediated PCR (LM-PCR)

LM-PCR was performed as described previously (Garrity and Wold, 1992; McPherson et al., 1993; Weinmann et al., 1999) with following modifications. All PCR reactions were carried out with *PfuTurbo* DNA polymerase (Stratagene) and 10% DMSO. Ligation reactions were performed in ligation buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP, 10 mM dithiothreitol, 25 µg/ml BSA) from New England Biolabs with 10% polyethylene glycol (PEG) 8000 at 17°C for 16-h. Immediately before use, add 5 µl of unidirectional linker (25 pmol, annealing between linker sense: 5'-GGGGTGACCCGGGAGAATCTGAATTC and linker antisense: 5'-GAATTCAGATC) and 4 units of T4 DNA ligase (New England Biolabs). For *Hae*III accessibility assay, the nested primers used were DH-1, 5'-CCTCCTCCGCCCCGGTCTGA; DH-2, 5'-GGTCGACCGCCCGCAGTGCT, and DH-3, 5'-ACCGCCCGCAGTGCTCGCCCAAG. For *Msp*I accessibility assay, the nested primers were DM-1, AAAGGCGTCCGAGAGGTTGACG; DM-2, AGAGGTTGACGAGGGGCGAGGAC; and DM-3, GAGGGGCGAGGACTGCAGCTCCGC. The PCR amplification was as follows: a hot start at 95°C for 4 min, 18 cycles of 1 min at 95°C, 2 min at 64°C, and 3 min at 76°C, final extension for 10 min at 76°C. The condition of the labeling PCR was: 4 min at 95°C followed by 6 cycles of 1 min at 95°C, 2 min at 68°C, and 3 min at 76°C; and a final elongation step for 10 min at 76°C. The PCR products were separated by electrophoresis on a 6% polyacrylamide gel (National Diagnostics), visualized by PhosphoImager Storm 840 (Molecular Dynamics).

Chromatin Immunoprecipitation (ChIP) and PCR amplification

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ChIP assay was performed according to the instructions from Upstate Biotechnology. Sonicated chromatin fragments typically ranged in size from 0.2 to 1 kb. 5 or 10 μ l of antibodies from Upstate were used in the immunoprecipitation. 2-5 μ l of immunoprecipitated DNA were used in the PCR amplification. PCR primers used for amplification of the endogenous mDOR promoter were 5'-TCCAGGTTCTTCTGACTCCGA and 5'-CGTGTCCGTCTCCACCGTG. In some experiments, Neuro2A cells were transfected with *Hpa*II- or *Sss*I-methylated or mock-methylated pD262 plasmids and ChIP assay was performed as before. In these cases the 5' primer for PCR (5'-CCGTGGCCTCCGTTTCC) corresponded to the 3' end of the mDOR promoter and the 3'-primer (5'-CCAGCGGTTCCATCTTCCAG) to a sequence in the pGL3-Basic vector (Promega). Conditions of linear amplification were determined empirically for all primer combinations. In some experiments, PCR amplification from immunoprecipitated DNA for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was carried out using 5'-ATCACTGCCACCCAGAAGACTGTGGA and 5'-GAGCTTGACAAAGTTGTCATTGAGAGC as the 5' and 3' primers to serve as an internal control. A hot start was performed (2 min, 94 °C) followed by 32-34 cycles of PCR with a final extension of 10 min at 72 °C. Reaction products were separated by electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, and photographed. The intensities of PCR products were determined from digital image using Scion Image software (Scion Corp.).

RESULTS

DNA methylation correlates with chromatin compaction within mDOR promoter region.

NS20Y and Neuro2A are mouse neuronal cells with different δ -opioid receptor expression levels as confirmed by RT-PCR analysis (Fig. 1E). We previously reported a strong

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negative correlation between the methylation status of the mDOR promoter and the mDOR gene expression levels in these two cell lines (Wang et al., 2003). Therefore, these cell lines represent a useful model system for studying the interplay of DNA methylation, chromatin structure and mDOR gene expression.

To determine whether chromatin structure near mDOR promoter plays a role in the regulation of mDOR gene expression, we first analyzed the nuclease sensitivity of the mDOR promoter using the *HaeIII* nuclear accessibility assay. The nuclei from NS20Y and Neuro2A cells were partially digested with the restriction enzyme *HaeIII* *in vivo*. DNA extracted from the nuclei was then completely digested with *StuI* *in vitro*. Mouse genomic DNA from NS20Y cells was digested with *StuI* alone and served as a control. Purified DNA was amplified by LM-PCR, as described in the *Experimental Procedures*. Nested primers were designed to amplify the mDOR promoter region encompassing two *HaeIII* sites (Fig. 1A, DH-1, DH-2 and DH-3). NS20Y cells showed chromatin accessibility, indicating the presence of open chromatin in mDOR promoter region (Fig. 1B, *lane* 1 and 2). In contrast, the same region in Neuro2A cells was more refractory to digestion than that of NS20Y cells, suggesting a more closed chromatin conformation (Fig. 1B, *lane* 3 and 4). The unidentified bands (marked as *) were non-specific primer annealing sites within mouse genome, since purified mouse genomic DNA digested with *StuI* alone also generated these bands (Fig 1B, *lane* 5). Taken together, the accessibility of *HaeIII* restriction digestion correlates well with the mDOR expression level and the apparent reduction of mDOR gene expression in Neuro2A cells is associated with an altered chromatin structure, suggesting that the chromatin structure of mDOR promoter region plays a role in the regulation of mDOR gene expression.

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To assess directly whether DNA methylation in the promoter region of mDOR gene generates an altered chromatin structure, we further characterized the nuclease sensitivity using the *MspI* nuclear accessibility assay. Although *MspI* is not sensitive to the methylation of internal cytosine within its recognition site, CCGG, methylated CCGG sites located within intact nuclei are resistant to digestion, presumably because of the formation of a methylation-dependent closed chromatin structure (Antequera et al., 1989). LM-PCR analysis of genomic DNA isolated from *MspI*-digested nuclei from NS20Y and Neuro2A cells revealed similar pattern in the sensitivity in the mDOR promoter region as *HaeIII* digestion. Neuro2A cells were substantially less sensitive to *MspI* digestion than NS20Y cells at the same site (Fig 1B and 1C). In addition, when the genomic DNA isolated from *MspI* digestion was further incubated with *HpaII*, a methylation sensitive restriction enzyme that can not cut DNA at its recognition site CCGG if the internal cytosine is methylated, three of the *MspI*-digested fragments (-154, -190 and -239) were almost abolished in both cell lines (Fig. 1D, lane 4 and 5). This indicates that most, if not all, of the genomic DNA that can be digested with *MspI* in their nuclei context is unmethylated. In other words, when DNA within mDOR promoter region is methylated, it can form a chromatin confirmation that is resistant to nuclease digestion. These results directly demonstrate that DNA methylation of mDOR gene promoter associates with the formation of a relatively compact chromatin structure.

Association of methylated mDOR promoter with reduced acetylation of histone H3 in a transient transfection assay in Neuro2A cells.

Recent studies indicate that the structure of the chromatin and the activity of the associated genes are mediated by a distinct set of covalent modifications of histone proteins,

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including phosphorylation, acetylation, ubiquitination, and methylation (Jaenisch and Bird, 2003; Strahl and Allis, 2000). For example, acetylation of lysine residues on histone H3 is associated with the formation of open chromatin structure and transcriptionally active gene, whereas deacetylation is linked with repressed chromatin. The array and combination of these specific modifications of various histones may constitute a distinct code that directs gene expression (Jenuwein and Allis, 2001).

We previously reported that methylation of mDOR promoter represses its activity as confirmed in a transient transfection assay in Neuro2A cells (Fig 2A and Fig 4C, *column* 1, 2 and 3). Repression of mDOR promoter activity is methylation-density dependent, such that a low level of methylation (*Hpa*II methylation) inhibits gene expression significantly, whereas extensive methylation (*Sss*I methylation) completely blocks mDOR promoter activity (Fig 2A and Fig 4C, *column* 1, 2 and 3). To determine whether DNA methylation is coupled to acetylation of histones bound to the mDOR promoter, we analyzed the immunoprecipitated plasmid DNA from Neuro2A cells transfected with *Hpa*II or *Sss*I methylated mDOR promoter construct pD262 using ChIP assay. Anti-acetyl-histone H3 (Anti-AcH3) was used to immunoprecipitate DNA and PCR was carried out using primers specific for the mDOR promoter construct. The amount of mDOR promoter sequence was significantly decreased in the immunoprecipitated DNA from fully methylated mDOR promoter construct relative to that from unmethylated or partially methylated reporter (Fig 2B, *lane* 5, 7 and 9; and Fig 2C). Little or no mDOR promoter sequence was detected by PCR in the absence of anti-AcH3 antibody, indicating the specificity of the ChIP analysis. These results demonstrated that methylation of mDOR promoter decreases the association of acetylated histone H3 in chromatinized plasmid.

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Differential modification of histone H3 and H4 within mDOR promoter region in NS20Y and Neuro2A cells.

In view of reduced level of acetylation in histone H3 associated with the methylated promoter relative to the unmethylated promoter in chromatinized plasmid (see above), we used ChIP assay to investigate whether the endogenous mDOR promoter in NS20Y and Neuro2A cells are associated with different amount of acetylated histone H3. It is evident that NS20Y cells showed significantly higher amount of acetylated histone H3 binding than that of Neuro2A cells (Fig 3A, *lane 3 and 9*). Furthermore, higher level of enrichment of acetylated histone H4 (AcH4) and acetylated histone H3 at lysine 9 (AcH3K9) was detected in the mDOR promoter in NS20Y cells relative to Neuro2A cells (Fig 3A, *lane 4 and 10, lane 5 and 11, respectively*). The enrichment of histone H3 methylated lysine 4 (MeH3K4), which is preferentially localized to transcriptionally active promoters, was also higher in the mDOR promoter region in the unmethylated NS20Y cells relative to the methylated Neuro2A cells (Fig 3A, *lane 6 and 12*). These results demonstrate that the DNA methylation of mDOR promoter is associated with an altered pattern of histone modification.

Cooperative interaction between MBD2 and histone deacetylase in the repression of methylated mDOR promoter in Neuro2A cells.

Methyl-CpG binding protein MBD2 has been found to be associated with methylated mDOR promoter in an electrophoresis mobility shift assay (EMSA) (Wang et al., 2003). ChIP analysis of MBD2 from Neuro2A cells transfected with *HpaII*- or *SssI*-methylated mDOR promoter construct pD262 indicated that MBD2 was more tightly associated with mDOR promoter when mDOR promoter is fully methylated. Little or no mDOR promoter sequence was

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detected by PCR when mDOR promoter is unmethylated or partially methylated (Fig 4A). In addition, CHIP analysis also showed the association of MBD2 with endogenous mDOR promoter in Neuro2A cells (Fig 4B). It has been implicated that MBD2 may participate in DNA methylation-related chromatin modification by recruiting histone deacetylase (Ng et al., 1999). In view of reduced level of histone acetylation associated with the methylated mDOR promoter relative to the unmethylated promoter (Fig 2), we determined whether MBD2 can account for histone deacetylation-dependent repression of methylated mDOR gene using a transient transfection and reporter assay. Neuro2A cells were transfected with unmethylated, partially methylated or fully methylated mDOR promoter construct pD262. Trichostatin A (5 nM), which is a specific histone deacetylase inhibitor that increases the level of histone acetylation, could overcome transcriptional repression caused by partial methylation of the mDOR promoter after 24-h treatment (Fig 4C, *column 4*), indicating that deacetylation is involved in the methylation-induced repression. However, trichostatin A could not restore full promoter activity even at a higher concentration (50 nM) when the mDOR promoter is fully methylated (Fig 4C, *column 5 and 6*). Furthermore, transcription of partially methylated mDOR promoter was completely abolished after transiently expressing MBD2 fused to the Gal4 DNA-binding domain (Gal4-MBD2) (Fig 4C, *column 7*). The inhibition could only be partially reversed by trichostatin A treatment (Fig 4C, *column 8 and 9*). Take together, these results indicate that MBD2 and histone deacetylase interact cooperatively with each other to repress mDOR promoter activity, and decreased methylation is a prerequisite for effective transcription following histone deacetylase inhibition.

Inhibition of histone deacetylase increases mDOR expression in Neuro2A cells.

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To further test whether histone acetylation plays a role in the regulation of mDOR gene expression, Neuro2A cells were treated with different concentration of trichostatin A for 24-h. Total RNA was isolated and RT-PCR was carried out to amplify the reversed transcribed mRNA. Compared with the untreated cells, the amount of mDOR mRNA after trichostatin A treatment was increased in a concentration dependent manner (Fig 5A). Our previous studies showed that the demethylating agent 5-aza-2'-deoxycytidine (Adc) can upregulate mDOR gene expression in Neuro2A cells after 3-day treatment (Wang et al., 2003). To further investigate the roles of histone deacetylation and DNA methylation in the regulation of mDOR gene, we induced partial demethylation of mDOR gene in the presence or absence of histone deacetylase inhibition. A lower dose of Adc (100 nM) produced little or no increase of mDOR expression after 3-day treatment (Fig 5B, *lane 3*). However, we observed increased expression of mDOR after addition of 5 nM trichostatin A for 24 h following 100 nM Adc treatment (Fig 5B *lane 2* and 4). The data suggest that histone acetylation regulates the mDOR gene transcription activity and active histone deacetylation together with DNA methylation has a role in silencing mDOR gene expression.

DISCUSSION

The mDOR expression is under temporal and spatial control. Elucidating the molecular mechanisms for the regulation of mDOR expression will benefit both basic and clinical perspectives. Our previous report showed that repression of mDOR gene in Neuro2A cells was linked to the presence of methylated CpGs in the promoter region (Wang et al., 2003). In the current study, the molecular mechanisms underlying the methylation induced mDOR promoter repression were further investigated. We demonstrate here the first time

that DNA methylation-related chromatin modification plays an important role in the regulation of mDOR gene expression.

The connection between DNA methylation and chromatin structure has been known for many years (Keshet et al., 1986). It has long been implicated that DNA methylation results in the formation of inactive chromatin structure that is relatively refractory to endonucleases digestion (Antequera et al., 1989; Groudine et al., 1981). In the current study, it has been found that the accessibility of mDOR promoter region correlated well with the methylation state within this region. In the absence of methylation, mDOR promoter in NS20Y cells displayed more accessibility to digestion when compare to Neuro2A cells that have partially methylated mDOR promoter (Fig 1). Our observations suggest that methylation induced silencing of mDOR involves the generation of a modified chromatin structure that has limited promoter accessibility.

The organization of DNA into chromatin plays a central role in the regulation of gene expression. In accessing the genetic material during transcription, the respective cellular machineries have to modify the chromatin structure to ensure that the genes are switched on and off as the respective proteins are needed for their diverse cellular functions. Chromatin structure can be altered by specific modification of histones, such as acetylation, methylation, phosphorylation and ubiquitylation (Strahl and Allis, 2000). One of the intensively studied among histone modifications is histone acetylation, which is carried out by histone acetyltransferases (HATs) and reversed by histone deacetylases. In general, increases in histone acetylation have been associated with an open chromatin structure and enhanced gene expression. Using ChIP assay, we demonstrated that methylation of mDOR promoter was associated with decreased levels of acetylated histone H3 and H4 at mDOR promoter region

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(Fig 3). This is also confirmed by our results showing that fully methylated mDOR promoters in reporter constructs were associated with reduced amount of acetylated histone H3 than the unmethylated promoters (Fig 2). Furthermore, inhibition of histone deacetylation by trichostatin A partially relieved transcriptional repression of the methylated mDOR promoter plasmid in a transient transfection assay (Fig 4) and upregulated mDOR mRNA level in Neuro2A cells (Fig 5). Taken together, these results clearly demonstrated that histone acetylation and deacetylation are involved in the DNA methylation mediated repression and DNA methylation may play a role in setting up the chromatin structure by recruiting HATs and histone deacetylases. Recent evidence from transgenic experiment also indicates that the methylation pattern established in early embryogenesis is both necessary and sufficient to direct the assembly of DNA into closed chromatin structure with deacetylated histone H3 and H4 (Hashimshony et al., 2003).

One mechanism by which DNA methylation could influence histone modification involves methyl CpG binding domain proteins (MBDs) (Razin, 1998). Our previous work showed that MBD2 binds mDOR promoter in a methylation dependent manner (Wang et al., 2003). Recent evidence indicates that certain MBDs, such as MBD2, interact with multiprotein repression complex that has histone deacetylase activity (Ng et al., 1999). Our observations that transient expressing MBD2 completely repressed partially methylated mDOR promoter activity and inhibition of deacetylation by trichostatin A could overcome transcriptional repression due to partial methylation of mDOR promoter are consistent with this model (Fig 4). Interestingly, trichostatin A could only partially reactivate the mDOR promoter in a fully methylated constructs. One possible explanation is that extensive methylation induces an unusual chromatin structure by binding to MBD2. At least partial

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demethylation and subsequent disassociation of MBD2 from the mDOR promoter region are required to reactivation of the gene. This notion is supported by the observation that trichostatin A could only partially reverse the repression caused by partial methylation and transient expression of MBD2 (Fig 4). Similar phenomena have been shown in other genes as well. For example, the methylated and silent fragile X mental retardation gene (FMR1) could not be reactivated by trichostatin A alone, but only after Adc treatment (Coffee et al., 1999).

The combination of our results allows us to propose a model to explain the mechanisms of DNA methylation-related chromatin modification in the regulation of mDOR gene expression. In our model, DNA methylation of mDOR promoter region plays a dominant role in regulation of mDOR gene expression. First, methylated mDOR promoter binds to MBD2 within a chromatin context. After that, histone deacetylase can reach chromatin through its association of MBD2, leading to histone deacetylation and subsequent alteration of chromatin structure.

The mechanisms establishing methylation patterns during development are still largely unknown. Current data suggest that methylation of DNA can be promoted by short RNAs derived via Dicer cleavage of double-strand RNA (dsRNA), i.e. the RNA-directed DNA methylation (RdDM) (Matzke et al., 2004). Whether this is the case for mDOR gene need to be further investigated.

In summary, we have shown that methylation of mDOR promoter induces the modification of chromatin structure within mDOR promoter region, leading to a more close chromatin configuration which represses the mDOR gene expression. DNA methylation, proteins able to bind specifically to methylated DNA such as MBD2, and chromatin modifications play a dynamic role in determining chromatin structure suitable for gene

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transcription or silencing (Bird and Wolffe, 1999). Further studies are required to fully identify the interaction between DNA methylation and chromatin modification in the regulation of mDOR gene expression.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. DNA methylation correlates with chromatin compaction within mDOR promoter region.

A, Schematic representation of the mDOR gene minimal promoter (-262 to -141) and its flanking region. The *vertical lines* indicate the position of each CpG dinucleotide. The restriction enzyme sites analyzed are indicated by *vertical arrows*. The cleavage position of each enzyme relative to the ATG start codon is indicated by the number inside the *parentheses*. The nested primer (DH-1, DH-2 and DH-3 for *HaeIII*; DM-1, DM-2 and DM-3 for *MspI*) binding sites are indicated. The radio labeled primer is indicated by an *asterisk*. **B**, *In vivo* analysis of *HaeIII* nuclear accessibility within mDOR promoter region by LM-PCR. Nuclei from NS20Y or Neuro2A cells were treated with 6 units/10 μ l (*lane 1 and 3*) or 12 units/10 μ l (*lane 2 and 4*) *HaeIII* for 20 min at 37°C. After purification, DNAs were digested with *StuI* to provide internal controls. Genomic DNA purified from NS20Y cells was also digested with *StuI* alone (*lane 5*). PCR products were analyzed using 6% polyacrylamide denaturing gels and exposed to PhosphorImager screens. The phiX174 DNA digested with *HinfI* (*lane 6*) was used as size standard (M). **C**, *In vivo* analysis of *MspI* nuclear accessibility within mDOR promoter region by LM-PCR. Nuclei from NS20Y or Neuro2A cells were subjected to a series of digestion with increasing concentrations of *MspI* (0, 2, 4 and 8 units/10 μ l) for 30 min at 37°C. After purification, the genomic DNAs were digested with *BglII* to provide internal controls. PCR products were analyzed using 6% polyacrylamide denaturing gels and exposed to PhosphorImager screens. The phiX174 DNA digested with *HinfI* (*lane 1*) was used as size standard (M). **D**, Nuclei from NS20Y (*lane 2, 4 and 6*) and Neuro2A (*lane 3, 5 and 7*) were digested with (*lane 2-5*) or without (*lane 6 and 7*) 8 units/10 μ l *MspI* for 30 min at 37°C. After purification the genomic DNAs were further digested with *BglII* followed by

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HpaII (lane 4-7) or mock digestion (lane 2 and 3). Primer DM-1, DM-2 and radio labeled primer DM-3 were used for LM-PCR. PCR products were analyzed using 6% polyacrylamide denaturing gels and exposed to PhosphorImage screens. The phiX174 DNA digested with *HinfI* (lane 8) was used as size standard (M). **E**, RT-PCR analysis of mDOR expression in NS20Y and Neuro2A cells. 1 µg of total RNA were analyzed by RT-PCR with (+) or without (-) reverse transcriptase (RT). Aliquots of the PCR products were resolved on 1.2% agarose gels. Expression of GAPDH was used as an internal control. (B)

Fig. 2. Association of methylated mDOR promoter with reduced acetylation of histone H3 in a transient transfection assay in Neuro2A cells.

A, Inhibition of mDOR promoter activity by CpG methylation in Neuro2A cells. The mDOR luciferase plasmid pD262 was partially methylated (*HpaII*), fully methylated (*SssI*) or mock methylated (Mock) for transfection of Neuro2A cells, as described in the *Experimental Procedures*. The data present the average \pm standard deviation (SD) of three independent experiments performed in duplicate. The luciferase activity of the mock methylated pD262 (Mock) is designated as 100%. Relative promoter activity is presented as the percentage of the unmethylated promoter activity. **B**, ChIP assay of the mDOR promoter sequence bound to acetylated histone H3 (AcH3) in Neuro2A cells transfected with mock methylated (Mock), partially methylated (*HpaII*), or fully methylated (*SssI*) mDOR promoter constructs pD262. The PCR product of the mDOR promoter is indicated. Lane 1, 2 and 3, total chromatin before immunoprecipitation from cells transfected with unmethylated, partially methylated or fully methylated mDOR promoter, respectively (Input); lane 4, 6 and 8, no antibody control (-); lane 5, 7 and 9, immunocomplex of anti-AcH3 antibody (+); GAPDH, internal control. **C**, Quantitative data from (B). The total density of the ChIP-PCR bands was calculated using Scion

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Image for Windows software from Scion Corp. after normalized to the density of the GAPDH chromatin bands. The *histograms* represent the means of percentage of immunoprecipitates to the GAPDH chromatin (designated as 100%) from three independent experiments. The *error bars* indicate the range of standard deviations.

Fig. 3. Differential modification of histone H3 and H4 within mDOR promoter region in NS20Y and Neuro2A cells.

A, Chromatin immunoprecipitation (ChIP) analysis of the mDOR promoter region. Chromatin from NS20Y and Neuro2A cells was immunoprecipitated with various antibodies: acetyl-histone H4 (Anti-AcH4), acetyl-histone H3 (Anti-AcH3), acetyl-histone H3 lysine 9 (Anti-AcH3K9), methyl-histone H3 lysine 4 (Anti-MeH3K4). No antibody immunoprecipitates (NAC) were used as control for ChIP assay specificity. The DNA that was immunoprecipitated with each antibody was subjected to PCR and visualized on a 1.2% agarose gel. Input, 1% of total chromatin used in ChIP. **B**, The total density of the ChIP-PCR bands was calculated using Scion Image for Windows software from Scion Corp. after normalized to the density of the input chromatin bands. The *histograms* represent the means of percentage of immunoprecipitates to the input chromatin from three independent experiments. The *error bars* indicate the range of standard deviations.

Fig. 4. Involvement of MBD2 in deacetylase-dependent repression of methylated mDOR promoter in Neuro2A cells.

A, MBD2 associates with methylated mDOR promoter construct pD262. Neuro2A cells were transfected with unmethylated (Mock, *lane* 1, 4 and 5), partially methylated (*HpaII*, *lane* 2, 6 and 7) or fully methylated (*SssI*, *lane* 3, 8 and 9) pD262 and ChIP assay was performed as mentioned in the *Experimental Procedures*. 10 μ l of anti-MBD2 antibody was used (+). No antibody

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immunoprecipitates (-) were used as control for ChIP assay specificity. **B**, MBD2 associates with mDOR promoter region *in vivo* in Neuro2A cells. Chromatin from Neuro2A cells was immunoprecipitated with 5 μ l or 10 μ l of MBD2 antibody. No antibody immunoprecipitates (NAC) were used as control for ChIP assay specificity. The DNA that was immunoprecipitated with each antibody was subjected to PCR and visualized on a 1.2% agarose gel. Input, 1% total chromatin used in ChIP. (C) Transcription of the transiently transfected pD262 constructs is repressed by methylation of *Hpa* II or *Sss* I sites. This repression could be fully reversed by 5 nM trichostatin A treatment when the pD262 was partially methylated and partially relieved by 5 or 50 nM trichostatin A treatment when the pD262 was fully methylated. Cotransfection of a construct encoding MBD2a fused to the Gal4 DNA-binding domain (GAL4-MBD2) together with *Hpa* II methylated pD262 construct into Neuro2A cells cause further repression. The trichostatin A (5 or 50 nM) could only partially relieve the repression of mDOR promoter. TSA: trichostatin A.

Fig. 5. Inhibition of histone deacetylase increases mDOR expression in Neuro2A cells.

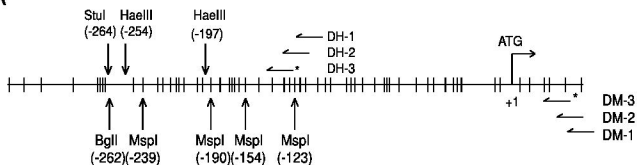
(A) Neuro2A cells were treated with increasing concentration of trichostatin A for 24 hr and total RNA was used for RT-PCR. GAPDH is shown below as a control for the amount of RNA amplified. The total density of the RT-PCR bands was calculated using Scion Image for Windows software from Scion Corp. after normalized to the density of the GAPDH bands. The relative amount of mDOR mRNA from the untreated sample (trichostatin A concentration is 0) was designated as 1. The *histograms* represent the means of three independent experiments. The *error bars* indicate the range of standard deviations. (B) The mDOR expression in Neuro2A cells was analyzed by RT-PCR after treatment with Adc (100nM, *lane* 3) or trichostatin A (5 nM, *lane*

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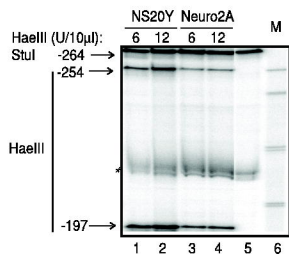
2) alone, or both (*lane* 4). The quantitative data were calculated as mentioned above. TSA:
trichostatin A.

Figure 1

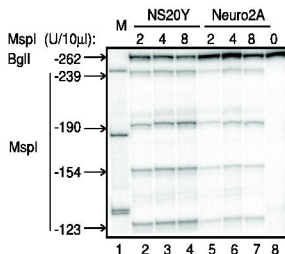
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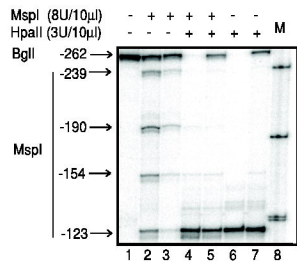
B



C



D



E

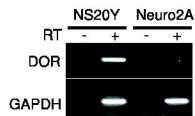
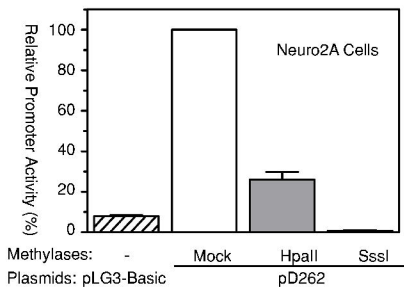
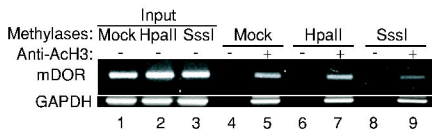


Figure 2

A



B



C

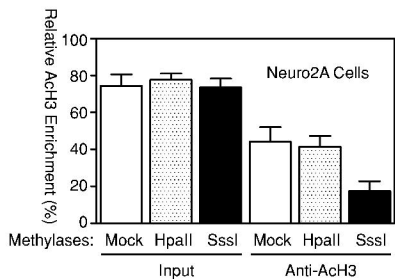
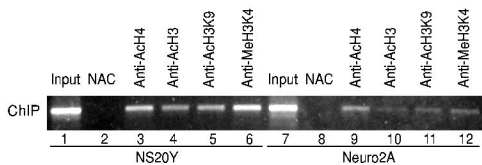


Figure 3

A



B

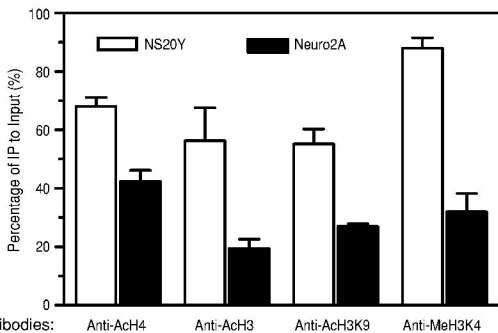
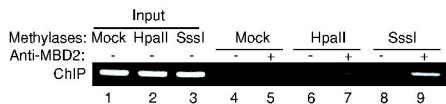
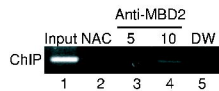


Figure 4

A



B



C

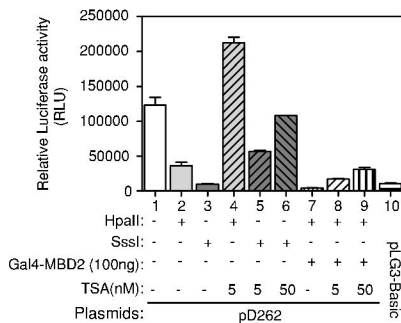
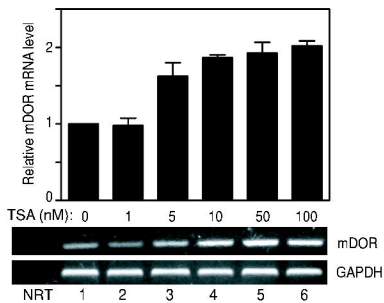


Figure 5

A



B

