Transcriptional Regulation of Mouse δ-Opioid Receptor Gene by CpG Methylation

IN INVOLVEMENT OF Sp3 AND A METHYL-CpG-BINDING PROTEIN, MBD2, IN TRANSCRIPTIONAL REPRESSION OF MOUSE δ-OPIOD RECEPTOR GENE IN Neuro2A CELLS

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Opioid receptors are expressed in a cell type-specific manner. Here we show that the mouse δ-opioid receptor (mDOR) methylation is regulated by promoter region CpG methylation. The mDOR promoter containing a putative CpG island is highly methylated in Neuro2A cells, correlating with the repression of this gene in these cells. This is in contrast with the unmethylated state of the mDOR promoter in NS20Y cells, which express a high level of mDOR. Repression of mDOR transcription in Neuro2A cells could be partially relieved by chemically induced demethylation with 5-aza-2′-deoxycytidine. In addition, in vitro methylation of the luciferase reporter gene driven by the mDOR promoter resulted in an inhibition of transcription in NS20Y cells. Methyl-CpG-binding protein complex 1 (MeCP1) has been implicated in methylation-mediated transcriptional repression of several genes. Electrophoretic mobility shift assays showed that fully methylated, but not unmethylated, mDOR promoter fragment formed a MeCP1-like protein complex that contained methyl-CpG-binding domain protein 2 (MBD2) and Sp3. Furthermore, the expression level of Sp3 was decreased when Neuro2A cells were demethylated with 5-aza-2′-deoxycytidine, and increasing Sp3 levels in Schneider’s Drosophila line 2 cells led to the repression of mDOR promoter activity when the promoter was methylated. These results demonstrate that Sp3 and MBD2 are involved in the transcriptional repression of mDOR in Neuro2A cells through binding to the methylated CpG sites in the promoter region and may play a role in the cell type-specific expression of mDOR.

Opioids display strong analgesic effects as well as addictive properties through the activation of three major types of G-protein-coupled receptors referred to as μ-, δ-, and κ-opioid receptors (1). Whereas all three opioid receptors mediate opioid-induced analgesia, each receptor type exerts a distinct pharmacological profile as well as a unique cell type-specific expression pattern (2, 3). The expression of the δ-opioid receptor (DOR) is mainly confined to certain regions of the adult central nervous system, although it is found in the peripheral nervous system and some immune cells. It has been reported that the expression levels of DOR determine the activities of the DOR agonist, and the localization of DOR generally matches the pharmacological action sites of δ-opioids. Thus understanding the molecular mechanisms underlying the cell type-specific expression of DOR will provide insights into the regulation of the pharmacological benefits of δ-opioids by manipulation of the DOR expression levels in certain cell types.

We reported previously (4) the isolation of mouse genomic clones of DOR (mDOR), and we identified transcription upstream stimulatory factors Sp1/Sp3 and Ets-1 that functionally interact with the mDOR promoter and activate it in a mouse neuronal cell line NS20Y (5, 6). As yet, no data are available on the mechanisms involved in the cell type-specific expression of mDOR.

Methylation of the cytosine residue in the 5′-CpG-3′ sequence is an epigenetic modification involved in the establishment and maintenance of cell type-specific gene expression (7). Methylation patterns of tissue-specific genes are unequivocally different from tissue to tissue (7–9). Several genes with (G + C)-rich promoters have been shown to be regulated by CpG methylation. The mDOR gene core promoter has a G + C content of more than 80%, raising the possibility that the CpG methylation may play a role in the regulation of cell type-specific expression of mDOR. Here we report that the suppression of mDOR expression in Neuro2A cells is a result of promoter region CpG methylation and that Sp3 and MBD2 are involved in transcriptional repression of the mDOR promoter through binding of methylated CpGs and may play a role in mDOR cell type-specific expression.

MATERIALS AND METHODS

Cell Culture and 5-Aza-2′-deoxycytidine (AdC) Treatment of Cells—The 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. Schneider’s Drosophila line 2 (SL2) cells were grown at 22–24 °C in Schneider’s Drosophila medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (complete medium). For AdC treatment experiments, cells were split to low density (103 cells/well of a 6-well culture plate) 24 h before treatment. Cells were then treated with AdC (12 or 10 μM) or mock-treated with the same volume of phosphate-buffered saline for 72 h changing the medium every 24 h. Cells were harvested on day 4 for RNA determinations and Western blots.

RNA Isolation and Reverse Transcription-PCR—Total RNA was prepared using the TRIreagent Kit (Molecular Research Center) according to the manufacturer’s instructions. The reverse transcription reaction hydro-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; MBD, methyl-CpG-binding domain protein; MeCP, methyl-CpG-binding protein; HDAC, histone deacetylase; RT, reverse transcriptase.
was carried out using SuperScript II reverse transcriptase from Invitrogen following the recommendations of the manufacturer. The PCR settings were as follows: 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C for 30 cycles. The primer sequences for the amplification of mDOR and GAPDH fragments are shown in Table I (DOR and GAPDH, respectively). The products were resolved on a 1.2% agarose gel. The total density of the mDOR mRNA bands was calculated using Scion Image for Windows software from Scion Corp. and normalized to the density of the GAPDH bands.

In Vitro Methylation of Reporter Plasmid—Methylation Soel and HpaII were used to methylate mDOR promoter/luciferase reporter constructs following the recommendations of the manufacturer (New England Biolabs). Complete methylation was determined by digesting the DNA constructs with methylation-sensitive restriction enzyme HpaII (New England Biolabs) and running the products on an agarose gel. Only the DNA that was completely methylated was used.

DNA Methylation Assay—The construction of all luciferase fusion plasmids (pD262, pD1300, and pD262/141) and the Drosophila expression vectors (pPacSp1 and pPacSp3) used in this study has been described previously (5, 10, 11).

NS20Y and Neuro2A cells were plated 24 h prior to transfection at a density of 3 × 10^5 cells/well onto 6-well culture plates. Transfection was carried out using the SuperFect Transfection reagent or Effectene Transfection reagent (Qiagen) as described by the manufacturer. Cells were washed and lysed with lysis buffer (Promega) 48 h after transfection. To correct for differences in transfection efficiency, one-fifth molar ratio of a pCH110 plasmid (Amersham Biosciences) containing β-galactosidase gene under the SV40 promoter was included in the transfection and used as a normalization control. After incubation with the lysis buffer (48 h), reporter activity was normalized to protein concentration using a BCA protein assay kit (Pierce).

Preparation of Genomic DNA and Bisulfite Treatment of DNA—Genomic DNA from the NS20Y and Neuro2A cells was isolated using Wizard Genomic DNA Purification Kit (Promega) and linearized with the restriction enzyme EcoRI. Bisulfite treatment of DNA was carried out as described previously (12). Briefly, digested DNA (10 μg) was denatured in 0.3 M NaOH for 30 min at 37 °C and precipitated. Denatured DNA was then incubated with 3.1 M sodium bisulfite, 0.5 mM hydroquinone, pH 5.0, at 55 °C for 4 h under mineral oil. Bisulfite Sequencing and Methylation-specific PCR—Bisulfite-modified DNA was amplified by PCR. The primer sequences for the amplification of mDOR (bisulfite sequencing primers) are listed in Table I. PCR conditions are as follows: 94 °C for 2 min, after which 35 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min, and finally 10 min at 72 °C. The PCR mixture contained 1× PCR Buffer with MgCl₂ (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3) from Roche Applied Science, 10 pmol of each primer, and 0.2 mM dNTPs. After PCR amplification, the PCR products were purified using a gel extraction kit (Qiagen) and cloned into pcR2.1-TOPO vector (Invitrogen) according to the manufacturer’s instructions. Sequencing reactions were performed with Applied Biosystems model 377 DNA Sequencers by the Advanced Genetic Analysis Center at the University of Minnesota by using M13 reverse primer.

After bisulfite treatment, DNA was also amplified by methylation-specific PCR (13). Two pairs of PCR primers, as shown in Table I, were designed to amplify specifically methylated or unmethylated DNA using a web-based primer design program (14). The PCR mixture contained 1× PCR Buffer with MgCl₂ (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3) from Roche Applied Science, 10 pmol of each primer, and 0.2 mM dNTPs. PCR was carried out as follows: 2 min at 94 °C for one cycle, then 30 s at 94 °C, 30 s at 65 °C, and 1 min at 72 °C for 30 cycles. PCR products were visualized by running a 1.2% agarose gel.

Electrophoretic Mobility Shift Assay (EMSA)—Molar excess extracts were prepared by using method the described previously (15). The probes used were CG11 (15), a 1350-bp fragment containing 20 CGCG sites and 7 CCGG sites, and 262/141, a 122-bp fragment from 262 to 141 bp upstream of mDOR promoter region. Each probe was labeled using T4 polynucleotide kinase and [γ-32P]ATP. Assay conditions were as described previously (15), using 2 μg of Micrococcus lysodeikticus genomic DNA (Sigma) digested with Sau3AI (Roche Applied Science) as a non-specific competitor. For oligonucleotide competition analysis, a 100-fold (or as indicated in figures) molar excess of competitor oligonucleotides was also added to the mixture. For antibody supershift/abolation assays, the following pre-immune sera or antibodies were added to the mixture: mouse monoclonal antibody to MD2 (Imgenex) and Sp1 (sc-420X, Santa Cruz Biotechnology), goat polyclonal antibody to Sp3 (sc-644X, Santa Cruz Biotechnology), and goat pre-immune IgG (Santa Cruz Biotechnology) before the addition of probe to nuclear extracts. The DNA-protein complexes were visualized by autoradiography using a Storm 840 PhosphorImager (Amersham Biosciences).

Western Blot Assay—Whole cell extracts were prepared by resuspending cells in lysis buffer containing 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Triton X-100, and Complete Protease Inhibitor Cocktail (Roche Applied Science), and incubated on ice for 30 min. After centrifugation of the lysates at 12,000 × g for 5 min at 4 °C, supernatants (cell extracts) were resolved on denaturing polyacrylamide gels and transferred to nitrocellulose. Membranes were incubated with 5% milk in 0.1% TTBS (3.025 g/liter Tris, 8.76 g/liter NaCl, pH 7.6, 1 ml/liter Tween 20) overnight at 4 °C. The membranes were incubated with anti-Sp3 antibody (SC-644, Santa Cruz Biotechnology), goat pre-immune IgG (Santa Cruz Biotechnology), and rabbit alkaline phosphatase-conjugated secondary antibody (Bio-Rad) diluted in 0.1% TTBS (1:1000). The blots were washed and then incubated with 12 ml/liter alkaline phosphatase substrate buffer (Bio-Rad) for 2 h. After development, the blots were washed with water and then exposed to X-ray films. The following pre-immune sera or antibodies were added to the mixture: mouse monoclonal antibody to MBD2 (Imgenex) and Sp1 (sc-420X, Santa Cruz Biotechnology), goat polyclonal antibody to Sp3 (sc-644X, Santa Cruz Biotechnology), and goat pre-immune IgG (Santa Cruz Biotechnology) before the addition of probe to nuclear extracts. The DNA-protein complexes were visualized by autoradiography using a Storm 840 PhosphorImager (Amersham Biosciences).

RESULTS

Expression of mDOR in NS20Y and Neuro2A Cells—NS20Y and Neuro2A are mouse neuronal cells with different mDOR expression levels. Northern blot analysis demonstrated the presence of an mDOR transcript in NS20Y cells but not in Neuro2A cells (16). This expression profile was confirmed by RT-PCR analysis (Fig. 1A). Although a high level of mDOR mRNA was detected in NS20Y cells, only a low expression level was seen in Neuro2A cells. The detection of an mDOR transcript in Neuro2A cells indicates that these cells have no defect in the basal transcription machinery that is required for the expression of the mDOR gene. This is further confirmed by the results using a transient transfection assay, where the mDOR promoter construct pD262 was introduced into Neuro2A and NS20Y cells (Fig. 1B). In contrast with the data obtained by RT-PCR analysis (Fig. 1A), Neuro2A and NS20Y cells showed almost the same level of luciferase activity of the core promoter construct pD262.

Methylation Status of the mDOR Gene Promoter Region in NS20Y and Neuro2A Cells—Because we could not detect any obvious defect in the mDOR gene nor in the transcriptional
megalocytic, we explored the possibility that expression of mDOR in Neuro2A cells is epigenetically regulated by CpG machinery. Sequence analysis revealed that the promoter region of mDOR is (G + C)-rich with a putative CpG island between −491 and −50, relative to the ATG start codon (Fig. 2A, gray area). This region fulfills the criteria of a CpG island on the basis of its size (>200 bp), GC content (>50%), and CpG dinucleotide frequency (observed/expected >0.6).

CpG islands in several tissue-specific genes are often methylated in many human and mouse cell lines. Hypermethylation of normally unmethylated CpG islands correlates with transcriptional repression. To obtain information about the methylation status of the mDOR promoter region, bisulfite genomic sequencing was carried out (12). Genomic DNA isolated from NS20Y and Neuro2A cells was treated with bisulfite under the conditions described under “Materials and Methods.” Fig. 2B shows the methylation pattern of the mDOR core promoter region of the indicated cell lines. In NS20Y cells where prominent mDOR expression was detected, the mDOR promoter was completely unmethylated, whereas this gene was partially methylated in Neuro2A cells that showed a low level of mDOR expression. The occurrence of methylated CpGs in Neuro2A cells was less frequent than that of unmethylated CpGs.

We obtained results consistent with the above using methylation-specific PCR (13). As shown in Fig. 2C, the mDOR promoter was fully unmethylated in NS20Y cells, whereas both methylated and unmethylated alleles existed in Neuro2A cells. Most of the genomic DNA from Neuro2A was unmethylated as indicated by the amount of PCR product amplified.

These data showed a strong negative correlation between the methylation status of the mDOR promoter and mDOR gene expression in NS20Y and Neuro2A cells.

In Vitro Methylation of the Promoter Region Induces the Repression of the mDOR Promoter Activity—In order to determine whether the mDOR promoter activity is regulated by CpG methylation, reporter gene assays were performed. Luciferase fusion plasmids containing 1.3 kb (pD1300) or 262 bp (pD262) of upstream regulatory sequences of the mDOR gene (5) were methylated by either partial methylase HpaII or full methylase SssI in vitro and subsequently transfected into NS20Y cells. Partial methylation of the luciferase constructs induced about 60% reduction in promoter activity compared with the mock-methylated constructs, whereas full methylation of the constructs completely suppressed the promoter activity (Fig. 3), suggesting that methylation of the promoter can contribute to the repression of mDOR gene transcription in a methylation density-dependent manner.

Increase of mDOR Expression after AdC Treatment in Neuro2A Cells—To test further the potential role of DNA methylation in the regulation of cell type-specific mDOR expression, demethylation was induced by treating cells with a DNA methyltransferase inhibitor, AdC. Neuro2A cells were grown for 72 h in the presence of 2 or 10 μM AdC. This treatment increased the transcription of mDOR as shown by RT-PCR (Fig. 4, A and B), suggesting that the demethylation of the mDOR gene is critical for its expression.

Methylated mDOR Core Promoter Region Sequences Bind to a Methyl-CpG-binding Protein Complexes 1 (MeCP1)-like Complex in Nuclear Extracts from Neuro2A Cells—Direct binding of specific methyl-CpG-binding proteins to methylated DNA appears to be a major mechanism of methylation-induced transcriptional repression (17). Therefore, we examined whether the transcriptional repression conferred by the methylated mDOR promoter correlates with its affinity for methyl-CpG-binding proteins.

The known proteins and complexes that bind preferentially to methylated CpG dinucleotides include methyl-CpG-binding protein complexes 1 and 2 (MeCP1 and MeCP2) (17). MeCP1 was the first one to be identified as a nuclear factor that could discriminate between methylated and unmethylated DNA (15) and bind in vitro to DNA containing at least 12 symmetrically methylated CpGs, whereas MeCP2 could bind to a single methylated CpG. Because the mDOR promoter sequence has multiple CpGs that were found to be completely methylated in genomic DNA from Neuro2A cells in which the gene is repressed, we turned our attention toward MeCP1 as a possible mediator of transcriptional repression. End-labeled methylated CG11 probe (Me-CG11), which is known to bind to MeCP1 in vitro (15), was incubated with nuclear extracts from Neuro2A cells and then subjected to EMSA. On autoradiography, an MeCP1 complex with doublet DNA-protein bands was observed with Me-CG11 (Fig. 5A), suggesting that nuclear extracts from Neuro2A contain MeCP1 complex. The formation of MeCP1 complex in Neuro2A nuclear extracts was competed more effectively by Me-CG11 itself than by methylated mDOR promoter fragment (Me-262/141) but was not competed by a 100-fold molar excess of cold, unmethylated CG11 or 200-fold molar excess of cold, unmethylated mDOR promoter fragment (262/141). These data suggest that the binding of the MeCP1 complex depends not only on the DNA sequence but also on the methylation status of that sequence.

Because Me-262/141 effectively competed for MeCP1 complex binding by Me-CG11, it raises the possibility that it may directly form a complex with MeCP1. End-labeled Me-262/141 probes were used in an EMSA with Neuro2A nuclear extracts. A similar complex was observed with Me-262/141 (Fig. 5B), and this complex was competed effectively by cold Me-262/141 but not by unmethylated 262/141. In addition, this complex was effectively competed by Me-CG11, but not by unmethylated CG11. These results indicate that methylated mDOR promoter...
region DNA sequence Me-262/141 can form a protein complex that is similar, but not identical, to the MeCP1 formed by Me-CG11 after incubation with Neuro2A nuclear extracts. MeCP1 has been shown to contain MBD2 as a major DNA binding component to mediate the methylation-induced repression (18). We further determined whether the complex formed by Me-262/141 also contains MBD2. As shown in Fig. 6A, incubation with an anti-MBD2 antibody blocked the complex formation. These data further suggest that protein complex formed by Me-262/141 is MeCP1-like.

**Involvement of Sp3 in the MeCP1-like Complex Formed by Me-262/141**—Modification of DNA by methylation has been proposed to interfere with transcription directly by modifying the binding sites of transcription factors so that they can no longer bind their cognate sequences (19). Several transcription factors have been shown to bind to the mDOR promoter region and play a critical role in the regulation of mDOR promoter activity (Fig. 2A) (5, 6). Among these transcription factors, Sp1/Sp3 accounts for about 70% of the promoter activity in a transient transfection assay (5). To determine whether methylation can block Sp1/Sp3 binding to the methylated mDOR promoter, we incubated the end-labeled Me-262/141 with antibodies against Sp1 and Sp3. To our surprise, we found that the anti-Sp3 antibody, but not anti-Sp1 antibody, could ablate the MeCP1-like complex formation (Fig. 6B), indicating that Sp3 can still bind to the methylated mDOR promoter and may be involved in MeCP1-like complex formation in Neuro2A cells.

**A Role for Sp3 in Transcriptional Repression of Methylated mDOR Gene Promoter Activity**—The finding that Sp3 was involved in MeCP1-like complex formation in the gel shift exper-

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**Fig. 2. Methylation status of the promoter region of mDOR gene in NS20Y and Neuro2A cells.** A, 5'-flanking region of mDOR gene is GC-rich with a putative CpG island from -491 to -50. The top portion of the figure represents the quantitation of the CpG ratio (observed/expected) and percentage (G+C). The estimated values for CpG (% observed/% expected, indicated as trace b in the figure) and % GC (indicated as trace a in the figure) were plotted against the position in the analyzed sequence using the "CpG Plot/CpG Report" utility from the EMBL European Bioinformatics Institute. The putative CpG island was indicated as the gray area. The bottom portion of the figure represents the distribution of each CpG dinucleotide of the mDOR gene. The vertical lines indicate the position of each CpG site. The minimal promoter region (-262 to -141) was indicated by the horizontal line. Three known cis-elements (GC box, EBS, and E-box) were indicated by the open boxes. B, methylation status of the mDOR gene in NS20Y (the 1st row of squares) and Neuro2A (the rest of squares) determined by bisulfite genomic sequencing. Each row of squares represents a single cloned allele, and each square indicates a single CpG site at a specific location. Black squares correspond to methylated cytosines and open squares to unmethylated cytosines. The numbers in parentheses indicate the numbers of identical clones sequenced. C, amplification of bisulfite-treated DNA from NS20Y and Neuro2A cells by methylation-specific PCR (MSP). Primer sets used for amplification are designated as unmethylated (U) or methylated (M).
iments (Fig. 6B) prompted us to examine the Sp3 expression level in Neuro2A cells, with and without AdC treatment. We found all three isoforms of Sp3 were down-regulated after AdC treatment, and the levels of Sp3 proteins correlated with the levels of mDOR mRNA (Fig. 7A).

Following these results, we hypothesized that Sp3 may act to repress mDOR transcription by competing with Sp1 binding to the methylated mDOR promoter. To examine this hypothesis, we transfected Sp3 and Sp1 cDNA with methylated promoter constructs into SL2 cells, a Drosophila cell line without endogenous Sp proteins. Transfections into SL2 cells have been used to analyze activation or repression properties of mammalian transcription factors (20, 21). SL2 cells are particularly suited to this task because they are devoid of many ubiquitous mammalian transcription factor activities, and thus their transcrip-

**DISCUSSION**

The mDOR gene promoter resembles promoters for the so-called housekeeping genes, characterized by a (G+C)-rich region, multiple GC boxes, and a lack of TATA or CCAAT sequences. In view of this, the fact that the mDOR gene is expressed in a tissue- and cell type-specific manner is rather surprising, because the housekeeping genes are usually ubiquitously expressed. Previous studies demonstrated that the mDOR promoter region from −262 to −141 containing Sp1/
Sp3, upstream stimulatory factor, and Ets-1-binding sites is critical for its basal promoter activity (5, 6). Given that these transcriptional factors are also ubiquitously expressed, the mechanisms involved in the regulation of cell type-specific expression of mDOR are not clear. We undertook the present study to determine whether methylation provides a molecular mechanism by which the mDOR gene is silenced in certain cells. In general, hypermethylation of normally unmethylated CpG islands correlates with transcriptional repression, leading to inactivation of gene expression. Here we provide evidence that mDOR expression is repressed by promoter region DNA methylation in Neuro2A cells.

An inverse correlation between promoter region methylation and mDOR gene expression levels was observed in two types of neuronal cell lines, NS20Y and Neuro2A. In vitro methylation of the CpGs in the core promoter region of mDOR gene reduced the promoter activity in a transient transfection assay (Fig. 3). Furthermore, demethylation with AdC increased the mDOR mRNA level in Neuro2A cells (Fig. 4). These results satisfy two well known criteria for a role for DNA methylation in cell type-specific gene repression: (i) the promoter of mDOR is densely methylated in low-expressing cells, and (ii) induced demethylation results in activation of mDOR. It is noticed that the elevated mDOR mRNA level in AdC-treated Neuro2A cells is not as high as that in the high expressing NS20Y cells. This would indicate that demethylation alone is not sufficient to release repression of mDOR in Neuro2A cells. Our subsequent observation of Sp3 being involved in the repression is consistent with this notion. We conclude that the transcriptional repression of mDOR gene is possibly mediated by multiple factors in Neuro2A cells, and promoter region CpG methylation is one of them.

The connection between the methylation status of gene promoters and the transcriptional activity of the respective genes has been established (9). Several mechanisms have been proposed for the methylation-induced gene repression. Among these, direct binding of methylation-specific repressors appears to be a major mechanism of transcriptional repression (22). A conserved family of ubiquitously expressed proteins that selectively bind methylated CpGs has been identified (23). They bind methylated CpG sites with little apparent specificity for flanking nucleotide sequences. MeCP1 was the first one to be identified as a nuclear factor that could discriminate between methylated and unmethylated DNA (15) and repress transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes (24). Here we show that a similar methylated DNA binding complex forms efficiently with the methylated mDOR promoter sequence by using nuclear extracts from Neuro2A cells. Furthermore, blocking of MBD2
with anti-MBD2 antibody nearly abolished the formation of MeCP1-like complex (Fig. 6A). Our results confirm the previous report showing MBD2 as a component of the MeCP1 or related complexes, and support the notion that MBD2 is responsible for the methyl-CpG-binding activity of the MeCP1. Full characterization of these large protein complexes is needed to determine whether the complex formed between the methylated mDOR promoter and Neuro2A nuclear extracts is identical to MeCP1. In addition, whether this MeCP1-like complex blocks transcription in this system will require further investigation.

Alternatively, it is possible that methylation of the mDOR gene promoter directly interferes with the binding of one or more specific transcriptional activating factors that have CpGs in their DNA recognition sites. Methylation of specific binding sites for certain transcription factors, e.g. e-Myc, can block their binding to these sites in vitro (19, 25). On the other hand, the general transcriptional factor Sp1 binds equally well to methylated and non-methylated sites (26) and stimulates transcription from both methylated and non-methylated templates (27). These are consistent with our cotransfection study using Drosophila SL2 cells, which do not have endogenous Sp proteins. As shown in Fig. 7B, Sp1 alone could activate both the methylated and unmethylated mDOR luciferase constructs in a similar extent. However, when Sp1 cotransfected with Sp3, increasing Sp3 levels inhibited the Sp1-mediated promoter activity of methylated pD262 construct. Furthermore, our EMSA study also showed the CpG methylation blocked the binding of Sp1, but the binding of Sp3 to methylated promoter fragment was not affected (Fig. 6). This is surprising, because Sp1 and Sp3 share similar domain structures with three zinc fingers and bind specifically to the same GC-rich elements such as the GC box (GGGGCCGGG) (28). Our previous data also demonstrated that Sp1 and Sp3 could bind to the same non-methylated DNA sequence from mDOR promoter region (5). One possible explanation is that Sp3 has a higher binding affinity to the methylated mDOR promoter than Sp1. Because Sp1 and Sp3 compete for the same binding site within mDOR promoter region, increased binding affinity of Sp3 to the methylated mDOR promoter could then hinder the binding of Sp1 to the same methylated DNA sequence. How Sp3 has a higher affinity to methylated mDOR sequence than Sp1 is not known. In our current study, because Sp3 and MBD2 coexisted in the same MeCP1-like complex, and blocking of either Sp3 or MBD2 with antibody nearly abolished the complex formation (Fig. 6), we speculate that Sp3 may interact with certain methylation-specific binding proteins like MBD2 to stabilize its binding to the methylated DNA sequence. The exact mechanism requires further investigation.

Several members of the Sp family, including Sp1, Sp2, Sp3, and Sp4, are generally known as activators of gene transcription, whereas Sp3 is also considered to be a repressor of gene transcription (28). It has been shown that Sp3 represses gene expression via the titration of the Sp1/Sp3 ratio (29–31). In the present study, cotransfection of Sp3 with methylated mDOR promoter constructs could repress the Sp1-mediated promoter activation in SL2 cells (Fig. 7B). Our results support the idea that increased binding affinity of Sp3 to the methylated DNA sequences results in the change of Sp1/Sp3 ratio bound to the mDOR promoter region, leading to the repression of Sp1-mediated activation. On the contrary, cotransfection of Sp3 with unmethylated mDOR constructs increased the promoter activity. These suggest that methylation of DNA is required for repression by Sp3. Different functions of Sp3 on the methylated and unmethylated mDOR may also come from the interactions between Sp3 and some methylation-related transcription factors such as MBDs and HDACs. It has been shown that Sp3 can recruit HDAC (32), which can then modify the regional chromatin structure of a gene and repress gene expression. Whether the Sp1/Sp3 ratio or the interaction between Sp3 and MBD/HDAC is involved in the Sp3-mediated repression of mDOR remains to be determined.

Because the known MBD proteins bind methylated DNA with little sequence specificity, the mechanisms involved in the recruitment of these proteins to particular regions in the genome are unknown (22). Our current study demonstrated for the first time that the sequence-specific transcription factor Sp3 is involved in the formation of the methylated transcriptional protein complex MeCP1 in Neuro2A cells. It may interact with the methyl-CpG-binding proteins and direct them to certain genes with sequence selectivity and methylation sensitivity.

In summary, our experiments demonstrate that mDOR gene expression is repressed by CpG methylation in Neuro2A cells. Sp3/MBD2 binding to the mDOR promoter region might be involved in transcriptional repression of the methylated gene in Neuro2A cells and play a role in the regulation of cell type-specific expression of mDOR.

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