

Up-Regulation of the μ -Opioid Receptor Gene Is Mediated through Chromatin Remodeling and Transcriptional Factors in Differentiated Neuronal Cells

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ABSTRACT

The effects of morphine are mediated mainly through the μ opioid receptor (MOR). Expression of the MOR is up-regulated during neuronal differentiation in P19 embryonal carcinoma cells and epigenetic changes play an important role in MOR up-regulation. This study investigates the basis for differentiation-dependent alterations of MOR chromatin by studying the recruitment or dissociation of several factors to the remodeled chromatin locus. Chromatin immunoprecipitation assays were used to demonstrate the recruitment of the transcriptional activator Sp1 and the chromatin remodeling factors Brg1 and BAF155 to this promoter, as well as the dissociation of repressors [histone deacetylases, mSin3A, Brm, and methyl-CpG-binding protein 2 (MeCP2)]. Histone modifications (acetylation, induction of histone H3-lys4 methylation, and reduction of H3-lys9 methylation) were

consistently detected on this promoter. Overexpression of Sp1 strongly enhanced MOR promoter activity, and the histone deacetylase inhibitor trichostatin A also increased promoter activity. In vitro DNA CpG-methylation of the promoter partially blocked binding of the Sp1 factor but induced MeCP2 binding. Coimmunoprecipitation studies also found novel evidence of an endogenous MeCP2 interaction with Sp3 but a weaker interaction with Sp1. Overall, the results suggest that during neuronal differentiation, MeCP2 and DNA methylation mediate remodeling of the MOR promoter by chromatin remodeling factors (Brg1 and BAF155) from a compacted state to a conformation allowing access for transcriptional factors. Subsequent recruitment of the activating transcription factor Sp1 to the remodeled promoter results in MOR up-regulation.

Opioid drugs, including endogenous opioid peptides and the analgesic drug morphine, exert their pharmacological and physiological effects through binding to opioid receptors. The three major types of opioid receptors (μ , δ , and κ) all belong to the G protein-coupled receptor superfamily. On agonist binding, these receptors couple to G proteins and affect several signal transduction pathways, including inhi-

bition of adenylyl cyclases and Ca^{2+} channels, activation of inward-rectifying K^{+} channels, transient increases of intracellular Ca^{2+} levels, and activation of phospholipase C and the mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 (Law et al., 2000).

The μ opioid receptor (MOR) is expressed mainly in the central nervous system with densities varying greatly in different brain regions displaying different functional roles (Mansour et al., 1995). The MOR-expressing brain regions are involved in motivating and rewarding behaviors mediated by opiates and other drugs of abuse. Furthermore, response to morphine varies in different neuronal cells and brain regions depending on receptor levels. Several studies suggest that the MOR plays a key role in mediating the major

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ABBREVIATIONS: MOR, μ opioid receptor; MeCP2, methyl-CpG-binding protein 2; RA, all-*trans* retinoic acid; TSA, trichostatin A; UD, undifferentiated; AP2d, intermediately differentiated; AP4d, fully differentiated; MNase, micrococcal nuclease; ChIP, chromatin immunoprecipitation; Brm, Brahma protein; Brg1, Brm-related gene 1; BAF155, Brg1-association factor 155; HDAC, histone deacetylase; PP, proximal promoter; DP, distal promoter; AS, antisense; S, sense; bp, base pair(s); PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; LM-PCR, ligand-mediated polymerase chain reaction; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; Pol II, polymerase II.

clinical effects of morphine and the development of tolerance and physical dependence on prolonged administration (Kieffer and Evans, 2002; Law et al., 2004). The pharmacological effects of morphine are blocked in MOR knockout mice (Matthes et al., 1996; Sora et al., 1997; Loh et al., 1998), suggesting that the *in vivo* activities of morphine depend on the levels of the MOR. These observations suggest that the regulation of MOR levels has a critical role in the cellular responses to long-term drug exposure.

MOR expression is also regulated temporally. In mice, MOR can be detected as early as embryonic day 8.5. After embryonic day 8.5, MOR expression increases significantly throughout development and reaches maximal levels in the adult (Zhu et al., 1998; Ko et al., 2002). Similar time-dependent regulatory patterns of MOR expression are seen in mouse embryonal carcinoma P19 cells during neuronal differentiation, increasing gradually to a maximum at day 4 of differentiation (Chen et al., 1999; Hwang et al., 2007). Overall, MOR expression seems to be regulated both spatially and temporally by finely tuned mechanisms.

MOR activity is regulated at different levels, including epigenetic (Hwang et al., 2007, 2009), transcriptional (Wei and Loh, 2002; Law et al., 2004), posttranscriptional (Pan et al., 2001; Choi et al., 2006; Kim et al., 2008), translational (Song et al., 2007, 2009a,b) and even at the protein level by receptor phosphorylation and desensitization (Arden et al., 1995; El Kouhen et al., 2001). The MOR gene contains many specific regulatory elements upstream of the promoter region, including regions mediated by Oct-1 (Liang and Carr, 1996), PU.1 (Hwang et al., 2004), interleukin-4 (Kraus et al., 2001), Sox (Hwang et al., 2003), Sp1 (Ko et al., 1998), nuclear factor- κ B (Kraus et al., 2003), cAMP response element-binding protein (Lee and Lee, 2003), Poly(C) binding proteins (Choi et al., 2007, 2008), and neuron-restrictive silencer factor (Kim et al., 2004, 2006). Although the above several studies have reported that the MOR gene is regulated by various *cis*- and *trans*-acting factors, critical questions remain unanswered, such as changes in the chromatin conformation of the endogenous MOR gene locus in a physiologically relevant cell model, and the dynamics of transcription factors and chromatin remodelers during the activation process of this gene during neuronal differentiation.

Epigenetic features such as DNA methylation and histone modifications in particular play an important role in MOR gene activation in mouse P19 cells undergoing neuronal differentiation (Hwang et al., 2007). In undifferentiated P19 cells, DNA hypermethylation of the MOR promoter and interactions with methyl-CpG-binding protein 2 (MeCP2) down-regulate the MOR gene at the transcriptional level. Likewise, the histone proteins of this chromatin region are mostly deacetylated, indicative of a heterochromatin structure. In differentiated neurons, the level of DNA methylation is reduced, and histone acetylation is correspondingly increased on the MOR promoter, thereby activating its transcription. Therefore, MOR gene-silencing is repressed primarily at the proximal promoter (PP) by MeCP2 in a methylation-dependent manner; its activation requires an epigenetic change elicited by DNA demethylation.

This study examined whether DNA methylation and histone modifications of the MOR promoter region are concurrent with chromatin remodeling of the promoter region to provide access for transcription factors to the DNA. The

chromatin structure of the MOR promoter in P19 cells was examined during neuronal differentiation to determine whether differentiation induces chromatin remodeling of the promoter and, if so, which of the factors were involved. The results indicate that differentiation induces recruitment of chromatin remodeling factors to the promoter and transcription factor involvement, ultimately leading to histone dissociation and subsequent activation of transcription.

Materials and Methods

Cell Culture and Transfection. The procedures to differentiate P19 cells have been described previously (Chen et al., 1999; Hwang et al., 2007). In brief, P19 cells were cultured in minimal essential medium containing 7.5% newborn calf serum and 2.5% fetal calf serum at 37°C in a humidified atmosphere of 5% CO₂. Cells were aggregated in bacteriological Petri dishes in minimal essential medium containing 5% heat-inactivated fetal bovine serum and 0.5 μ M all-*trans* retinoic acid (RA) for 2 days; the treatment was repeated for another 2 days. After aggregation, the cells were dissociated using DNase I and trypsin/EDTA and plated in tissue culture dishes in the absence of RA. Cytosine arabinoside (10 μ M) was added to the culture medium 24 h after the cells were plated to maintain only postmitotic neuronal cells and to eliminate proliferating glial cells. Cells were harvested at different time points for protein, DNA, and total RNA purifications. Treatments with 100 nM trichostatin A (TSA) (Sigma-Aldrich, St. Louis, MO) for 24 h were performed as described previously (Hwang et al., 2007).

Neuroblastoma NMB cells were grown in RPMI 1640 medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in an atmosphere of 5% CO₂/95% air at 37°C. Transfections into NMB cells for promoter analyses were performed as described previously (Hwang et al., 2003). MOR promoter plasmid (p189) contains the DNA sequence from -450 to -189 bp upstream of the mouse MOR gene (relative to the translation start site at +1) in the luciferase reporter plasmid, pGL3-basic (Promega, Madison, WI).

Preparation of Nuclei and Nuclease Digestion. These procedures were carried out as described previously (Gui and Dean, 2001; Park et al., 2005) with some modifications. Undifferentiated and differentiating P19 cells (1–4 days after plating) were cross-linked with 1% formaldehyde for 10 min followed by the addition of 1.47 ml of 1 M glycine per 10 ml of culture medium to stop cross-linking. Populations consisted of undifferentiated (UD), intermediately differentiated (2 days after plating; AP2d), or fully differentiated (4 days after plating; AP4d) P19 cells. Nuclei isolated from approximately 10⁸ cells (from four 10-cm dishes) were suspended in 1 ml of wash buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, and 8.5% sucrose) and digested with micrococcal nuclease (MNase; 20–120 U; Worthington Biochemical, Freehold, NJ) or DNase I (50–100 U/ml; Invitrogen) for 6 min at 37°C. Reactions were stopped by adding 100 μ l of 5 M NaCl and 100 μ l of stop solution (10% SDS, 125 mM EDTA, and 1 mg/ml proteinase K) and incubating for 3 h at 50°C. The temperature was increased to 65°C for at least 4 h to reverse cross-linking. Purified genomic DNA was analyzed for MNase digestion by Southern blot hybridization using PCR-generated probes (1, 2, and 3; Fig. 1B) labeled with [α -³²P]dCTP by the random priming method.

Ligation-Mediated PCR. The procedures were performed as described previously (Park et al., 2005; Carey et al., 2009) with minor modifications. The primer and linker oligonucleotides used are listed in Table 1. For nucleosome mapping, 1 μ g of MNase-digested DNA was phosphorylated and then ligated with annealed linker DNA (linkers 1 and 2) overnight at 16°C. Ligated DNAs were amplified with PCR using primer 1 and linker 1, as follows: 25 cycles, 1 min (4 min for the first cycle) at 95°C, 2 min at 63°C, 3 min at 76°C, and an additional 5-min extension at 76°C. Labeling was accomplished by adding [γ -³²P]ATP-3'-end-labeled primer 2 to the PCR reaction and

performing three more cycles as follows: 1 min (4 min for the first cycle) at 95°C, 2 min at 68°C, and 10 min at 76°C. Amplified and labeled DNA fragments were resolved in a 6% polyacrylamide/urea gel followed by exposure to a PhosphorImager screen (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Ligand-mediated PCR (LM-PCR) using DNase I-digested nuclei was performed as described previously (Park et al., 2005).

PCR-Based Survey of Nucleosomes on the MOR Promoter. Nuclei were treated with 120 U of MNase at 37°C for 30 min. Genomic DNAs corresponding to a mononucleosome (i.e., a 147-bp monomer) were cut from a 1.4% agarose gel and purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA). The purified DNA was identified and quantified on an agarose gel. PCR amplification was performed by 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C) using several primer sets (Table 1). Purified monomers from UD, AP2d, and AP4d cells were used as template DNAs for real-time quantitative PCR with primer sets designed to detect the position of nucleosome N1.

A Micrococcal nuclease-Southern blot assay

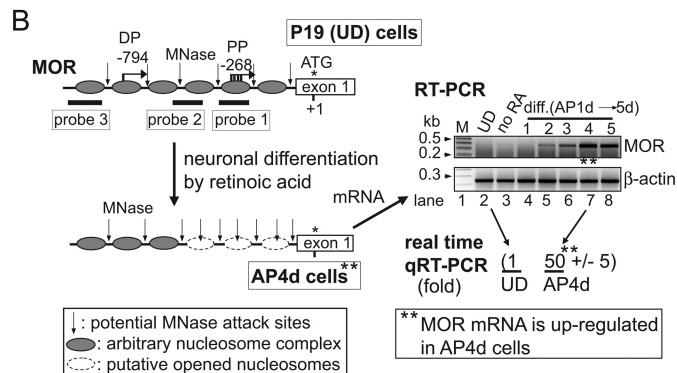
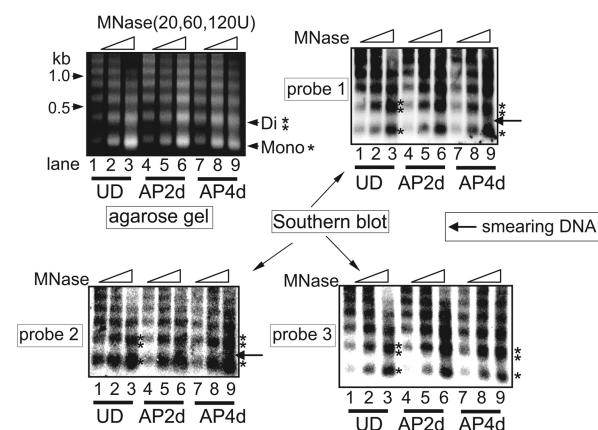


Fig. 1. MNase-Southern blot analyses. A, nuclei were isolated from UD, AP2d, or AP4d cells and treated with different concentrations of MNase. Top left, ethidium bromide-stained agarose gel of isolated genomic DNA. Southern blots using α - 32 P-labeled probes as indicated are also shown. Asterisks (one or two) indicate mono- or dinucleosomal DNAs, respectively, in UD or AP4d cells. Arrows indicate smeared DNA in AP4d cells using probes 1 and 2. B, schematic of the MOR gene promoters (DP and PP) and the first exon. The minor DP (-794, relative to the translation start codon at +1) has a single transcription initiation site (Ko et al., 1997), whereas the major PP has four (Min et al., 1994). Locations of the probes used in A are indicated. The downward arrows represent potential MNase attack sites. Right, enhanced expression of the MOR gene in differentiating P19 cells. Levels of MOR mRNA were determined at different developmental stages induced by RA using RT-PCR and real time qRT-PCR. The identities of the PCR products were confirmed by sequencing. Lane 1 (M), 1-kb-plus size marker (Invitrogen); lane 2, UD cells (control); lane 3, P19 cells cultured in parallel without *trans*-RA treatment; lanes 4 to 8, P19 cells cultured 1 to 5 days after plating (AP).

Electrophoretic Mobility Shift Assay. Nuclear extracts from P19 cells were prepared using a modification of Dignam's procedure (Dignam et al., 1983; Hennighausen and Lubon, 1987). A DNA fragment covering the MOR promoter was amplified by PCR using primers S-606 and AS-210 (Table 1) and P19 genomic DNA as a template. The resulting PCR product (Me-1 probe) was confirmed by agarose gel electrophoresis and DNA sequencing. Methylated DNA probe (methyl Me-1) was generated by a CpG methyltransferase reaction (M.SssI; New England Biolabs). Both methyl Me-1 and unmethylated Me-1 probes were end-labeled separately with [γ - 32 P]ATP. End-labeled DNA probes were incubated with nuclear extracts in a final volume of 20 μ l of electrophoretic mobility shift assay (EMSA) buffer [10 mM Tris-HCl, pH 7.5, 5% glycerol, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mg/ml poly(dI-dC)] at room temperature for 30 min. For DNA competition analyses, a 100-fold molar excess of unlabeled competitor was added to the mixture before adding the probe. To detect a specific factor's complex, samples were incubated with 1 μ l of each antibody for 30 min before adding the probe. The reaction mixtures were electrophoresed in a nondenaturing 4% polyacrylamide gel in 0.5 \times buffer (45 mM Tris borate and 1 mM EDTA) at 4°C and visualized by PhosphorImager scanning (Storm 860; GE Healthcare). Anti-MeCP2 and anti-Sp1 antibodies were purchased from Abcam Inc. (Cambridge, MA) and Millipore (Billerica, MA), respectively.

Reverse Transcription-PCR and Real-Time Quantitative RT-PCR. Total RNA was isolated using TRI Reagent and analyzed by reverse transcription-polymerase chain reaction (RT-PCR) using the MOR gene-specific primers mMOR-S1 and mMOR-AS1 (covering exons 1 and 2, respectively; see Table 1) (Hwang et al., 2007). RT-PCR was performed using a QIAGEN OneStep RT-PCR kit. A similar reaction was carried out using primers for β -actin as an internal control (Hwang et al., 2007). PCR products were analyzed by electrophoresis on 2% agarose gels.

For quantitative RT-PCR (qRT-PCR), 5 μ g of total RNA was treated with DNase I and reverse-transcribed using reverse transcriptase (Roche Diagnostics, Indianapolis, IN) and primers combined with oligo(dT) and random hexamer. One fortieth of this reaction was used for real-time qRT-PCR analysis of gene expression. Real-time qPCR was performed in an iCycler (Bio-Rad Laboratories, Hercules, CA) using SYBR Green I (Quantitect SYBR Green PCR kit; QIAGEN). The primer sequences used were MOR_E3-S and MOR_E4-AS, covering exons 3 and 4, respectively (Table 1). The specificity of RT-PCR primers was determined using a melt curve after the amplification to show that only a single species of qPCR product resulted from the reaction. Single PCR products were also verified on an agarose gel. The RT-PCR and real-time qRT-PCR experiments were repeated two or three times to obtain statistical significance.

Coimmunoprecipitation and Chromatin Immunoprecipitation Assays. Coimmunoprecipitation was performed as described previously (Kim et al., 2006; Hwang et al., 2009). In brief, P19 cells were lysed in a buffer containing 1% Triton X-100, 10 mM Tris, 5 mM EDTA, 50 mM NaCl, and 50 mM NaF supplemented with protease inhibitors (Roche Diagnostics). Approximately 1 mg of each clarified cell lysate was incubated overnight at 4°C with antibodies. Immunoprecipitates were recovered on protein G-agarose beads, washed extensively, and separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P (polyvinylidene difluoride; Millipore) membranes for Western blotting.

Chromatin immunoprecipitation (ChIP) assays were performed as reported previously (Hwang et al., 2004, 2007; Kim et al., 2004). A total of 10^6 cells was used for each ChIP assay. Cells were cross-linked with 1% formaldehyde. Sonicated cell extracts, normalized to contain equivalent amounts of protein, were precipitated with 2 μ g of antibody at 4°C overnight, followed by the addition of protein G beads for 1 h. Two percent of each lysate was reserved as the input (i.e., preprecipitation control), and the residual lysate was subjected

to immunoprecipitation. The antibodies used were against MeCP2 (kindly provided by Dr. Weidong Wang, National Institutes of Health, Bethesda, MD); Sp1, Brg1-association factor 155 (BAF155), mSin3A, and RNA polymerase II (Pol II) (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA); acetylated histone H3, acetylated histone H4, Lys4 dimethylated histone H3, Lys9 dimethylated histone H3 Brm-related gene 1 (SNF2 β /Brg1), and histone deacetylase 1 (HDAC1) (all from Millipore); and Brahma protein (SMARCA2/Brm) and histone deacetylase 3 (HDAC3) (all from Abcam). All ChIP assays were controlled by performing parallel pull-down experiments with either no antibody, normal rabbit serum, or nonspecific Gal4 antibody (Santa Cruz Biotechnology). Each immunoprecipitated DNA sample was analyzed by PCR using the indicated PCR primers (Table 1 and Figs. 4 and 5). Each ChIP experiment was repeated at least twice.

Western blot analyses were performed on protein samples prepared as described previously (Hwang et al., 2007). Proteins transferred to Immobilon-P membranes were incubated with primary antibodies and detected using an ECF Western detection kit (GE Healthcare). The antibodies used were anti-MeCP2 (Abcam), Anti-Sp1 (Millipore), anti-Sp3 (Santa Cruz), and anti- β -actin (Cell Signaling Technology, Danvers, MA). The membrane was scanned using a PhosphorImager (GE Healthcare).

Results

Chromatin Structure of the Mouse MOR Gene. To examine the chromatin structure of MOR promoter before and after neuronal differentiation, limited MNase digestion coupled with Southern blot analyses were performed to map the location of nucleosomes in this region (Fig. 1). Nuclei isolated from UD, AP2d, and AP4d cells were treated with increasing amounts of MNase (Fig. 1A, top left). Southern blots using probes 1 and 2 (covering the PP and its upstream region, respectively; Fig. 1A, top right, bottom left) detected smearing of MOR chromatin in AP4d (lane 9 of each) but relatively regular nucleosome arrays in both UD and AP2d samples (lanes 3 and 6, respectively, of each). However, nu-

cleosomal arrays covering the distal promoter (DP; detected by probe 3) were not significantly different between the samples (Fig. 1A, bottom right). These data show that the chromatin structure of the MOR PP region becomes dissociated or opened in differentiated cells compared with undifferentiated or partially differentiated cells. RT-PCR analyses using primers covering exons 1 and 2 revealed that transcription of MOR mRNA resulting from the chromatin structure alterations increased gradually up to day 3 (Fig. 1B, bottom right, lanes 2–6), reached a maximum on day 4 (lane 7), and stayed at the same level on day 5 (lane 8), as described previously (Hwang et al., 2007), whereas β -actin mRNA was relatively constant. These results indicate that changes in the chromatin structure of the MOR promoter in differentiated neuronal cells lead to increased MOR transcription. The increased MOR expression was also confirmed by real-time qRT-PCR using UD and AP4d cells showing approximately 50-fold increase in AP4d compared with UD cells (Fig. 1B).

MNase Hypersensitivity of the MOR Gene Promoter Region. To map the location of nucleosomes on the MOR promoter, LM-PCR was performed using specific sets of primers and linkers to amplify MNase-treated P19 genomic DNA (Fig. 2A). As the concentration of MNase increased from 20 to 60 units, the LM-PCR produced a band of ~142 bp from the P19 cells (lanes 6 and 7), whereas nonspecific or weaker bands lower than the 142-bp band were detected throughout all the samples. These nonspecific bands were also detected with differing degrees of intensity in control samples using naked DNA treated with increasing amounts of MNase, suggesting that they were generated by nonspecific PCR reactions. Negative control experiments (i.e., no template) showed no bands (lane 12). Taking the 25-bp linker into account, the 142-bp PCR product contains a 117-bp fragment derived from nucleosome N1. This fragment mapped to the 5' border of N1, at the -345 position relative to the translation initiation site (Fig. 2A). The 142-bp product seen in undiffer-

TABLE 1

List of primers used in this study

The location (starting at the 5' end) of each primer is designated relative to +1 (as the ATG start codon).

Name	Primer (5' → 3')	Location	Notes
mMOR-S1	CCCTCTATTCTATCGTGTGTGT	+218	Located at exon 1
mMOR-AS1	AGAAGAGAGGATCCAGTTGCA	+568	Located at exon 2
MOR_E3-S	CATCAAGCACTGATCAGCATTC	+903	Located at exon 3
MOR_E4-AS	TAGGGCAATGGAGCAGTTTCTGC	+1196	Located at exon 4
AS-210	CCACCTTAGAAGTACACGGAGGC	-210	LM-PCR
AS-233	GCATCCTTAGCATCCCCAAAGCGCCACTCT	-233	LM-PCR
AS-260	TCTCTGAGTGAAGGAGGGAGCAT	-260	LM-PCR, L2 ^a
AS-288	CTGTGAGGGATCCAGAGGCTAGAGGGGAGG	-288	LM-PCR
Linker 1	GCGGTGACCCGGGAGATCTGAATTC		
Linker 2	GAATTCAGATC		
S-731	CTTTGAACAGGTTTGTGGGGTTG	-731	N3 ^a
AS-623	TTACCCACATCCCCATATCTGA	-623	N3 ^a
S-606	CCCTCCAGTCCTAATTAATTGC	-606	L3 ^a
AS-473	TCTTTGTAACAACCATGCATTAGGT	-473	L3 ^a
S-497	ACCTAATGCATGGTTGTACAAAG	-497	N2 ^a
AS-393	AAGGAGTGTAATTTGGGGTAGG	-393	N2 ^a
S-381	GCATAGAGACTGAGGAGGCTGATTCTG	-381	L2 ^a
S-342	CACAATCCACTCCTTCTCTCCTC	-342	N1 ^a
AS-229	AGGCGCATCCTTAGCATCCCCA	-229	N1 ^a
S-328	TCTCTCTCCTCCCTCCCTCTAGCCTCTGG	-328	L1 ^a
AS-178	GTCCGATATTCTCCTCTGCTGTAG	-178	L1 ^a
AS-182	GATATTCTCCTCTGCTGTAGCCC	-182	
S-408	CCAATTTACACTCCTTTACACGGAAGT	-408	Real-time qPCR primer for MOR
AS-285	GGGCTGTGAGGATCCAGAGGCTAG	-285	Real-time qPCR primer for MOR

S, sense; AS, antisense.

^a Names of products from nucleosome-specific PCR (see Fig. 3A).

entiated P19 cells decreased in cells undergoing neuronal differentiation (asterisks, lanes 9 and 11). It is possible that less PCR product was generated from these samples because dissociation of the nucleosome increased the ability of MNase to digest chromatin in this region (compared with nucleosome-compacted regions).

Another LM-PCR using primer set B (located upstream of primer set A) identified a specific band at 82 bp in the UD sample (Fig. 2B, lane 2), which calculated to the same position at the 5'-border of N1 shown in Fig. 2A. This band also decreased in intensity as neuronal differentiation occurred. It is noteworthy that this N1 region contains a known core promoter site for the MOR gene that has minimal promoter activity by itself (Ko et al., 1997).

The nonspecific bands in Fig. 2B (arrowhead) were similar in intensity for all the three samples, suggesting equivalent amounts of starting chromatin input for the LM-PCR. However, in Fig. 2A the intensity of the nonspecific bands varied somewhat (arrowheads). To confirm that the same amount of input was used for each sample, semiquantitative PCR was performed. Intact chromatin DNA was isolated from the same samples used for LM-PCR, diluted serially, and used as PCR templates with two sets of primers covering the DP upstream region and the PP region. The PCR bands gener-

ated were similar for each dilution of DNA with either primer regardless of stage of differentiation, confirming that nearly equivalent amounts of chromatin were used for the above LM-PCR (Fig. 2A, top right).

DNase I-mediated LM-PCR analysis was performed because differential characteristics of enzymatic cleavage on chromatin between DNase I and MNase have been described previously (Carey et al., 2009). The location of the 5'-border of nucleosome N1 at position -346 (Fig. 2C, lanes 1 and 2) is almost the same as that seen in results from MNase-mediated LM-PCR (Fig. 2, A and B). In addition, a reduction in band intensity similar to that seen in MNase-mediated LM-PCR was observed in differentiated cells (lanes 3 and 4, asterisks) relative to undifferentiated cells (lanes 1 and 2), again suggesting a higher level of nucleosome dissociation in the differentiated cells.

When P19 cells were treated with the HDAC inhibitor TSA, nucleosome N1 seemed to dissociate and shift from its original position to position -262 (Fig. 2D, lane 5), suggesting that nucleosome N1 behaves differently in TSA-treated UD cells and differentiated cells. This might depend on whether histone acetylation (induced by the HDAC inhibitor) acts alone or whether other epigenetic features (e.g., DNA demethylation and histone acetylation seen in differentiated

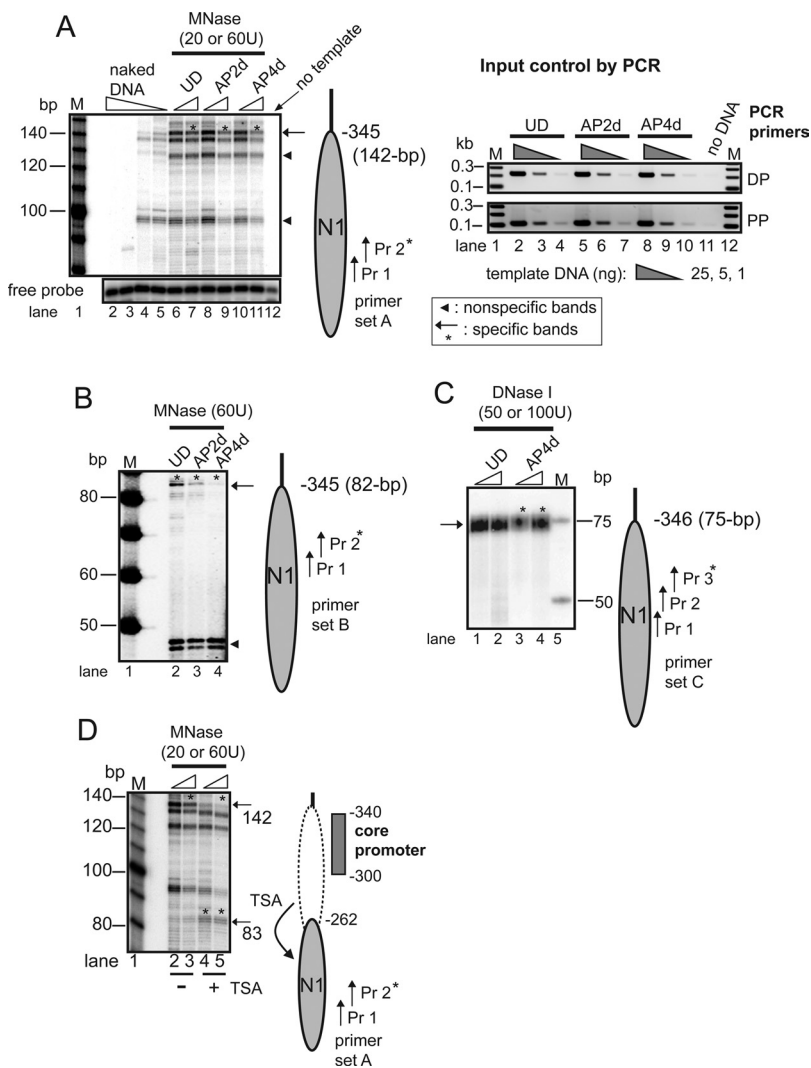


Fig. 2. Mapping of the nucleosome sites in the MOR promoter. A, MNase-mediated LM-PCR analysis. Left, nuclei isolated from UD, AP2d, and AP4d cells were treated with MNase. Primer (Pr) set A (Pr 1, AS-210; Pr 2, AS-233) and the N1 location are illustrated (right); Pr 2 starts at position -233 relative to the translation site and is end-labeled with γ - 32 P. The amplified fragments extend 142 bp to the 5'-border of N1 (position -345 relative to the translation site). Controls include purified chromosomal DNA (naked DNA) treated with 100, 25, 5, or 1 U MNase (lanes 2-5, respectively). Free probe was also present in all samples (below, indicating equal amounts of labeled primers). Right, genomic nuclear DNA analyzed by semiquantitative PCR show equal amounts of input DNA used for MNase-mediated LM-PCR analysis. Input DNA was diluted serially. PCR primers (Table 1) were chosen to detect either distal (DP-MOR: S-1318 and AS-1099) (Hwang et al., 2007) or proximal (PP-MOR: S-342 and AS-229) MOR promoter regions. B, LM-PCR analysis with primer set B (Pr 1, AS-260; Pr 2, AS-288 end-labeled with 32 P). Nuclei were treated with 60 U MNase. C, DNase I-mediated LM-PCR analysis with primer set C (Pr 1, AS-260; Pr 2, AS-285; Pr 3, 32 P-end-labeled AS-296). Genomic DNA from DNase I-treated nuclei was isolated and digested with EcoRI/PvuII restriction enzymes for LM-PCR analysis. D, LM-PCR analysis of nuclei isolated from P19 cells with (+) or without (-) 100 nM TSA for 24 h were treated with MNase. Primer set A was used for analysis. \square , MOR core promoter region (positions -340 to -300). The positioning of the 5' border of the N1 nucleosome at position -262 is induced by TSA (compared with its location at -345 in untreated controls). All LM-PCR experiments were performed at least twice independently.

cells) are also occurring. The details of this mechanism remain to be addressed in further studies.

Disassembly of Nucleosomes on the MOR Promoter in Differentiated Neuronal Cells. Having determined the exact 5' border of nucleosome N1 on the MOR promoter, we confirmed the presence of other nearby nucleosomes using specific PCR primer sets (Fig. 3A). In eukaryotes, with very few exceptions, one nucleosome complex occupies 147 bp with a 50-bp spacer (also known as the linker region bound by histone H1) between nucleosomes (Olins and Olins, 2003). Nucleosome ladders therefore produce fragments separated by approximately 200 bp after nuclease digestion. We therefore designed several PCR primer sets with some overlapping the nucleosome area (Fig. 3A). Cell nuclei were isolated, completely digested with MNase, and separated on an agarose gel. Mononucleosomal fragments were isolated from an agarose gel (Fig. 3B, left) and amplified by PCR using primer pairs specific to a particular nucleosome. As a positive control, intact genomic DNA was used as template. All PCR products amplified at their expected sizes (Fig. 3A, top gel).

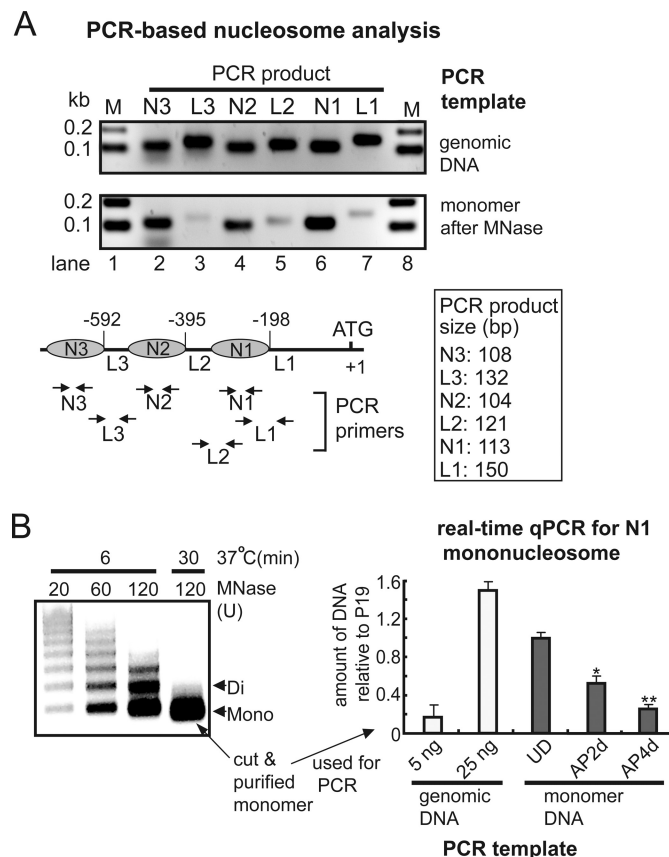


Fig. 3. PCR-based analyses of nucleosomes on the MOR promoter. **A**, purified monomer DNA (see **B**) was used as a template with several primer sets (below, and see Table 1). As a control, intact genomic DNA was used as a template with the same primer sets. Each primer set was designed according to the predicted nucleosome positions. The expected sizes of the amplified products for each primer set are indicated on the right of box. **B**, left, MNase-digested nucleosomes separated on an agarose gel before purification. Mono, mononucleosome; Di, dinucleosomes. Right, purified monomers from UD, AP2d, and AP4d cells were used as template DNA for real-time qPCR with a primer set designed to detect nucleosome N1's position (A and Table 1). Each experiment was performed at least twice independently. Genomic DNA (5 or 25 ng) was used as a positive control. Asterisks indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$) relative to the UD sample.

However, using purified monomer DNA as a PCR template, only PCR products from primer sets covering nucleosomes N3, N2, and N1 (Fig. 3A, bottom gel, lanes 2, 4, and 6, respectively) were amplified. Little or no PCR products were generated from primer sets covering the linker regions (L1, L2, and L3). These results demonstrated the exact nucleosome locations surrounding the MOR promoters.

To examine mononucleosome alterations in the promoter during neuronal differentiation, we used real-time qPCR to assess the relative amount of mononucleosomal DNA derived from the N1-specific genomic DNA region. This method allows rapid assessment of the relative amounts of specific nucleosomes formed on a particular genomic DNA segment. Nucleosome N1 of the PP (i.e., the region around the major transcription initiation site) was detected at its highest levels in UD cells and decreased significantly in AP2d and AP4d cells (Fig. 3B, right), suggesting that "loosening" or dissociation of nucleosome N1 occurs during neuronal differentiation, consistent with increased accessibility to MNase and DNase.

Involvement of Sp Transcription Factor Family Members with the MOR Promoter Region in Differentiated Neuronal Cells during MOR Gene Activation.

Epigenetic changes such as CpG DNA methylation are associated with chromatin remodeling. The N1 and promoter regions contain several GC boxes (Fig. 4A). These GC boxes are binding sites for the transcription factor Sp1 (Ko et al., 1998, 2003). To determine whether these transcription factors are affected by DNA methylation and whether transcription factor exchanges occur on this promoter during DNA methylation, we used in vitro-methylated MOR promoter DNA or unmethylated DNA as probes with nuclear extracts from P19 cells in an EMSA assay. When unmethylated DNA was used as a probe (Fig. 4A), the addition of Sp1 (lane 5) but not MeCP2 antibody (lane 4) reduced the top two bands relative to the control (lane 2), indicating that the Sp1 factor bound selectively to the unmethylated MOR promoter DNA, whereas MeCP2 did not. However, when methylated DNA was used as a probe (lanes 6–10), Sp1 antibody decreased the top band (relative to the control) but increased the second band. Likewise, adding MeCP2 antibody caused the top band to disappear and increased the intensity of the second band (lane 9, arrowhead), indicating strong binding of MeCP2 to the methylated DNA. There is a GC box (approximately at position –317) that does not overlap the methylated GC sites. Sp1 factor might bind to this GC box even when the probe DNA is methylated at its GC sites. It is noteworthy that in coimmunoprecipitation studies, Sp3 and its isoforms interacted strongly with MeCP2 (Fig. 4B, lane 4), whereas Sp1 exhibited little or no binding to MeCP2 (lane 2). It is possible that Sp3 and its isoforms function as repressors when bound to MeCP2. Alternatively, Sp1 could function exclusively as an activator of transcription through binding to unmethylated DNA.

The specificity of the antibodies against MeCP2, Sp1, and Sp3 and the expression levels of the proteins were demonstrated by Western blotting (Fig. 4C). Expression of Sp1 and Sp3 gradually decreased during differentiation, whereas the expression level of MeCP2 increased slightly throughout differentiation. Despite their presumed functional roles in the P19 cell system, protein expression levels did not associate closely with MOR mRNA levels, suggesting that absolute

protein levels are not as important as the timing and pattern of expression.

Differentiation Induces Sp1 Binding to GC Boxes and Reduces MeCP2 Binding in the MOR Promoter. ChIP assays were performed to examine the binding patterns of transcription factors on the PP-MOR. It is noteworthy that although the Sp1 level decreased in differentiated cells (Fig. 4C, lane 5), Sp1 binding to the GC boxes of the PP-MOR actually increased in the differentiated cells (Fig. 5A). Dissociation of nucleosome N1 presumably makes the GC boxes more accessible for Sp1 binding. This is consistent with the hyperacetylation of histone H3 observed at N1 in differentiated cells (lane 10).

Additional ChIP assays were performed to examine several signature regulators in gene regulation. Interaction of MeCP2 decreased in differentiated cells (Fig. 5B), consistent with previous results (Hwang et al., 2007). Interactions of HDAC1 and HDAC3 as well as mSin3A (a known corepressor for HDACs) also decreased in differentiated cells. The presumed opening of the chromatin structure on

the PP-MOR in differentiated cells prompted us to examine the SWI/SNF family of chromatin remodeling complexes (Fig. 5B). The ATP-dependent chromatin-remodeling factor Brg1, but not Brm, was recruited to PP-MOR after differentiation. BAF155 and Pol II were also recruited to this promoter during differentiation.

Histone Modifications on Nucleosomes N1 and N2 of the MOR Promoter. Because histone interactions are relatively low in N1 and the MOR promoter region extends into N2, we examined histone modifications by performing ChIP assays using PCR primers covering both nucleosomes (Fig. 5C). Antibodies against acetylated histones H3 and H4 immunoprecipitated more DNA from differentiated than from UD cells (Fig. 5C), suggesting that the acetylated forms are enriched on the MOR promoter in differentiated cells. Further ChIP analyses (Fig. 5C) showed reduced methylation of histone Lys9 dimethylated histone H3 (a hallmark of gene inactivation), concurrent with increased dimethylation at lysine 4 (a hallmark of gene activation) (Berger, 2001) in the differentiated cells. These results indicate that the histone

A Electrophoretic mobility shift assay

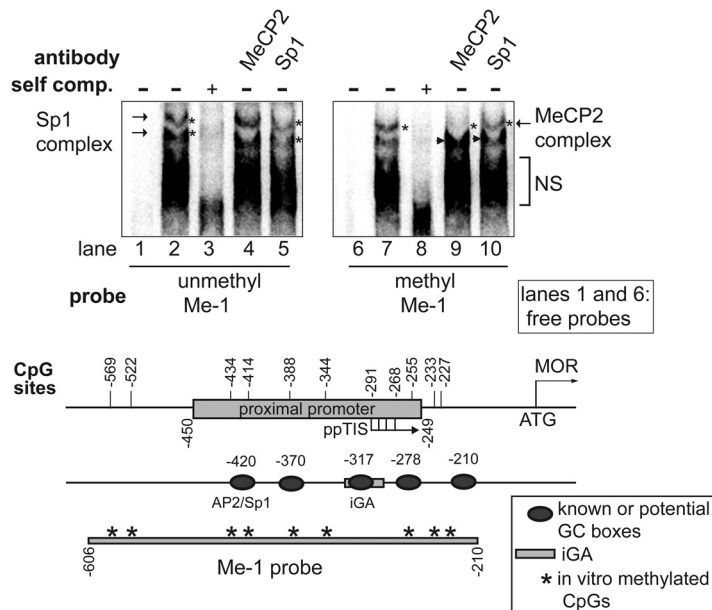
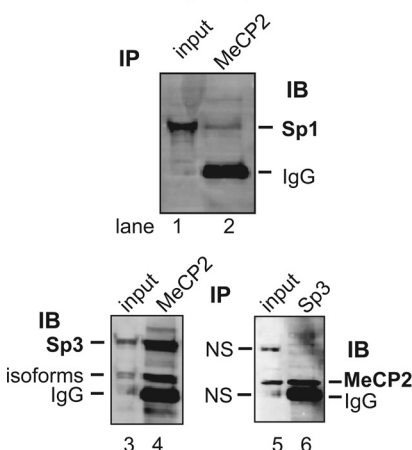
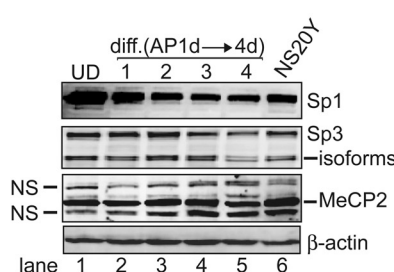


Fig. 4. Selective binding of Sp1 factor to unmethylated MOR promoter DNA confirmed by EMSA. A, γ - 32 P-end-labeled probes for native (unmethyl Me-1) and methyl Me-1 (as shown below) were incubated with the nuclear extracts from P19 cells. Antibodies (MeCP2 and Sp1) were used to detect each factor's complex. Major protein-DNA complexes are indicated by arrows. Arrowheads indicate bands shifted by the addition of each antibody. Asterisks indicate specific protein-DNA bands. NS, nonspecific band. Lanes 1 and 6, probe alone (free probes); lanes 2 and 7, control reactions; lanes 3 and 8, 100-fold excess of unlabeled self-competitor; lanes 2 to 5, labeled unmethyl Me-1 probe + nuclear extracts; lanes 7 to 10, labeled methyl Me-1 probe + nuclear extracts. Bottom, schematic of probe location on the MOR promoter. Black ovals, GC boxes for Sp transcription factor binding (Ko et al., 1998, 2003); grey rectangle, iGA site (Ko et al., 1998); asterisks, CpG sites for DNA methylation (Hwang et al., 2007). Numbers indicate the site location, relative to the translation start site (+1). B, interaction of MeCP2 with the Sp factors analyzed by coimmunoprecipitation. Five micrograms of MeCP2 antibody was used to immunoprecipitate and to visualize the interaction with Sp1 (lane 2) and Sp3 (lane 4) in P19 extracts. Lane 6, reverse immunoprecipitation using Sp3 antibody for immunoprecipitation and MeCP2 antibody for immunoblotting. IP, immunoprecipitation; IB, immunoblotting antibody. Input lanes contain one tenth of each IP reaction. C, Western blot analysis of Sp1, Sp3, and MeCP2 stage-specific expression in the UD, AP2d, AP4d P19, and NS20Y cells. Anti- β -actin was used as a control. NS, nonspecific band.

B Co-immunoprecipitation



C Western blot



code is in an active state and that DNA demethylation occurs at the PP in differentiated cells with active MOR expression.

Sp1 Activates MOR Promoter Activity. Overexpressing Sp1 in MOR-positive NMB cells increased the cotransfected MOR promoter activity approximately 5-fold relative to controls (Fig. 5D). Treatment of NMB cells with the HDAC inhibitor TSA resulted in approximately a 2.4-fold increase in promoter activity, indicating an association between histone acetylation and active transcription of the MOR promoter, consistent with the ChIP data.

Discussion

In eukaryotes, DNA methylation and histone modifications represent the major epigenetic mechanisms implicated in the regulation of gene transcription. Methylation of DNA is associated with gene silencing in conjunction with histone core modifications, probably through chromatin remodeling (Berger, 2001; Martinowich et al., 2003).

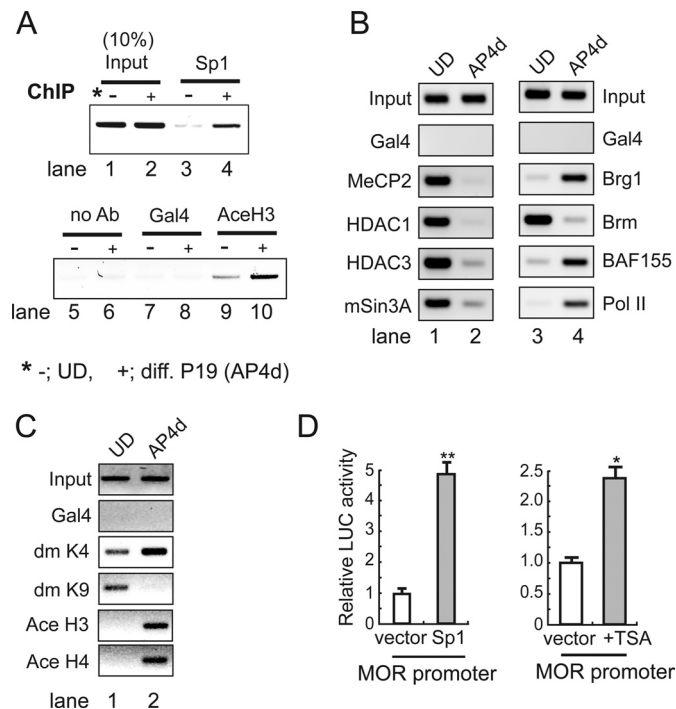


Fig. 5. ChIP analyses of chromatin modifications and nuclear factor interactions. **A** and **B**, primers specific for nucleosome position N1 of the MOR gene promoter (S-342 and AS-229; Table 1) were used to amplify genomic DNA sequences present in immunoprecipitates by ChIP-PCR. Amplification of soluble chromatin before precipitation was used as an input control. Antibodies for each ChIP reaction are indicated at the top (**A**) or the side (**B**) of each gel image. Parallel controls were performed without antibody or using a nonspecific antibody (anti-Gal4). Images are representative of at least two experiments. **C**, ChIP analyses to detect chromatin modification on the N1 and N2 positions of the MOR promoter. Primers specific for the N1 and N2 locations (S-497 and AS-182; Table 1) were used to amplify genomic DNA sequences that were present in each immunoprecipitate by ChIP-PCR. Conditions for the ChIP assays were similar to those used for the ChIP experiments (**A** and **B**). **D**, transcriptional effect of Sp1 factor on the MOR promoter of NMB cells. NMB cells were cotransfected with Sp1 expression plasmid along with the MOR promoter construct p189. NMB cells transfected with the MOR promoter construct also were exposed to TSA. Cells were harvested 48 h after transfection, lysed, and assayed for luciferase activity. Promoter activity was normalized by protein concentration. Graphs indicate the averages from at least two representative experiments. Asterisks indicate statistically significant findings (*, $p < 0.05$; **, $p < 0.01$) relative to the vector control. Error bars indicate the range of standard errors.

Chromatin remodeling, especially in the promoter region, is a key regulatory step in transcription, providing DNA accessibility for transcription (or nuclear) factors and transcription complexes (Gilbert et al., 2004). The nuclear factors' ability to access DNA can be regulated by different chromatin remodeling complexes, which alter DNA-histone contacts by using the energy of ATP hydrolysis (Lusser and Kadonaga, 2003; Gilbert et al., 2004).

Transcription of the mouse MOR gene is regulated during the differentiation of neuronal P19 cells. The proximal promoter, a major promoter of the MOR gene, is silenced in P19 stem cells. During neuronal development, the MOR promoter becomes activated through demethylation of the promoter DNA and dissociation of MeCP2, a methyl-CpG-binding protein (Hwang et al., 2007). An epigenetic study of the mouse brain (Hwang et al., 2009) showed hypermethylation of the MOR promoter, high levels of MeCP2 bound to the promoter, and corresponding chromatin modifications in the cerebellum, the region of the brain in which MOR is expressed at its lowest levels. In addition, the highest levels of methylated DNA in the MOR promoter occur in mouse neuroblastoma NS20Y cells, which have the lowest expression levels of the MOR gene among all cultured cells tested in our laboratory (Hwang et al., 2009). These studies suggest that DNA methylation of the region around the MOR promoter plays an important role in MOR gene inactivation. Several other studies have reported that DNA methylation of the promoter region is closely associated with changes in chromatin structure such as histone modifications and chromatin remodeling. Although such epigenetic features in the MOR promoter were revealed in previous studies, the exact locations of the histone complexes in the MOR promoter were not known. It also was not known how the chromatin structure is altered during MOR gene activation to allow access of active transcriptional factor complexes to the promoter. This study attempts to determine the changes in the chromatin structure of the MOR gene promoter during neuronal differentiation and to delineate the molecular events coinciding with the disassembly of nucleosomes on this promoter.

MNase-Southern blots confirmed that the nucleosome complex dissociates from the major MOR promoter region in differentiated P19 cells (i.e., MOR-positive cells), suggesting that this process is necessary for activation of transcription. MNase digestion-coupled and DNase I-mediated LM-PCR analyses defined the 5' border of nucleosome N1 to position -345 relative to the translation start site. This places the 5' border 54 bases upstream of the first of four known transcription initiation sites (Min et al., 1994). It is noteworthy that nucleosome N1 (-345 to -198) covers the MOR core promoter region (-340 to -300), a region that is itself responsible for 70% of the activity of the whole promoter (Ko et al., 1997). Furthermore, nucleosome N1 dissociation allows access for transcription factors when the MOR gene is active in differentiated cells. That is, in its inactive state, the major MOR promoter region is covered by nucleosome N1, but when active, the promoter is free of nucleosome complexes, thus exposing binding sites for transcription factors and permitting gene activation. It was therefore important to determine whether other epigenetic changes of the MOR gene promoter were also involved in chromatin remodeling, in addition to DNA demethylation and histone modifications.

We examined factors involved in chromatin alteration of

N1 and its surrounding regions because these regions overlapped with several GC boxes and the transcription initiation sites of the MOR gene. Among the five GC boxes studied, all of which serve as binding sites for Sp family members, three are located inside N1. Transcriptional regulation by members of the Sp family of transcription factors has been reviewed extensively (Suske, 1999; Kaczynski et al., 2003). Sp1 functions mainly as a transcriptional activator, whereas Sp3 is a bifunctional factor with three different isoforms, and its activities can be regulated in part by the relative expression of these isoforms (Kennett et al., 2002; Hernandez et al., 2004). *Drosophila melanogaster* SL2 cells do not endogenously express Sp family proteins (Schneider, 1972; Courey and Tjian, 1988). When these cells were cotransfected with Sp factors and MOR promoter plasmids, Sp1 and Sp3 stimulated MOR promoter activity 4.4- and 2-fold, respectively (Choi et al., 2005). It is noteworthy that the Sp3 isoforms M1 and M2 (i.e., shorter forms of the full-length Sp3) repressed promoter activity to 60 and 70%, respectively, of control values. Furthermore, Sp1 and Sp3 (along with its isoforms) share the same binding site. When a fixed amount of Sp1 plasmid was cotransfected into SL2 cells with varying amounts of Sp3, M1, or M2, both isoforms inhibited Sp1-mediated activation of the MOR promoter in a dose-dependent manner, with M1 being the stronger inhibitor of the two. In contrast, cotransfecting higher amounts of full-length Sp3 actually increased the Sp1-mediated activation of the MOR promoter. In conjunction with the current data, these results suggest that Sp1 activates the MOR promoter and that Sp1-mediated activation is suppressed by the Sp3 isoforms but not by the full-length Sp3. These findings suggest that the function of Sp3 on the activation state of the MOR gene might be similar to its effects on other genes (Kennett et al., 2002; Hernandez et al., 2004). Expression of the multiple isoforms of Sp3 is controlled by alternative translation initiations, with each isoforms having a unique role leading to either activation or repression of the MOR gene.

There is increasing evidence that chromatin-remodeling factors are involved in several biological events, including cancer development (Guasconi and Ait-Si-Ali, 2004; Dey, 2006; Medina et al., 2008), ovarian germ and somatic cell development and differentiation (Pépin et al., 2007), neuronal differentiation (Seo et al., 2005; Lessard et al., 2007), DNA damage repair (Pena and Pereira-Smith, 2007), embryonic stem cell neuronal differentiation (Meshorer, 2007), aging (Anastasiou and Krek, 2006), and immune cell development (Winandy, 2005). These findings indicate that chromatin remodeling plays an important role in gene regulation. Alteration of nucleosomes by ATP-dependent remodeling complexes represents a critical step in the regulation of transcription. Therefore, we investigated the involvement of chromatin remodeling factors from the human SWI/SNF family in MOR gene-activated cells. This family is composed of complexes that contain either Brg1 or hBrm as the central ATPase. Brg1, an ATP-dependent chromatin-remodeling factor, and BAF155 were recruited to the MOR promoter in MOR-activated cells. Surprisingly, binding of Brm to the MOR promoter was lower in MOR-activated cells than in MOR-negative cells. However, differential roles for these two factors have been suggested in the κ opioid receptor promoter (Park et al., 2005). Although Brm is generally known as a global transcriptional

activator of homeotic genes (Tamkun et al., 1992), it can also act as a transcriptional suppressor (Zhang et al., 2000; Brumby et al., 2002). One recent report showed that Brm interacts with MeCP2, a major epigenetic regulator, suggesting Brm's role in epigenetic repressor complexes (Harikrishnan et al., 2005). However, another group has reported contradictory results (Hu et al., 2006). In our laboratory, MeCP2 did not interact with Brm but rather with Brg1 (Hwang et al., 2009). Brm also interacted in vitro with mSin3A (Hwang et al., 2009), another component of transcriptional repressor complexes, showing a direct association between chromatin remodeling complexes and proteins involved in gene repression (Sif et al., 2001). Although Brm and Brg1 have very high homology in their protein sequences, they have differential expression profiles in human tissues (Reisman et al., 2005), suggesting that they might have different functions in chromatin remodeling complexes.

In summary, here we report the exact location of the nucleosome complexes surrounding the major MOR promoter region. These nucleosomes dissociate when the gene is transcriptionally active, concurrent with histone modifications and interactions with chromatin remodeling factors. Based on the results of the present study, we propose the following model (Fig. 6): In normal P19 cells, the chromatin of MOR is "closed" (i.e., inactive). MeCP2 binds to hypermethylated DNA (CpG) of the MOR promoter, coincident with interactions with several factors (HDACs, mSin3A, and Brm). This compacted chromatin structure, characterized by histone H3–Lys9 dimethylation and histone H3 and H4 deacetylation, silences the MOR gene in these cells. Interactions of Sp3, probably through its isoforms (known to act as repressors), with MeCP2 might assist the formation of the histone-associated repressor complex. During RA-induced neuronal differentiation, nearly complete demethylation of the CpGs

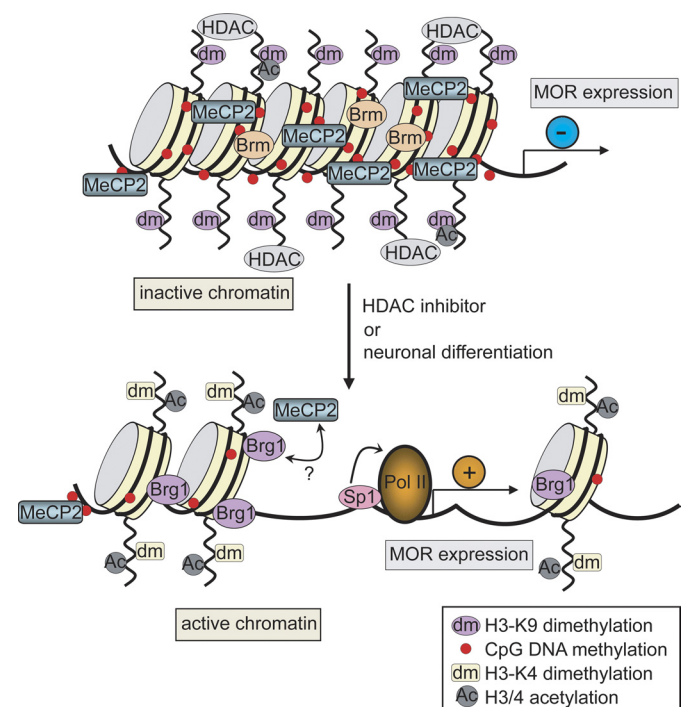


Fig. 6. Proposed molecular mechanism for MOR gene regulation through chromatin remodeling.

in the promoter coincides with dissociation of MeCP2 and its corepressors (HDACs). The ATP-dependent chromatin-remodeling factor Brm also dissociates, but another remodeling factor, Brg1 is recruited concurrent with histone H3–Lys4 dimethylation and histone H3 and H4 acetylation. These result in the opening of the inactive chromatin. The transcription factor Sp1 is recruited to the opened region and the RNA Pol II preinitiation complex forms, leading to active transcription of the MOR gene in the cells. Treatment of P19 cells with the HDAC inhibitor TSA also leads to the opened chromatin state. Future studies are needed to determine how chromatin remodeling of the MOR promoter is triggered during MOR gene activation. For example, a direct physical and functional interaction between MeCP2 and Brg1 still remains to be clearly demonstrated.

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