

**Transcriptional Regulation of Mouse μ Opioid Receptor Gene: Sp3 Isoforms (M1, M2)
Function as Repressors in Neuronal Cells to Regulate the μ Opioid Receptor Gene**

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The abbreviations used are: MOR, μ opioid receptor; EMSA, electrophoretic mobility shift assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MAP, mitogen activated protein; bp, base pair

Abstract

The 5'-flanking region of the mouse μ opioid receptor (MOR) gene has two promoters, referred to as distal and proximal. MOR mRNA is predominantly initiated by the proximal promoter. Previously, several important *cis*-elements and *trans*-factors have been shown to play a functional role in the proximal promoter of the MOR gene. In this study, we defined another functional, negative regulatory element located in the -219 to -189 bp (translational start site designed as +1) region of the proximal promoter. It is designated as the Sp binding sequence for its sequence homology to the consensus Sp binding element. Mutation of the Sp binding element led to a 100% increase of MOR promoter activity in MOR positive cells (NMB cells), confirming the negative role of the Sp binding sequence. Surprisingly, electrophoretic mobility shift analysis (EMSA) and chromatin immunoprecipitation (ChIP) assays revealed that Sp3 and its isoforms (M1 and M2) were specifically bound to the Sp binding sequence. In co-transfection assays of *Drosophila* SL2 cells using cDNA encoding Sp1, Sp3, and the M1 and M2 isoforms of Sp3, The M1 and M2 isoforms *trans*-repressed the MOR promoter, while Sp1 and Sp3 *trans*-activated the MOR promoter. Significantly, ectopic expression of the M1 and M2 isoforms of Sp3 led to repression of the endogenous MOR gene transcripts in NMB cells. These results suggest that the binding of the M1 and M2 isoforms of the Sp3 transcription factor to the Sp binding sequence may play a role in mouse MOR gene expression.

Introduction

Opioids are used as potent clinical analgesics for pain, but have serious limitations such as tolerance and dependence. The opioid receptors are classified into three major types (μ , δ and κ), studied by numerous pharmacological reports and molecular cloning (Min et al., 1994; Wei and Loh, 2002). All three types of opioid receptors belong to the superfamily of G-protein-coupled receptors. The μ opioid receptor (MOR) is known to play roles in morphine-induced analgesia, tolerance, and dependence, as indicated from pharmacological studies and analysis of MOR knock-out mice (Kieffer, 1995; Kieffer, 1999). Upon the binding of opioids, MOR is able to couple to G-proteins and to regulate adenylyl cyclase, intracellular calcium, inwardly rectifying potassium channels, MAP kinase, and other messengers, which further trigger a cascade of intracellular events (Law et al., 2000).

MOR is mainly expressed in the central nervous system (CNS), with receptors varying in densities at different regions perhaps playing different roles (Maldonado et al., 1997); (Mansour et al., 1995). MOR is also expressed in other tissues, such as immune cells (Sedqi et al., 1995). The presence of MOR in immune cells can elucidate the reason why drug abusers (opioid and heroin) become more susceptible to external pathogens after weakening the body's immune system by chronic drug use, especially well-described in HIV-infected opiate users (Donahoe and Vlahov, 1998; Nyland et al., 1998; Sheng et al., 1997). The mouse MOR gene spans about 250 kb and consists of multiple exons (Pan, 2002; Pan et al., 2001). Several MOR isoforms have been reported. Among them, MOR-1 is the most abundant form, and is encoded by exons 1, 2, 3 and 4. In contrast, the other isoforms, which make use of exons 6-10 and/ or 11-14, are relatively rare (Pan, 2002; Pan et al., 2001).

Two different promoters (distal and proximal) of the mouse MOR gene have been reported, which are located within 1 kb upstream of the translational start site (ATG) (Ko et al., 1997). The

distal promoter initiates MOR transcription from a single transcription initiation site, located 794 bp upstream of translation start site. The proximal promoter initiates MOR transcription from four major transcription initiation sites located in a region ranging from 291 to 268 bp upstream of translation start site. The mouse MOR promoter contains a 5'-DPRS (5'-distal promoter regulatory sequence), a 34-bp *cis*-acting element which possesses a strong inhibitory effect against the transcriptional function of the distal promoter (Hwang et al., 2003). Recently our laboratory has found that the PU.1 transcription factor interacts with the negative 34-bp *cis*-acting element in the MOR distal promoter and represses the MOR distal promoter in immune and neuronal cells (Hwang et al., 2004). Both promoters exhibit characteristics of housekeeping genes lacking a TATA box, and the distal promoter is known to 20-fold less active than the proximal promoter, based on quantitative RT-PCR using adult and embryonic mouse brain (Ko et al., 2002).

The *cis*-elements and *trans*-factors in the -250 to +1 bp regulatory region for the MOR proximal promoter are not well studied. In this study, we investigated the functional elements and binding factors of this region. The requirement of a negative element for proximal promoter activity was demonstrated by transient transfection using serial 3'-deletional promoter constructs. Binding of the M1 and M2 isoforms of the Sp3 transcription factor to the negative element of MOR promoter was examined by EMSA. We found that the M1 and M2 isoforms of the Sp3 transcription factor may tune expression levels of the MOR gene in neuronal cells.

Materials and Methods

Plasmid construction and *In vitro* translation. Luciferase fusion plasmids were constructed containing the DNA sequence from -450 bp upstream of the mouse MOR gene (p1 construct; -450 to +1 bp related to the translation start site as +1) to various lengths of 3'-short downstream regulatory sequences. The 3'-deletion constructs (p29, p66, p90, p100, p121, p189, p220, and p249) were generated by recombinant polymerase chain reaction (PCR) with the same upstream primer at -450 bp bearing a SacI site and the corresponding downstream primers each bearing a HindIII site as indicated in Fig.1. All the PCR fragments were cloned into pGL3-basic (Promega). The DNA sequences of all constructs were confirmed by automated DNA sequencing. Mutant constructs of the p189 plasmid were also generated by PCR using the corresponding mutant PCR primers as described in Fig. 3A. *In vitro* translation was carried out with pCR-M1/flu and pCR-M2/flu in a reaction mixture containing [³⁵S] methionine (Amersham) using a TNT quick coupled transcription/translation system (Promega). Plasmids pPacSp1 and pPacSp3 were obtained from Robert Tjian (Courey and Tjian, 1988; Schneider, 1972). pPacM1, pPacM2, pCR-M1/flu, and pCR-M2/flu were obtained cordially from Dr. J.M. Horowitz (Kennett et al., 2002; Kennett et al., 1997). The labeled proteins were then electrophoresed in 12% SDS-PAGE and their sizes were compared with the predicted sizes. pcDNA-M1/flu and pcDNA-M2/flu were constructed by subcloning BamHI fragments of M1/flu and M2/flu into the BamHI site of pcDNA 3.1. The flu denotes the influenza hemagglutinin (HA) epitope.

Cell culture. Human neuroblastoma NMB and SHSY-5Y cells were grown in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FBS, Life Technologies) in an atmosphere of 5% CO² and 95% air at 37°C. Mouse neuroblastoma NS20Y cells were grown in Dulbecco's minimal essential medium (DMEM) with 10% heat-inactivated fetal calf serum (FBS, Life Technologies). Schneider's *Drosophila* line 2 (SL2) cells purchased from ATCC were grown at 22-

24°C in Schneider's *Drosophila* medium (Life Technologies) containing 10% heat-inactivated fetal calf serum.

Transient transfection and reporter gene assay. Cells were plated in 6-well dishes at a concentration of 1×10^6 cells/well and cultured overnight before transfection. Various plasmids at equimolar concentrations were used with Effectene transfection reagent (Qiagen) as described previously (Hwang et al., 2003). Briefly, for luciferase analysis of MOR promoters, 0.5 μ g of the reporter plasmids was mixed with the Effectene transfection reagent for 10 min before being added to various cells. Forty-eight hours after transfection, cells grown to confluence were washed once with 1x phosphate-buffered saline and lysed with lysis buffer (Promega). To correct for differences in transfection efficiency, a one-fifth molar ratio of pCH110 (Amersham) containing the β -galactosidase gene under the SV40 promoter was included in each transfection for normalization. The luciferase and β -galactosidase activities of each lysate were determined as described by the manufacturers (Promega and Tropics). SL2 transient transfection followed as described previously (Wang et al., 2003).

Nuclear extract preparation. Nuclear extracts were prepared from NMB and SHSY-5Y cells as described previously (Hwang et al., 2003). Briefly, cells were grown to confluence, harvested and washed with phosphate-buffered saline. All of the following steps were performed at 4 °C. The cells were resuspended in sucrose buffer (0.32 M sucrose, 3 mM CaCl_2 , 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, and 0.5% NP-40). The lysate was microcentrifuged at 500xg for 5 min to pellet the nuclei, which were washed with sucrose buffer NP-40. The nuclei were resuspended in low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.02 M KCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF), followed by addition of high salt buffer to extract the nuclei, with incubation for 20 min on a rotary platform. Diluent (2.5 vol. of 25 mM HEPES, pH 7.6, 25% glycerol, 0.1 mM EDTA, 0.5 mM DTT, and 0.5

mM PMSF) was added and the sample was microcentrifuged at 13,000xg. Aliquots of supernatant (nuclear extracts) were stored at -80 °C.

Electrophoretic mobility shift assay (EMSA). The upper and lower strands of each probe (5'-TTCTAAGGTGGGAGGGGGCTACAAG-3' and 5'-CTTG TAGCCCCCTCCACCTTAGAA-3') were annealed and the double-strand oligonucleotides were then end-labeled with [γ -³²P] ATP. The end-labeled DNA probes (1×10^4) were incubated with nuclear extract (10 μ g of protein) in a final volume of 20 μ l EMSA buffer (10 mM Tris, pH 7.5, 5% glycerol, 1 mM EDTA, pH 7.1, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 0.1 mg/ml poly (dI-dC)) at room temperature for 20 min. For oligonucleotide competition analysis, a 100-fold molar excess of cold competitor oligonucleotide was added to the mixture prior to adding the probe. For antibody supershift assays, 2 μ g of anti-Sp1 (sc-59X), Sp3 (sc-644X), p50 (NF κ B, sc-1190X), or p65 (NF κ B, sc-372X) (Santa Cruz) antibody was added to the mixture. The reaction was then incubated at 4 °C for 30 min. The reaction mixtures were electrophoresed in a 4% polyacrylamide nondenaturing gel in 0.5 X TBE (45 mM Tri-borate and 1 mM EDTA) at 4 °C and visualized by autoradiography.

Chromatin Immunoprecipitation Assay (ChIP). M1 and M2 overexpressed NS20Y cells and NS20Y cells were used for ChIP assays. ChIPs were performed using a modified protocol from Upstate Biotechnology (Lake Placid, NY). Cells in a 10-cm dish (70% confluent) were treated for 10 min with 1% formaldehyde at 37 °C. The cells were lysed in cell lysis buffer (5 mM HEPES, pH 8.1, 85 mM KCl, 0.5% Triton X-100), and the nuclei were resuspended in nuclei lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS). The lysate was sonicated under conditions yielding fragments ranging from 200 to 1,000 bp. One-tenth of the diluted lysate was used for input, and the residual lysate was subjected to the following immunoprecipitation. Samples were subsequently precleared at 4 °C with recombinant protein A-agarose beads (Upstate Biotechnology) coated with salmon sperm DNA. Precleared lysate (100 μ l) diluted in immunoprecipitation buffer (0.01% SDS,

1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) was used for overnight immunoprecipitation with 10 μ g of Sp3 (sc-644X) and HA (HA.11, COVANCE) antibody at 4 °C. Complexes were collected for 4 h by using recombinant protein A-agarose beads coated with salmon-sperm DNA. After washing and elution, formaldehyde cross-linking was reversed with a 6-h incubation at 65 °C. Following the reverse cross-linking step, DNA was precipitated following a phenol/chloroform extraction and dissolved in 30 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). PCR reaction mixtures contained 4 μ l of the immunoprecipitated chromatin sample with the following primers spanning the mouse MOR promoter region, in 50 μ l of total volume. The mouse MOR primers, up-1 and down-1, produce a 196-bp PCR product, up-1 forward, 5'-TCTGGATCCCTCACAGCCCAT-3' and down-1 reverse, 5'-TAGTGGAAACCAGAGAAGAG-3'. A set of primers specific to mouse β -actin (5'-TGGCCTTAGGGTGCAGGGGG-3' and 5'-GTGGGCCGCTCTAGGCACCA-3') was used for β -actin amplification. After 35 cycles of amplification, 10 μ l of the PCR product was analyzed on a 2% agarose gel.

Western blot analysis. Nuclear extracts (10 μ g of total proteins) from NMB cells and NS20Y cells were incubated with treatment buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and boiled for 5 min. The treated extracts were electrophoresed through SDS-10% polyacrylamide gel. The gel was electroblotted onto polyvinylidene difluoride membrane (Amersham) in transfer buffer (48mM Tris-HCl, 39 mM glycine, 20% methanol). The membrane was blocked in blocking solution (10% dry milk, 0.1% Tween-20 in Tris-buffered saline) at 4 °C for overnight. Western blotting with the anti-Sp3 antibody (1:1000, anti-Sp3) was performed following manufacturer's instructions (Amersham). The signals were detected using a Molecular Dynamic Storm 840 phosphorimager system.

Reverse transcription-PCR of MOR gene and heterologous expression of Sp3 isoforms (M1 and M2). Total RNA was isolated according to the supplier's protocol (TRI Reagent,

Molecular research Center) and analyzed by reverse transcription-PCR (RT-PCR) using primers, as follows. Primers specific to total MOR mRNA are 5'-GATCATGGCCCTCTACTCCA-3' (located at position 216 in exon 1) and 5'-GCATTTTCGGGGAGTACGGAA-3' (located at position 557 in exon 2 to avoid amplification of genomic DNA). Primers to human β -actin are 5'-AGACCTCTATGCCAACACAGT-3' and 5'-GACACACCTAACCACCGAGAT-3'. The pcDNA-M1/flu, and pcDNA-M2/flu plasmids harboring the coding sequences of Sp3 isoform M1, and Sp3 isoform M2, respectively, were transfected into human NMB cells using Effectene transfection reagent (Qiagen). To test the endogenous MOR gene regulation by those Sp3 isoforms (M1 and M2), total RNAs were isolated from NMB cells transfected with each factor (Sp1, Sp3, Sp3 isoform M1, and Sp3 isoform M2), as described in RNA isolation of "Materials and Methods". RT-PCRs were performed using Qiagen OneStep RT-PCR Kit, and MOR PCR primers and β -actin primers as described in RT-PCR of "Materials and Methods". The PCR products were electrophoresed in a 2% agarose gel and quantified by ImageQuant version 5.2 software (Amersham).

Results

Identification of a regulatory element (-250 to +1) for the proximal promoter of the mouse μ opioid receptor (MOR) gene. Previously, several papers indicated that the region of -250 to +1 bp in the mouse MOR proximal promoter is important for regulation of mouse MOR (Ikeda et al., 2001; Lee and Lee, 2003). The molecular mechanisms responsible for MOR regulation in this region are not well known and therefore this study focuses on this regulatory region. In order to identify the *cis*-element and *trans*-acting factors required for promoter activity, 3'-deletional analysis was performed using this region.

Chimeric constructs (p1, p29, p66, p90, p100, p121, p189, p220 and p249) (Fig. 1) containing various lengths of the proximal promoter of mouse MOR were cloned into a pGL3-basic plasmid (promoterless luciferase reporter vector). They were tested in MOR-expressing cells (NMB) using transient transfection assays. The promoter activity of each construct was presented as n-fold of the promoterless plasmid (pGL3-basic) activity. As shown in Fig. 1, the p1, p29, p66, p220 and p249 constructs showed similar promoter activities, but the p90, p100, p121, p189 constructs showed low promoter activities relative to the above constructs. The p189 (containing the region of -450 to -189) construct relative to the p220 construct (-450 to -220) displayed a significant reduction in promoter activity (approximately 100%), suggesting the involvement of the -219 to -189 bp region in negative regulation of proximal promoter activity. The p66 (-450 to -66) construct relative to the p90 construct (-450 to -90) displayed a significant increase in promoter activity (approximately 80%), suggesting the involvement of the -89 to -66 bp region in the positive regulation of proximal promoter activity. Collectively, these results demonstrated that the region between -219 and -189 contains a negative *cis*-acting element and the region between -89 and -66 bp contains a positive *cis*-acting element. A search for transcription factor binding sites over these regions was undertaken using the TRANSFAC v4.0 program (<http://www.cbil.upenn.edu/cgi-bin/tess>). The results revealed

that the negative element contains a Sp binding sequence (5'-GGGAGGGGGC-3') located at nucleotide -211 to -202 (Fig. 2) and the positive element contains an Ets (5'-GAGGAA-3', -87 to -82 bp) and an Sp binding sequence (5'-GGGGCGG-3', -76 to -70 bp). Among them, the MOR negative regulatory element region is highly conserved, especially the Sp binding sequence (5'-GGGAGGGGGC-3'), with only a nucleotide difference among human, mouse and rat species (Fig. 2). The positive regulatory region is under further investigation.

Functional analysis of the negative regulatory element in the mouse MOR proximal promoter. To verify the contribution of the negative regulatory element to the functional activity of the proximal promoter, the negative regulatory element of the p189 construct was mutated as shown in Fig. 3A. Because Sp transcription factors are known to bind to various consensus sequences, including the GC box (Courey and Tjian, 1988), the GT element (Hwang et al., 2001), and mor iGA (Ko et al., 1998), we simply replaced GG and GC sequences of the negative regulatory element with CC (p189-m3), GA (p189-m1), TTAA (p189-m4) and TG (p189-m2) (Fig. 3A). The mutant constructs p189-m1, p189-m2 and p189-m4 displayed similar promoter activity to the wild type construct (p189) (the wild type promoter activity is arbitrarily defined as 100%). The mutant construct p189-m3 displayed higher promoter activity (200%) than that of the wild type construct. Therefore, this suggests that the core negative regulatory sequence in the MOR proximal promoter is GGGAGG, based on our functional promoter assays above and previous Sp consensus sites. It was previously shown that the single nucleotide change between GGGGGCT and GGGGGAT sequence in the MOR negative regulatory element resulted in differing size and expression levels of MOR mRNA between CXBK and B6 mice (Ikeda et al., 2001). However, our data indicates that the regulatory sequences GGGGGCT (as in the wild type p189 construct) and GGGGGAT (as in the p189-m1 construct) show similar promoter activity in neuroblastoma NMB cells, suggesting that the base transition from C to A is less important in MOR gene regulation in NMB cells.

Sp3 protein binds specifically to the negative regulatory element in the mouse MOR proximal promoter. To identify the *trans*-acting factor that binds to the negative regulatory sequence and verify its contribution to the promoter activity, electrophoresis mobility shift assays (EMSA) was performed using a 25 bp oligonucleotide, designated NS as negative regulatory sequence and containing the Sp binding sequence (5'-TTCTAAGGTGGGAGGGGGCTACAAG-3'; -220 to -196), as the probe and the nuclear extracts from either NMB or SHSY-5Y cells. SHSY-5Y cells are human neuroblastoma cell and express both mu- and delta-opioid receptors (Kraus et al., 2003). As shown in Fig. 4, two major bands (indicated by arrows) and several minor protein/DNA complexes were observed (Fig. 4, lane 2). A 100-fold molar excess of unlabeled NS oligonucleotide (Fig. 4, lane 3) and consensus Sp1 binding sequence (Fig. 4, lane 5) completely inhibited the complex formation, while mutated negative regulatory sequence (mNS) (Fig. 4, lane 4) did not inhibit the complex formation in NMB and SHSY-5Y cells, indicating sequence specificity of these major protein/DNA complex bands. Thus, this suggests that the negative regulatory sequence contains Sp binding sequence and the major complexes might contain Sp proteins. To identify the exact transcription factors binding to negative regulatory element, we performed a supershift assay. As shown in Fig. 5, the major band were the Sp3 complexes indicated by arrows and major supershift bands in panels A and B were observed using anti-Sp3 (indicated by asterisk in lane 7), minor supershift bands in B was observed using anti-Sp1 (indicated by asterisk in lane 6) and Sp1 was bound very weakly, whereas no supershift bands were observed using anti-p50 or anti-p65 (NF- κ B) antibodies. These results demonstrated that Sp3 was the major *trans*-acting factor binding to the negative regulatory element.

***In vitro* translated M1 and M2 proteins bind specifically to the negative regulatory element of the MOR promoter.** It was reported that Sp3 encodes three distinct gene products as follows: a full-length protein (Sp3) that is an activator of transcription and its two isoforms (M1 and

M2) derived from internal translational initiation sites that function as transcriptional repressors in other genes (Kennett et al., 2002; Kennett et al., 1997). Therefore, we hypothesized that the Sp3 isoforms (M1 and M2) may also act to repress the mMOR proximal promoter. To address whether the two factors M1 and M2 are present in neuronal NMB cells, western blot analysis was carried out using nuclear extract from NMB cells. Protein bands of approximately 115, 80, 78 kDa were observed when the blot was incubated with anti-Sp3 antibody (Fig. 6A). The molecular weights of Sp3, M1, and M2 transcription factors are consistent with sizes in previous reports (Kennett et al., 1997). Thus both Sp3 isoforms cDNA, M1 and M2, are present in the cells. As expected, *in vitro* translation of an HA-tagged M1 cDNA construct led to synthesis of the 80 and 78 kDa, whereas HA-tagged M2 cDNA directed synthesis of only the 78 kDa isoform of Sp3 *in vitro* (Fig. 6B). Thus, *in vitro* translated isoforms cDNAs of M1 and M2 produce the same molecular weight proteins (80 and 78 kDa) as Sp3 produces *in vivo* by internal translational initiation. To confirm whether the *in vitro* translated M1 and M2 protein can indeed bind to the negative regulatory element, EMSAs were carried out using *in vitro* translated M1 and M2 proteins. The M1 and M2 proteins shifted the target NS oligonucleotide probe (Fig. 6C, lane 3, 10). The specificity of these DNA-protein interactions was verified by competitive inhibition in the presence of cold self-competitor using the M1 and M2 proteins in lane 6 and 13 of Figure 6C, respectively. To further confirm the specificities of M1- and M2-DNA interactions, we performed supershift assays using *in vitro* translated HA-tagged M1 or M2 protein with the HA antibody (Santa Cruz Biotech) (Fig. 6C, lane 4, 11). The protein-DNA complexes of both M1 and M2 were supershifted with the addition of HA antibody in lane 4 and 11 of Figure 6C, respectively, while retaining the complexes in the presence of preimmune serum (lane 5 and 12). This indicates the specificity of the HA antibody for the complexes. These results suggest that the *in vitro* translated M1 and M2 proteins can bind to the NS probe.

Protein bands of approximately 115, 80, 78 kDa were observed in NS20Y cells when the blot was incubated with anti-Sp3 antibody (Fig. 6D). To further support that Sp3 isoforms, M1, and M2 regulate endogenous MOR transcription, a chromatin immunoprecipitation (ChIP) assay was carried out to determine the interaction of M1 and M2 proteins on the mouse MOR promoter (Fig. 6F). After cross-linking of proteins and DNAs with formaldehyde, cell lysates from NS20Y cells and NS20Y cells containing overexpressed M1 and M2 were subjected to immunoprecipitation with Sp3 and HA antibody. The precipitated DNA fragments were amplified with primer spanning a 196-bp region covering the M1 and M2 binding site of the mouse MOR promoter. As shown in Fig. 6D, ChIP PCR product were detected with Sp3 and HA antibody in NS20Y cells, but not detected with preimmune serum (PI) as a control. This suggests that the endogenous M1 and M2 specifically bind to negative regulatory region of the MOR promoter. This is consistent with the *in vitro* results of our supershift assays in Fig. 6C.

Effects of Sp3 and Sp3 isoforms (M1 and M2) on the mMOR proximal promoter and the Sp1-mediated trans-activation of the mMOR proximal promoter in *Drosophila* SL2 cells.

We hypothesized that the Sp3 isoforms (M1 and M2) may also act to repress the mMOR proximal promoter. To examine this hypothesis, cDNA encoding Sp1, Sp3, and the Sp3 isoforms (M1, M2) were each co-transfected with the p189 plasmid containing the mMOR proximal promoter 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, especially Sp family proteins (Courey and Tjian, 1988; Schneider, 1972). SL2 cells are particularly suited to this task because they are devoid of many ubiquitous mammalian transcription factor activities, and thus their transcriptional properties can be investigated in the absence of interference by endogenous factors. As shown in Fig. 7A, Sp1 and Sp3 could activate the p189 plasmid luciferase construct while Sp3 isoforms (M1, M2) could inhibit the promoter activity of the p189. However, the M1 and

M2 isoforms were not able to inhibit the promoter activity of the p189-m3, which contains a mutated Sp binding site (data not shown). Based on the data in the EMSA of Fig. 5 and the functional assay of Fig. 7A, Sp1 binds (although weakly) to this regulatory region and increases the mMOR promoter activity of p189 more than 4-fold, compared to vector-only co-transfection with p189, indicating that a small amount of Sp1 factor would be enough to regulate the MOR proximal promoter. Since Sp1, M1, and M2 share the binding site, we performed a co-transfection with a fixed amount of Sp1 plasmid (0.5 μ g) and various amounts (0-1 μ g) of Sp3, M1, and M2 in SL2 cells (Fig. 7B). Both isoforms M1 and M2 were able to inhibit Sp1-mediated *trans*-activation of the p189 promoter, and the trans-activation is more sensitive to expression of M1 than M2. Sp3 did not show any significant effect on Sp1-mediated *trans*-activation of the p189 promoter activity. To evaluate whether the M1 and M2 were involved in the repression of the mMOR proximal promoter in NMB cells, we cotransfected a M1 and M1 expression plasmids with reporter construct p1. As shown in Fig.7C, when the p1 construct was transfected into NMB cells in the presence or absence of M1 and M2, MOR promoter activity in the presence of M1 and M2 was repressed. These results suggest that the Sp3 isoforms (M1 and M2) are involved in the repression of the mMOR proximal promoter.

Effect of Exogenous Sp3 isoforms (M1 and M2) on MOR gene expression. To evaluate whether transiently overexpressed Sp3 isoforms (M1 and M2) may result in down-regulation of endogenous MOR transcript, reverse transcriptase-PCR (RT-PCR) analysis using MOR specific primers was performed with total RNA from NMB cells transfected with varying amounts (0-4 μ g) of pcDNA-M1/flu or pcDNA-M2/flu, as well as with the pcDNA3.1 vector. Transfecting cells with pCMV-Sp1 or pCMV-Sp3 did not effect MOR mRNA expression (data not shown). Since NMB cells were known to produce saturated levels of Sp1 and Sp3 proteins, these exogenous Sp1 and Sp3 constructs were not able to effect any significant changes on endogenous MOR gene expression

in NMB cells. In Fig. 8A and B, both M1 and M2 could down-regulate the endogenous MOR gene expression in a dose-dependent manner with a little greater expression of M2. All these results indicate that the M1 and M2 isoforms of Sp3 may play an important role in the regulation of MOR gene expression.

Discussion

Precise transcriptional regulation of opioid receptor genes in the brain is crucial for normal neuropharmacological function. Several classes of nuclear proteins are intricately involved in controlling expression of these genes (Wei and Loh, 2002). The 5'-flanking region of the mouse μ opioid receptor (MOR) gene has two promoters, referred to as distal and proximal, and the activities of each in the brain are quite different from the other (Ko et al., 2002). The distal promoter of the mouse MOR gene is known to be regulated via various *cis*-elements and *trans*-factors (Hwang et al., 2003; Im et al., 2001). The negative *cis*-acting element (34 bp containing consensus PU.1 binding site) of the distal promoter of the mouse MOR gene exerts a strong repressor function on the MOR distal promoter and the heterologous SV early promoter (Choe et al., 1998). The proximal promoter of the mouse MOR gene is also known to be regulated via various *cis*-element and *trans*-factors (Ko et al., 2003; Ko et al., 1998), all of which are important for proximal promoter activity. Each of these *cis*-elements and *trans*-factors contribute a portion (25%-50%) of the total promoter activity. This is a unique feature of the proximal promoter of mouse MOR, as compared to the other opioid receptor gene promoters (κ - and δ -). The two latter promoters (κ - and δ -) contain a single *cis*-element which controls the major promoter activity (approximately 80-90%) in either a positive or negative manner (Ko et al., 1997). Thus the proximal promoter of mouse MOR gene is under a comparatively fine tune and tight control (Ko et al., 2002; Ko et al., 2003; Ko et al., 1998; Ko and Loh, 2001; Ko et al., 1997).

Transient transfections of deletion constructs suggest that the negative regulatory sequence located in the -219 to -189 bp region plays a role in the regulation of the proximal promoter (Fig. 1). This sequence has homology to the Sp binding sequence and is highly conserved among mouse, rat, and human species (Fig. 2). A mutation of the negative regulatory sequence (5'-GGGA-3'→5'-GCCA-3') increased the promoter activity (approximately 100%) (Fig. 3 B). Recently, it was

reported that a similar probe of the regulatory region contained NF- κ B subunits which bind to this region and function as inducible factors in human immune and neuronal cells (Kraus et al., 2003). However, in neuronal cells, such as NMB and SHSY-5Y cells, NF- κ B complexes were not observed (Fig. 5 A and B). The binding of NF- κ B to this regulatory region requires stimulation such as TNF treatment in neuronal cells (Kraus et al., 2003). Since we used non-stimulated conditions, the NF- κ B binding on this region was not observed. However, this remains under further investigation. The NF- κ B binding sequence has been shown to bind Sp-related proteins (Sp1, Sp3 and Sp4) because of the similarity of both DNA binding sequences (Mao et al., 2002). So we may hypothesize that in non-stimulated cells, this regulatory sequence binds Sp3 isoforms (M1 and M2) to repress MOR promoter activity. However, under stimulated conditions, such as TNF treatment, this sequence binds NF- κ B subunits to activate the MOR promoter activity (Kraus et al., 2003). The negative regulatory sequence contains a C/A polymorphism at -202 bp (C nucleotide in CXBK mice, but A nucleotide in B6 mice) of the MOR gene which results in different amounts and sizes of transcripts between these two mouse strains (Ikeda et al., 2001). However, our results showed that the base change from C to A did not affect the MOR promoter activity in NMB cells.

To determine which nuclear proteins recognize the negative regulatory sequence, this element was used as the probe in EMSA and supershift assays. We identified a major protein /DNA complex containing Sp3, but Sp1 binding was very weak. (Fig. 4 and 5). Based on these data, we conclude that Sp3 is a major transcription factor bound to the negative element in NMB and SHSY-5Y cells. Sp3 is a member of the Sp family of transcription factors and binds to Sp consensus DNA with similar affinity and specificity to that of Sp1. Sp3 has been shown to be a bifunctional transcriptional factor that can both activate and repress transcription, and encodes three distinct gene products as follow: a full-length protein (Sp3) that is an activator of transcription, and two isoforms (M1 and M2) derived from internal translational initiation which function as

transcriptional repressors (Ammanamanchi and Brattain, 2001; Majello et al., 1997; Won et al., 2002). The minimal region required for transcriptional repression by M2 consists of the amino terminal 93 amino acids of the M2 (Kennett et al., 2002). In regard to Sp3, co-transfection experiments in *Drosophila* SL2 cells indicated that the overexpression of Sp3 can *trans*-activate the proximal promoter of the MOR gene, and overexpression of the Sp3 isoforms, M1 and M2, can repress both the MOR proximal promoter and Sp1-mediated activation of MOR promoter activity (Fig. 7). To determine whether M1 and M2 recognize the negative regulatory sequence, this element was used as the probe in EMSA and supershift assays using *in vitro* translated M1 and M2 proteins. We identified that major protein/DNA complexes contain M1 and M2 (Fig. 6), indicating that the M1 and M2 isoforms of Sp3 are nuclear factors interacting with the negative regulatory sequence. It has been shown that Sp1, Sp3, M1, and M2 proteins interact with TAF_{II} basal transcription factors (eg., TAF_{II} 70 and TAF_{II} 40) (Kennett et al., 2002). We propose that Sp1/Sp3 activators and M1/M2 repressors may compete for interactions with TAF_{II} basal transcription factors and this competition may determine the expression level of the Sp-dependent mouse MOR gene. Significantly, ectopic M1 and M2 expression led to repression of endogenous MOR gene expression in NMB cells. This data showed that overexpression of M1 and M2 proteins in NMB cells by transfection of pcDNA-M1/flu and pcDNA-M2/flu resulted in repression of endogenous MOR gene expression (Fig. 8). Thus, transcriptional control of the MOR gene may be dependent upon the ratio between M1/M2 and Sp1/Sp3. The amino-terminal 93 amino acids of M2 harbor the first 25 amino acids of the glutamine-rich domain and serine/threonine-rich region which were required for gene repression (Kennett et al., 2002). Sp3, M1, and M2 appear to be expressed in all mammalian cells and tissues at equivalent levels, but dependent on growth status or induction by extracellular stimuli. In contrast to full-length Sp3, M1 and M2 function as potent repressors of Sp-mediated transcription. M1 and M2 proteins were bound strongly to the negative regulatory sequence of the mouse MOR promoter

and thus this sequence confers repression of the mouse MOR gene. Our findings may promote a better understanding of the molecular mechanisms underlying MOR gene expression.

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Footnotes

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Figure legends

Figure 1. 3'-deletion analysis of proximal promoter activity of μ opioid receptor gene. A schematic diagram representing the proximal promoter region of the mouse μ opioid receptor gene (MOR) gene from nucleotide -450 to +1. The translation start site (ATG) is designated as +1. Fragments of various lengths of the promoter region were inserted into the promoterless luciferase vector, pGL3-basic (Promega). The numbers on the right of each construct refer to the number of the nucleotide at the 3'-end of each inserted promoter fragment. The numbers on the left of each construct refer to the number of the nucleotide at the 5'-end of each construct, which is -450 bp. Transient transfections and luciferase assays of various constructs were performed in NMB cells as described in the Materials and Methods. The proximal activity of each construct was expressed as *n*-fold activation of pGL3-basic promoter activity. The histograms represent the mean values of fold activation from three independent transfection experiments with two different plasmid preparations. Error bars indicate the range of standard errors. TIS, transcription initiation site.

Figure 2. Sequence comparison of mouse, rat and human MOR negative regulatory region in the proximal promoter. (A) Negative regulatory sequence of MOR promoter. The Sp binding region is underlined. (B) Sequences represent the mouse (-219 to -189, the translational initiation site designated as +1), rat (-220 to -190) and human (-219 to -189) MOR negative regulatory region in the proximal promoter. The matched sequences of negative regulatory regions are marked by asterisks

Figure 3. Mutational analysis of the putative Sp binding site in the mouse MOR promoter. (A) One Sp consensus binding site in p189 is underlined and the mutated sequences in mutant constructs p189-m1, p189-m2, p189-m3, and p189-m4 are also underlined. (B) The luciferase

activity of the mutant constructs in NMB cells is expressed as a luciferase/ β -galactosidase activity ratio. The histograms represent mean values of three independent transfection experiments with two different plasmid preparations. Error bars indicate the range of standard errors.

Figure 4. Binding of nuclear proteins to the negative regulatory element. (A) Negative regulatory sequence (NS) was used as a probe in EMSA analysis. NS and consensus Sp1 sequence were used as competitors and mNS (Sp3 binding site of NS) was mutated as underlined. EMSA was performed with nuclear extracts (NE) from NMB (B) and SHSY-5Y (C) cells separately. The major protein/DNA complexes are indicated by arrows. Double-stranded NS was selected as a probe and was ^{32}P -labeled. All lanes contained NS probe and 5 μg of nuclear extracts (NE) from either NMB or SHSY-5Y cells. Lane 1 is NS probe alone. Lane 2 is a control reaction in the absence of competitor. Lanes 3, 4, and 5 contain 100 molar ratio excess of unlabelled NS (lane 3), mNS (lane 4) and consensus Sp1 sequence (lane 5) as specific competitors.

Figure 5. Binding of the Sp3 factor to the negative regulatory element. NS sequence was used as a probe in EMSA analysis. The nuclear extracts were preincubated with p50 (NF- κB), p65 (NF- κB), Sp1, or Sp3 antibody, or preimmune serum (PI) as indicated. EMSA was performed with nuclear extracts (NE) from either NMB (A) or SHSY-5Y (B) cells. The major protein/DNA complexes are indicated by arrows. The supershift bands are indicated by *. EMSA was performed as in Fig. 4B. All lanes contained NS probe and 10 μg of nuclear extracts (NE) from NMB cells or SHSY-5Y cells. Lane 1 is NS probe alone. Lane 2 is a control reaction in the absence of competitor. Lane 3 contains 100 molar ratio excess of unlabelled NS as a self competitor. Lane 4, p50 antibody; lane 5, p65 antibody; lane 6, Sp1 antibody; lane 7, Sp3 antibody; lane 8, preimmune serum (PI). Right figures are more optimized EMSA analysis.

Figure 6. Supershift assay of *in vitro*-translated HA-tagged Sp3 isoforms (M1 and M2) using the anti-HA antibody and ChIP assay using Sp3 and HA antibodies. (A) Western blot analysis was carried out with nuclear extracts from NMB cells and anti-Sp3 antibody. Three different isoforms of Sp3 with different molecular weight are indicated. M.W stands for molecular weight in kilodaltons (KDa). (B) *In vitro* translated M1 and M2 proteins were radiolabeled with [³⁵S] methionine using Promega's *in vitro* translation kit. (C) EMSAs were performed as indicated in Materials and Methods. Lanes 2 and 9 are Reticulocyte (RBC) EMSA reactions without antibody. Lanes 3 and 10 are the control EMSA reactions without antibody. The supershift bands are indicated by <. Lanes 4 and 11 are the EMSA reactions with the HA antibody. Lanes 5 and 12 are reactions with preimmune serum (PI). Lanes 6 and 13 are self-competitor controls without antibody. Lanes 7 and 14 are non-specific competitor (Sox binding sequence; 5'-ACAATTGTTTCTTTGAACAGGTTT-3') controls without antibody. All lanes contained 5 µl of *in vitro* translated M1 and M2 products from a 50 µl reaction volume (Promega's *in vitro* translation kit). *In vitro* translated M1 and M2 proteins were incubated with the monoclonal HA antibody, preimmune serum (PI), or self competitor as indicated. (D) Western blot analysis was carried out with nuclear extracts from NS20Y cells and anti-Sp3 antibody. Three different isoforms of Sp3 with different molecular weight are indicated. M.W stands for molecular weight in kilodaltons (KDa). (E) Chromatin immunoprecipitation analysis of Sp3 isoforms, M1, and M2 binding interaction at MOR promoter. ChIP assay was performed using a Sp3 and HA antibody in NS20Y cells. Immunoprecipitated DNA was analyzed by PCR using primers that amplified a 196 bp region of the MOR promoter, which encompasses a negative regulatory sequence. Lane 1, input; lane 2 and lane 3, 10 µg of HA antibody; lane 4, lane 7, 10 µg of Sp3 antibody; lane 5, pre-immune serum (PI); lane 6, no antibody; Lane 7, a negative control of PCR for Sp3 immunoprecipitation

using specific primers for mouse β -actin gene.

Figure 7. Effects of Sp1, Sp3, and Sp3 isoforms (M1, M2) on mMOR promoter in SL2 cells and effects of M1 and M2 on mMOR promoter in NMB cells. (A) *Trans*-activation of the mMOR promoter by Sp1 and Sp3 and repression of the mMOR promoter by the Sp3 isoforms (M1 and M2). SL2 cells were cotransfected with 0.5 μ g of the mMOR promoter construct p189 and 0.5 μ g of Sp transcription factors (pPacSp1, pPacSp3, pPacM1, and pPacM2). (B) Effect of Sp3 isoforms (M1, M2) on the Sp1-mediated *trans*-activation of mMOR promoter. SL2 cells were cotransfected with 0.5 μ g of pPacSp1 and different amounts of Sp transcription factors (pPacSp3, pPacM1, and pPacM2) along with 0.5 μ g of the mMOR promoter construct p189. (C) Effect of Sp3 isoforms (M1, M2) on mMOR promoter in NMB cells. NMB cells were cotransfected with 1 μ g of pcDNA-M1/flu and pcDNA-M2/flu along with 0.5 μ g of the mMOR promoter construct p1. Cells were harvested 48 h after transfection, lysed, and assayed for luciferase activity and results from at least two representative experiments were averaged. The promoter activity was normalized by protein concentration. Error bars indicate the range of standard errors.

Figure 8. MOR mRNA expression levels in Sp3 isoform (M1, and M2) cDNA-transfected NMB cells. (A) Total RNAs from NMB cells transfected with a varying amount of pcDNA-M1/flu plasmid was reverse-transcribed into cDNA that was used as a template for PCR using MOR gene-specific PCR primers and β -actin primers as indicated. (B) pcDNA-M2/flu was instead of pcDNA-M1/flu. PCR products were electrophoresed on a 2 % agarose gel, and the relative densities of MOR mRNA were normalized to β -actin. Lane 1, vector DNA only transfected; lane 2, 0.5 μ g of each plasmid DNA was transfected; lane 3, 1 μ g of each plasmid DNA was transfected; lane 4, 2 μ g of each plasmid DNA was transfected. lane 5, 4 μ g of each plasmid DNA was transfected. The total

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amount of DNA transfected was equalized by using vector pcDNA3 DNA. The results of at least three independent transfection experiments performed in triplicate plus S.E.M. are shown. *: $p < 0.05$.

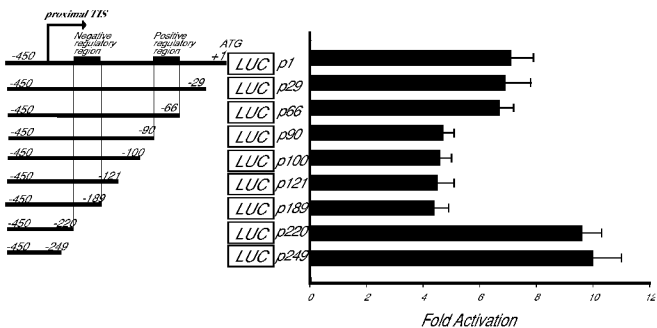


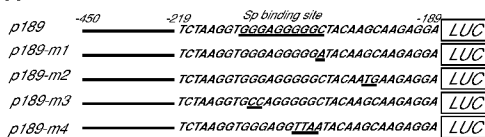
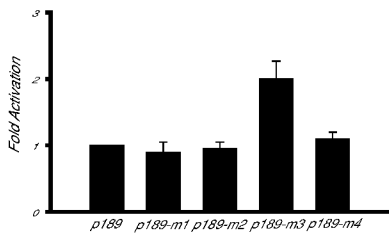
Fig. 1

A

⁻²¹⁹
TCTAAGGTGGGAGGGGGCTACAAGCAGAGGA⁻¹⁸⁹
Sp binding sequence

B

⁻²¹⁹
mMOR **TCTAAGGTGGGAGGGGGCTACAAGCAGAGGA**⁻¹⁸⁹
rMOR **TCTAAGGTGGGAGGGGGCTACAAGCAGAGGA**
hMOR **ACTAAGGTGGGGGGGGGGCTATACGCAGAGGA**

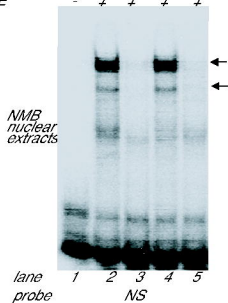

A**B**

A

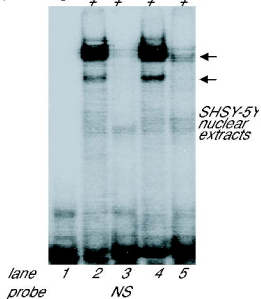
NS (-220) **TTCTAAGGTGGGAGGGGGCTACAAG** (-196)
mNS **TTCTAAGGTGCCAGGGGGCTACAAG**
Sp1 **CCCTTGGTGGGGGCGGGGCTAAGCTGCC**

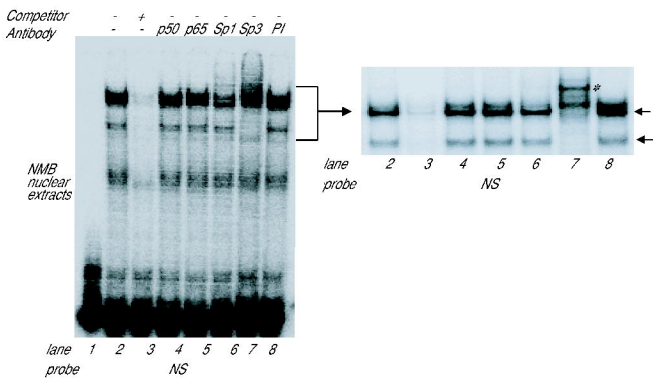
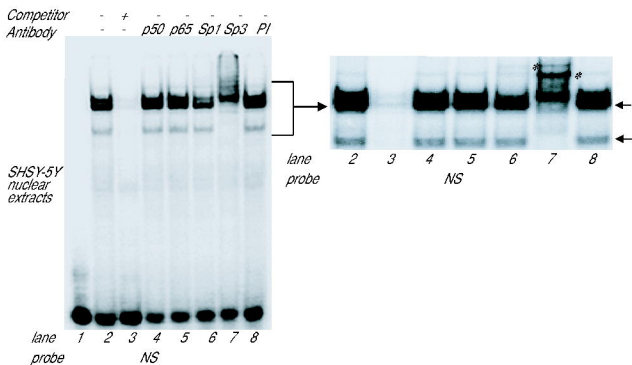
B

Competitor - - NS mNS Sp1
 NE - + + + +

**C**

Competitor - - NS mNS Sp1
 NE - + + + +



A**B****Fig. 5**

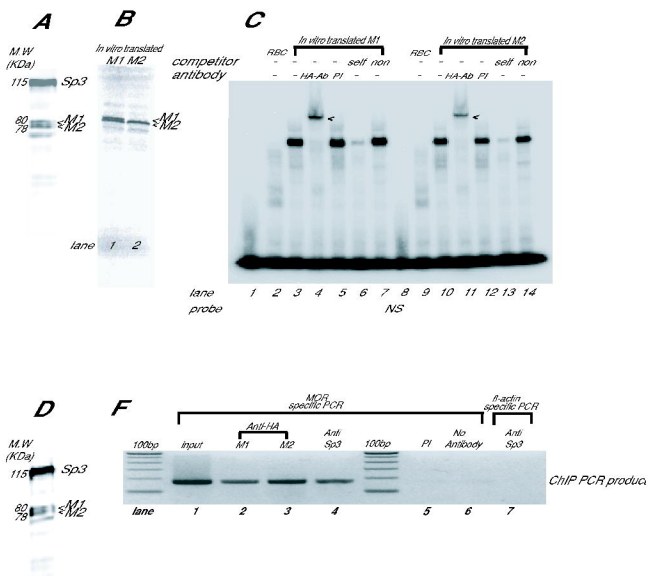


Fig. 6

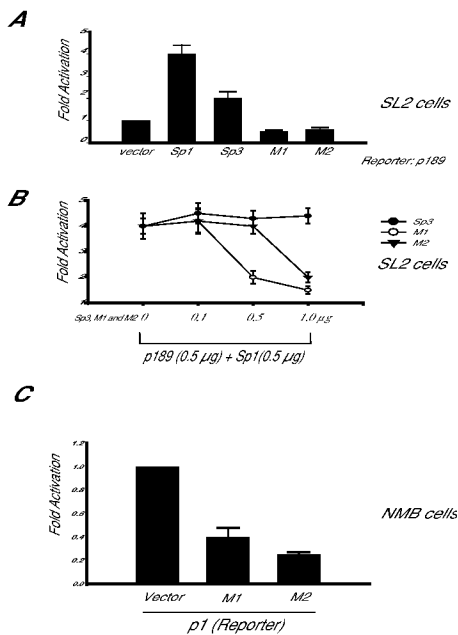
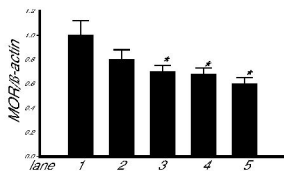
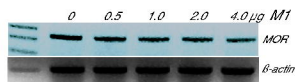


Fig. 7

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A



B

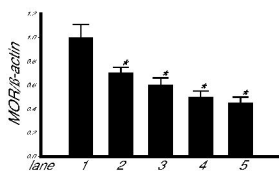
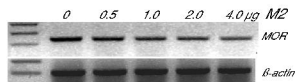


Fig. 8