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# The Role of Transcription Factors Sp1 and YY1 in Proximal Promoter Region in Initiation of Transcription of the Mu Opioid Receptor Gene in Human Lymphocytes

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**Abstract** Although previous studies have shown that the mechanism of the lymphocyte mu opioid receptor (MOR) gene expression was distinctly different from that in the central nervous system, and is involved in several disparate aspects of the immune response, its precise molecular mechanism is still undefined. In this study, we analyzed the proximal promoter region of the MOR gene in lymphocytes to identify the influences of potential *trans*-acting factors in activating the initiation of the expression of the MOR gene in lymphocytes. The electrophoretic mobility shift assay showed that two transcription factors, Sp1 and YY1, were able to bind the promoter region. Using sequence overlapping probes and mutation assays, we determined that the CCC sequence of Sp1 and the GGC sequence of YY1 binding elements were core sequences, and replacement of these sequences lead to substantial loss of promoter activity. Stimulation with morphine was capable of up-regulating the intracellular level of Sp1 and YY1 proteins. Chromatin immunoprecipitation assays showed that the blockage of naloxone is achieved through down-regulation of transcription factor YY1. Furthermore, coimmunoprecipitation and transfection assays confirmed that the functional interaction of Sp1 and YY1 transcription factors was a crucial step in the initiation of expression of the MOR in lymphocytes. Thus, we conclude that the cooperative interaction of Sp1 and YY1 transcription factors is the critical event triggering the initiation of transcription of the MOR gene in lymphocytes, and this finding will be helpful to understand the pharmacological effect of morphine on lymphocytes. J. Cell. Biochem. 104: 237–250, 2008. 

**Key words:** mu opioid receptor; lymphocytes; expression; transcription factors; morphine; promoter

The human mu opioid receptor (MOR) is the major molecular target of morphine and is predominantly expressed in the central nervous system. It is involved in analgesia, as well as the development of opioid tolerance and physical dependence [Waldhoer et al., 2004]. The novel splice variant of the MOR supports the presence of morphinergic signaling in animals [Cadet, 2004]. However, accumulated data in the past decade have also strongly suggested the involvement of the MOR in immune responses, including innate and acquired immune responses [Pampusch et al., 1998; McCarthy et al., 2001; Suzuki et al., 2002, 2003; Szabo et al., 2003; Royal et al., 2005]. To date, all studies indicate that the effects of morphine on the immune system are achieved through opioid receptors. MOR mRNA transcripts like those found in the brain were in fact reported in rat peritoneal macrophages and in a variety of human and monkey immune cells, human Raji B cells, human CD4+ and CD8+ cells, human monocytes/macrophages, human neutrophils, monkey peripheral blood mononuclear cells and monkey neutrophils [Sedqi et al.,

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1995; Beagles et al., 2004; Wang et al., 2005]. However, this conclusion is mainly derived from pharmacological and immunological experiments and the mechanism of expression of the lymphocytes MOR gene at the level of transcription is still unclear. It has been noted that expression of the MOR gene in lymphocytes can be up- and down-regulated in response to various stimuli though its low copy numbers of transcripts, indicating that the expression of the MOR gene in lymphocytes is subtly regulated [Suzuki et al., 2000; Börner et al., 2007].

Although there has been progress in the functional characterization of the MOR gene 5′-flanking region in neurons, there are substantial differences in lymphocytes. For example, nerve cells lines have multiple transcription initiation sites (TIS) as has been shown by various studies [Wang et al., 1994; Liang et al., 1995; Choe et al., 1998; Ko et al., 1998; Wendel and Hoehe, 1998; Andria and Simon, 1999; Xu and Carr, 2000; Choi et al., 2005]. However, in lymphocytes, there is only one transcription initiation site located at 110 bp upstream of the translation start codon as shown by data from our recent investigations [Wei et al., 2005]. The latter results may partly account for the lower number of copies of the mu transcripts that result in difficult detection of mu mRNA in lymphocytes, and may also explain why different cell types use the alternative promoter to create more diversity in regulating developmental and tissue specific gene expression. This diversity and complexity of the MOR gene was reviewed recently [Pan, 2005].

Our previous studies on the MOR gene in lymphocytes have also demonstrated that the regions from base pair −372 to −253 (translational start site designed as +1) located in the 5′ regulatory sequence of the MOR gene in lymphocytes contains one critical enhancer. In addition, deletion of 119 bp from the 5′-terminal of the promoter has been shown to result in a remarkable decrease of firefly luciferase activity [Wei et al., 2005]. Further analysis of this sequence has shown that it contains two potential binding sites for transcription factors, Sp1 and Ying Yang 1 (YY1). The binding of Sp1 is necessary for a significant transcription rate [Nelson et al., 1995; Zaid et al., 1999]. Although Sp1 is known to mainly promote the constitutive expression of housekeeping genes, analysis of several other types of genes also shows a strong regulatory influence by Sp1 in various models of physiological adaptation, accompanied and probably mediated by increased Sp1 phosphorylation. In addition, Sp1 is known to play a role in the regulation of genes lacking a functional TATA box. The sequence analysis of the lymphocyte MOR promoter region has revealed that the expected region 25–30 bases upstream of TIS lacks a classic TATA box. Like Sp1, the transcription factor YY1 is a general transcription regulator controlling a great number of genes ranging from viral genes to structural proteins such as α-actin [Goffart and Wiesner, 2003]. Therefore, it is speculated that the binding sites for Sp1 and YY1 in this region, and the interaction between trans-factors might play a role in the expression of the MOR gene in lymphocytes.

The expressional and regulatory properties of human MOR gene in lymphocytes have not yet been clearly reported. Two questions need to be clarified: (1) how the expression of MOR gene in lymphocytes is regulated since the evidences on interaction of protein–DNA is lacked in lymphocytes and (2) how opioids, such as morphine, impact the regulatory profile after all the effect of opioids in immune system is different from nerve system. The overall objective of this study is to shed light on the potential regulatory mechanism for the expression of the MOR gene in lymphocytes. In this study, we will further analyze the function of the positive regulatory element located at −372 to −2 bp in the 5′-flanking region of the MOR in lymphocytes. In addition, we also verify the impact of morphine stimulation on the interrelationship of *cis*-acting elements and transcription factors in the initiation of the MOR gene expression. It is hoped that clarification of the regulatory mechanism of the MOR expression in lymphocytes will provide further insights for understanding the alterations of the immune system in morphine tolerance, dependence, and addiction, particularly in cases of drug abuse associated with disease, such as AIDS [Hu et al., 2005; Mahajan et al., 2005].

## MATERIALS AND METHODS

### Cell Culture

$1 \times 10^6$ cells of the human lymphocyte cell line (CEM x174) were cultured in RPMI 1640 medium (containing 10% fetal calf serum,

100 U/ml penicillin and 100 mg/L streptomycin) at 37°C in a humidified atmosphere with 5% $CO_2$ for 48 h. To observe the effects of morphine on the influence of transcriptional factors Sp1 and YY1 on the expression of the MOR gene in lymphocytes, cells ($5 \times 10^5$ cells per ml) were treated with 10 μM morphine chloride, which has proved to be an optimal dose according to dose response curves as reported in our previous experiments [Li et al., 2003]. In the naloxone blocking assay, the CEM x174 cells were preincubated with 10 μM naloxone for 30 min and subsequently treated with 10 μM of morphine for 12 h.

### EMSA and Supershift EMSA

Nuclear extracts from CEM x174, SY5Y and Hela cells were prepared with the method as described by Koga et al. [2005]. Briefly, $1 \times 10^5$ cells per ml were cultured in RPMI 1640 medium at 37°C in a humidified atmosphere with 5% $CO_2$ for 48 h. After collection from the flask, cells were suspended in a solution containing 20 mM Hepes (pH 7.9), 25% glycerin, 0.02 mM KCl, 1.5 mM $MgCl_2$, 0.05 mM dithiothreitol and 0.2 mM PMSF. The process was repeated as necessary to lyse the cells. The pellets were then suspended in high salt buffer containing 20 mM Hepes (pH 7.9), 25% glycerin, 1.2 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.2 mM PMSF to extract the nuclear protein, which was collected as a supernatant after centrifugation at 25,000*g* for 30 min and dialyzed for 6 h to remove the salt. After centrifugation at 25,000*g* for 20 min the supernatant was stored in aliquots at −80°C. The concentration of the solubulized nuclear protein was conventionally measured by the Coomassie brilliant blue method [Bradford, 1976]. Oligonucleotides of different sizes containing Sp1 and YY1 transcription factor binding sites were used as probes for EMSA. An oligonucleotide (120 bp) was derived from *Pst*I/*Xho*I digestion on a fragment (419 bp, location at bases −372 to −2) of the MOR gene 5′-flanking region amplified from CEM x174 cells by PCR assay using primers P419U and P419D. PCR for amplifying the 419 bp fragment was started at 94°C for 5 min, and subsequent conditions were: 30 s at 94°C, 30 s at 58°C, and 45 s at 72°C for 30 cycles, followed by a final extension for 10 min at 72°C. The PCR product (120 bp) was analyzed by 2% agarose gel electrophoresis. A 120 bp probe was used to verify the potential Sp1 and YY1 binding sites in the region of interest. The other synthetic oligonucleotides (AuGCT Biotech Co., China) used in these experiments are listed in Table I (the nucleotides altered in mutant oligonucleotides are indicated in boldface). Partially

**TABLE I. Sequences of Oligonucleotides Used as Primers or Probes for PCR Amplification and EMSA**

| Constructs | Sequences |
|---|---|
| Probes for EMSA | |
| S30 sense | 5′-ACCCCTTTTCCCTCCTCCCTCCCTTCCAGC-3′ |
| S30 antisense | 5′-GCTGGAAGGGAGGGAGGAGGGAAAAGGGGT-3′ |
| Y25 sense | 5′-CTCCGAATCCCGCATGGCCCACGCT-3′ |
| Y25 antisense | 5′-AGCGTGGGCCATGCGGGATTCGGAG-3′ |
| $S30^m$ sense | 5′-ACCCCTTTTCCCTCCT**GGT**TCCCTTCCAGC-3′ |
| $S30^m$ antisense | 5′-GCTGGAAGGGA**ACC**AGGAGGGAAAAGGGGT-3′ |
| $Y^m1$ sense | 5′-CTCCGAATCCCGC**TA**GGCCCACGCT-3′ |
| $Y^m1$ antisense | 5′-AGCGTGGGCC**TA**GCGGGATTCGGAG-3′ |
| $Y^m2$ sense | 5′-CTCCGAATCCCGCAT**AAT**CCACGCT-3′ |
| $Y^m2$ antisense | 5′-AGCGTGG**ATT**ATGCGGGATTCGGAG-3′ |
| OP1 sense | 5′-ACCCCTTTTCCCTC-3′ |
| OP1 antisense | 5′-GAGGGAAAAGGGGT-3′ |
| OP2 sense | 5′-CCCTCCTCCCTCC-3′ |
| OP2 antisense | 5′-GGAGGGAGGAGGG-3′ |
| OP3 sense | 5′-TCCCTCCCTTCCAGC-3′ |
| OP3 antisense | 5′-GCTGGAAGGGAGGGA-3′ |
| Primers for site-directed mutagenesis and EMSA | |
| P419U | 5′-ACCTCGAGAAGAGTGCCCAGTGAAG-3′ |
| P419D | 5′-ACAGTACCGGAATGCCAAGC-3′ |
| MS1-sense | 5′-GGTTTTCCCTCCT**GGT**TCCCTTCCAGCC-3′ |
| MS2-antisense | 5′-GGCTGGAAGGGA**ACC**AGGAGGGAAAAG-3′ |
| MY1-sense | 5′-GAATCCCGCAT**AAT**CCACGCTCCCC-3′ |
| MY2-antisense | 5′-GGGGAGCGTGG**ATT**ATGCGGGATTC-3′ |
| Primers for ChIP assay | |
| S142 | 5′-ACTCCTTGGATCGCTTTGC-3′ |
| A142 | 5′-CCTCCCACCTTAGTAGTTCACA-3′ |

The mutated bases are in boldface.

sequence-overlapping probes were used to identify the precise Sp1 binding sites. Double-stranded oligonucleotides were generated by annealing the synthetic oligonucleotides with respective complementary sequences. Complementary oligonucleotides of equal quantity (2.5 mM each) were annealed in a thermocycler (Techgene, UK) at the following temperatures: 88°C, 2 min; 65°C, 10 min; 37°C, 10 min and 25°C, 5 min.

The 5′-ends of the gel shift probes were radiolabeled with $\gamma$ $^{32}$P-ATP using polynucleotide kinase (Promega). After labeling at 37°C for 10 min, the probes were purified with disposable columns containing Sephadex G-25 medium (Sigma Chemical Co.). Five micrograms of the nuclear extract was mixed with 2 μg of salmon sperm DNA, 2 μl of 5× binding buffer (50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 2.5 mM dithiothreitol) and $H_2O$ to a total volume of 9 μl for 10 min. Approximately 0.2 μmol of the labeled DNA oligoprobe was then added to the nuclear mixture above to a final volume of 10 μl, and incubated for 20 min at room temperature. Subsequently, the DNA–protein complexes were separated from the unbound DNA by electrophoresis via a 6% nondenaturing polyacrylamide gel in 0.5× TBE buffer at 350 V for 30 min. The gels were dried under vacuum and exposed for autoradiography for 24 h at −80°C. Competition experiments included the addition of a 125-fold excess of unlabeled DNA oligonucleotides, while supershift analysis included the addition of 2 μg of the antibody against either Sp1 or YY1 (Santa Cruz Biotechnology) to the reaction mixture for 30 min at room temperature before the addition of probes. A 125-fold excess of unlabeled nonspecific probe was used as a nonspecific reaction control. Mutated probes were used to determine which base sequences were potential sites for protein binding in *cis*-elements.

### Verification of Sp1 and YY1 Capability for DNA Binding by the Chromatin Immunoprecipitation (ChIP) Assay

CEM x174 cells were treated with 10 μM morphine or/and 10 μM naloxone for 48 h. The chromatin immunoprecipitation (ChIP) assay was performed as described by Yin et al. [2004]. CEM x174 cells ($\sim 2 \times 10^7$ in a 75 ml culture flask) were fixed with 1% formaldehyde for 12 min at 37°C. The cell pellet was washed twice with cold PBS and re-suspended in cell lysis buffer (1% SDS, 5 mM EDTA, 50 mM Tris–HCl at a pH of 8.1) containing 1% protease inhibitor cocktail (Sigma Chemical Co.). The chromatin was sonicated (Ultrasonic Processor from Xinzhi Tech Ins, China) on ice with five pulses of 10 s each with a 20 min interval to an average length of about 200–750 bp as determined by resolving the purified DNA on a 1.5% agarose gel. The sample was then centrifuged at 4°C in an Eppendorf centrifuge (10 min at 15,000 rpm) to remove the cell debris from the crude chromatin lysate. Twenty microliters of the lysate was diluted with 80 μl of dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–Cl at a pH of 8.1) and set aside as the input chromatin. The sheared chromatin from CEM x174 cells was diluted at 1:10 and mixed with 50% protein A sepharose 4B (Sigma Chemical Co.; about 50 μl in 1 ml chromatin), which was blocked with 2 μg of salmon sperm DNA at a concentration of 2 μg/ml. After shaking 2 h, the sample was centrifuged at 1,600 rpm for 5 min at 4°C. The supernatant was collected and added either Sp1 or YY1 polyclonal antibody to 2 μg/ml and shaken overnight at 4°C. The sample was then mixed again with 50% protein A sepharose 4B (about 50 μl in 1 ml sample) and 2 μg of salmon sperm DNA to a final concentration of 2 μg/ml. After shaking 1 h, the sample was centrifuged at 1,600 rpm for 5 min to remove the supernatant. Pellets were consecutively washed with 1 ml of TSE I (0.1% SDS, 1% TritonX100, 2 mM EDTA, 20 mM Tris–HCl and 150 mM NaCl at a pH of 8.1), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA 20 mM Tris–HCl and 500 mM NaCl at a pH of 8.1) and buffer III (1% NP40, 1% Deoxycholic acid, 1 mM EDTA, 10 mM Tris–HCl and 0.25 M LiCl at a pH of 8.1) for 10 min each on a shaker at 4°C. Antibody–protein–DNA complexes were eluted from protein A sepharose 4B with 100 μl of elution buffer (0.1 M $NaHCO_3$, 1% SDS) by shaking on a rotatory platform for 10 min. Eluted complexes, as well as the input chromatin, were incubated in a water bath at 65°C for 8 h to remove protein. DNA fragments were purified with a Qiaquick PCR purification kit (QIAGEN) and stored at −20°C until use. The PCR employed in the ChIP assay consisted of 25 μl of the PCR reaction mix containing 2 μl of the DNA template, 0.5 μl of each primer (S142 and A142; 0.5 μM), 2.5 μl of

PCR buffer (10×), 0.5 μl of 10 mM of dNTPs and 1 μl of Taq polymerase (2.5 U/μl) which was subjected to amplification in a thermocycler. The PCR parameters for the Sp1 and YY1 binding regions were initially at 95°C for 2 min, followed by 30 cycles at 94°C for 30 s for denaturation, 52°C for 30 s for annealing, and 72°C for 30 s to extend the DNA. The final PCR amplified product was identified on a 2% agarose gel together with the 100 bp DNA ladder (Promega). The predicted size was 142 bp corresponding to bases −218 to −359 bp of the 5′-flanking region of the MOR gene in lymphocytes. To further verify the effects of morphine on the influence of transcriptional factors Sp1 and YY1 on the expression of the mu receptor, cells were treated with 10 μM morphine chloride and/or 10 μM naloxone for 12 h before treatment with formaldehyde and analyzed as described above.

### Coimmunoprecipitation and SDS–PAGE/Western Blot Analysis

Coimmunoprecipitation (CoIP) experiments to evaluated the interaction of Sp1 and YY1 were performed on cells solubilized in 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, and proteinase inhibitor cocktail. For CoIPs of Sp1 (or YY1), cells were solubilized in medium containing 1% NP-40 in place of Triton and SDS. Lysates were clarified by centrifugation (50,000 rpm for 10 min with a TLA120.2 rotor) and then incubated overnight at 4°C with antibody against YY1 (or Sp1; Santa Cruz Biotechnology) bound to protein A-Sepharose. Non-immune rabbit IgG was also used as a control at the step. Immune complexes were washed three times in solubilization medium without DOC and SDS, washed once in phosphate-buffered saline, and eluted in sample buffer containing 0.2 M DTT for SDS–PAGE. The samples were evaluated by 12% SDS–PAGE electrophoresis and Western blot analysis. Briefly, transfer of proteins from gels onto nitrocellulose membrane (Amersham, UK) was electrophoretically mediated in a transblotting cell at 4°C for 2 h. Membranes were blocked by immersion for 1 h in 5% non-fat milk (w/v)/PBS, and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Promega) at room temperature for 1 h. Immunocomplexes resolved by electrophoresis were visualized by incubation of the membranes with Enhanced Chemiluninescence (Zhongshang Boil Tech Co., Beijing) and exposure on an X-ray film.

### Plasmid Promoter Constructs and Site-Directed Mutagenesis

Creation of 5′ deleted MOR-Luc fusion constructs with the Luciferase Reporter Gene was described in our previous report as "Luc-5," which containing binding sites for Sp1 and YY1 [Wei et al., 2005]. Mutated MOR-Luc constructs of Sp1 and YY1 were created by PCR using the mutation primers MS1/MS2 and MY1/MY2 (Table I). All constructs were verified by DNA sequencing.

### Transient Transfection and Luciferase Reporter Gene Assay

All the plasmids used in these transfection experiments were prepared by the Large-scale Purification Kit (Vigorous, China) following the manufacturer's recommended protocol. Transfection efficiency was monitored by cotransfection of the pRL-SV 40 promoter driven Renilla luciferase (Promega). CEM x174 cells were transfected by Lipofectamine 2000 Reagent Invitrogen as described in our previous report [Wei et al., 2005]. Briefly, the cells were pelleted and resuspended in 50 μl of lysis buffer (Promega) at 36 h after transfection. After one freeze/thaw cycle, the cell lysate (20 μl) was mixed with 100 μl of LAR II reagent and the firefly luciferase activity was measured as light output (for a 10 s interval) in a Centro LB 960 luminometer (Berthold, Germany). The Renilla luciferase activity was estimated from the same lysates by the addition of 100 μl of Stop and Glo reagent (Berthold), and the light output (for a 10 s interval) was measured separately. The corrected pLuc promoter driven luciferase activity was expressed as the ratio of pLuc promoter driven luciferase activity to renilla luciferase activity. The promoter-less luciferase reporter vector (pLuc-basic) served as the negative control. To observe the influence of morphine on expression of lymphocyte MOR, CEM x174 cells transfected with Luc 5 was incubated with 10 μM morphine chloride and/or 10 μM naloxone for 12 h before determination of luciferase.

## RESULTS

### Identification of the Putative Sp1 and YY1 Binding Region in the Lymphocyte MOR Promoter

To characterize the MOR promoter region in lymphocytes, we amplified a 419 bp DNA

fragment containing the potential Sp1 and YY1 binding region upstream of the human MOR gene (GenBank accession number: AJ000341). The amplified fragment covers the region 377 bp upstream of the start codon which contains putative Sp1 and YY1 elements (Fig. 1A). An oligonucleotide (120 bp) derived from *Pst*I/*Xho*I digestion of the 419 bp fragment was labeled with [$\gamma$-$^{32}$P] and used as a probe to detect the putative binding sites for Sp1 and YY1 (Fig. 1B). A slow migrating band was observed in the EMSA experiment which could be specifically abolished by competition with a 50-fold molar excess of unlabeled oligonucleotide, but not by non-specific probe (Fig. 1C). The results demonstrated the possibility of the existence of binding sites for Sp1 and YY1 proteins in MOR promoter region in lymphocytes. Also, complexes in various densities could be visualized with nuclear extracts from SY5Y neuronal cells (high expression of the MOR) and Hela cells (no expression of the MOR; Fig. 1D).

### Analysis of Binding of Sp1 and YY1 to the MOR Promoter in Lymphocytes

To identify whether Sp1 element is able to bind to the proximal promoter of lymphocytes, complex formation with nuclear extracts from CEM x174, SY5Y and Hela cells were compared. The results showed that one complex was

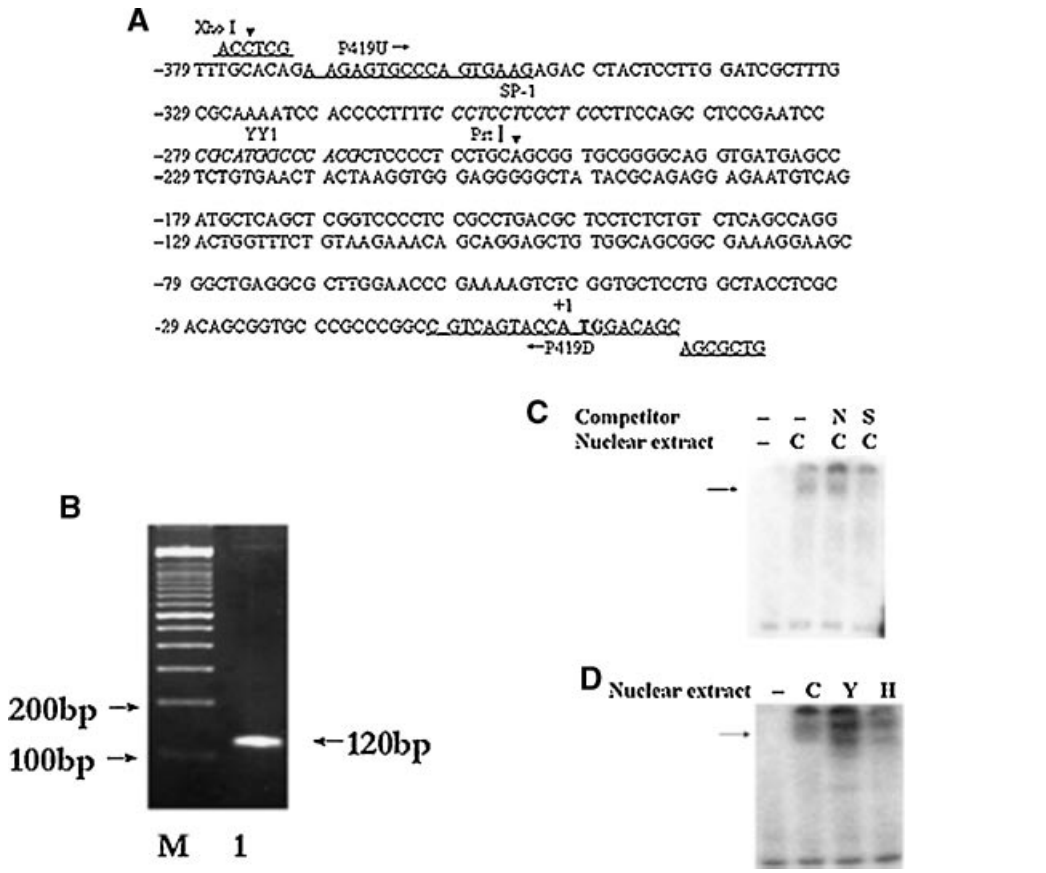


**Fig. 1.** Verification of putative Sp1 and YY1 binding site in the proximal promoter of the MOR gene in lymphocytes. **A**: Nucleotide sequence of the 5′-region from the MOR gene. Nucleotide +1 corresponds to the translation start codon, the boldfaced ATG. Underlined sequences represent the nucleotides used as primers for PCR. Additional nucleotides with restriction sites in the ends of primers are shown above or below the sequence. The putative Sp1 and YY1 *cis*-acting transcription elements are boxed and presented in italicized letters. Restriction sites used for making the 120 bp probe are indicated by the closed triangles. **B**: A fragment of 120 bp was visualized by amplification from CEM x174 cells by PCR assay. M: DNA ladder; lane 1: PCR product. **C**: EMSA experiment was performed with nuclear extracts from CEM x174 cells with a 120 bp probe. **Lane 1**, probe alone; **lane 2**, probe plus 5 μg of total protein of nuclear extract; **lane 3**, 50-fold molar excess of unlabeled non-specific probe (N) plus 5 μg of total protein of nuclear extract; **lane 4**, 50-fold molar excess of unlabeled double-stranded competitor (S) plus 5 μg of total protein of nuclear extract. Weak bands are indicated by horizontal arrows. **D**: Binding comparison of 120 bp probe in different cell lines. ''C'' represents CEM x174 cells; ''Y'' represents SY5Y cells; ''H'' represents Hela cells. All images were representative of an experiment that was repeated three times.

visualized only in the lane of CEM x174 cells (Fig. 2A). Whereas, neither SY5Y neuronal cells nor Hela cells showed any detectable bands. Non-specific probe was unable to abolish formation of the Sp1-complex (Fig. 2B). As shown in Figure 2C, the Sp1-complex could be abolished by anti-Sp1 antibody, indicating that Sp1 proteins could bind to the region within −298 to −310 bp of the MOR promoter. To more specifically identify the binding sites, three sequence overlap probes (OP1, OP2, and OP3) were designed as competitors. Since only critical bases in probe could bind Sp1 protein in nuclear extract, the precise Sp1 binding sites could hereby be identified. The results showed that the Sp1 complex could be abolished by OP2 and OP3, but not by OP1, indicating that the CCC, the overlap region of OP2 and OP3, was critical for the binding of Sp1 protein. Interestingly, if OP3 was used as the competitor, another abolished region could be observed, indicating the existence of an additional, as yet unidentified, factor modulating the promoter activity (Fig. 2C). We mutated the CCC of the OP2 probe to GGT and observed the effect of the mutated probe as a competitor ($S30^m$). However, a 125-fold molar excess of unlabeled $S30^m$ was unable to compete with the binding site for the Sp1 complex, confirming the role of CCC within the Sp1 binding site (Fig. 2D).

To analyze the role of the YY1 site in lymphocyte MOR promoter activity, we generated a double-strand oligonucleotide (25 bp) as a probe, which contained the YY1 binding site and spanned the region between −268 and −286 bp. The comparison of the formation of YY1 complex showed that a dense band was observed in the lane of CEM x174 cells compared with that of SY5Y and Hela cells (Fig. 3A). Combined with the results of Sp1, it was

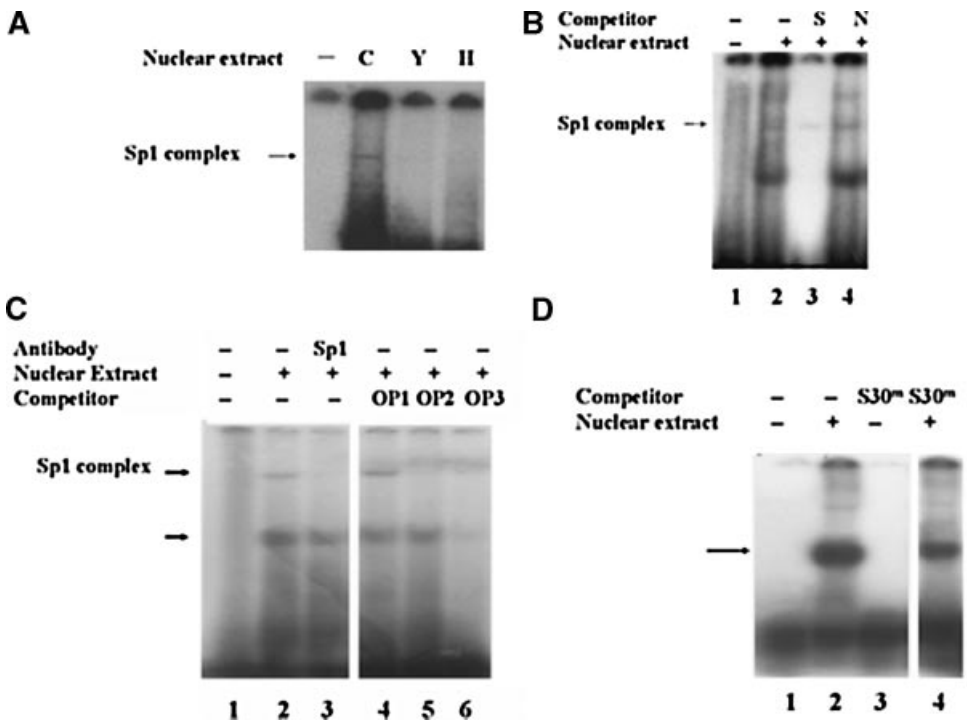


**Fig. 2.** Binding of Sp1 transcription factor in the lymphocyte MOR promoter. Five micrograms of total proteins of nuclear extract from CEM x174 cells were used in the EMSA experiment. **A**: Comparison of probe S30 binding to Sp1 from different cell lines. ''C'' represents CEM x174 cells; ''Y'' represents SY5Y cells; ''H'' represents Hela cells. **B**: **lane 1**, probe S30 alone; **lane 2**, probe S30 plus nuclear extract; **lane 3**, probe S30 with nuclear extract plus 125-fold molar excess of unlabeled double-stranded competitor; **lane 4**, probe S30 with nuclear extract plus 125-fold molar excess of unlabeled non-specific probe. ''S'' represents unlabeled competitor; ''N'' represents unlabeled non-specific probe. **C**: **lane 1**, probe S30 alone; **lane 2**, S30 plus nuclear extract; **lane 3**, probe with nuclear extract plus 2 μg of anti-Sp1 antibody; **lanes 4–6**, probe with nuclear extract plus various sequence-overlapping probes as competitors. Upper arrow indicates the major complex. Lower arrow indicates the unidentified complex. **D**: The CCC of the OP2 was mutated to GGT as a competitor ($S30^m$). **Lane 1**, OP2 probe only; **lane 2**, OP2 plus nuclear extract; **lane 3**, $S30^m$ with OP2; **lane 4**, $S30^m$ with OP2 and nuclear extract. All images were representative of an experiment that was repeated three times.

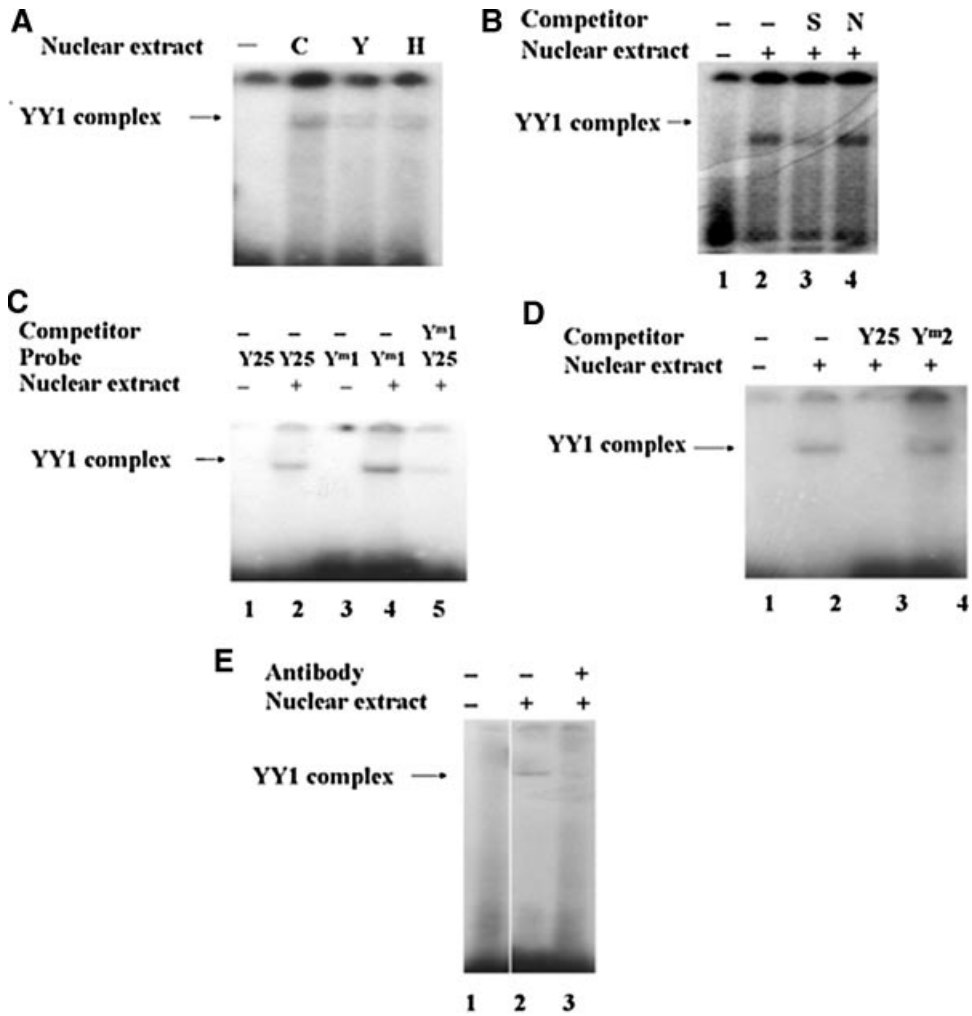


**Fig. 3.** Binding of transcription factor YY1 in the lymphocyte MOR promoter. Five micrograms of total protein of nuclear extract from CEM x174 cells were used in this EMSA experiment. **A**: Comparison of probe Y25 binding to YY1 from different cell lines. ''C'' represents CEM x174 cells; ''Y'' represents SY5Y cells; ''H'' represents Hela cells. **B**: **lane 1**, probe Y25 alone; **lane 2**, probe Y25 plus nuclear extract; **lane 3**, probe Y25 with nuclear extract plus 125-fold molar excess of unlabeled double-stranded competitor; **lane 4**, probe Y25 with nuclear extract plus 125-fold molar excess of unlabeled non-specific probe. ''S'' represents unlabeled competitor; ''N'' represents unlabeled non-specific probe. **C**: **lane 1**, probe Y25 alone; **lane 2**, probe Y25 plus nuclear extract; **lane 3**, mutated YY1 probe ($Y^m1$) alone; **lane 4**, probe $Y^m1$ plus nuclear extract; **lane 5**, probe Y25 with nuclear extract plus 125-fold molar excess of unlabeled probe $Y^m1$. **D**: **lane 1**, probe Y25 alone; **lane 2**, Y25 plus nuclear extract; **lane 3**, probe Y25 with nuclear extract plus 125-fold molar excess of unlabeled Y25 competitor; **lane 4**, probe Y25 with nuclear extract plus 125-fold molar excess of unlabeled $Y^m2$ competitor. **E**: **lane 1**, probe Y25 alone; **lane 2**, Y25 plus nuclear extract; **lane 3**, probe Y25 with nuclear extract plus 2 μg of anti-YY1 antibody. YY1 complex was indicated by an arrow in each panel. All images were representative of an experiment that was repeated three times.

speculated that the mechanism in regulation of the expression of the MOR gene in lymphocytes was different from other cell lines. Figure 3B shows the specific complex which could be abolished by unlabeled competitor probe (125-fold excess), but not by non-specific probe (Fig. 3B). Since the sequence for the binding of YY1 has been listed as having AGATGGC as the consensus base sequence 9 [Emanuele et al., 1998], we generated two different mutations of the probe ($Y^m1$ and $Y^m2$) to verify the functional bases in the YY1 binding site. If $Y^m1$ (mutated from AT to TA) was used as probe, YY1 complex was still detected. Accordingly, unlabeled $Y^m1$ could abolish the binding of wild-type probe, indicating the AT was not necessary for the binding of YY1 (Fig. 3C). However, the mutation from GGC to AAT when tested as a competitor was unable to efficiently compete with the formation of the complex of the wild-type

sequence, indicating the importance of GGC in the binding of YY1 (Fig. 3D). Supershift EMSA was performed to determine the specificity of the binding of YY1 protein to its putative binding sites. The data showed that the retardation caused by labeled probe could be significantly reduced by the addition of YY1 antibody (Fig. 3E). In concert, the data from the EMSA and supershift EMSA analysis confirmed that Sp1 and YY1 proteins bind to their putative binding sites in the MOR gene promoter.

### Effects of Morphine on the Formation of Sp1 and YY1 Complex

To investigate the regulatory mechanisms of morphine on the expression of the MOR in lymphocytes, 10 μM morphine chloride or/and 10 μM naloxone (30 min before the addition of morphine) was added into cultured CEM x174 cells for 12 h before nuclear extraction. As shown in Figure 4A,B, morphine significantly

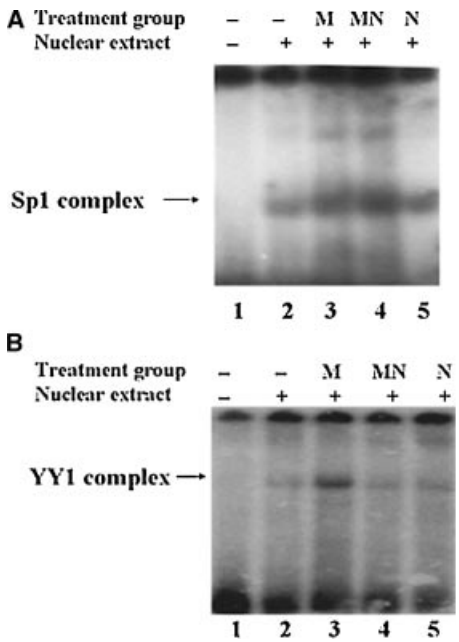


**Fig. 4.** Effect of morphine on the formation of Sp1 and YY1 complexes. Five micrograms of total protein of nuclear extract from CEM x174 cells was used in this EMSA experiment. **A**: **lane 1**, probe S30 alone; **lane 2**, probe S30 plus nuclear extract; **lane 3**, treated with 10 μM morphine; **lane 4**, treated with 10 μM morphine and 10 μM naloxone; **lane 5**, treated with 10 μM naloxone alone. **B**: **lane 1**, probe Y25 alone; **lane 2**, probe Y25 plus nuclear extract; **lane 3**, treated with 10 μM morphine; **lane 4**, treated with 10 μM morphine and 10 μM naloxone; **lane 5**, treated with 10 μM naloxone alone. YY1 complex is indicated by an arrow in each panel. M: morphine treated group; N: naloxone treated group; MN: morphine plus naloxone treated group. All images were representative of an experiment that was repeated three times.

elevated the formation of Sp1 and YY1 complexes. However, compared with the antagonistic effect of naloxone on YY1 (Fig. 4B), no detectable change in Sp1 was observed (Fig. 4A). It seems like that naloxone does not always antagonize the effect of morphine, particularly at the molecular level. An additive rather than antagonistic effect of naloxone with morphine on the expression of some genes has also been previously reported [Xu et al., 2004].

### Effects of Morphine on the Binding of Sp1 and YY1 to the Lymphocyte MOR Promoter

Chromatin immunoprecipitation analysis was performed in order to confirm that the effect of morphine on the binding of Sp1 and YY1 to the MOR promoter occurs in vivo. For these experiments, CEM x174 cells were treated with morphine or/and naloxone as describe above for 12 h, and then cross-linked with formaldehyde to fix the DNA–protein complexes. Next, cells were sonicated to shear the DNA fragments, and then immunoprecipitation with antibodies specific to Sp1 and YY1 was performed. Finally, the cross-links were reversed, and the DNA was purified and used as template for PCR amplification. Primers S142 and A142 which contained the putative Sp1 and YY1 binding sites were used and listed in Table I. The data from the ChIP assay showed that there was more Sp1 protein binding its antibody after treatment with morphine. Consistent with the results of the EMSA, the antagonistic effect of naloxone was not apparent (Fig. 5A). Similarly, the effects of morphine on YY1 could be blocked by naloxone (Fig. 5B). The fact that the Sp1 and YY1 antibodies could immunoprecipitate the Sp1 and YY1 proteins binding to the MOR promoter region indicated that both Sp1 and YY1 transcription factors were involved in the expressional regulation of the MOR gene in CEM x174 cells.

### Evaluation of Interaction of Transcription Factor Sp1 and YY1

To reveal the occurrence of an interaction between Sp1 and YY1 at the protein level, CoIP analysis was performed. Putative Sp1–YY1 protein complexes were immunoprecipitated with anti-Sp1 or (anti-YY1) antibody and analyzed by Western blotting. Immunoprecipitation with anti-YY1 led to the detection of a Sp1 band and vice versa (Fig. 6, lanes 3 and 6). Whereas no Sp1 or YY1 was detected when we

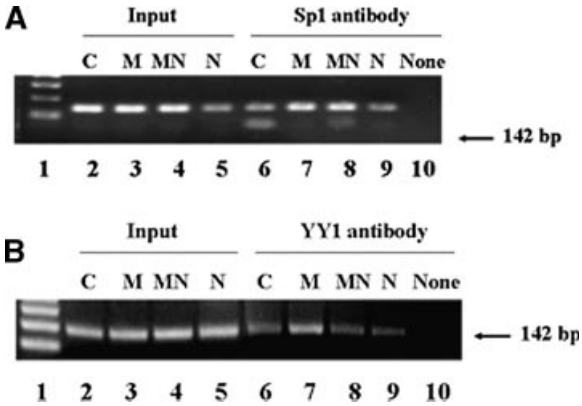


**Fig. 5.** Confirmation of Sp1 and YY1 binding to the lymphocyte MOR promoter region with the Chromatin Immunoprecipitation assay (ChIP). Formaldehyde cross linked chromatin from CEM x174 cells treated with 10 μM morphine or/and 10 μM naloxone was immunoprecipitated with anti-Sp1 or anti-YY1 antibody and subjected to PCR as described under Materials and Methods Section. PCR products were identified in a 2% agarose gel using primers containing the Sp1- or YY1-binding region of the lymphocyte MOR proximal promoter. Input represents total chromatin applied for immunoprecipitation. **A**: **lane 1**, 100 bp DNA ladder; **lanes 2–5**, input of groups treated with 10 μM morphine or/and 10 μM naloxone; **lanes 6–9**, PCR product detected in the CEM x174 cells treated with 10 μM morphine or/and 10 μM naloxone, from which cellular chromatin complexes were immunoprecipitated with anti-Sp1 antibody; **lane 10**, 'None' represents a negative control of the CHIP assay without chromatin. Amplified product (142 bp) is indicated by an arrow. **B**: **lane 1**, 100 bp DNA ladder; **lanes 2–5**, input of groups treated with 10 μM morphine or/and 10 μM naloxone; **lanes 6–9**, PCR product detected in the CEM x174 cells treated with 10 μM morphine or/and 10 μM naloxone, from which cellular chromatin complexes were immunoprecipitated with anti-YY1 antibody; **lane 10**, 'None' represents a negative control of the ChIP assay without chromatin. The 142 bp amplified product is indicated by an arrow. C: control; M: morphine treated group; N: naloxone treated group; MN: morphine plus naloxone treated group. All images were representative of an experiment that was repeated three times.

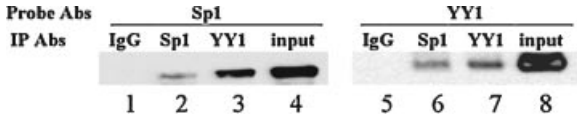


**Fig. 6.** Coimmunoprecipitation analysis of the interaction of transcription factors Sp1 and YY1. Lysates from CEM x174 cells were immunoprecipitated (IP) with antibodies to Sp1 or YY1 protein, or non-immune rabbit IgG (as indicated above each lane) and separated on a SDS/PAGE gel. Coimmunoprecipitated complexes were transferred to a nitrocellulose membrane, immunoblotted with anti-Sp1 (**lanes 1–4**) or anti-YY1 (**lanes 5–8**) antibody and exposed to X-ray film. Lanes 1 and 5, coimmunoprecipitated Sp1–YY1 complex with protein A-Sepharose beads precoated with non-immune rabbit IgG and probed with antibody to Sp1 (lane 1) or YY1 (lane 5); lanes 2 and 6, coimmunoprecipitated Sp1–YY1 complex with protein A-Sepharose beads precoated with anti-Sp1 antibody and probed with antibody to Sp1 (lane 2) or YY1 (lane 6); lanes 3 and 7, coimmunoprecipitated Sp1–YY1 complex with protein A-Sepharose beads precoated with anti-YY1 antibody and probed with antibody to Sp1 (lane 3) or YY1(lane 7); lanes 4 and 8, total protein immunoblotted with antibody to Sp1 (lane 4) or YY1(lane 8) as input. Image was representative of an experiment that was repeated three times.

used a non-immune rabbit IgG as a control for the immunoprecipitation (Fig. 6, lanes 1 and 5), indicating the specificity of the interaction between Sp1 and YY1 proteins. These results suggested the possibility of the interaction in the regulation of expression of the MOR gene in lymphocytes.

### Functional Analysis of the Mutation of Sp1 and YY1 Elements on the Activity of the Lymphocyte MOR Promoter

To investigate the underlying role of Sp1 and YY1 elements in the lymphocyte MOR promoter, we introduced either a Sp1 mutation or a YY1 mutation, or both into a MOR-Luc construct containing the MOR promoter sequence (Fig. 7A). Constructs with wild-type and mutated sites for Sp1 or/and YY1 were transfected into CEM x174 cells. Promoter activity was determined by measuring luciferase levels in transfected cells and normalized with cotransfected pRL-null Renilla luciferase activity. Data showed that Luc-5 with wild-type Sp1 and YY1 binding sites expressed a higher level of luciferase activity in CEM x174 cells (Fig. 7A). However, promoter activity from a construct containing a mutation either in the Sp1 or YY1 element was significantly less than with the wild type construct. A construct mutated in both Sp1 and YY1 binding sites resulted in the lowest level of luciferase activity and compared with Luc-5, the decrease in activity was approximately 60% ($P < 0.01$). These results implied that the interaction of transcription factors Sp1 and YY1 was a necessary event in the initiation of transcription of the MOR gene in lymphocytes. Morphine treatment was able to stimulate the activity of luciferase in Luc-5 transfected cells, which was reversed by naloxone (Fig. 7B). The result indicated that the region within −298 to −310 bp upstream of the start codon containing Sp1 and YY1 elements might be one of sites morphine affected through influencing Sp1 and YY1. All finding taken together revealed that the bases CCT of Sp1 and the bases GGC of YY1 elements in the promoter region were critical

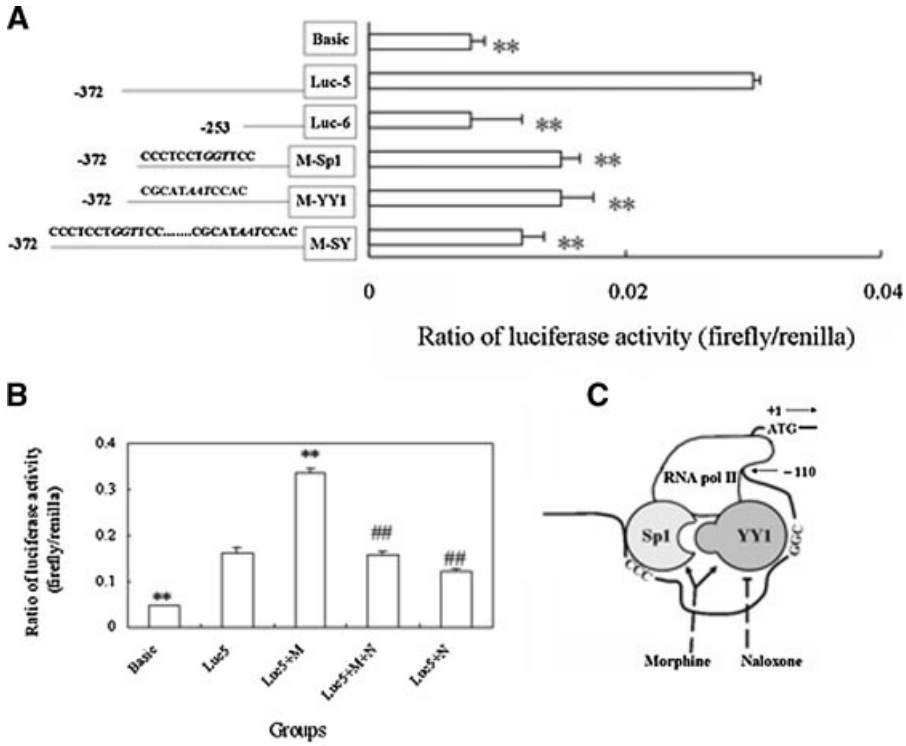


**Fig. 7.** Lymphocytes MOR promoter activity in transient transfection assays. A series of MOR promoter/luciferase constructs were prepared and introduced into CEM x174 cells. After culture for 24 h, cells were harvested for the luciferase activity assay. **A**: Various constructs containing MOR promoter with deleted or mutated Sp1 or YY1 binding sites. The sequences represent the potential Sp1 or YY1 *cis*-acting elements. Mutated bases are italicized. M-Sp1: mutated Sp1 binding site; M-YY1: mutated YY1 binding site; M-SY: mutated in both Sp1 and YY1 binding sites. The promoter activity of each construct was standardized by cotransfection of the internal control plasmid, renilla luciferase, and was expressed as the ratio of pRL-SV 40 promoter driven renilla luciferase activity to the pLuc promoter driven firefly luciferase activity. **B**: The activity of luciferase in Luc-5 transfected CEM x174 cells stimulated by morphine. Luc5 + M: morphine treated group; Luc5 + M + N: morphine plus naloxone treated group; Luc5 + N: naloxone treated group. The bars in panel A and B showed the mean + SD of the results from four independent transfection experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ was considered statistically significant between construct Luc-5 and all other constructs (A) or between morphine treated group and all other groups (B). $^{\#\#}P < 0.01$ between morphine treated group and morphine plus naloxone or naloxone treated group (B). **C**: Schematic representing the role of transcription factors Sp1 and YY1 in the initiation of the MOR gene expression in lymphocytes. Both Sp1 and YY1 bind to their *cis*-elements in the proximal promoter of the lymphocyte MOR gene and interact with each other. This interaction presumably is related to the activity of RNA polymerase II and the transcription of the MOR gene in lymphocytes is thereby triggered. The present bases represent the core sequences for binding of transcription factors. ATG represents the start codon. Upper arrow indicates the direction of transcription. Lower arrow indicates the possible initiation site of transcription about 110 bp upstream of the translation start codon [Wei et al., 2005]. The possible effects of morphine and naloxone are indicated.

bases for the protein–DNA interaction (Fig. 7C). Morphine was capable to regulate the expression of lymphocyte MOR through affecting the binding of Sp1 and YY1 to their elements in promoter region.

## DISCUSSION

Over the past decade there has been a substantial increase in our understanding of the general genomic structure of the MOR gene and its 5′ regulatory region [Liang et al., 1995; Wendel and Hoehe, 1998]. All of the advances in this field have been helpful in explaining the characteristic effects of the mu receptor interacting with its ligands in the nervous system, which include reward, tolerance, dependence and analgesia. However, the evidence explaining immune regulation by opioid at the level of transcription is still limited. It is likely that a dissimilar regulatory mechanism for the expression of the MOR exists in lymphocytes. For example, as compared with the multiple transcription initiation sites found in rodent brain cells and in human neuroblastoma SK-N-SH cells, only one initiation site has been detected in lymphocytes, which might be the direct reason accounting for the lower number

of copies of the MOR transcripts in lymphocytes and the difficulty in detection of MOR mRNA in lymphocytes [Min et al., 1994; Liang et al., 1995; Andria and Simon, 1999; Madden et al., 2001; Wei et al., 2005]. In addition, two *cis*-acting elements, namely Sp1 and YY1 boxes, were predicted to reside immediately upstream of the translation start site of the MOR gene in CEM x174 cells, and were identified as critical for controlling promoter activity of the MOR gene in lymphocytes [Wei et al., 2005].

To clarify the mechanism underlying the morphine-stimulated transcription of the MOR gene in lymphocytes, we focused on analysis of these two elements. The EMSA data showed that the putative Sp1- and YY1-binding sites in the region of −372 to −2 bp identified in these studies are required for Sp1 and YY1 binding. The fact that the binding of transcription factors to the proximal promoter is different in various cells can partially explain the expressional difference of the MOR gene. It has been reported that the promoter of the MOR gene lacks a consensus TATA box and initiator [Gill and Tjian, 1992; Javahery et al., 1994]. Other analysis also showed that this promoter does in fact possess a canonical Sp1 binding site and it has been proven that the Sp1 protein can initiate transcription through binding the Sp1 element in a TATA-less promoter [Papadodima et al., 2005; Xia et al., 2005]. Using sequence-overlapping oligonucleotide as probes, we determined that CCC was the critical base sequence for Sp1 binding, and the mutation of this sequence resulted in a loss of Sp1 binding. These results demonstrated the precise binding site of transcription factor Sp1 in lymphocytes, which were not described by other similar work in nerve cell lines.

YY1 is a multifunctional transcription factor that exerts positive and negative control on a large number of cellular and viral genes by binding to sites overlapping the transcription start site [Goffart and Wiesner, 2003]. It is reported that YY1 regulates transcription in three ways. It can activate, repress, or initiate gene transcription depending on the promoter which is involved [Breslin and Vedeckis, 1998]. Furthermore, the activity of YY1 in activation of transcription may be related to the presence of a switch region. Deletion of this region causes YY1 to act as a repressor of promoter activity [Bauknecht et al., 1995]. Several proposals for models of YY1-mediated transcription of gene have been put forward [Thomas and Seto, 1999; Sui et al., 2004]. Although the presence of the YY1 element has been identified within the proximal promoter of the MOR gene in lymphocytes, its precise function in the initiation of transcription, particularly in regard to its interaction with other *trans*-factors, is as yet not understood in detail. Our data from mutation assay of EMSA and transfection experiments has shown that transcription factor YY1 functions as an initiator of the MOR gene in lymphocytes with the core sequence GGC rather than AT as has been reported elsewhere [Gill and Tjian, 1992].

The latter part of our study was designed to define the mechanism by which morphine triggers the interaction of Sp1 and YY1, hereby initiating the transcription of the MOR gene in lymphocytes. Despite accumulated knowledge regarding the effect of morphine in lymphocytes, neither the pharmacological and immunological findings, nor clinical studies in drugs abuse account fully for the mechanism of the MOR gene expression triggered by morphine in lymphocytes. Our previous work demonstrated the relationship between morphine treatment dose and the level of expression of the MOR gene in lymphocytes, which was found to be related to the critical elements within the proximal promoter [Wei et al., 2005]. In this study, the level of both Sp1 and YY1 proteins could be up-regulated by morphine. It is of interest that the blocking effect of naloxone on morphine could be observed on the YY1 but not on the Sp1 protein. These results were consistent in data from both EMSA and ChIP assays. Thus, these results suggest that the counteraction of the morphine-induced expression of the MOR gene by naloxone is achieved through down-regulation of the YY1 protein, accounting partially for the blocking mechanism of naloxone. The precise mechanism by which this occurs will be a major focus of our future research.

Our previous work demonstrated the importance of the proximal promoter containing Sp1 and YY1 boxes on the transcriptional activity of the lymphocyte MOR promoter. There has previously been description of the physical interaction of these two transcription factors in other work [Lee et al., 1993], and in fact, this interaction in the MOR proximal promoter in lymphocytes was existed as indicated in the present study by CoIP analysis. However, the functional interaction of these two molecules

remains as the focus of our ongoing study. The functional significance of the critical bases on Sp1 and YY1 binding sites was confirmed in the current study by transfection assay of mutated constructs. As the double mutation of critical bases on both Sp1 and YY1 binding sites resulted in a decrease of luciferase activity, it is likely that the transcription of the MOR gene in lymphocytes is regulated by a cooperative interaction of Sp1 and YY1 transcription factors.

As reported previously, the MOR in lymphocytes could be up-regulated by morphine [Suzuki et al., 2000]. However, the precise molecular mechanism was not clarified. Our data that showed a significant elevation in the formation of Sp1 and YY1 complexes by morphine indicated one of possible explanations. Interestingly, the fact that the effect of morphine on the level of Sp1 was not abolished by naloxone indicated that the expression of the MOR gene in lymphocytes is elaborately regulated.

In summary, the present study reveals the mechanism affecting the activity of the proximal promoter on transcription of the MOR gene in lymphocytes. By identifying the functional bases of Sp1 and YY1 binding sites, the relationship between protein–protein interaction and transcriptional activity of the MOR promoter, and also the effect of morphine on Sp1 and YY1, we have been able to describe a part of the complicated mechanism for regulation of morphine on the expression of the MOR gene in lymphocytes.

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