Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 345 (2006) 530–537
BBRC
www.elsevier.com/locate/ybbrc

Interplay of Sps and poly(C) binding protein 1 on the μ-opioid receptor gene expression ☆

Aida Rivera-Gines [a], Ryan J. Cook [a], Horace H. Loh [b], Jane L. Ko [a,*]

[a] *Department of Biology, Seton Hall University, South Orange, NJ 07079, USA*
[b] *Department of Pharmacology, University of Minnesota, Medical School, Minneapolis, MN 55455, USA*

Received 19 April 2006
Available online 2 May 2006

Abstract

The proximal promoter of mouse μ-opioid receptor (MOR) gene is the dominant promoter for directing MOR-1 gene expression in brain. Sp1/Sp3 (Sps) and poly(C) binding protein 1 (PCBP) bind to a *cis*-element of MOR proximal promoter. Functional interaction between Sps and PCBP and their individual roles on MOR proximal core promoter were investigated using SL2 cells, devoid of Sps and PCBP. Each factor contributed differentially to the promoter, with a rank order of activity Sp1 > Sp3 > PCBP. Functional analysis suggested the interplay of Sps and PCBP in an additive manner. The in vivo binding of individual Sps or PCBP to MOR proximal promoter was demonstrated using chromatin immunoprecipitation (ChIP). Re-ChIP assays further suggested simultaneous bindings of Sps and PCBP to the proximal promoter, indicating physiologically relevant communication between Sps and PCBP. Collectively, results documented that a functional coordination between Sps and PCBP contributed to cell-specific MOR gene expression.

Keywords: Functional interplay; Sp1; Sp3; Poly(C) binding protein 1; Chromatin immunoprecipitation; MOR gene expression

Opioids are the major class of analgesics used in the management of moderate to severe pain. Repeat administration of these analgesics, such as morphine, may cause the development of tolerance and physical dependence [1,2]. Opioids also produce euphoria in humans and function as a positive reinforcer [1]. The rewarding effects of these drugs make a major contribution to compulsive drug seeking and addiction [3]. All of these effects are mediated through opioid receptors. Using pharmacological studies and molecular cloning, these receptors have been classified into three major types: μ, κ, and δ [4]. Pharmacological studies originally demonstrated the μ-opioid receptor (MOR) to be the main site of interaction for morphine-induced analgesia, tolerance, and physical dependence, and this conclusion has been confirmed by the MOR gene knockout studies [5].

MOR is temporally and spatially expressed mainly in the central nervous system, with receptors varying in densities in different regions and playing different roles [6]. Deciphering the structural organization of the MOR gene has enabled investigators to study the mechanisms responsible for gene regulation and expression. The mouse MOR gene is over 100 kb in length and consists of several exons [7,8]. Three different promoters have been reported, with the proximal promoter the principal one directing the expression of MOR-1 (the most abundant form) gene in mouse adult brain [9] and during development [10]. Several transcription factors, such as poly(C) binding protein 1 (PCBP), Sp1, and Sp3, have been shown to bind to the MOR proximal promoter region and regulate MOR gene expression [11–15].

Sp1 and Sp3 belong to the Sp family of transcription factors and are ubiquitous zinc finger proteins that contain

☆ *Abbreviations:* MOR, μ-opioid receptor; PCBP, poly(C) binding protein 1; ss, single-stranded; KH domain, K homology domain; hnRNP K, heterogeneous nuclear ribonucleoprotein K; ChIP, chromatin immunoprecipitation.

* Corresponding author. Fax: +1 973 313 6396.
E-mail address: kojane@shu.edu (J.L. Ko).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.04.117

similar DNA binding domains [16]. Sp1 and Sp3 may compete for common target sequences; their effect depends on promoter structure and cellular environment [17], as well as their potential interaction with other transcription factors [18]. Therefore, Sp1 and Sp3 can differ in their capacity to regulate transcription. For example, Sp1 can activate transcription of a large number of regulated and constitutively expressed genes whether the promoter contains a TATA box or not [19]. It has been shown that Sp1 can transactivate many TATA-less promoters by interacting with general transcription machinery, such as the TATA binding protein (TBP) and TBP associated factors [16]. Although Sp1 is considered to be associated with constitutive expression, it has recently been shown to participate in activities such as cell differentiation [20], the cell cycle [21], development [22], and the enhancement of drug resistance [23].

Unlike Sp1, Sp3 contains an inhibitory region between the second glutamine-rich activation domain and zinc finger region [24]. Sp3 can activate or repress transcription but the exact molecular event that controls this dual function remains unclear. It has been suggested that cellular context plays a role in the activation of transcription by Sp3. For example, Sp3 stimulated transcription from the HERV-H terminal repeat in the teratocarcinoma cell line, NTera2-D1, but acted as a repressor in HeLa cells [25]. Whether Sp3 acts as an activator or repressor might also depend on the promoter [24,26]. The extent of post-translational modification also influences the role Sp3 plays in transcription [27]. For example, inducing acetylation of Sp3 stimulated promoter activity and enhanced transcriptional activity in MCF-7E breast cancer cells [28]. Therefore, the role of Sp3 in transcription is dependent on a variety of molecular factors.

In addition to the Sp factors, a ssDNA binding protein, PCBP, obtained from yeast one-hybrid screening system using a mouse cDNA library, has also been shown to participate in the MOR gene regulation [15,29]. PCBP belongs to the K-homology (KH) domain superfamily, the initially identified member of which was heterogeneous nuclear ribonucleoprotein K (hnRNP K). The hnRNP K protein has been shown as a RNA binding protein and is involved in telomere regulation, transcription, pre-mRNA splicing, mRNA export, and translation [30–33].

Previous studies demonstrated that MOR promoter activity can be regulated by Sp1, Sp3 or PCBP, raising the question of whether and how the interplay between Sp proteins and PCBP may regulate the MOR gene expression. The functional interaction of PCBP, Sp1, and Sp3 on the MOR proximal core promoter activity was therefore investigated here. The in vivo dynamic status of these factors binding the MOR proximal promoter was also examined using the chromatin immunoprecipitation (ChIP) assay and re-CHIP assay. Our results suggested that the functional cooperation between Sp proteins and PCBP may contribute to differences in levels of MOR gene expression in different cells in vivo.

Materials and methods

Plasmid construction. The full-length cDNA fragment of poly(C) binding protein 1 (PCBP) was cloned into the *Xho*I cloning site of the drosophila expression vector Ppac, to create the Ppac-PCBP plasmid. Ppac-Sp1 and Ppac-Sp3 were generous gifts from Dr. Tijan (University of California, Berkeley) and Dr. Gantrum Suske (University of Marburg, Germany), respectively.

Cell culture. Human neuroblastoma NMB cells were grown in RPMI Medium with 10% heat-inactivated fetal calf serum in an atmosphere of 5% CO_2 and 95% air at 37 °C. Drosophila Schneider line 2 (SL-2) cells, purchased from ATCC, were grown in Schneider's Drosophila medium (Invitrogen) supplemented with 10% FBS at room temperature.

Transient transfection and reporter gene activity assay. SL2 cells were transfected using the Superfect (Qiagen, Valencia, CA) lipofection method. Briefly, cells at approximately 40% confluence were transfected with each test plasmid. The amount of DNA used was within the linear range of the relationship between the luciferase activity and the amount of DNA. Forty-eight hours after transfection, cells grown to confluence were washed with PBS and lysed with lysis buffer (Promega, Madison, WI). Normalization of samples followed by the method described by Conn et al. [34]. All transfection experiments were repeated at least three times with similar results, utilizing constructs that were independently prepared at least twice. The luciferase activity of each lysate was determined as described by the manufacturers (Promega). Cell lysates were mixed with luciferin substrate mixture, and the light emission from the reaction was measured and recorded in relative light units (RLUs) by a luminometer (Lumat LB 9507, Berthold Technologies, Drescher, PA).

Western blot analysis. Ppac-Sp1, Ppac-Sp3, Ppac-PCBP or Ppac plasmid transfected SL2 cells were harvested 48 h after transfection, and lysed using 1% SDS containing protease inhibitors. The cell lysate (20 μg of total protein) was then incubated with the treatment buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and boiled for 2 min. The treated extracts were then subjected to SDS–10% polyacrylamide gel. The gel was electroblotted onto polyvinylidene difluoride membrane (GE healthcare) in transfer buffer (48 mM Tris–HCl, 39 mM glycine, and 20% methanol). The membrane was incubated with blocking solution (10% dried milk, 0.1% Tween 20 in Tris-buffered saline) overnight at 4 °C. Western blot with individual anti-Sp1, Sp3 or PCBP antibody (Santa Cruz Biotechnology, Inc.) was performed following the manufacturer's instruction (GE healthcare). The signals were detected using a Molecular Dynamic Storm phosphorimager system.

Chromatin immunoprecipitation (ChIP). The procedure was performed using a kit purchased from Upstate Biotechnology, according to the protocol by the manufacturer. Briefly, cells were incubated with formaldehyde at a final concentration of 1% at 37 °C for 10 min. Cells were washed twice with ice-cold PBS and were collected by centrifugation at 4 °C, and resuspended in the cell lysis buffer (50 mM Tris–HCl, pH 8, 10 mM EDTA, 1% SDS, and protease inhibitors). Cell lysates were sonicated to give a DNA size range from 300 to 600 bp, and supernatants were diluted with dilution buffer (16.7 mM Tris–HCl, pH 8, 1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, and protease inhibitors). The solutions were precleared with salmon sperm DNA/protein G agarose slurry and then treated with antibody overnight at 4 °C. Immune complexes were collected by adding a salmon sperm DNA/protein G agarose slurry. The beads were washed sequentially in the following buffers: low salt wash buffer (20 mM Tris–HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100); high salt wash buffer (20 mM Tris–HCl, pH 8.1, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100); LiCl wash buffer (10 mM Tris–HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, and 1 mM EDTA), and Tris–EDTA buffer. Immuno-complexes were extracted from the beads with 1% SDS and 0.1 M $NaHCO_3$. Cross-linking was reversed by heating the eluates at 65 °C for 4 h. The eluants were then digested with proteinase K at 45 °C for 1 h, and then further subjected to the phenol/chloroform extraction. The DNA was purified by ethanol precipitation.

The −491 to −214 bp region of the human MOR proximal promoter (translation start site designated as +1) was amplified by polymerase chain reaction (PCR). The pair of primers used in the PCR included the sense primer: 5′-GGCGCTGGAAAATTGAGTGATGTTAGC-3′ and the antisense primer: 5′-CCTTAGTAGTTCACAGAGGCTCATC-3′. Re-ChIP assays utilized a similar protocol, except that the eluant of the primary immuno-complex obtained with the first antibody was diluted with dilution buffer (16.7 mM Tris–HCl, pH 8, 1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, and protease inhibitors), and then further subjected to immunoprecipitation with the second antibody.

Results

Comparison of the transactivation acitivity of Sp1, Sp3, and PCBP on the MOR proximal promoter

Several transcription factors, such as Sp1, Sp3, and poly(C) binding protein1 (PCBP), have been reported to bind to the mouse MOR proximal promoter region, −450 to −249 bp (relative to the translation start site, which is designated as +1) [12–15,29]. PCBP was found to regulate the MOR gene by binding to the sense strand of polypyrimidine sequence located in polypyrimidine/polypurine region (PPy/u), which can adopt single-stranded (ss) DNA conformation [14,15]. Interestingly, the iGA motif that both Sp1/Sp3 (double-stranded binding protein) bind to is located within the PPy/u region, and PCBP (single-stranded binding protein) is able to bind to the same 14 bp region located at the 3′-end of the PPy/u region [12,14,15].

We previously also demonstrated that the core promoter region (−340 to −300 bp) was essential for the proximal promoter activity [12]; importantly, it contains only the PPy/u region and the overlapping iGA motif. Therefore, the MOR core promoter construct (pL340/300 plasmid) was selected, allowing us to simply compare the transactivation activity of individual Sps and PCBP protein on the same element.

Functional analysis was carried out to investigate the transactivation activity of Sp1, Sp3 or PCBP on the MOR core promoter. The co-transfection was performed using the Sp1 (pPacSp1), Sp3 (pPacSp1) or PCBP (pPacPCBP) expression plasmid and the luciferase reporter gene driven by the MOR core promoter region (pL340/300 plasmid) in SL2 cells, which is devoid of Sps factors and PCBP, which were confirmed using the Western blot analysis (Fig. 1A). The relative transactivation activity was compared to those of the pPac expression plasmid containing no insert at the same concentration, which was arbitrarily defined as 100%.

As shown in Fig. 1B, all three factors, Sp1, Sp3, and PCBP, displayed significant transactivation activities in a dose-dependent manner on the MOR proximal core promoter (in a linear regression range, $p < 0.0001$ for Sp1 and Sp3 and $p < 0.0019$ for PCBP), with the Sp1 displaying the most potent transactivation activity and a moderate activity with Sp3. The PCBP provided the least transactivation activity.

Taken together, results demonstrated that Sp1, Sp3, and PCBP transactivated the MOR proximal promoter differentially.

Investigation of functional interactions of Sp1/Sp3 and PCBP

Sp1/Sp3 and PCBP bind to the same region of the proximal promoter, indicating a possible interplay between Sp1/Sp3 and PCBP. Having determined their individual contributions on the MOR promoter activity; we next examined their combinatory effect on MOR gene expression. Combinations of Sp1 (pPacSp1) or Sp3 (pPacSp3) expression plasmids with that of PCBP (pPacPCBP) were co-transfected with the pL340/300 plasmid containing the MOR proximal core promoter into SL2 cells.

Sps were transfected at a constant level of 0.5 μg with varying amounts of PCBP. The promoter activity in the presence of various amounts (0.1, 0.5, and 1 μg) of the empty pPac plasmid (indicated as white bars) along with a constant amount of 0.5 μg pPacSp1 was arbitrarily defined as 100%. As shown in Fig. 2A, the addition of increasing amounts (0.1, 0.5, and 1 μg) of PCBP (indicated as hatched bars) resulted in the increase of transactivation in a dose-dependent manner, and the PCBP effect at each dose was additive with the effect of Sp1 alone.

Although Sp3 and Sp1 belong to the same family, Sp3 contains its unique inhibitory domain. In order to examine the functional interaction of Sp3 and PCBP, a similar experiment was carried out. As shown in Fig. 2B, the combination of various amounts of PCBP (gray bars) and a constant amount of 0.5 μg Sp3 also demonstrated an additive effect on the MOR core promoter activity in a dose-dependent manner, as compared to those of activities in the presence of the empty pPac plasmid (indicated as white bars; arbitrarily defined as 100%).

Collectively, these results showed that PCBP enhanced either Sp1 or Sp3 transactivation of the MOR promoter activity in an additive manner.

Chromatin immunoprecipitation assays of Sps and PCBP proteins

The above studies demonstrated the functional interaction of Sps and PCBP on the MOR proximal promoter. Although the exact mechanism underlying the regulation of the MOR gene expression by Sps and PCBP is still unclear, the recruitment of these factors to the MOR proximal promoter region in vivo is essential. To determine directly the in vivo physical interaction of these factors to the MOR proximal promoter, we performed chromatin immunoprecipitation assays (ChIP). The sequence of the MOR core promoter region is highly homologous with that of human (Fig. 3A); we therefore performed the ChIP assay using human neuronal NMB cells, which express MOR endogenously.

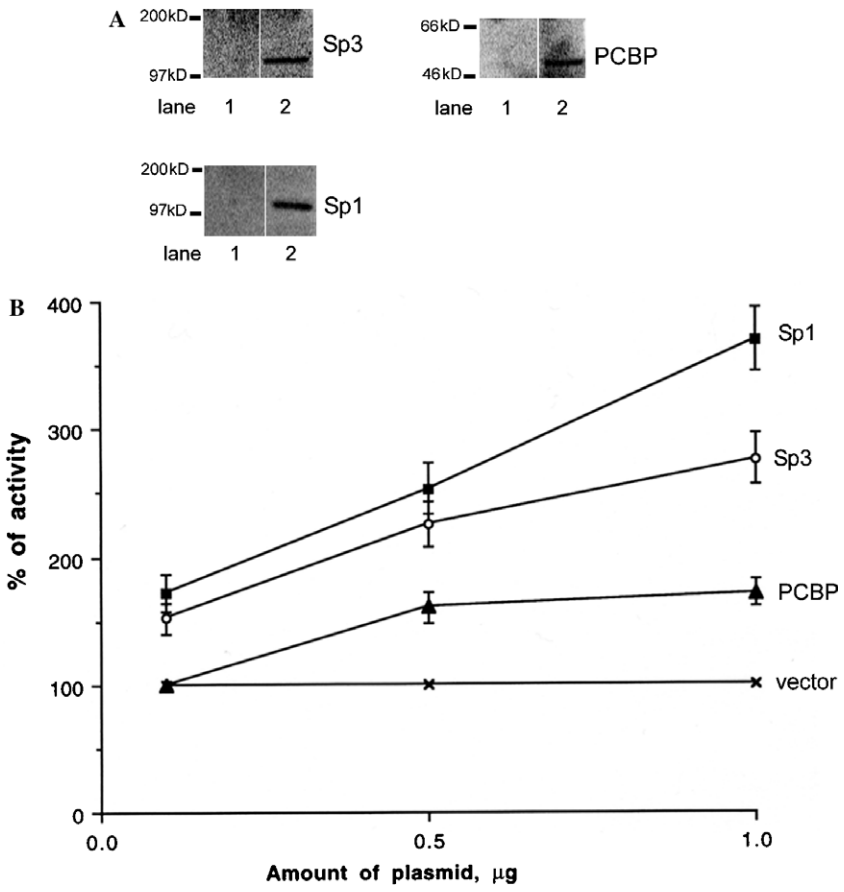

Fig. 1. Western blot and functional analyses of individual Sp1, Sp3, and poly(C) binding protein 1 (PCBP) in SL2 cells. (A) Western blot analysis was performed using the cell lysate from SL2 cells transfected with pPacSp1, pPacSp3, pPacPCBP or pPac expression plasmid and anti-Sp1 (indicated as Sp1), Sp3 (indicated as Sp3) or PCBP (indicated as PCBP) antibodies. Lane 1, 20 μg cell lysates from the empty vector pPac transfected cells; lane 2, 20 μg cell lysates from pPacSp1 (Sp1), pPacSp3 (Sp3) or pPacPCBP (PCBP) transfected cells. Numbered lines indicate the size of protein standards. (B) SL2 cells were transiently co-transfected with indicated amounts of pPacSp1 (closed squares), pPacSp3 (open circles), pPacPCBP (closed triangles) or pPac vector (x), containing no insert, and the pL300/340 plasmid, a luciferase reporter driven by the MOR proximal core promoter. The percentage of activation was calculated as the percentage of the activity of each reporter in the presence of indicated amount of each expression vector divided by the activity of reporter with pPac plasmid (arbitrarily defined as 100%).

As shown in Fig. 3B, PCR employed a pair of primers spanning −491 to −214 bp of the human proximal promoter region (translation start site designated as +1), and thus encompassing the PPy/u region (indicated by a box) bound by Sps and PCBP. Cells were cross-linked by formaldehyde, lysed, sonicated, and then subjected to immunoprecipitation using anti-Sp1, Sp3 or PCBP antibody, respectively. As shown in Fig. 3C, all three antibodies precipitated the 278 bp fragments of the proximal promoter region of MOR gene, indicating the association of Sp1, Sp3, and PCBP with this MOR proximal promoter region individually. In summary, ChIP analysis suggested that individual Sp1, Sp3 or PCBP could be recruited to and bind to the MOR proximal promoter region in vivo.

Co-localization of Sps and PCBP proteins on the MOR proximal promoter

The recruitment of different transcription factors to a promoter is in a dynamic status; since the interaction of Sps or PCBP on the MOR proximal promoter was observed, we therefore examined the possibility of Sps and PCBP could be simultaneously localized at the MOR proximal promoter in vivo using the re-ChIP assay. The cross-linked chromosome from NMB cells was immunoprecipitated with the first antibody, and the resulting immuno-complex was then eluted and further subjected to immunoprecipitation with the second antibody or no antibody (indicated as "−").

As shown in Fig. 4A, re-ChIP results showed that the proximal promoter region of MOR gene was present in the first immuno-complex using anti-Sp1 antibody, and the immuno-complexes were further pulled down again by either the anti-PCBP (indicated as PCBP) or anti-Sp3 (indicated as Sp3) antibody. These results suggested that Sp1 and PCBP as well as Sp1 and Sp3 were simultaneously present at the MOR proximal promoter. Similarly, in Fig. 4B, the anti-Sp3 antibody immunoprecipitated immuno-complexes could be further pulled down using anti-Sp1 (indicated as Sp1) or anti-PCBP (indicated as PCBP)

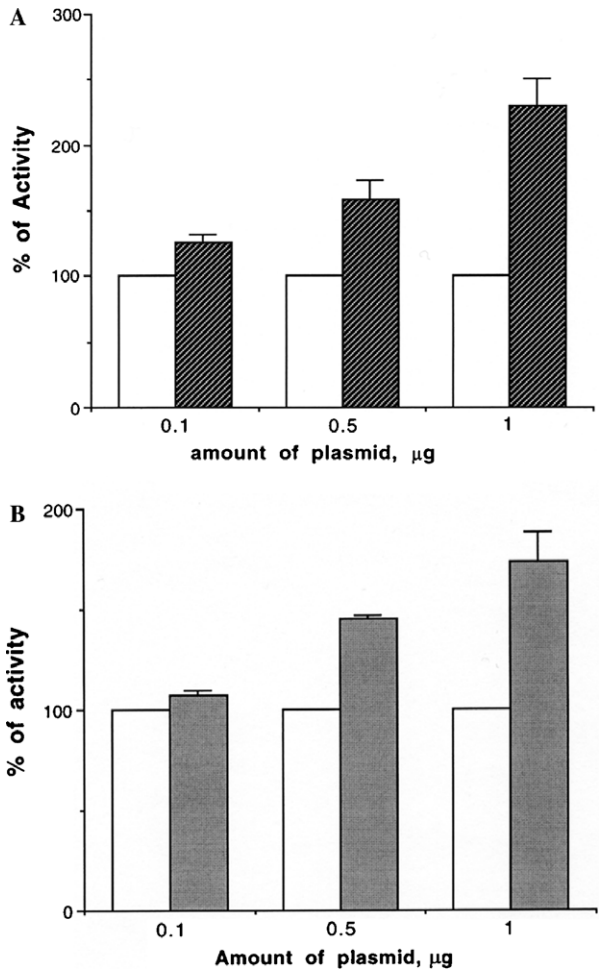

Fig. 2. Functional interaction of Sps and PCBP on the MOR core promoter. SL2 cells were transiently co-transfected with a constant amount of 0.5 μg pPacSp1 (A) or pPacSp3 (B) and various amounts (0.1, 0.5, and 1 μg) of pPacPCBP [hatched bars in (A); gray bars in (B)] or pPac vector (open bars) along with the pL300/340 plasmid, a luciferase reporter driven by the MOR proximal core promoter. The percentage of activation was calculated as the percentage of the activity of each reporter in the presence of indicated amount of each expression vector divided by the activity of reporter with pPac plasmid (arbitrarily defined as 100%).

antibody. In Fig. 4C, the MOR proximal promoter region was precipitated with anti-PCBP antibody, and then further pulled down by anti-Sp1 (indicated as Sp1) or anti-Sp3 (indicated as Sp3) antibody. Taken together, these results confirmed the simultaneous recruitment of Sps and PCBP to the MOR proximal promoter region, implicating that they may be associated at the same element and all participated in the MOR gene regulation in vivo.

Discussion

Three different factors, Sp1, Sp3, and poly(C) binding protein 1 (PCBP), are required for the activation of MOR proximal promoter, and the Sps bind to an element overlapping that binding of PCBP in the region of the core promoter [12,14,15]. In here, we first demonstrated that individual Sps and PCBP each transactivated the MOR proximal core promoter with Sp1 displaying the strongest effect and PCBP displaying the least efficacy in SL2 cells, which is devoid of Sps and PCBP factors, and thus provide a null background to study the relationship between Sp and PCBP factors.

Although the exact mechanism underlying the differential regulation of individual Sp1, Sp3, and PCBP on the core promoter is not clear, Sp1 has been reported to tether the general transcriptional machinery [35]. Therefore, it is reasonable to envision that Sp1 possesses a strong transactivation activity. Sp3 and Sp1 belong to the same Sp family, and Sp3 has also been shown to communicate with the basal transcriptional machinery [36], but it contains an inhibitory domain [24], which may result in its lower transactivation activity as compared to the effect of Sp1 on the MOR core promoter. PCBP belongs to the KH family as the hnRNP K, which has also been shown to directly interact with the general transcriptional machinery [31]. However, whether PCBP interacts with transcriptional machinery needs to be further examined empirically. The weak transactivation activity of PCBP on the MOR core promoter suggested that it may not efficiently tether the transcriptional machinery.

The possible functional interaction between the Sps, which bind to double-stranded DNA, and PCBP factor, which binds to single-stranded DNA, was first suggested by their bindings to an overlapping DNA element. Functional analysis further demonstrated that combinations of Sp1 and PCBP were in an additive manner. The combination of Sp3 and PCBP also demonstrated an additive effect on the MOR proximal promoter. Although there has been no previous report of a combinatory effect between PCBP and Sps, the additive phenomenon has been observed in the combination effect of other factors on various promoters. For example, a cooperative interaction between Sps and hnRNP K, the prototype of KH domain family, has been shown in the nicotinic acteylcholine receptor promoter [37], and the hnRNP K has been reported to functionally interact with other transcription factors, such as Zik1 [38].

The mechanism underlying this additive effect is currently unclear. In particular, whether this combinatory effect is mediated via a direct physical interaction between Sps and PCBP or via a third factor needs to be further examined. In addition, the possible involvement of histone acetylation/deacetylation is also an interesting focus for investigation. For example, PCBP may be able to enhance the interaction between Sp3 and p300, which possesses the acetyltransferase activity [39,40]. Another possibility is that the combination of Sp and PCBP may enhance the recruitment of histone acetylase, which results in the histone acetylation and hence increases the transcription activity. Overall, the combinatory results suggested that a functional interaction between Sps and PCBP could participate in the regulation of MOR proximal promoter activity over a broad range of activities, with different levels of Sps and/or PCBP present in different cells and/or the interaction between Sps and PCBP possibly contributing to variation in MOR gene expression levels in different cells and tissues.

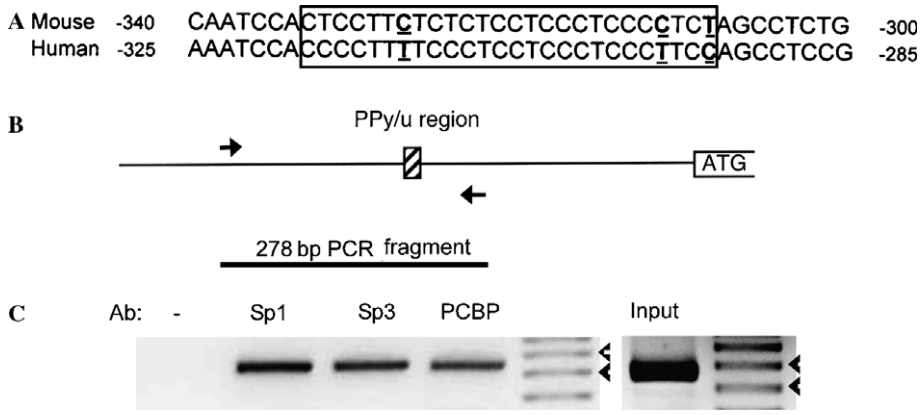

Fig. 3. Association of Sps or PCBP with the human MOR proximal promoter. (A) Sequence comparison between mouse and human MOR core promoter regions. Sequences of the core proximal promoter region of MOR gene are highly homologous (approximately 88%) between mouse (−340 to −300 bp) and human species (−325 to −285 bp). The key *cis*-acting element, PPy/u region, which is critical for the core promoter activity, is indicated by a box. A total of 3 bp (indicated by bold letters and underlined) out of 26 bp in the PPy/u region contain a C to T conversion between mouse and human species. (B) Schematic representation of the human MOR core promoter and the location of a primer pair used in the PCR. The expected size of PCR product is 278 bp, which is depicted by a solid line. (C) ChIP assay was performed using the chromatin prepared from NMB cells, a MOR expressing cell line. The soluble chromatin extracts were immunoprecipitated with anti-Sp1, Sp3, and PCBP antibodies (as indicated individually) or with no antibody (indicated as "−"), and then followed by PCR. "Input" indicated the crude chromatin extracts prior to immunoprecipitation was also analyzed. The arrowheads indicate the 200 and 300 bp DNA markers, respectively.

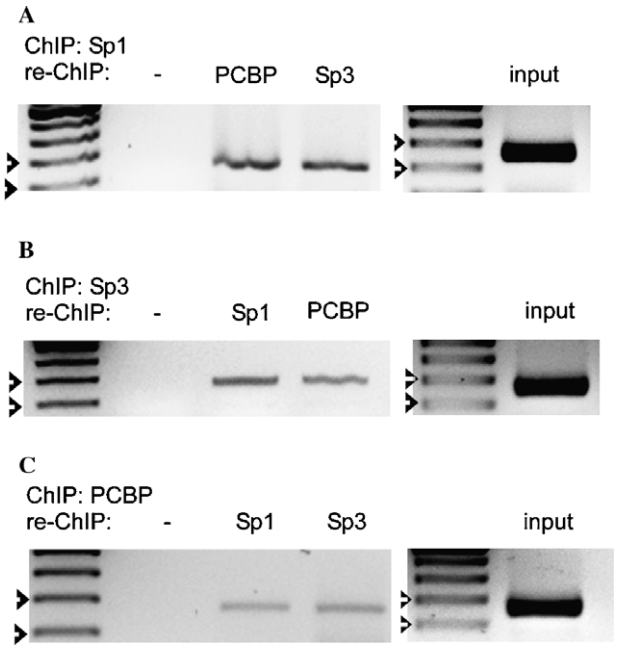

Fig. 4. Simultaneous recruitment of Sps and PCBP to the MOR proximal promoter. (A) ChIP was first performed using the chromatin prepared from NMB cells, a MOR expressing cell line, and the anti-Sp1 antibody. The eluant of immuno-complex was further subjected to immunoprecipitation using anti-PCBP, Sp3 antibody or with no antibody (indicated as "−"). The precipitated chromatin was amplified by PCR using a pair of primers (as indicated in Fig. 3B) flanking the PPy/u region in the MOR proximal promoter. Inputs indicated the chromatin extracts prior to the re-ChIP were also analyzed. Arrowheads indicate the 200 and 300 bp DNA markers, respectively. (B) ChIP was first performed using anti-Sp3 antibody. The eluant of immuno-complex was then subjected to re-ChIP using anti-PCBP, Sp1 antibody or with no antibody (indicated as "−"). (C) The soluble chromatin extracts were first immunoprecipitated with anti-PCBP antibody, and then was subjected to re-ChIP using anti-Sp1, Sp3 antibody or with no antibody (indicated as "−").

Direct evidence for the functional association between Sps or PCBP and MOR promoter was provided by chromatin immunoprecipitation (ChIP) assays, which confirmed the in vivo binding of Sp1, Sp3 or PCBP to the MOR proximal promoter. The dynamic status of Sps and PCBP simultaneously associated with the MOR proximal promoter in vivo was further suggested by re-ChIP analysis, because we found that PCBP was present in the immunocomplexes which also contained Sp1 or Sp3. These results suggest that PCBP and Sps may be recruited to the MOR proximal promoter region containing the overlapping element located in PPy/u region. In addition, the re-ChIP analysis agreed with ChIP results that Sps and PCBP associated at the MOR proximal promoter region may exist, at least, in a close proximity. These results also suggested the existence of functional coordination of Sps and PCBP factors for the promoter activity. However, it remains to be elucidated if Sps and PCBP can interact directly.

In conclusion, this study demonstrated the important role of functional interactions between Sps and PCBP, which can contribute to the differences in levels of the cell-specific MOR gene expression. The association of Sps and PCBP at the active MOR proximal promoter in vivo, as shown by re-ChIP analysis, also implicated that the cooperation between Sps and PCBP, presumably via the direct or indirect protein–protein interaction (such as mediated via a third factor present in the complex), may facilitate the basal transcription machinery.

Different expression levels of opioid receptors in different cells under the physiological or pathological situations may in turn result in variations of opioid pharmacological responses [6,41,42]. Therefore, pathological situations may alter the transcription factor levels in certain cells, and

subsequently alter the opioid receptor expression level in cells. It is reasonable to suggest that an alteration at the transcriptional level may at least partially provide a molecular explanation for the variable pharmacological responses. The study of MOR gene regulation will provide a better understanding of the essential and major determinants of the cell-specific MOR gene expression, which may lead to opportunities for the strategic development of therapeutic targets by modulation of the gene expression at the transcription level.

Acknowledgments

This research was supported by NIH research Grant DA-016673. We thank Dr. Hsien-Ching Liu and Dr. Andrew P. Smith for their intellectual contribution and helping preparation of the manuscript.

References

[1] B.L. Kieffer, C.L. Evan, Opioid tolerance—in search of the holy grail, Cell 108 (2002) 587–590.
[2] P.Y. Law, H.H. Loh, L.N. Wei, Insight into the receptor transcription and signaling: implications in opioid tolerance and dependence, Neuropharmacology 47 (2004) 300–311.
[3] J.M. Van Ree, M. Gerrits, L. Vanderschuren, Opioids, reward and addiction: an encounter of biology, psychology, and medicine, Pharmacol. Rev. 51 (1999) 341–396.
[4] P.Y. Law, H.H. Loh, Regulation of opioid receptor activities, J. Pharmacol. Exp. Ther. 289 (1999) 607–624.
[5] B.L. Kieffer, C. Gaveriaux-Ruff, Exploring the opioid system by gene knockout, Prog. Neurobiol. 66 (2002) 285–306.
[6] A. Mansour, C.A. Fox, H. Akil, S.J. Watson, Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications, Trends Neurosci. 18 (1995) 22–29.
[7] B.H. Min, L.B. Augustin, J.A. Felsheim, J.A. Fuchs, H.H. Loh, Genomic structure and analysis of promoter sequence of a mouse μ opioid receptor gene, Proc. Natl. Acad. Sci. USA 91 (1994) 9081–9085.
[8] Y.X. Pan, J. Xu, L. Mahurter, E. Bolan, M. Xu, G.W. Pasternak, Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene, Proc. Natl. Acad. Sci. USA 98 (2001) 14084–14089.
[9] J.L. Ko, S.R. Minnerath, H.H. Loh, Dual promoters of mouse mu-opioid receptor gene, Biochem. Biophys. Res. Commun. 234 (1997) 351–357.
[10] J.L. Ko, H.C. Chen, H.H. Loh, Differential promoter usage of mouse mu-opioid receptor gene during development, Mol. Brain Res. 104 (2002) 184–193.
[11] P.Y. Law, H.H. Loh, L.N. Wei, Insights into the receptor transcription and signaling: implications in opioid tolerance and dependence, Neuropharmacology 47 (2004) 300–311.
[12] J.L. Ko, H.C. Liu, S.R. Minnerath, H.H. Loh, Transcriptional regulation of mouse mu-opioid receptor gene, J. Biol. Chem. 273 (1998) 27678–27685.
[13] J.L. Ko, H.C. Liu, H.H. Loh, Role of an AP-2-like element in transcriptional regulation of mouse mu-opioid receptor gene, Mol. Brain Res. 112 (2003) 153–162.
[14] J.L. Ko, H.H. Loh, Single-stranded DNA-binding complex involved in transcriptional regulation of mouse mu-opioid receptor gene, J. Biol. Chem. 276 (2001) 788–795.
[15] J.L. Ko, H.H. Loh, Poly C binding protein, a single-stranded DNA binding protein, regulates mouse mu-opioid receptor gene expression, J. Neurochem. 93 (2005) 749–761.
[16] G. Suske, The Sp-family of transcription factors, Gene 238 (1999) 291–300.
[17] P. Bouwman, S. Philipsen, Regulation of the activity of Sp1-related transcription factors, Mol. Cell. Endocrinol. 195 (2002) 27–38.
[18] K. Roder, S.S. Wolf, K.J. Larkin, M. Schweizer, Interaction between the two ubiquitously expressed transcription factors NF-Y and Sp1, Gene 234 (1999) 61–69.
[19] A. Courey, R. Tijan, in: S. McKnight, K. Yamamoto (Eds.), Transcription Regulation, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992, pp. 743–769.
[20] R.W. Legget, S.A. Armstrong, D. Barry, C.R. Mueller, Sp1 is phosphorylated and its DNA binding activity down-regulated upon terminal differentiation of the liver, J. Biol. Chem. 270 (1995) 25879–25884.
[21] A.R. Black, D. Jensen, S.Y. Lin, C. Azizkhan, Growth/cell cycle regulation of Sp1 phosphorylation, J. Biol. Chem. 274 (1999) 1207–1215.
[22] J.D. Saffer, S.P. Jackson, M.B. Annarella, Developmental expression of Sp1 in the mouse, Mol. Cell. Biol. 11 (1991) 2189–2199.
[23] V. Noe, C. Alemany, M. Nicolas, C.J. Ciudad, Sp1 involvement in the 4β-phorbol 12-myristate 13-acetate (TPA)-mediated increase in resistance to methotrexate in chinese hamster ovary cells, Eur. J. Biochem. 268 (2001) 3163–3173.
[24] J. Denning, M. Beato, G. Suske, An inhibitor domain in Sp3 regulates its glutamine-rich activation domains, EMBO J. 15 (1996) 5659–5667.
[25] E. Sjottem, S. Aderssen, T. Johansen, The promoter activity of long terminal repeats of the HERV-H family of human retrovirus-like elements is critically dependent on Sp1 family proteins interacting with a GC/GT box located immediately 3′ to the TATA box, J. Virol. 70 (1996) 188–198.
[26] M.J. Birnbaum, A.J. Van Wijnen, P. Odgren, T.J. Last, G. Suske, G. Stein, J. Stein, Sp1 trans-activation of cell cycle regulated promoters is selectively repressed by Sp3, Biochemistry 34 (1995) 16503–16508.
[27] A. Sapetschnig, F. Koch, G. Rischitor, T. Mennenga, G. Suske, Complexity of translationally controlled transcription factor Sp3 isoform expression, J. Biol. Chem. 279 (2004) 42095–42105.
[28] S. Ammanamanchi, J.W. Freeman, M.G. Brattain, Acetylated Sp3 is a transcriptional activator, J. Biol. Chem. 278 (2003) 35775–35780.
[29] S.S. Kim, K.K. Pandey, H.S. Choi, P.Y. Law, L.N. Wei, H.H. Loh, Poly C binding protein family is a transcription factor in mu-opioid receptor gene expression, Mol. Pharm. 68 (2005) 729–736.
[30] O. Denisenko, K. Bomsztyk, Yeast hnRNP K-like genes are involved in regulation of the telomeric position effect and telomere length, Mol. Cell. Biol. 22 (2002) 286–297.
[31] E.F. Michelotti, G.A. Michelotti, A.I. Aronsohn, D. Levens, Heterogeneous nuclear ribonucleoprotein K is a transcription factor, Mol. Cell. Biol. 16 (1996) 2350–2360.
[32] A. Expert-Bezancon, J. P Le Caer, J. Marie, Heterogeneous nuclear ribonucleoprotein (hnRNP) K is a component of an intronic splicing enhancer complex that activates the splicing of the alternative exon 6A from chicken beta-tropomyosin pre-mRNA, J. Biol. Chem. 277 (2002) 16614–16623.
[33] A. Ostareck-Lederer, D.H. Ostareck, C. Cans, G. Neubauer, K. Bomsztyk, G. Superti-Furga, M.W. Hentze, c-Src-mediated phosphorylation of hnRNP K drives translational activation of specifically silenced mRNAs, Mol. Cell. Biol. 22 (2002) 4535–4543.
[34] K.J. Conn, C.B. Rich, D.E. Jenson, M.R. Fontanilla, M.M. Bashir, J. Rosenbloom, J.A. Foster, Insulin-like growth factor-I regulates transcription of the elastin gene through a putative retinoblastoma control element. A role for Sp3 acting as a repressor of elastin gene transcription, J. Biol. Chem. 271 (1996) 28853–28860.
[35] G. Gill, R. Tjian, Eukaryotic coactivators associated with the TATA box binding protein, Curr. Opin. Genet. Dev. 2 (1992) 236–242.
[36] Y. Zhang, M.L. Dufau, Repression of the luteinizing hormone receptor gene promoter by cross talk among EAR3/COUP-TFI, Sp1/Sp3, and TFIIB, Mol. Cell. Biol. 23 (2003) 6958–6972.
[37] Q. Du, I.N. Melnikova, P.D. Gardner, Differential effects of heterogeneous nuclear ribonucleoprotein K on Sp1- and Sp3-mediated

transcriptional activation of a neuronal nicotinic acetylcholine receptor promoter, J. Biol. Chem. 273 (1998) 19877–19883.

[38] O.N. Denisenko, B. O'Neill, J. Ostrowski, I. Van Seuningen, K. Bomsztyk, Zik1, a transcriptional repressor that interacts with the heterogeneous nuclear ribonucleoprotein particle K protein, J. Biol. Chem. 271 (1996) 27701–27706.

[39] P.R. Thompson, D. Wang, L. Wang, M. Fulco, N. Pediconi, D. Zhang, W. An, Q. Ge, R.G. Roeder, J. Wong, M. Levrero, V. Sartorelli, R.J. Cotter, P.A. Cole, Regulation of the p300 HAT domain via a novel activation loop, Nat. Struct. Mol. Biol. 11 (2004) 308–315.

[40] S. Ammanamanchi, J.W. Freeman, M.G. Brattain, Acetylated Sp3 is a transcriptional activator, J. Biol. Chem. 278 (2003) 35775–35780.

[41] D.E. Selley, L.J. Sim, R. Xiao, Q. Liu, S.R. Childers, mu-Opioid receptor-stimulated guanosine-5′-*O*-(gamma-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy, Mol. Pharmacol. 51 (1997) 87–96.

[42] E.J. Nestler, Historical review: molecular and cellular mechanisms of opiate and cocaine addiction, Trends Pharmacol. Sci. 25 (2004) 210–218.