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Poly(C) Binding Protein Family Is a Transcription Factor in μ-Opioid Receptor Gene Expression

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ABSTRACT

The mouse μ-opioid receptor (MOR) gene has two promoters, referred to as distal and proximal promoter. Previously, our colleagues reported that a 26-base pair (bp) *cis*-acting element of the mouse MOR gene activates MOR gene expression. Here, we report the cloning of four members of the poly(C) binding protein (PCBP) family and show that the 26-bp polypyrimidine stretch in MOR proximal promoter interacts with these PCBPs and activates MOR transcription. The PCBPs bind not only to single-stranded but also to double-stranded DNA. The nuclear run-off assay and semiquantitative RT-PCR shows that PCBPs enhance the transcription rate of MOR gene. Furthermore, we performed refined mapping to elucidate the core region (−317/−304) involved in mediating the PCBP-induced MOR promoter activity. Decoy oligonucleotides against the polypyrimidine stretch inhibit the PCBP-induced MOR promoter activity, thereby reconfirming the role of this element in regulating MOR promoter activity. Chromatin immunoprecipitation assay confirmed the interaction of PCBPs with MOR promoter in vivo. In conclusion, we demonstrate that PCBPs act as a transcription factor and positively regulate MOR gene expression in NMB cells.

Opioids exert their pharmacological and physiological effects through binding to their endogenous receptors. There are at least three major types of opioid receptors: μ, δ, and κ (Kieffer, 1995). These receptors belong to the superfamily of G protein-coupled receptors. Expression of opioid receptors is mainly limited to the central nervous system, and each receptor type has a distinct distribution pattern (Bausch et al., 1995; Minami and Satoh, 1995). These receptors regulate responses to pain, stress, and emotions when being activated by structurally related endogenous opioid peptides. The μ-opioid receptor (MOR) is of particular interest because it is the receptor that mediates important opioid effects such as analgesia and addiction (Chaillet et al., 1984). The mouse knockout studies have also concluded MOR to be the main opioid receptor involved in analgesia (Kieffer, 1999).

Ever since the cloning of the μ-opioid receptor (Chen et al., 1993; Thompson et al., 1993; Wang et al., 1993), several laboratories, including ours, have been working toward understanding the MOR gene regulation. The MOR gene uses two TATA-less promoters: the distal and proximal promoters, both of which are approximately 500 bp apart and are located within 1 kb of the translation start site. Recently, another promoter was reported that is located approximately 10 kb upstream to these dual promoters (Pan, 2002). The proximal promoter is used preferentially, accounting for approximately 95% of total MOR transcripts (Ko et al., 1997). Transcription of MOR from the distal promoter starts at 794 bp upstream of the translation start site (Liang et al., 1995); however multiple transcription sites located between 268 and 291 bp upstream to the start codon have been observed for proximal promoter (Min et al., 1994). The proximal promoter is regulated by several *cis*-elements and *trans*-acting factors, including a neuron-restrictive silencer element (Kim et al., 2004), an activator protein-2–like element (Ko et al., 2003), and canonical Sp binding sites (Ko et al., 1998). Recently, we reported a 26-bp polypyrimidine-rich *cis*-element in the MOR proximal promoter located at −334/−308, very close to the transcription initiation sites (Ko and Loh, 2001). This stretch of pyrimidine-rich bases is able to form a single-stranded DNA-like structure and can bind to single-strand

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ABBREVIATIONS: MOR, μ-opioid receptor; PCBP, poly(C) binding protein; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; siRNA, small inhibitory RNA; bp, base pair(s); nt, nucleotide(s); kb, kilobase(s); PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; SS, single stranded; DS, double stranded.

nucleic acid binding proteins. Later, we identified the interacting protein with the polypyrimidine rich element to be a poly(C) binding protein (PCBP) (Ko and Loh, 2005).

PCBPs belong to a family of RNA-binding proteins that are characterized by high-affinity and sequence-specific interaction with poly r(C). These PCBPs have been divided into two sets in mammalian cells: hnRNP K/J (Matunis et al., 1992) and the αCP proteins (α-complex proteins). The widely studied PCBPs are hnRNP K, αCP-1, and αCP-2. The latter two proteins are also known as PCBP1 and PCBP2 or hnRNP E1 and hnRNP E2, respectively (Kiledjian et al., 1995; Leffers et al., 1995). Recently, two other members of the PCBP family were discovered and named PCBP3 and PCBP4 (Makeyev and Liebhaber, 2000). All members of the PCBP family are evolutionary related, and the common feature of all PCBPs is the presence of three hnRNP K homology domains: two of them located at the N terminus, and a third located at the C-end (Makeyev and Liebhaber, 2002). The hnRNP K homology domains are approximately 70 amino acid-long RNA binding modules; however, they do not provide binding specificity to poly(C) (Dejgaard and Leffers, 1996). Members of this PCBP family are involved in multiple functions through their poly(C) binding nature. They can act as the mRNA stabilizer through interaction with 5′-untranslated region (Weiss and Liebhaber, 1995; Holcik and Liebhaber, 1997) or a translational silencer by binding to 3′-untranslated region (Ostareck et al., 1997; Collier et al., 1998).

In this study, we investigated the interaction of PCBPs with the polypyrimidine tract in MOR proximal promoter region and the role of PCBPs in regulating MOR gene expression in human neuroblastoma NMB cells. We showed that PCBPs bound to the −317/−304 region in MOR proximal promoter. PCBPs could also bind to double-stranded DNA sequence. More importantly, we found PCBPs could enhance transcription of MOR gene in nuclear runoff experiences. The overexpression of PCBPs resulted in an increase of endogenous MOR expression as well as nascent MOR mRNA synthesis. We concluded that PCBPs act as the transcription activator in regulating MOR gene expression in NMB cells.

Materials and Methods

Cell Culture. Human neuroblastoma NMB cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 5% CO_2 at 37°C.

Cloning of PCBP Family. Total RNA was isolated from mouse brain. The isolated RNA was treated with RNase-free DNase (Promega, Madison, WI), according to the manufacturer's instructions. RT-PCR was performed using OneStep RT-PCR kit (QIAGEN, Valencia, CA). PCR was performed with the primers (for PCBP1: forward, 5′-GAATTCATGGACGCCGGTGTG-3′, and reverse, 5′-CTCGAGCTAGCTGCACCCCAT-3′; for PCBP2: forward, 5′-GAATTCATGGACACCGGTGTG-3′, and reverse, 5′-CTCGAGCTAGCTGCTCCCCAT-3′; for PCBP3: forward, 5′-GAATTCATGGAATCTAAGGTCTCG-3′, and reverse, 5′-CTCGAGTTAGAGTACACCCATCCC-3′; and for PCBP4: forward, 5′-GAATTCATGAGCAGTTCAGATGCG-3′, and reverse, 5′-CTCGAGTCAGTAGGGGGAGAATTT-3′); PCR conditions used were 94°C for 15 min; 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. The primers were designed using GenBank. The RT-PCR product (approximately 1000 bp) was excised from a 2% agarose gel, purified using QiaQuick gel extraction kit (QIAGEN), and cloned in pCRII-TOPO vector (Invitrogen). Candidate plasmids containing the correctly sized inserts were confirmed by restriction digestions and dideoxy sequencing on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). For transient expression studies, the PCBPs were cloned in pcDNA3.1 vector (Invitrogen).

RT-PCR of MOR Gene. Total RNA was isolated using TRI reagent according to the manufacturer's instructions (Molecular Research Center. Cincinnati, OH). Primers used for MOR PCR were 5′-GATCATGGCCCTCTACTCCA-3′ (located at position 216 in exon 1) and 5′-GCATTTCGGGGAGTACGGAA-3′ (located at position 557 in exon 2). Selection of primers from two different exons helped to avoid the amplification of genomic DNA. Primers used to amplify human β-actin were 5′-GCCAACACAGTGCTGTCTGG-3′ and 5′-TACTCCTGCTTGCTGATCCA-3′. RT-PCR was performed using Qiagen OneStep RT-PCR Kit on GeneAmp PCR System 9600 (PerkinElmer Life and Analytical Sciences, Boston, MA). The PCR products were electrophoresed in a 2% agarose gel and quantified by ImageQuant 5.2 (Amersham Biosciences Inc., Piscataway, NJ).

Plasmid Construction for Reporter Assays. Luciferase fusion plasmids were constructed containing the DNA sequence from −450 bp upstream of the mouse MOR gene (pGL-450 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 construct, pGL-301, was generated by PCR with the upstream primer at −450 bp bearing a SacI site and the downstream primers bearing a HindIII site. The PCR fragment was cloned into pGL3-Basic (Promega), and the sequence of the insert was verified by DNA sequencing.

Transient Transfection and Reporter Gene Assay. NMB cells were plated in six-well plates at a concentration of 1×10^6 cells/well and cultured overnight before transfection. Each plasmid at equimolar concentrations was transfected in NMB cells with Effectene transfection reagent (QIAGEN) according to manufacturer's instructions. In brief, for luciferase analysis of MOR promoter activity, 0.5 μg of the reporter plasmids was mixed and incubated with the Effectene transfection reagent for 15 min at room temperature before being added to NMB cells. Twenty-four hours after transfection, cells were washed once with 1× phosphate-buffered saline and lysed with reporter lysis buffer (Promega). To correct for the differences in transfection efficiency, a one-fifth molar ratio of pCH110 (Amersham Biosciences) containing the β-galactosidase gene under the simian virus 40 promoter was added in each well. The luciferase and β-galactosidase activities of each lysate were measured using assays kits as described by the manufacturer (Promega).

In Vitro Translation. In vitro translation was carried out with pcDNA3.1-PCBP family in a reaction mixture containing [^{35}S]methionine (Amersham Biosciences) using a TNT quick-coupled transcription/translation system (Promega). The labeled proteins were then electrophoresed in 12% SDS-PAGE, and their sizes were compared with the predicted sizes (data not shown).

Nuclear Runoff Transcription. Effect of PCBPs on MOR transcription was checked by nuclear runoff assay as per standard protocol (Greenberg and Bender, 2002). PCBP1, PCBP2, PCBP3, and PCBP4 were individually transfected in NMB cells using Effectene transfection reagent (QIAGEN) according to manufacturer's instructions, and the nuclei were prepared after 24 h. Transcription was allowed to proceed for 30 min at 30°C in presence of [^{32}P]UTP and the endogenous DNA, and protein were removed by treating with DNase I followed by proteinase K. Runoff RNA products were purified by TRI reagent (Molecular Research Center) followed by ethanol precipitation and were resuspended in Ambion Ultrasensitive hybridization buffer (5×10^5 cpm/ml; Ambion, Austin, TX). RNA was hybridized at 42°C for 72 h to linearize and denature MOR (full-length plasmid DNA) or β-actin (PCR product) probes slot-blotted on Hybond-NX nylon membrane (Amersham Biosciences) using a slot-blot apparatus (Schleicher & Schuell, Keene, NH). Signal intensities were quantified using a PhosphorImager (Storm 860; Amersham Biosciences), and the MOR signal was normalized to β-actin.

Electrophoretic Mobility Shift Assay. The oligonucleotides were end-labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. To

check the interaction of PCBPs with double-stranded DNA, the complementary strands of the oligonucleotides were annealed and labeled with T4 polynucleotide kinase. The end-labeled DNA probes were incubated with TNT reaction products of PCBP expression constructs in a final volume of 20 μl of electrophoretic mobility shift assay (EMSA) buffer [10 mM Tris, pH 7.5, 5% glycerol, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol, 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 before adding the probe. The reaction mixtures were electrophoresed in a 4% polyacrylamide nondenaturing gel in 0.5 $\times$ Tris borate-EDTA (45 mM Tris-borate and 1 mM EDTA) at 4°C, and the gel was dried. Gel was exposed to a filmless autoradiographic analysis screen and scanned on a PhosphorImager.

Chromatin Immunoprecipitation Assay. NMB cells transiently transfected with HA-tagged PCBP were used for chromatin immunoprecipitation (ChIP) assay using the protocol from Upstate Biotechnology (Lake Placid, NY). Twenty-four hours after transfection of NMB cells with Effectene transfection reagent (QIAGEN), chromatin was cross-linked by the addition of formaldehyde to the culture medium at a final concentration of 1% and incubated for 10 min at 37°C. Cells were washed twice with phosphate-buffered saline containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Washed cells were counted, and $\sim 1 \times 10^6$ cells were resuspended in 200 μl of SDS lysis buffer (Zhang and Dufau, 2002). We determined conditions to produce sheared chromatin of average lengths between 0.5 and 1.5 kb using a cell destructor (Ultrasonic Inc., Plainview, NY). Each sample was sonicated using the cell destructor at 30% intensity for six 10-s bursts with a gap of 30 s between each burst. Cells were maintained on ice throughout. Sheared chromatin was centrifuged for 10 min at 14,000*g* at 4°C, after which the supernatant was diluted 10-fold with dilution buffer (Zhang and Dufau, 2002). As input control, 1% of each diluted supernatant was retained at this step. Chromatin was precleared for 1 h at 4°C with 75 μl of 50% protein G agarose (Invitrogen), 20 μg of sonicated and sheared salmon sperm DNA, and 50 μg of bovine serum albumin. Anti-HA antibodies (2 μg) (Roche) were added to precleared supernatant, and the immunoprecipitates were allowed to form overnight at 4°C with end-over-end rocking. Immune complexes were incubated for 1 h at 4°C with 60 μl of 50% protein G agarose beads and 4 μg of sonicated and sheared salmon sperm DNA and then centrifuged gently for 1 min at 4°C. Beads were washed four times for 5 min and eluted twice in 250 μl of fresh elution buffer (1% SDS and 0.1 M $NaHCO_3$). Samples were heated at 65°C for 4 h to reverse cross-links, and the DNA samples were purified by phenol/chloroform extraction followed by ethanol precipitation. The purified DNA was resuspended in 50 μl of H_2O. PCR reaction mixture contained 2 μl of the DNA template with the MOR-specific primers (forward, 5′-CCAACCCTTCTCTCCATCTC-3′; reverse, 5′-TATAGCCCCCTCCCACCTTA-3′) spanning the human MOR promoter region in a total volume of 20 μl. After 35 cycles of amplification, 5 μl of the PCR product was analyzed on a 2% agarose gel.

Decoy Oligonucleotide Approach in NMB Cells. In the decoy oligonucleotide approach, double-stranded oligonucleotides with specific binding sequences for transcription factors (PCBPs) were transfected into cells to selectively disrupt the function of these binding factors. In this case, transcription factors interact with an excess of decoy oligonucleotides instead of binding to the natural regulatory motifs of genes (Bielinska et al., 1990; Kraus et al., 2003). The various concentrations of decoy oligonucleotides were directly cotransfected with either PCBP-expressing constructs or the carrier DNA (the promoterless cloning vector pGL-301). There was no chemical modification on oligonucleotides. To avoid any effect on transfection efficiency caused by different amounts of transfected DNA, the total amounts of both plasmid DNA and oligonucleotides were kept constant in the individual experiments. A nonspecific oligonucleotide (5′-ATTGCGCTCCTCCTTCTCTCTGATCGGGGCGGGGCAAGCTTATA-3′) was used as the negative control.

siRNA-Mediated Inhibition of PCBPs. To confirm the role of PCBPs in MOR regulation, we used siRNAs to inhibit the expression of PCBPs and see its effect on MOR expression level. One hundred nanomoles of siRNA duplexes (Dharmacon Research, Lafayette, CO: PCBP1, 5′-GAAAGUGGACUAAAUGUGAUU-3′; PCBP2, 5′-GAUUGAAGGUGGAUUAAAUUU-3′; PCBP3, 5′-GAUCUAAUAGGCUGCAU-AAUU-3′; and PCBP4, 5′-CGCCAGAUCUGCGCUGUUAUU-3′) were transfected into NMB cells using RNAiFect (QIAGEN) according to manufacturer's protocol. Mock transfection was performed with transfection reagent alone. RNA was isolated after 48 h, and the expression level of PCBP and MOR was checked by semiquantitative RT-PCR. The primers used for amplifying the PCBPs included the region corresponding to the siRNA sequence (PCBP1 forward, 5′-GGATGCCGGTGTGACTGAAAG-3′; reverse, 5′-TTACACCCGCCTTTCCCAATC-3′; PCBP2 forward, 5′-GAACACTGCTCGACATGGACAC3′; reverse, 5′-TCTCCTCGCGCAT-CTTCTTAAC-3′; PCBP3 forward, 5′-ACACTCCTCCGAAGAA-GCTC-3′; reverse, 5′-GGCGTTGATGAGATACTGGGC-3′; and PCBP4 forward, 5′-GGCTGGCACCAAGATCAAG-3′; reverse, 5′-GGGGACCGCATGGCTTGAG-3′). The MOR-specific primers used to check the MOR level were forward, 5′-GATCATGGCCCTCTACTCCA-3′, and reverse, 5′-GCATTTCGGGGAGTACGGAA-3′. β-Actin was used as the internal control in RT-PCR.

Results

Cloning of Poly(C) Binding Protein Family. Previously, we reported a 26-bp single-stranded *cis*-element (polypyrimidine tract) in mouse MOR proximal promoter interacts with a polypyrimidine binding protein and regulates the MOR gene expression (Ko and Loh, 2001). Later, by screening the mouse brain library, the polypyrimidine binding protein was identified as poly(C) binding protein. In this study, we investigated the role of PCBPs in regulating MOR transcription in human neuroblastoma NMB cells, a cell model that expresses endogenous MOR.

First, we cloned the PCBP family members. Full-length PCBPs were amplified using RT-PCR from mouse brain mRNA. Specific pairs of primers (see *Materials and Methods*) were used to amplify each of the PCBPs. PCR products were cloned into pCRII-TOPO (Invitrogen). The resulting clones were sequenced to ensure that the entire DNA sequences were identical with that of the mouse PCBPs in the database.

For the transient transfection and expression studies, we digested the above clones with EcoRI-XhoI to purify the full-length coding region of PCBPs and ligated into expression vector pcDNA3.1 at the corresponding restriction enzyme sites. The expression pattern of all four PCBPs was examined. The PCBP family was transcribed and translated in vitro using TNT kit (Promega) with [^{35}S]methionine, and the products were electrophoresed on a 12% SDS-PAGE. On the basis of the size comparison with the molecular weight markers, the molecular weight of the translated PCBP family was found to be approximately similar to the predicted molecular weight deduced from the full-length cDNA sequence (data not shown).

PCBP Family Proteins Bind to the MOR Proximal Promoter In Vitro. To verify the binding of PCBPs to the polypyrimidine stretch in MOR proximal promoter, we performed EMSA with single-stranded (SS) and double-stranded (DS) 26 nt long oligonucleotide sequence (Fig. 1A). The 26 nt long oligonucleotide containing the PCBP binding sequence (5′-TCTCTCCTCCCTCCCCTCTAGCCCTC-3′;

−326 to −301) was used as the probe, and PCBP proteins were translated in vitro using TNT kit. The binding assay was done at room temperature for 20 min, and the products were electrophoresed on a 4% nondenaturing PAGE. As shown in Fig. 1B, a specific protein-probe complex band was observed with PCBP1. A 100-fold molar excess of unlabeled DS or SS oligonucleotide completely inhibited the complex formation. There was no complex formation in the absence of the translated protein product (probe only). With DS oligonucleotides also, the PCBP1-DNA complex was evident, although the binding efficiency was much lower compared with the SS DNA oligonucleotides.

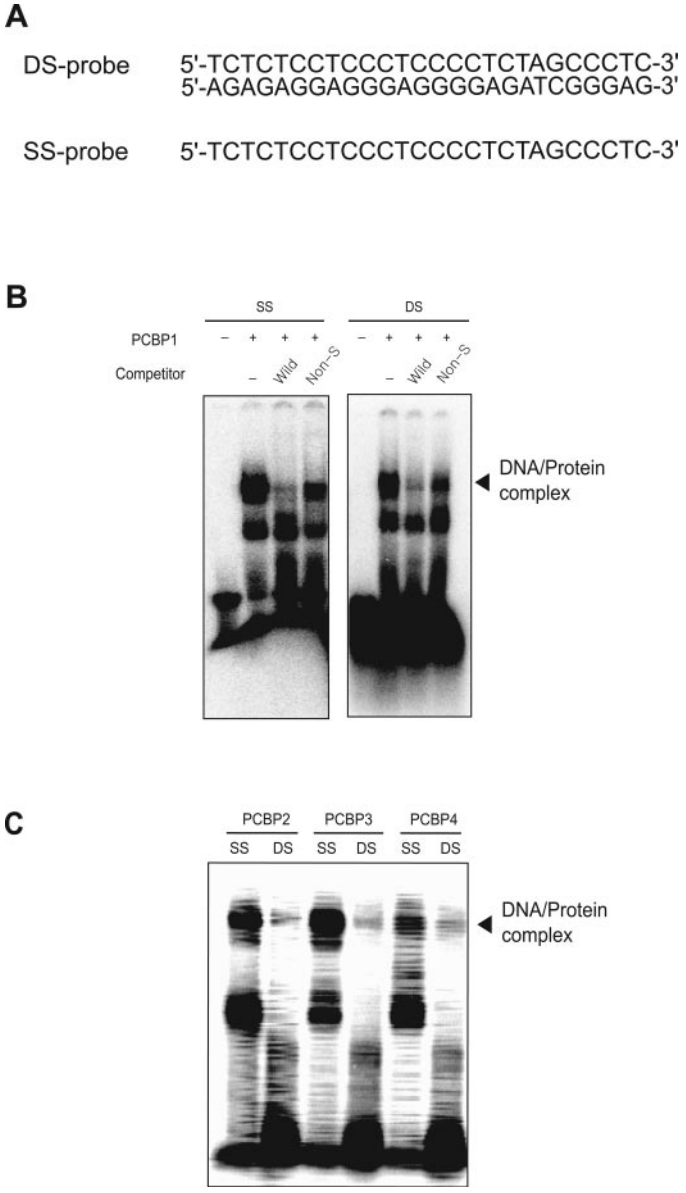

Fig. 1. Analysis of PCBPs binding to the proximal promoter region in vitro. A, single-stranded and double-stranded sequence was used as a probe in EMSA analysis. EMSA was performed using ^{32}P-labeled 26 nt long SS or DS sequence in polypyrimidine residues from MOR proximal promoter as a probe. B, EMSA of PCBP1 protein with SS and DS probes. The in vitro translated PCBP1 was incubated with probe. The protein/SS or protein/DS complex was shown. A 100-fold molar excess of competitor oligonucleotide was added to the mixture before adding the probe. Nonspecific oligonucleotide (5′-ATTGCGCTCCTCCTTCTCTCTGATCGGGGCGGGGCAAGCTTATA-3′) was used as the negative control. C, EMSA of PCBP2, PCBP3, and PCBP4 proteins with SS and DS probes. The in vitro translated PCBP2, PCBP3, and PCBP4 were also incubated with SS- or DS-labeled probe; the DNA/probe complexes formed are shown.

EMSA with other PCBP family members (PCBP2, PCBP3, and PCBP4) and the above probe yielded results similar to those observed with PCBP1 (Fig. 1C). These results clearly demonstrate that PCBPs bind to the MOR proximal promoter region and bind to DS as well as SS DNA in vitro.

Specific Binding of PCBP Family Proteins to the MOR Promoter in Vivo. To support the EMSA findings that PCBPs interact with the MOR proximal promoter region in vitro, we performed the ChIP assay to test weather the PCBP protein interacts with the MOR promoter in vivo. NMB cells were transiently transfected with HA-tagged PCBP expression construct. As shown in Fig. 2, a specific 217-bp ChIP-PCR product was detected with the specific primer in HA-tagged PCBP–transfected cells when the samples were immunoprecipitated using anti-HA antibodies but not in samples immunoprecipitated with preimmune serum or anti-Flag antibodies. The result suggests that the PCBP specifically binds to the region containing the polypyrimidine tract in the MOR promoter in NMB cells.

PCBPs Act as Transcription Factor and Induce the Expression of Endogenous MOR. Earlier experiments suggested that PCBPs bind to the MOR proximal promoter region, thereby inducing the proximal promoter activity in reporter assays. Our next aim was to determine the role of PCBPs in regulating endogenous MOR expression in NMB cells. We transfected each expression construct of PCBP family or vector (pcDNA3.1) alone into the human NMB neuroblastoma cells. Twenty-four hours after transfection, the total RNA was isolated, and RT-PCR was done to determine the MOR mRNA level using a pair of MOR-specific primer. As shown in Fig. 3A, endogenous MOR mRNA expression increases significantly in PCBP-transfected cells. All four members of the PCBP family were able to induce the MOR expression by 2.5- to 4.5-fold.

To convincingly show that PCBPs can directly enhance the rate of transcription of MOR gene, a nuclear runoff in vitro

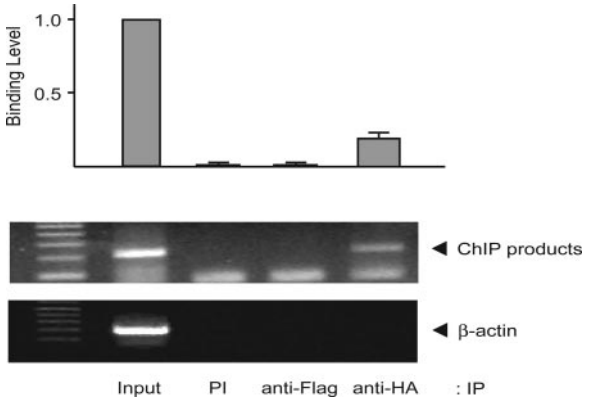

Fig. 2. Analysis of PCBPs binding to the proximal promoter region in vivo. ChIP assay was performed in NMB cells using HA-tagged PCBP. Lysates from PCBP-transfected cells were immunoprecipitated with anti-HA monoclonal antibody, nonspecific antibodies (anti-Flag or preimmune serum), and the bound DNA was analyzed by PCR using primers that amplified a 217-bp region of the MOR promoter spanning the PCBP binding site. Lane 1, 100-bp DNA marker; lane 2, input; lane 3, preimmune serum; lane 4, anti-Flag; lane 5, anti-HA. β-Actin was used as the negative control. The band intensity in lanes 2 to 4 was analyzed using ImageQuant 5.2 software.

transcription assay was performed. We transfected each of the PCBPs in neuroblastoma NMB cells and determined the rate of nascent MOR mRNA transcripts. The nascent mRNA transcripts elongated in the isolated nuclei were labeled with [α-^{32}P]UTP and hybridized to MOR or actin-specific probes. Our results showed that overexpression of PCBPs causes a significant increase in nascent MOR mRNA synthesis (2- to 4-fold compared with vector-transfected control), whereas the rate of β-actin mRNA synthesis remains largely unchanged (Fig. 3B). These results suggest that PCBPs act as a transcriptional factor and enhance the transcription rate of MOR.

Effect of PCBP Family on MOR Proximal Promoter Activity. To determine the function of the PCBP family on MOR gene expression, we checked the proximal promoter activity by cotransfection. Cotransfection was performed using each type of PCBP expression construct and the reporter construct containing the proximal promoter region (−450/+1) fused to the luciferase gene in NMB human neuroblastoma cells. The MOR promoter activity in PCBP-cotransfected cells was increased (approximately 5-fold) compared with vector (pcDNA3.1)-only transfected control cells (arbitrarily defined as 1-fold) (Fig. 4). These findings suggest that all four PCBP family members can directly activate the MOR proximal promoter activity at a varying rate.

Identification of the Region Required for PCBP-Mediated MOR Promoter Activation. The EMSA results showed the binding of PCBPs to the −326/−301 region of mouse MOR promoter. To do more refined mapping of the region essential for PCBP-mediated MOR proximal promoter activation, we mutated the 26 nucleotides at the 3′-end in pGL-301 as shown in Fig. 5. pGL-301 contains the −450/−301 region of MOR proximal promoter. We simply replaced triple nucleotide sequences step by step and cotransfected with PCBP-expressing construct in NMB cells. The mutant constructs pGL-301-M2, pGL-301-M3, pGL-301-M4, and pGL-301-M5 displayed 30% promoter activities compared with the wild-type construct (pGL-301-wild) (data not shown). On the other hand, mutant constructs including pGL-301-M1, pGL-301-M6, pGL-301-M7, and pGL-301-M8 displayed promoter activity comparable with pGL-301-wild (Fig. 5). Therefore, this result suggests that the core site required for PCBP-mediated MOR promoter activation resides in the region from −317/−304.

Use of Decoys to Inhibit the PCBP-Mediated Induction of Proximal Promoter Activity. We also used a decoy oligonucleotide approach to confirm the PCBPs binding spe-

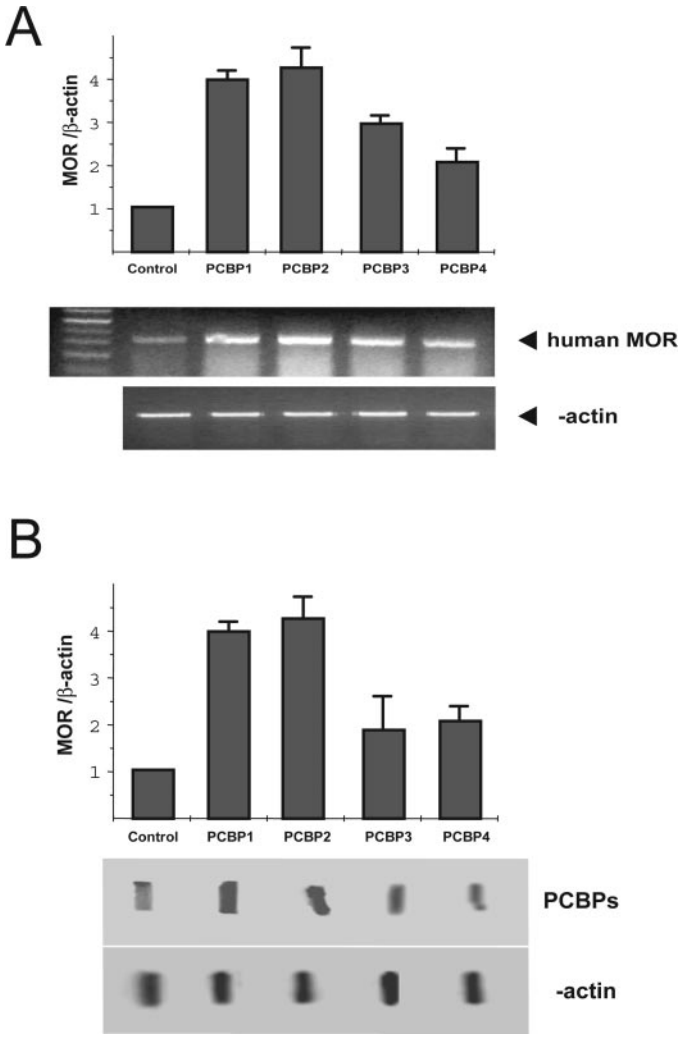

Fig. 3. PCBP family acts as a transcription factor in inducing the expression of MOR gene in NMB cells. A, endogenous MOR mRNA level of PCBP-transfected cells using RT-PCR. NMB cells were transfected with PCBP-expressing constructs, and the expression level of MOR was determined by RT-PCR. β-Actin was used as a control. B, PCBP-transfected cells were used for nuclear runoff assay. NMB cells were transfected with PCBPs or pcDNA3.1 (control), and the nuclei were isolated after 24 h. The rate of nascent mRNA synthesis was determined by in vitro transcription in the presence of [^{32}P]UTP and by hybridizing the nascent mRNA to the MOR or actin-DNA blotted on a nylon membrane. Fold increase in MOR transcription compared with the control was plotted. The results are a representation of three independent experiments.

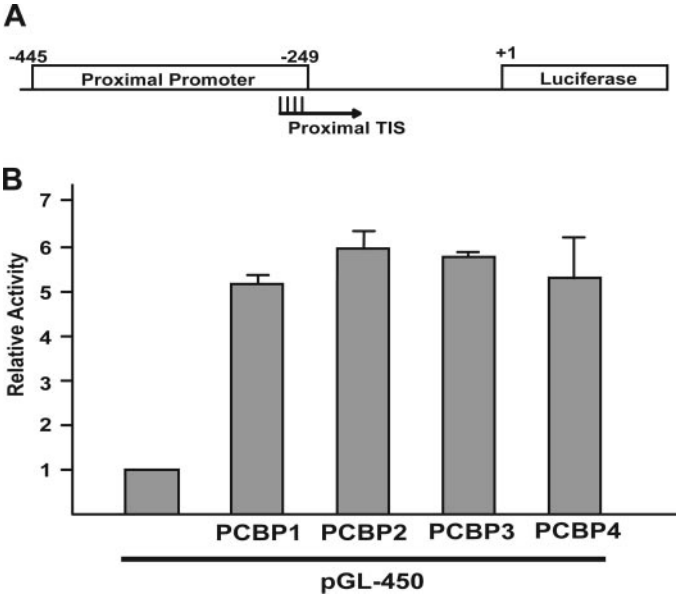

Fig. 4. PCBP enhances the proximal promoter activity of μ opioid receptor gene. A, schematic diagram of mouse MOR proximal promoter region. Nucleotide +1 corresponds to the translation start site (ATG). TIS, transcription initiation site. B, promoter activity assay of PCBP-transfected cells. PCBP-expressing constructs were cotransfected in NMB cells and with pGL-450 in NMB cells, and the luciferase activity was measured. Relative activity was calculated by comparing with pGL-450 alone. The results from at least three representative experiments were averaged. The promoter activity was normalized by β-galactosidase.

cifically to the polypyrimidine tract in MOR promoter. For this purpose, a luciferase reporter gene construct under MOR promoter fragment containing PCBP binding region was cotransfected with various concentrations of decoy oligonucleotide in NMB cells. The decoy oligonucleotide binds to the PCBPs and decreases the ability of PCBPs to bind to their cellular target sequences. Hence, decoy-induced inhibition of PCBP-mediated induction of promoter activity was measured (Fig. 6A). The decoy oligonucleotide was able to block the PCBP-induced MOR promoter activity by approximately 80%. The specific interaction of PCBP family protein with the decoy molecule is shown in Fig. 6B. The cotransfection of MOR promoter reporter construct with mutated decoy oligonucleotide did not inhibit PCBP-mediated induction of MOR proximal promoter activity. Wild-type PCBP binding site decoy oligonucleotides were much more effective competitors than the mutated decoys for inhibiting PCBP-induced MOR promoter activation. Thus, from these results, it was confirmed that PCBPs binds to a specific region of MOR proximal promoter region (−326/−301).

siRNA-Mediated Inhibition of PCBP Expression. Our data showed that PCBPs bind directly to the MOR proximal promoter region and increase transcription activity. siRNAs have been used extensively to study or confirm the gene function in the mammalian system. We used duplex siRNAs against PCBPs individually and in combination to inhibit the expression of PCBP family and studied their effects on MOR gene expression. There was no effect on the MOR expression level when the cells were transfected with siRNA against one type of PCBP (Fig. 7B), even though the expression of that particular type of PCBP was decreased (Fig. 7A), indicating a redundancy in PCBP functions. Only the inhibition of all PCBP expression by siRNA treatment caused a decrease in MOR mRNA level (Fig. 7B).

Our results indicate that multiplexing of siRNAs for all four PCBPs causes significant inhibition of MOR mRNA levels as evidenced by RT-PCR (Fig. 7), indicating that perhaps all PCBPs may mainly contribute to the regulation of MOR gene expression in NMB cells.

Discussion

Our group reported previously that a 26-nt *cis*-element rich in polypyrimidine residues in MOR proximal promoter can adopt SS DNA structure and interact with the nuclear proteins. In this study, we demonstrated that this polypyrimidine tract interacted with a family of PCBPs (Ko and Loh, 2005). Despite their recognized function as RNA binding proteins, PCBPs seemed to also function to directly activate gene transcription of the MOR gene.

The proximal promoter of the mouse MOR gene is known to be regulated by various *cis*-elements and *trans*-factors, and all of these elements are important in regulating proximal promoter activities. These features are unique in the proxi-

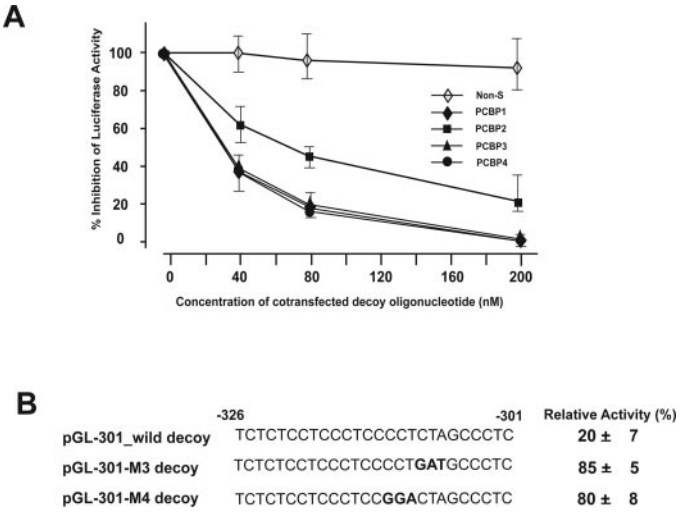

Fig. 6. Decoy oligonucleotides corresponding to PCBP binding element inhibit proximal promoter activity. NMB cells were cotransfected with pGL-301 and PCBP-expressing constructs in the presence of wild-type or mutant decoy oligonucleotides. A, wild-type decoy oligonucleotide attenuated PCBP-induced activity (reported as the percentage of decrease) of a reporter gene construct containing a PCBP binding site in NMB cells. B, the reporter construct was cotransfected with wild or mutant decoy oligonucleotides (80 nM), and the effect on PCBP-induced luciferase activity was measured. The results shown were from at least two independent transfection experiments performed in triplicate.

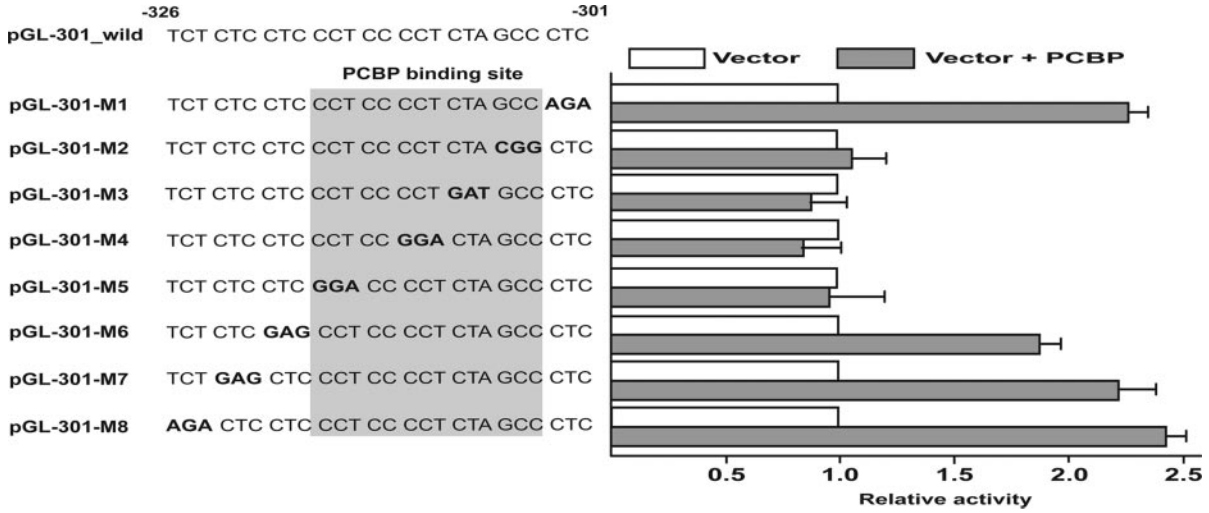

Fig. 5. Identification of essential PCBP-binding site The mutated sequences in mutant constructs from pGL-301-M1 to pGL-301-M8 are shown in boldface letters. The luciferase activity of the mutant constructs in NMB cells was measured as described under *Materials and Methods* and is expressed as a luciferase/β-galactosidase activity ratio. The proximal activity of each construct was expressed as *N*-fold activation of vector-only cells. Histograms represent mean values of three independent transfection experiments with two different plasmid preparations.

mal promoter of mouse MOR compared with the promoter of other opioid receptor genes (κ and ä). Thus, the proximal promoter regulation of mouse MOR gene is under a fine and tight control (Law et al., 2000; Ko and Loh, 2001; Kraus et al., 2001; Ko et al., 2003). The −340/−301 region in MOR proximal promoter is rich in pyrimidine residues, especially the cytosines. PCBPs belong to a family of nucleic acid binding proteins that interact with poly(C) sequences with high affinity and specificity (Makeyev and Liebhaber, 2002). Because there is no mouse neuronal cell line endogenously expressing MOR available so far, we performed all of our studies in a human neuroblastoma cell line NMB, which expresses MOR endogenously.

PCBPs have been involved in a spectrum of post-transcriptional controls. First insights into the functions were obtained from an investigation of human α-globin mRNA stabilization (Weiss and Liebhaber, 1995). Stabilization of α-globin mRNA is tightly linked to the formation of a binary complex between a single molecule of PCBPs and pyrimidine-rich region within the α-globin. We performed a nuclear runoff assay in PCBP-transfected NMB cells and determined the nascent mRNA synthesized with MOR probe. MOR nascent mRNA synthesis increased (~2.5- to 4-fold), indicating that PCBPs enhance MOR gene transcription. This result provides direct evidence that PCBP can act as a transcription factor that binds to MOR proximal promoter and thereby enhances MOR gene transcription. Further supports were obtained from more functional studies discussed as follows below.

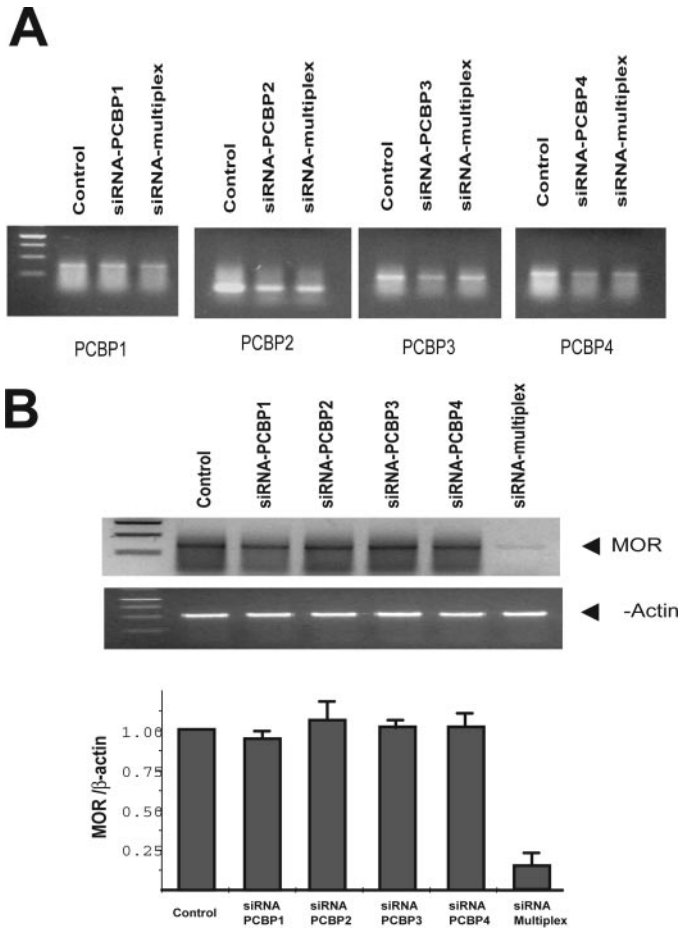

Fig. 7. siRNA-mediated inhibition of MOR gene expression. siRNA of PCBPs decreased MOR transcription in NMB cells. A, NMB cells were transfected with siRNAs for PCBPs (singly or multiplexed as indicated), and the cells were harvested after 48 h. RT-PCR was done to determine the level of PCBP expression. B, the effect of siRNA PCBP treatment (singly or in multiplex) over the expression of MOR gene in NMB cells was checked by RT-PCR; β-actin was used as the internal control for RT-PCR. The results are representative of two independent experiments.

Decoy oligonucleotide studies showed that decoy oligonucleotides inhibited the PCBP-induced MOR promoter activity. Mutations in decoy oligonucleotides (pGL-301-M3 and -M4) resulted in the loss of their ability to inhibit the MOR promoter activity. Inhibition of expression of PCBPs by siRNA treatment caused a decrease in MOR mRNA level when the siRNA against all four PCBPs was multiplexed. There was not much effect on the MOR expression level when the cells were transfected with siRNA against one type of PCBP, even though the expression of that particular type of PCBP was decreased, indicating a redundancy in PCBP functions.

The functions of the PCBPs are mediated through its poly(C) binding nature. There should be other factor(s) that provide the specificity for a particular type of function to the PCBPs. The topology of the poly(C) element and the structure of the particular PCBP might be involved in providing the specific function. Another possibility is the involvement of additional proteins that could facilitate the interaction (directly or indirectly) of PCBPs with the poly(C) element in MOR promoter. hnRNPK, a well-studied poly(C) binding protein, was shown to interact directly with TATA binding protein, a component of RNA polymerase complex (Michelotti et al., 1995). In a recent study using PCBP2, it was shown that PCBP2 can interact with 160 different types of mRNA and regulate a number of cellular processes (Waggoner and Liebhaber, 2003).

In summary, we conclude that the PCBP family regulates endogenous mouse MOR gene expression by acting as a transcriptional activator. Further studies are required to understand the actual mechanism by which PCBPs interact with the proximal promoter and regulate the expression of the MOR gene. Studying the interaction of PCBPs with the RNA polymerase II and other components of the transcription complexes may shed more light on the mechanism of MOR gene regulation.

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