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# Multiple KH Domains of Poly C Binding Protein are Required for its Trans-Activation on the Proximal Promoter of the Mu-Opioid Receptor Gene

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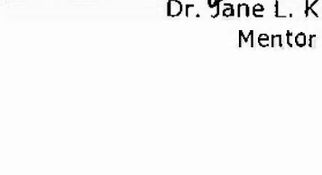

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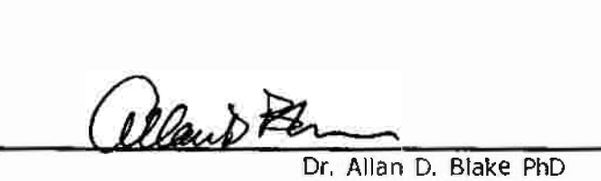

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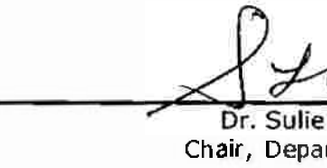

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**Abstract**

The mu-opioid receptor (MOR) has been shown to mediate morphine induced analgesia, addiction, and tolerance. The expression of the major product of the MOR (MOR-1) gene has been found to be driven by two promoters, a distal promoter and a proximal promoter. The proximal promoter region has been shown in previous studies to be the major promoter for the regulation of MOR gene transcription in the adult mouse brain and embryonic development. Recent studies have shown that single-stranded DNA binding proteins (such as Poly C Binding Protein) are involved in transcriptional regulation of mouse MOR gene. Poly C binding protein (PCBPs) is categorized in the family of hnRNP's, containing multiple K-Homology (KH) domains, which is involved in RNA stabilization, translational activation, and gene silencing. In this study, we attempted to determine the trans-activation domains of PCBP and examine the regulatory effects of each trans-activation domain of PCBP in MOR transcription. Mouse neuronal cells (N2A) were transiently transfected with various truncation constructs of PCBP to determine which domains were important in trans-activation. Our preliminary data suggests that at least two KH domains were required for PCBP to display its trans-activation activity.

## Introduction

Opioids have a spectrum of pharmacological and physiological effects that include sedation, euphoria, respiratory depression, and analgesia (Rossi et. al, 1994), along with tolerance and dependence upon chronic administration (Law et al, 1999). Three different opioid receptor types have been identified that can interact with opioids, which include the mu (μ), kappa (κ), and delta (δ) opioid receptors. These receptors belong to the seven transmembrane domain receptor superfamily coupled to guanine nucleotide regulatory proteins (G-proteins), also called the GPCR (G-protein coupled receptor) superfamily (Kieffer et al, 1995). Each opioid receptor mediates its effects by first activating heterotrimeric G-proteins that couple to intracellular effector enzymes and channels (Brillet et al, 2003). Opioid receptors undergo receptor phosphorylation, desensitization, internalization, and recycling upon agonist activation. Of the three opioid receptors, the mu opioid receptor (MOR) is mainly distributed in the central nervous system and in large part involved in morphine induced analgesia, addiction, tolerance and dependence (Keiffer et al, 1996).

The MOR gene shows approximately 50-70% homology to the genes encoding for the δ-(DOR-1), κ-(KOR-1) and orphan (ORL1) receptors (Koch et al, 1998). The MOR gene is over 200 kb in size and several different splice variants have been identified, such as differing only in the presence or absence of a set of amino acids at the intracellular carboxyl terminus. The splice variants exhibit differences in their rate of onset and recovery from agonist-induced internalization, but their pharmacology does not appear to differ in ligand binding assays (Pan et al, 2002). Two MOR splice variants, MOR-1A and MOR-1B, were identified shortly after cloning

of the MOR-1 gene (Bare et al., 1994, Zimprich et al., 1995). MOR-1A was identified first in the human cell line and a similar murine variant has been isolated. MOR-1B, encoded by exons 1, 2, 3, and 4 in the murine variant was first isolated from the rat and differs from MOR-1A in that it contains an alternatively spliced exon 5 instead of the original exon 4. Of the various splice variants, the MOR-1 gene is the most prominent form and is encoded by four exons, exons 1, 2, 3 and 4 (Min et al, 1994). The MOR-1 is a seven transmembrane protein that spans over 356 amino acids in size with its N-terminus (amino) facing the extracellular space and the C-terminus (carboxy) facing the intracellular space.

Previous studies have shown that the mouse MOR contains three different promoters, a distal and proximal promoter, located approximately within 1 Kb upstream of the translation start site (Ko et al, 1997) and a third promoter located approximately 10 KB upstream of the translation start site (Pan et al, 2002). Both the distal and proximal promoters lack a consensus TATA box sequence, a promoter sequence involved in gene regulation in eukaryotes (Ko et al, 1997). The distal promoter is located 794 bp upstream of the translation start site and initiates transcription from a single transcription initiation site (Liang et al, 1995). The proximal promoter initiates MOR transcription from four transcription initiation sites that are located in a region spanning from 291 to 268 bp upstream of the translation start site (Min et al, 1994). The third promoter has been shown to regulate the expression of MOR variants containing exon 11. This promoter contains a TATA box and is flanked by positive and negative regulatory elements (Pan et al, 2002). It has been shown using quantitative reverse transcriptase-PCR with mRNA from the adult mouse brain that the proximal promoter is approximately twenty-fold more active than the distal promoter (Ko et al, 1997).

Data from transfection analysis indicated that the proximal promoter directs neuronal subtype expression (Ko et al, 1997). Furthermore, it was shown that the MOR transcription is mainly initiated by the proximal promoter in the mouse adult brain and these results suggest that it is the major functional promoter (Ko et al, 1997).

It has been shown that several DNA binding proteins bind and regulate MOR gene expression. Sp factors (double stranded DNA binding protein) and mPy (mor polypyrimidine binding protein – an unidentified protein) have been shown to be critical in regulating transcription of MOR. In addition to double stranded DNA binding proteins, single-stranded DNA binding proteins may also be involved in the transcriptional regulation of the MOR gene (Ko et al. 2000).

A single-stranded DNA binding protein, named poly C binding protein (PCBP), was recently identified from a mouse brain library using the yeast one hybrid screening system (Ko and Loh, manuscript submitted). It has been shown that PCBP can trans-activate the MOR-gene by interacting with the proximal promoter of the MOR gene. The PCBP protein is categorized under the K-Homology (KH) domain superfamily of nucleic-acid binding proteins, one of which is a well-characterized protein known as heterogeneous nuclear ribonucleoprotein K (hnRNP K Homology). The KH domain has been shown to be an RNA-binding motif (Adinolfi et al, 1999). hnRNP K is an evolutionarily conserved factor that is found in the nucleus, cytoplasm and mitochondria. hnRNP's have been shown to be involved in chromatin remodeling (Denisenko and Bomsztyk, 2002), transcription (Michelotti et al, 1996; Du et al, 1998), pre mRNA splicing (Expert-Bezancon et al, 2002), mRNA export (Michael et al, 1997), and translation (Ostareck et al, 1997) along with attenuating steroid hormone receptor gene trans-activation (Chen et al, 2003). Many of these reactions

have been shown to be regulated by K protein phosphorylation that is either induced by changes in the extracellular environment or by activity of specific ligands (Ostrowski et al, 2003).

Poly(C)-binding proteins (PCBP) are approximately 37-39 kDa in size and belong to the KH domain superfamily. PCBPs can be divided into two groups, hnRNP K/J and alphaCPs (1-4). These proteins are involved in RNA stabilization (Weiss and Liebhaber, 1994,1995; Holcik & Liebhaber 1997), translational activation (Blyn et al, 1997; Gamarnik and Andino 1997), and translational silencing (Ostareck et al, 1997; Collier et al., 1998). hnRNPs are a family of over 20 proteins that contribute to a complex around nascent pre-mRNA and are thus able to modulate RNA processing. (Chen et al, 2003).

The presence of three KH domains in PCBP leads one to question which domain or combination of domains is essential for its optimal trans-activation of the MOR gene. In this study, we examine which domains of PCBP are important for its trans-activation activity on the mouse MOR proximal promoter.

## Methods and Materials

Plasmid construction and isolation –

The mammalian expression vector, pcDNA3 (Invitrogen, Carlsbad, CA), was used to construct the PCBP expression plasmids. The full length of a PCBP cDNA fragment was inserted into the multiple cloning site of the vector, which resulted in the pcDNA3-PCBP plasmid. For construction of ΔVR deletion construct, the pcDNA3-PCBP plasmid was digested with Kpn1 and Bgl II restriction enzymes (150 minutes at 37°C) and purified by agarose gel electrophoresis. Concurrently, another pcDNA3-PCBP fragment digestion with Nsi1 and Xho1 restriction enzymes (150 minutes at 37°C). The digested fragments were then purified by agarose gel electrophoresis and the fragments were isolated and purified via Gene Clean kit (Bio 101, Carlsbad, CA) and then relegated and then inserted into the pcDNA3 vector. KH23 was generated by utilizing ΔVR and subjecting it to EcoRV and Xho1 restriction enzyme digestion and purification for relegation into the pcDNA3 vector. For construction of other constructs (KH1, KH2, KH3, VR, KH23VR), PCR amplification (described later) was performed to prepare the desired insert fragment that was then inserted into the multiple cloning sites of the pcDNA-3 plasmid vector (Figure 1b). Constructs from PCR amplification were digested for 150 minutes at 37°C with Kpn1 and Xho1 restriction enzymes and subjected to gel electrophoresis and visualized by AlphaImager (Alpha Innotech, San Leandro, CA) for confirmation.

*E. Coli* Transformation –

Plasmid DNA was added to 100 μL of *E. coli* competent cells and incubated on ice for 30 minutes. The cells were then heat shocked for 1 minute in a 42 °C water bath

and then chilled for 2 minutes on ice. 250 µL of Luria Broth media was added to the cells and incubated for 60 minutes at 37 °C and then plated on Luria Broth agar plates with 10µg/µL ampicillin for positive selection. The plates were incubated overnight at 37 °C.

Large Scale Plasmid Preparation –

One milliliter (mL) of transformed cultured *E. coli* was added to 250 mL Luria Broth media with ampicillin and grown overnight at 37 °C. The large scale culture (250 mL) was then transferred to large centrifuge bottles and centrifuged at 6000 rpm for 15 minutes at 4 °C (RC5C). The plasmid DNA was extracted utilizing QIAGEN (Valencia, CA) Plasmid Maxi Kit where the *E. coli* pellet was resuspended in 10 mL of P1 (50mM glucose, 10mM EDTA (pH 8) 25mM Tris HCl (pH 8) with RNase A) buffer. The cells were then lysed by addition of 10 mL of P2 (0.2M NaOH with 1% SDS). Ten mL P3 (5 M potassium acetate, glacial acetic acid) buffer was added to neutralize the reaction and the 30 mL mixture was then centrifuged at 16,000 rpm at 4 °C for 35 minutes. The plasmid was then purified through a column and then washed twice with QC (100% Ethanol) wash buffer. The DNA was then eluted using 15 mL of QF Elution buffer and 11 mL of chilled isopropanol was then added. The mixture was centrifuged at 9000 rpm at 4 °C for 35 minutes. The pellet was then washed with 3 mL of 70% ethanol and then centrifuged on a tabletop centrifuge. The resulting DNA pellet was resuspended in 300 µL of TE solution.

Cell culture-

Neuronal mouse cells (N2A) (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat inactivated fetal

calf serum (FCS) in an atmosphere of 10% $CO_2$ at 37 °C and 90% air. Cell monolayers were grown in T-75 $cm^2$ flasks (Fisher Scientific, Tustin, CA) and passaged when the monolayers reached 70% confluence by visual examination. Cells were passaged by washing with 10 mL of DMEM serum free medium, incubating with 7.0 mL of 0.1% trypsin/EDTA in phosphate buffered saline (PBS) and resuspended after centrifugation in 10 mL of DMEM with 10% FCS. An aliquot of resuspended cells were transferred to a new T-75 $cm^2$ flask (Fisher Scientific, Tustin, CA) containing 25 mL DMEM with 10% FCS and incubated at 37 °C in an atmosphere of 10% $CO_2$ and 90% air.

Transient Transfection-

Cells were transfected following the manufacturer's protocol using a Superfect lipofection method (Qiagen, Valencia, CA). Neuronal mouse cells (N2A) were incubated overnight in 6-well plates at a concentration of $0.7x10^6$ cells per well prior to the beginning of the experiment. Cells were transfected with equimolar amount of each test plasmid (0.1 µg, 0.5 µg, 1.0 µg). The amount of DNA used was within the linear range of the relationship between luciferase activity and the amount of DNA transfected. Cells were washed twenty-four hours after transfection and 4 mL DMEM with 10% FCS was added to each well. Forty-eight hours after transfection, cells grown to confluence were washed twice with Phosphate buffered saline (PBS) and lysed with Reporter Lysis Buffer (Promega, Madison, WI). The resulting lysate was subjected centrifuged at 12000 rpm and the supernatant was separated from the cell debris and used for activity assay. To control for differences in transfection efficiency from dish to dish, one fifth molar ratio of pCH110 (internal control)

containing the β-galactosidase gene driven by the SV40 promoter was included in each transfection and used for normalization.

Luciferase and β-galactosidase assays-

Ten microliters of cell lysates were mixed with 100 µL of luciferin, and the light emission from the reaction was measured and recorded in relative light units (RLUs) by a Lumat LB 9507 machine (Berthold Technologies, Drescher, PA). For β-galactosidase activity assay, 7 µL of substrate and light emission accelerator mixture in a 1:100 dilution were added to 70 µL of cell lysate and incubated for 45 minutes at room temperature. The mixture was then mixed with 100 µL of substrate and the emitted light was measured and recorded by Lumat LB 9507 (Berthold Technologies, Drescher, PA).

*In vitro* coupled transcription and translation -

*In vitro* transcription and translation was carried out with a final reaction mixture of 20 µL using Promega TNT Coupled Transcription/Translation Systems Kit (Promega, Madison, WI). The mixture was comprised of 12.5 µL Rabbit Reticulocyte Lysate, 0.5 µg DNA, 25 units T7 RNA polymerase,10 µCi $^{35}S$-methionine, 0.5 µL 1 mM NTPs, 1.5 mM $MgCl_2$, and 0.4 µL 1 mM amino acid mix. The reaction mixture was incubated at 30 °C for 90 minutes and 1 µg of RNAse-A was added to stop reaction and destroy peptidyl-tRNA. The reaction was further incubated for 10 minutes at 30 °C. The resulting transcription and translated protein products were analyzed using SDS-PAGE.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)-

A 20% polyacrylamide gel was prepared. The samples were electrophoresed and the resulting gel was then dried using a BioRad 583 Gel Dryer (Bio-RAd, Hercules, CA). The gel was then exposed in a phosphoimager cassette overnight at room temperature. The signals on the cassette were then detected the following day using a Storm 840 Phosphorimager system (Molecular Dynamic Inc., Piscatway, NJ)

DNA Sequencing –

DNA plasmids were amplified using PCR with addition of 3' and 5' primers (M13 Rev - GGAAACAGCTATGACCATG, Sp6 – AGCTATTTAGGTGACACTATAG, T7 – TAATACGACTCACTATAGGG), along with Taq DNA polymerase. The samples were then subjected to electrophoresis utilizing a DNA sequencing gel (National Diagnostics, Atlanta, GA) and the resulting gel was dried using a BioRad 583 Gel Dryer (Bio-RAd, Hercules, CA). The gel was then exposed in a phosphoimager cassette overnight at room temperature. The signals were then detected the following day using a Storm 840 Phosphorimager system (Molecular Dynamic Inc., Piscatway, NJ)

Polymerase Chain Reaction (PCR) –

Polymerase Chain Reaction (PCR) amplification was performed utilizing 1x PCR buffer in reaction mix buffer containing 1 µL Taq DNA polymerase (Promega, Madison, WI), 0.5 µg pCBP cDNA template, 10 nM sense primer for KSP (5'-GGTACCATGGACGC-CGGTGTGACTG-3'), 10 nM antisense primer for KSP (5'-CTCGAGTTATCTAATGGGT-GGTTTGAGTAGATGC-3') in a final volume of 10 µL. PCR was carried out by 35 cycles with each round consisting of 1 minute at 93 °C, 30 seconds at 68 °C and 30

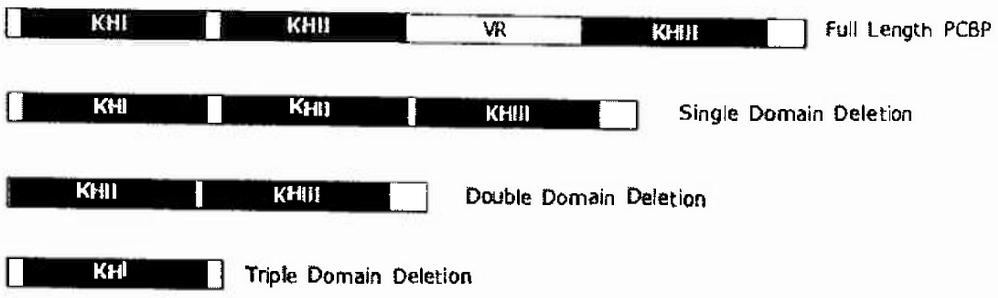
seconds at 72 °C. The resulting PCR product was resolved on 2% agarose gel and subjected to ethydium bromide staining and visualized by AlphaImager (Alpha Innotech, San Leandro, CA).

## Results

### *Construction of Plasmids*

We utilized a mammalian expression system to achieve our goal of determining which domains of PCBP were necessary for trans-activation. The mammalian expression vector, pcDNA3, was utilized and the full length cDNA fragment of PCBP (Figure 1A) was inserted into multiple cloning sites on the vector (Figure 1B). With the pcDNA3-PCBP plasmid constructed, we then created 3' and 5' deletion constructs. These deletion constructs were created either as single domain deletions, double domain deletions, or triple domain deletions (Figure 1A). For the generation of some constructs, PCR inserts were created and the insert size was confirmed using agarose gel electrophoresis (Figure 1B) before being isolated, purified, and subcloned into the multiple cloning site of the vector.

A



B

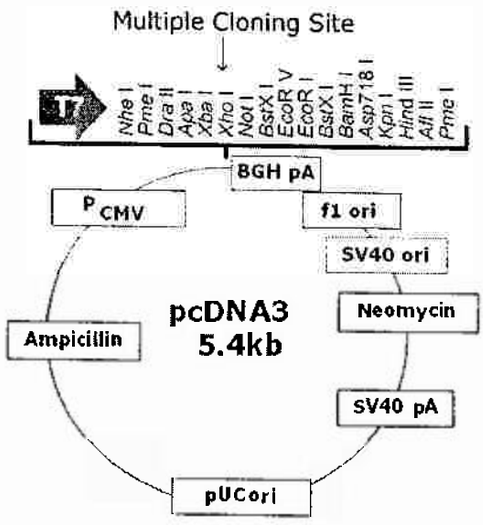



***Figure 1.*** *A. Schematic depiction of the full length PCBP fragment and its truncated constructs. B. The mammalian expression pcDNA3 vector (Invitrogen, Carlsbad, California) is driven by a CMV promoter. The full length of PCBP and truncated PCBP DNA fragments were inserted into the cloning site of this vector.*

**Confirmation of Identity of Constructs**

The identity of these deletion constructs were confirmed using restriction enzyme digestion (Asp718 and Xho1). The samples were then subjected to gel electrophoresis and analyzed to look for the insert band size located approximately 100 bp in size for triple domain deletions (Figure 2A). The identity of these constructs was further confirmed using DNA sequencing (Figure 2B).

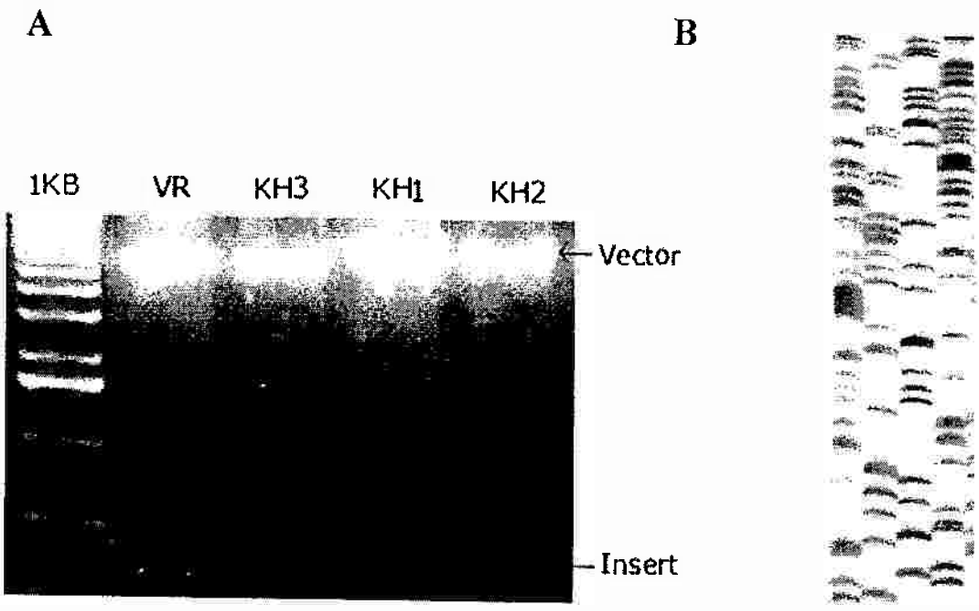



***Figure 2.*** *A. Electrophoretic analysis of a deletional constructs plasmids. The identity of deletion constructs was verified by confirming insert band size by restriction enzyme digestion. B. DNA sequencing analysis of various constructs was performed to verify the identity of the correct construct.*

**Molecular Weight Examination of *in vitro* Expressed PCBP**

The molecular weight of the proteins generated by *in vitro* transcription and translation method was then examined using SDS-PAGE. The result of SDS-PAGE analysis is shown in Figure 3. As shown, the single domain proteins were expressed at approximately 10 kDa with the full length PCBP shown at approximately 38 kDa. The construct missing one domain, the VR domain in this case, was shown to be about 27 kDa in size.

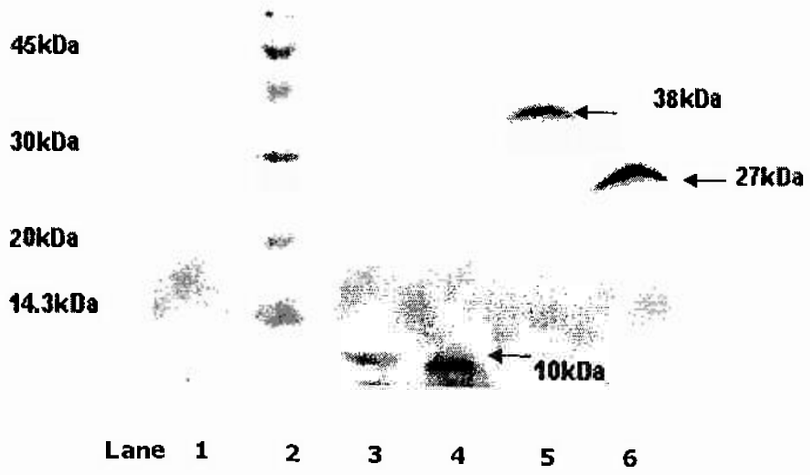



***Figure 3.*** *The full length (lane 5) or truncated forms of PCBP (lane 3-4 and 6) were translated using pcDNA3-PCBP or pcDNA3-PCBP deletion plasmids via in vitro transcription/translation method, individually. These translated proteins were then subjected to a 20% polyacrylamide gel electrophoresis and analyzed. Lane 1, pcDNA3 vector alone as a negative control. No translated protein was visualized; lane2, protein markers; lane 3, KH1; lane 4, KH3, lane 5, full length of PCBP and lane 6 ΔVR proteins. The gel was then dried and subjected to the autoradiography using a Molecular Dynamic Storm 840 Phosphorimager system.*

**Transfection Analysis**

We next setup the transfection system for determining the trans-activation activity of PCBP on the mouse MOR gene. Figure 4 shows the co-transfection analysis with various amounts of DNA. Here we show that with increasing amounts of DNA (0.1 μg, 0.5 μg, 1.0 μg), PCBP was able to trans-activate the reporter gene driven by the MOR promoter in a dose-dependent manner.

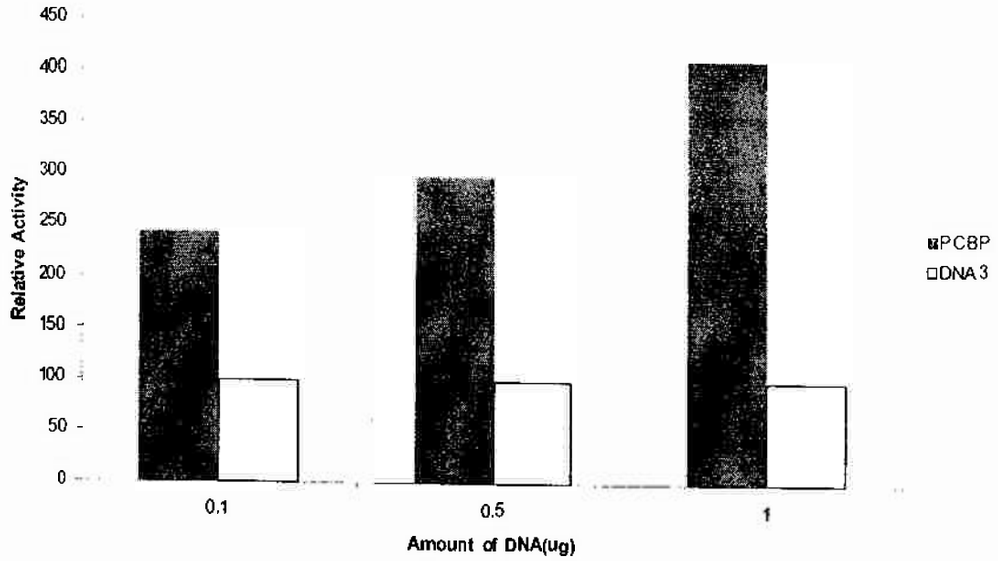



***Figure 4.*** *Mouse neuronal (N2A) were co-transfected with luciferase reporter plasmids driven by MOR promoter and the PCBP (pcDNA3-PBCP) construct. The trans_activation activity was presented as relative luciferase activities (RLU), with the activity of pcDNA3 (Vector) arbitrarily defined as 100%.*

**Transfection Analysis of Single Domain Deletions**

With the transfection system setup, we analyzed the trans-activation ability of various deletion constructs of PCBP in mouse neuronal cells (N2A) and examine their expression activities. Figure 5 shows the result of this transfection assay where pcDNA3 was used as a negative control and full length PCBP was used as the positive control. The preliminary data of transfection analysis shows that the deletion of one domain lead to a decrease in activity but did not result in a total loss of trans-activation activity.

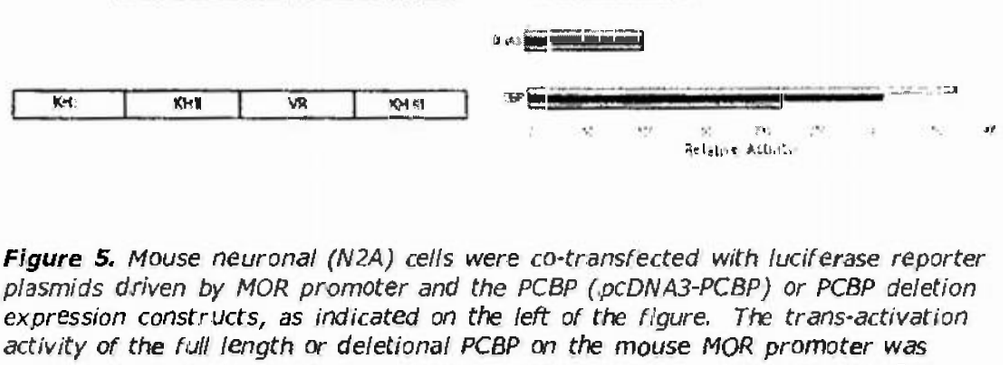


***Figure 5.*** *Mouse neuronal (N2A) cells were co-transfected with luciferase reporter plasmids driven by MOR promoter and the PCBP (pcDNA3-PCBP) or PCBP deletion expression constructs, as indicated on the left of the figure. The trans-activation activity of the full length or deletional PCBP on the mouse MOR promoter was presented as relative luciferase activities (RLU), with the activity of pcDNA3 arbitrarily defined as 100%. Each increasing bar represents a dose response from 0.1 μg (gray), 0.5 μg (black), and 1.0 μg (white) of the PCBP or respective PCBP deletional plasmid.*

**Transfection Analysis of Double Domain Deletion Mutants**

Furthermore, we sought to examine the trans-activation activity utilizing double domain deletion mutants. Figure 6 shows the transfection analysis with two domains of the PCBP deleted. Our preliminary data shows that double domain deletion resulted in a sharp decrease in the trans-activation activity that was lower than in single domain deletions (Figure 4).

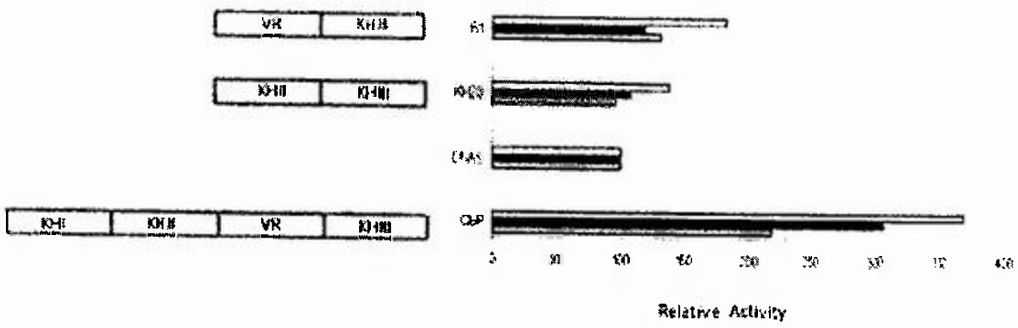



***Figure 6.*** *Mouse N2A cells were co-transfected with luciferase reporter plasmids driven by MOR promoter and the PCBP (pcDNA3-PCBP) or PCBP deletion expression constructs, as indicated on the left of the figure. The trans-activation activity of the full length or deletional PCBP on the mouse MOR promoter was presented as RLU, with the activity of pcDNA3 arbitrarily defined as 100%. Each bar represents a dose response from 0.1 μg (gray), 0.5 μg (black), and 1.0 μg (white) of the PCBP or truncated CPBP plasmid.*

**Transfection Analysis of Triple Domain Deletion Mutants**

Finally, the trans-activation activity of triple domain deletion constructs were examined. Figure 7 shows each of the single domain constructs missing three of other domains. Compared with the negative control, pcDNA3, it was shown that no construct containing a single domain had the ability to trans-activate the reporter gene compared to the construct with the full length PCBP.

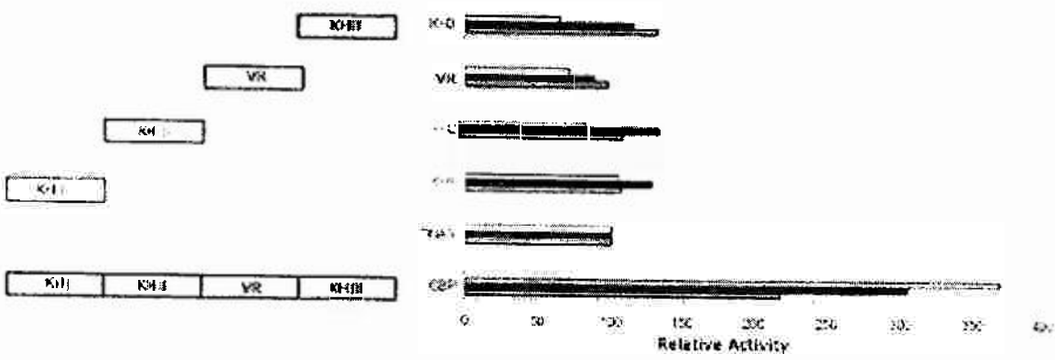



***Figure 7.*** *Mouse N2A cells were co-transfected with luciferase reporter plasmids driven by MOR promoter and the PCBP (pcDNA3-PCBP) or PCBP deletion expression constructs, as indicated on the left of the figure. The trans-activation activity of the full length or deletional PCBP on the mouse MOR promoter was presented as RLU, with the activity of pcDNA3 arbitrarily defined as 100%. Each bar represents a dose response from 0.1 μg (gray), 0.5 μg (black), and 1. 0 μg (white) of the PCBP or truncated CPBP plasmid.*

## Discussion

Previous studies from our laboratory have shown that Poly(C)-Binding Protein (PCBP) can trans-activate the proximal promoter of the mouse mu-opioid receptor (MOR) gene (Ko and Loh, manuscript submitted). PCBP is a well-known RNA binding protein; however, it has never been documented as a regulatory factor which is able to regulate a gene expression at the DNA level, nor has the molecular basis of how PCBP can act as a transcriptional regulator and regulate the MOR gene expression been investigated. In this report, we therefore investigated the molecular basis of how PCBP can regulate the MOR promoter activity by determining which domains of PCBP are necessary for the optimal trans-activation on the MOR gene at the DNA level.

The following is a diagram of the structure of PCBP where PCBP contains three copies of a KH motif (RNA-binding K homologous motif), which has been identified in a wide spectrum of RNA-binding proteins (Siomi et al, 1993). This motif was first described for hnRNP K, a member of the hnRNP family (Adinolfi et al, 1999).


PCBP is a member of hnRNP family or a KH domain family. The KH domain is identified as a RNA binding domain. The arrangement of each of the KH motifs within all members of the hnRNP family is similar where two KH domains are located at the N-terminus in a consecutive sequence with a third KH domain located at the C-terminus that is separated by a varying sequence (VR). Within PCBP, no other

RNA-binding motif has been found. The presence of three KH domains lends to the question of which domain(s) is(are) necessary for its regulatory role at the DNA level.

With the transfection system successfully established in a neuroblastoma cell model system (N2A), functional analysis study further confirmed that PCBP is in fact able to trans-activate the mouse MOR promoter with increasing amounts of the plasmid DNA of PCBP (Figure 4). Therefore, in order to determine which domains of PCBP were necessary for the optimal trans-activation, multiple deletion constructs, containing the KH domains in varying combinations, were generated and tested. Functional analysis using triple domain deletion mutants demonstrated the abolishment of all trans-activation activity of PCBP (Figure 7). This data suggests that one domain alone does not have the ability to induce trans-activation by PCBP itself. However, double domain deletion mutants displayed an increase in the promoter activity compared to triple domain deletions (Figure 6). An implication here could be that one domain may contain the binding domain, while the other contains the trans-activation domain. Furthermore, trans-activation activities of double domain deletion mutants are not as great as those using single domain deletion constructs (Figure 5), suggesting that multiple domains could be necessary for optimal activity. Collectively, these data demonstrated that the overall trans-activation is not dependent on just one domain but rather a combination of domains.

There are several possibilities of why multiple domains are needed. First, the trans-activation domain and DNA binding domain may be located at different regions of PCBP, therefore at least two domains are required. Second, the presence of multiple domains may result in increasing its binding affinity and/or protein stability, because each KH domain may be composed of a conserved yet different and

independent DNA binding unit. This notion may be further supported by the complexity of the RNA binding properties of KH domain family proteins. A previous study showed that KH domains of hnRNP K and PCBP can bind the poly r(C) RNA sequence, however, each KH domain differentially binds to RNA sequences (Dejgaard and Leffers, 1996). In addition, it has been shown that only the first KH domain of hnRNP K was able to function as an independent RNA binding unit (Silvera et al., 1999), whereas modification or deletions of each of the three KH domains in hnRNP K protein have been shown to diminish or decrease its RNA binding capability (Siomi et al, 1994), indicating the necessity for the presence of multiple KH domains. Third, the presence of multiple domains of PCBP may simply retain correct conformation for its interaction or increase the frequency of interaction with other factors. Our present study indicated that the structure of PCBP can be fine tuned by combination of different domains with sequence variations, which then results in its trans-activation activity and sequence-specific DNA binding profile.

It also can be noted that the presence of the VR region with the KH2 construct (Figure 6 and 7) resulted in an increase in trans-activation activity, which is further established with the increase in activity seen with the addition of VR region to the KH23 construct (Figure 5 and 6). These observations imply that the VR region, though not established as an RNA-binding motif, may contain some properties that allow it to take part in trans-activation. To further understand the molecular basis of PCBP as a transcription regulator on the MOR gene expression, future studies will focus on not only the construction of more deletion constructs to confirm which domains are crucial for trans-activation, but the identification of DNA binding domains of PCBP on the proximal promoter of the MOR gene will also be investigated.

## Conclusion

In summary, we have explored the necessary domains of PCBP that are required for optimal trans-activation of the proximal promoter on the mouse MOR gene. We have successfully constructed several deletion plasmids that were confirmed using gel electrophoresis, SDS-PAGE, and gene sequencing. Next, we were able to show that PCBP can trans-activate the MOR gene in a dose dependent manner in mouse neuronal (N2A) cells.

In this study, we were able to show that triple domain deletion constructs of PCBP had lost ability to trans-activate the MOR gene (Figure 7). The trans-activation activity was enhanced upon presence of another domain as shown by double domain deletion constructs (Figure 6). Our results also show that deletion of one domain lead to slight decrease in trans-activation activity of PCBP on the MOR promoter (Figure 5). Collectively, this data suggests that no single domain of PCBP has critical control over trans-activation. We hypothesize that multiple domains are necessary for optimal trans-activation. Also, it is possible that certain domains may contain the binding domains while others contain the trans-activation domain and deletion of either resulted in decrease of trans-activation activity.

Our preliminary data suggest that it is necessary for completion of more domain deletion constructs to further examine their effects on trans-activation. Also, the results seen in this study ought to be further confirmed using other trans-activation methods, such as yeast one hybrid systems. Lastly, identification of the DNA binding domains of PCBP will be helpful in characterizing the mechanism of action of the PCBP protein.

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