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# Determination of the Regions Related to Nuclear Localization of the Poly C Binding Protein

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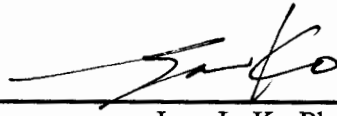
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**Determination of the Regions Related to Nuclear Localization  
of the Poly C Binding Protein**

Andrea M. Berry

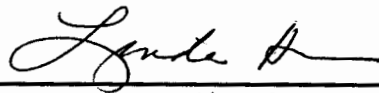
**Submitted in partial fulfillment of the requirements for the  
Degree of Masters of Science in Biology from the  
Department of Biology of Seton Hall University  
June 2005**

Approved By:



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Jane L. Ko Ph.D  
Mentor



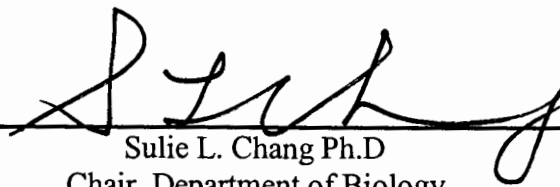
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## Abstract

Poly-C binding protein (PCBP), a single stranded DNA binding protein, belongs to the heterogeneous nuclear ribonucleoproteins (hnRNPs) family containing multiple KH homology (KH) domains. PCBP contains three KH domains (KHI, KHII and KHIII) and a variable domain located between the KHII and KHIII domains. Recently, PCBP has been shown to participate in the regulation of the  $\mu$  opioid receptor (MOR) gene, which is mainly expressed in the central nervous system. PCBP must be actively transported into the nucleus to exert its functions on MOR gene regulation in neuronal cells. A nuclear localization signal (NLS) within the sequence of the protein directs nuclear transportation through the nuclear pores. However, PCBP contains no classic NLS consensus sequences. Therefore, we aim to determine the NLS of PCBP in neuronal cells.

In order to determine the NLS regions of PCBP, we performed deletional analysis by generating single- or double-domain deletions of PCBP. The mammalian expression plasmid containing either the wild type or truncated form of PCBP fused with the enhanced green fluorescence protein (EGFP) was then made. These plasmids were individually transfected into mouse neuronal (N2A) cells, and their cellular distributions were examined using a laser scanning confocal microscope. Our results showed that the full length PCBP was mainly expressed in the nucleus, although a lesser amount of cytosolic distribution was also observed.

A single domain deletion construct devoid of KHI domain, the pK2v3 plasmid, was constructed and then transfected into the neuronal cells. This truncated PCBP displayed a stronger nuclear distribution as compared to its cytosolic distribution, similar to the wild type PCBP. This result suggested no critical NLS located in the KHI domain.

However, deletion of the variable region of PCBP, the p $\Delta$ V plasmid, resulted in a drastic decrease of nuclear distribution in transfected neuronal cells, suggesting that the variable region contains the important NLS, directing the nuclear localization of PCBP.

Furthermore, deletion of the KHIII domain of PCBP, the pK12v plasmid, resulted in less nuclear distribution than full length protein, but not as drastic a loss as seen in the variable domain deletion, indicating that in addition to the variable domain, KHII domain may also contain important NLS.

To further examine the functional roles of the variable domain and KHIII domain, various double-domain deletion constructs were also made and transfected into the neuronal cells. The dual-domain truncated PCBP which contained both the variable and KHIII domain, the pK3v plasmid, showed the highest expression in the nucleus. In contrast, deletion of both variable and KHIII domains, the pK12 plasmid, resulted in a drastic loss of nuclear staining. These results further confirmed that NLS of PCBP is located in both variable and KHIII domains. Furthermore, inclusion of only one essential NLS-containing domain of either variable or KHIII domain restored some degree of nuclear expression, but not as high as when both domains are present. In summary, these results clearly demonstrated the functional role of both the variable region and the KHIII domain in directing PCBP nuclear localization in neuronal cells.



## Introduction

Protein synthesis does not take place in the nucleus, but many proteins are required in the nucleus in order to access their targets to affect processes such as DNA synthesis, transcription and signal transduction pathways. Smaller proteins can diffuse freely through the nuclear membrane, but larger proteins require a specific sequence, known as the nuclear localization signal (NLS), to be actively transported into the nucleus through the nuclear pore complex (NPC) (Gasiorowski and Dean, 2003). The NPC spans the nuclear envelope and allows for selective entry of proteins into the nucleus. The NLS is recognized by karyopherins, members of the importin family of nuclear transport proteins (Gorlich et al. 1995; Wendler et al. 2004). More specifically, importin  $\alpha$  acts as a carrier protein that binds to the positively charged amino acids of the NLS. This complex then binds to transport receptor importin  $\beta$  and passes through the NPC (Nadler et al. 1997; McBride et al. 2002).

Nuclear transport also requires the Ran protein, which binds to a groove created by the importin  $\alpha/\beta$  complex (Conti et al. 1998; Ems-McClung et al. 2004). Ran is a small protein with a GTP binding region. RanGDP is at a higher concentration in the cytoplasm and RanGTP is found in much higher concentrations in the nucleus. The gradient created by the unequal distributions of these two proteins across the nuclear membrane drives protein movement through the NPC. Importin  $\beta$  associates more strongly with RanGDP. Once inside the nucleus, however, the GDP is replaced with GTP, based on the difference in GDP/GTP concentrations inside the nucleus. This displacement of GDP with GTP allows the importin complex to dissociate from the NLS

of the protein of interest. That protein is now free to move about the nucleus to find its target, and the importins are recycled through the membrane back into the cytoplasm.

Originally, nuclear localization signals were categorized as monopartite or bipartite. Monopartite, or, classical NLSs contain a single cluster of basic residues and was first identified in the SV40 large T antigen (Kalderon et al. 1984). Below is a comparison of the classical SV40 antigen NLS and three other proteins determined to possess a classical NLS (Welch et. al, 1999).

	NLS sequence	localisation
SV40	<b>PKKKRKV</b>	nuclear
Pho4p	<b>PYLNRKRGKP</b>	nuclear
<i>c-Myc</i>	<b>PAAKRVKLD</b>	nuclear
RanBP3	<b>PPVKRERTS</b>	nuclear

The general formula for the classical NLS sequence is **(K(K/R)X(K/R)** where *X* is any amino acid and bolded amino acids are critical for function.

Bipartite NLSs contain two clusters of residues separated by a linker of 5-20 unconserved amino acids. The standard NLS sequence was determined in nucleoplasmin. The NLS of nucleoplasmin is **KRPAATKKAGQAKKKKL**. The bolded amino acids are critical for NLS function (Robbins et al. 1991).

Recently, a novel, third class of NLS, called the M9 sequence, has been found. The following chart shows four consensus M9 sequences that have been determined for nuclear RNA binding proteins (Siomi et al. 1995).

		M9		
Human A1	258	S	N	Y
Human A2/B1	287	M	Y	N
Xenopus A1	312	G	E	N
Xenopus A2	328	M	Y	N

M9 sequences differ from classical NLSs in that they also contain a nuclear export signal, allowing proteins to exit the nucleus (Siomi and Dreyfuss 1995; Siomi 2000).

Transportin is able to bind directly to M9 sequence for nuclear import (Pollard et al. 1996; Gustin and Sarnow 2002). This M9 sequence alone allows for nuclear localization, suggesting that it does not require the importin complex for nuclear import and makes direct contact with the nuclear pore complex (Michael et al. 1997).

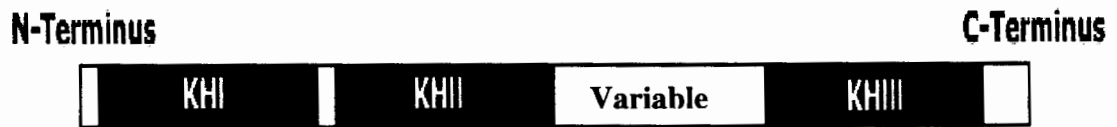
A general set of rules for classical NLSs was proposed and includes the following conditions. First, the core NLS most often exists as a hexapeptide with four arginines and lysines. A general set up for this hexapeptide is RXXKKR where X is any non-bulky amino acid. Secondly, bulky amino acids such as phenylalanine, tyrosine, and tryptophan, as well as negatively charged amino acids such as glutamic acid and aspartic acid, are not found in the NLS sequence. Thirdly, an NLS is usually flanked by a helix breaking domain, most often composed of aspartic and glutamic acids, along with a proline or glycine residue. Finally, in order for the NLS on the protein to be exposed to the receptor that will confer nuclear localization, the sequence should not contain hydrophobic amino acid residues in the core flanking region. These guidelines are theoretical generalizations and it is not required that an NLS sequence contain all four criteria in order to be considered a classical NLS (Boulikas 1994; Cokol et al. 2000).

We are interested in a poly C binding protein (PCBP), which is a single-stranded DNA binding protein. Recently, it has been shown to participate in the regulation of mouse  $\mu$ -opioid receptor gene expression (Ko and Loh 2005). PCBP belongs to the hnRNP superfamily, which contains over 20 known proteins involving in many levels of gene expression, including transcriptional regulation by hnRNP K (Michelotti et al. 1996;

Da Silva et al. 2002), translational regulation by hnRNP K and PCBP (Ostareck et al. 1997; Ostareck-Lederer et al. 2002), and RNA processing by SRC pyrimidine-binding protein (SPy) (Ritchie et al. 2003). Members of the hnRNP family also contribute to a complex around nascent pre-mRNA, thereby affecting RNA processing (Chen et al. 2003). Among members of the hnRNP superfamily, hnRNP K has been extensively studied (Michael et al. 1997) and its NLS has also been determined. The hnRNP K contains two KH domains located at the N terminus, separated from the third KH domain located at the C terminus by a nonconserved amino acid variable region (Chkheidze and Liebhaber 2003). The NLS of hnRNP K is composed of basic amino acids consistent with the consensus sequence for a classical bipartite-basic NLS and its sequence is KRPAEDMEEEQAFKRSR (Michael et al. 1995). This protein also contains the M9 sequence GFSADETWDSAIDTWSPSEWQMAY that allows nuclear localization upon the deletion of the known NLS sequence (Michael et al. 1997).

PCBP is also involved in RNA stabilization (Weiss and Liebhaber 1994, 1995; Sidiqi et al. 2005), translational activation (Blyn et al. 1997; Makeyev and Liebhaber 2002), and translational silencing (Ostareck et al. 1997; Makeyev and Liebhaber 2002) as well as transcriptional regulation (Ko and Loh 2005). This protein is a member of the hnRNP superfamily. Although PCBP also serves as a multifunctional protein similar to hnRNPK, PCBP does not contain an NLS similar to that found in hnRNP K.

In this study, we aim to determine the region or regions of PCBP that direct nuclear localization in neuronal cells. As seen in Fig. 1, PCBP consists of three consensus KH domains (KHI, KHII and KHIII). The KHII and KHIII domains are separated by a variable domain.



**Figure 1:** Schematic representation of the full length PCBP protein, containing the KHI domain at the N- terminus, followed by the KHII domain, followed by the variable domain, followed by the KHIII domain at the C-terminus.

In order to determine the location of NLS of PCBP in neuronal cells, deletional analysis and transfection assays were performed. We generated the enhanced green fluorescent protein (EGFP) fusion constructs containing one or two domain deleted PCBP protein which allow for visualization of the nuclear localization using a laser scanning confocal microscope. Our results demonstrated that the variable domain and KHIII domain are important for nuclear localization of PCBP in neuronal cells.

## Materials and Methods

### Plasmid construction and isolation –

For construction of the deletion constructs, a pcDNA3-PCBP plasmid (Invitrogen, Carlsbad, CA) was digested with restriction enzymes and purified by agarose gel electrophoresis to create constructs containing different combinations of KH domains.

The DNA fragments of the full length of PCBP or truncated PCBP were then isolated and purified via Gene Clean kit (Bio 101, Carlsbad, CA), re-ligated and inserted into the pEGFP-C1 (BD Bioscience, San Jose, CA) vector. The ligation resulted in the GFP-PCBP and GFP-truncated PCBP constructs.

### *E. Coli* Transformation -

Plasmid DNA was added to DH5 $\alpha$  *E. coli* competent cells and incubated on ice for 30 minutes. The cells were then heat shocked at 42 °C water bath and then chilled on ice. Luria Broth media was added to the cells and incubated for 60 minutes at 37 °C. The transformed cells were then plated on Luria Broth agar plates containing 1 $\mu$ g/ml kanamycin for positive selection. The plates were incubated overnight at 37 °C.

### Small scale plasmid preparation -

A 1.5 mL of the overnight cultures from the transformed *E. coli* was centrifuged at maximum speed in a tabletop centrifuge. The Luria broth was removed from the tube and the plasmid DNA was extracted utilizing the QIAGEN (Valencia, CA) Plasmid Mini Prep Kit, where the *E. coli* pellet was re-suspended in 250  $\mu$ L P1 buffer (50mM glucose, 10mM EDTA, pH 8, 25mM Tris HCl, pH 8 with RNase A). The cells were then lysed by

addition of 250  $\mu$ L of P2 buffer (0.2M NaOH with 1% SDS). A 350  $\mu$ L of P3 buffer (5 M potassium acetate, glacial acetic acid) was added to neutralize the reaction and the mixture was centrifuged at maximum speed in the tabletop centrifuge to remove the cell debris and chromosomal DNA. The supernatant was then loaded into a column and washed with PB and PE wash buffer. The plasmid DNA was eluted using 50  $\mu$ L of EB Elution buffer. The plasmid was subjected to the restriction enzyme digestion and then analyzed by gel electrophoresis, which was further visualized by AlphaImager (Alpha Innotech, San Leandro, CA) for confirmation.

#### Large Scale Plasmid Preparation –

A plasmid-transformed *E. coli* was grown overnight at 37 °C in 250 mL Luria Broth media with antibiotics. The overnight culture was then centrifuged at 6000 rpm for 15 minutes. The plasmid DNA was extracted utilizing QIAGEN (Valencia, CA) Plasmid Maxi Kit where the *E. coli* pellet was re-suspended in 10 mL of P1 buffer (50mM glucose, 10mM EDTA, pH 8, 25mM Tris HCl ,pH 8 with RNase A). The cells were then lysed by addition of 10 mL of P2 buffer (0.2M NaOH with 1% SDS). A 10 mL of P3 buffer (5M potassium acetate, glacial acetic acid) was added to neutralize the reaction and the mixture was then centrifuged at 16,000 rpm to remove the cell debris and chromosome DNA. The supernatant was then loaded onto a column and washed with QC wash buffer. The plasmid DNA was then eluted using QF Elution buffer, and a 11 mL of isopropanol was then added to the eluant. The plasmid DNA pellet was collected by centrifugation and was re-suspended in TE (10mM Tris, pH 8, 1mM EDTA, pH 8) 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<sub>2</sub> at 37 °C. Cell monolayers were grown in T-75 cm<sup>2</sup> flasks (Fisher Scientific, Tustin, CA) and passaged when the monolayers reached 100% confluence by visual examination using an inverted microscope at 60X magnification. An aliquot of re-suspended cells was transferred to a new T-75 cm<sup>2</sup> flask (Fisher Scientific, Tustin, CA) containing 25 mL DMEM with 10% FCS and incubated at 37 °C in an atmosphere of 10% CO<sub>2</sub>.

### Transient Transfection-

Cells were transfected with lipofect following the manufacturer's protocol (Qiagen, Valencia, CA). Neuronal mouse cells (N2A) at a concentration of 0.2x10<sup>6</sup> cells per well were incubated overnight in 6-well plates with a poly-L lysine coated coverslip placed in the bottom of each well. Cells were transfected with 1µg plasmid DNA. After twenty-four hours of incubation, the cells were washed and DMEM with 10% FCS was added to each well. Forty-eight hours after transfection, cells were grown to confluence, washed twice with phosphate buffered saline (PBS) and prepared for fixation.

### Fixation and Preparation for Confocal Microscopy-

Cells were fixed with 4% paraformaldehyde solution in PBS, and were then washed twice with PBS. The cells were therefore perforated with a 0.1% Triton X100 solution in 4% paraformadehyde, treated with RNase solution, and were then washed three times with



PBS solution before staining using 3 $\mu$ g/ml propidium iodide solution in de-ionized water. The cells were washed again with PBS solution. The coverslips were then placed onto a clean glass slide with a drop of anti-fade reagent (Vector Labs, Burlingame, CA) and sealed.

#### Confocal Microscopy-

The prepared slides were viewed with a laser scanning confocal microscope (Fluoview 1000, Olympus) with multi-line argon laser lines at emissions of 457nm, 488nm, and 514nm, and green NeHe laser lines at an emission of 543nm. The cells were viewed under phase contrast at 10X and fluorescence light using oil immersion at 40X for image capture.

## Results

### Construction of the expression vector containing the full length or truncated PCBP cDNA

In order to investigate the NLS region of PCBP, truncated cDNA fragments of PCBPs were generated. The mammalian expression vector, pEGFP-C1, driven by the CMV promoter, was used to express the green fluorescence protein (GFP)- PCBP or GFP- truncated PCBP fusion protein. The full length cDNA fragment of the PCBP was first inserted into the multiple cloning site of the vector to generate the pGFP-PCBP construct. Once the pGFP-PCBP construct was made, we were able to create the single and double domain of PCBP deletion constructs, as shown in Fig. 2. These constructs were then subjected to DNA sequencing to ensure that PCBP and its derivatives were in-frame.

GFP-PCBP	GFP	KHI	KHII	Variable	KHIII
GFP-K2v3	GFP	KHII	Variable	KHIII	
GFP-ΔV	GFP	KHI	KHII	KHIII	
GFP-K12v	GFP	KHI	KHII	Variable	
GFP-K3v		GFP	Variable	KHIII	
GFP-K12		GFP	KHI	KHII	
GFP-K2v		GFP	KHII	Variable	
GFP-K23		GFP	KHII	KHIII	

**Figure 2:** Schematic representation of the full length or truncated PCBP protein, which was fused with GFP of the pEGFP-C1 mammalian expression vector.

### **Subcellular localization of the full length PCBP protein**

The cellular distribution of full length PCBP in neuronal cells has not been studied. We therefore set out to determine the cellular localization of the wild-type protein using neuronal (N2A) cells. The pGFP-PCBP plasmids, containing the full length of PCBP fused with GFP, were transiently transfected into cells. The transfected cells were fixed, and the subcellular distribution of PCBP was localized using the laser scanning confocal microscope.

As shown in Fig. 3A, the expression of the full length PCBP protein (indicated by the green fluorescence) is observed throughout the nucleus and cytoplasm. However, its nuclear distribution is stronger than its cytoplasmic expression in neuronal cells. A punctated nuclear staining pattern can also be seen in the full length PCBP protein, as indicated by the arrows (Fig. 3A).

To verify the nucleus contains a higher amount of PCBP than the cytosolic portion, we performed the nuclear staining using propidium iodide, and then merged nuclear staining image with the image of PCBP expression of the same cell. Fig. 3B showed the nuclear location indicated by the red color, and Fig. 3C is a merged image of the green fluorescence image (Fig. 3A) and the nuclear staining image (Fig. 3B). The yellow color represented areas of co-localization of PCBP protein (in green color) and the nucleus (in red color). This result (Fig. 3C) confirmed that the higher expression of PCBP is indeed located in the nucleus region.

In summary, these results demonstrated that the full length PCBP is highly expressed in the nucleus, although it is also present throughout the neuronal cell.

**A**

**B**

**C**

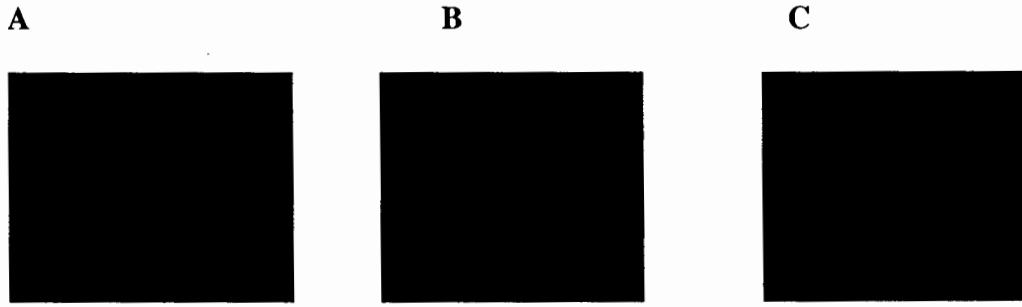


**Figure 3:** *Neuronal cells were transiently transfected with pGFP-PCBP plasmids. After two days of transfection, the cells were fixed and viewed using a laser scanning confocal microscope at 40X magnification. A, the subcellular distribution of the full length PCBP (indicated by the green color). The arrows indicate the punctated nuclear staining pattern. B, Nuclear region is shown in red color which was stained using propidium iodide. C, The merged image of A and B, showing co-localization (indicated as the yellow color) of the green fluorescence labeled PCBP (A) and nuclear staining (B).*

### **Single Domain Deletions:**

#### **The KHI domain is not critical for directing the nuclear localization of PCBP.**

In order to investigate the NLS regions of PCBP, we first carried out the 5'-deletion to remove the KHI domain from the full length PCBP. The resulting construct, pGFP-K2v3, containing KHII, variable and KHIII regions fused with GFP, was then transfected into neuronal cells. As shown in Fig. 4A, this truncated protein is located throughout the nucleus and cytoplasm with stronger expression seen in the nucleus. A punctated nuclear staining pattern is also seen with this truncated protein, as indicated by the arrows (Fig. 4A). Similarly, the yellow color in Fig. 4C showed the co-localization of the green fluorescent labeled protein (Fig. 4A) and the nuclear stain (Fig. 4B), confirming that the nuclear distribution of this truncated protein is higher than its cytosolic distribution, which is similar to the expression pattern of the full length of PCBP (Fig. 3A). These results suggested that the KHI domain is not essential for directing PCBP nuclear localization in the neuronal cells.

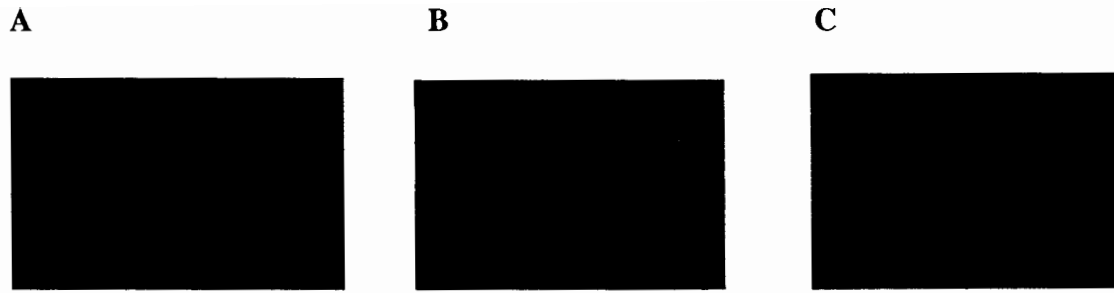


**Figure 4:** *Neuronal cells were transiently transfected with pGFP-K2v3 plasmid. After two days of transfection, the cells were fixed and viewed using a laser scanning confocal microscope at 40X magnification. A, Cellular expression (indicated in the green color) of the truncated PCBP protein, devoid of the KH1 domain. The arrows indicate the punctated nuclear staining pattern. B, Nuclear staining using propidium iodide. C, The merged image of A and B, showing co-localization (yellow color) of the green fluorescence labeled truncated protein and nuclear stain.*

**The variable region of PCBP is important for directing its nuclear localization.**

Among the KH domain superfamily, DNA sequences of the variable domain of PCBP is less conserved as compared to the KH domains. Therefore, it is critical to investigate the functional role of this variable domain in directing the nuclear localization of PCBP. The pGFP- $\Delta$ V plasmid was devoid of the variable domain of PCBP and was fused with GFP. This mutant plasmid was then transfected into neuronal cells. As seen in Fig. 5A, deletion of the variable domain of PCBP resulted in the mainly cytoplasmic expression of this truncated protein. The nuclear distribution of this protein was drastically reduced when compared to the full length PCBP protein (Fig. 3A). The drastic decrease of nuclear expression of this truncated protein was further confirmed in Fig. 5C, which lacked any yellow color, suggesting that there is little co-localization between this truncated protein (Fig. 5A) and the nuclear stain (Fig. 5B).

Taken together, these results demonstrated that the variable region of PCBP is critical in directing its nuclear localization in the neuronal cell.



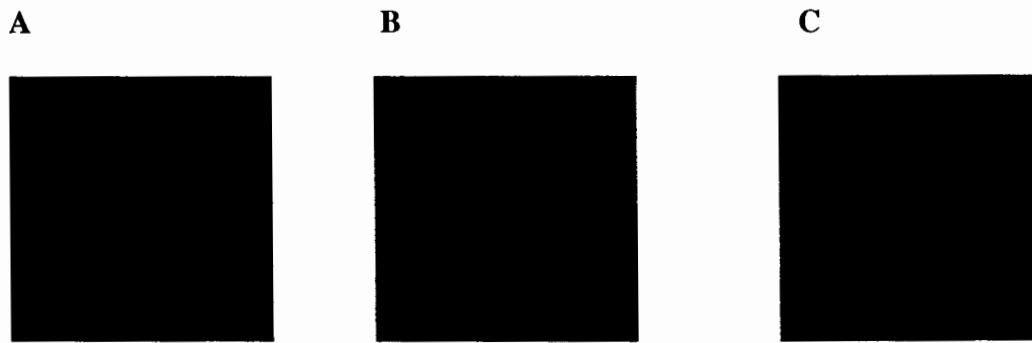
**Figure 5:** *Neuronal cells were transiently transfected with pGFP- $\Delta$ V plasmid. After two days of transfection, the cells were fixed and viewed using a laser scanning confocal microscope at 40X magnification. A, Cellular expression (indicated in the green color) of the truncated PCBP protein, devoid of the variable domain. B, Nuclear staining using propidium iodide. C, The merged image of A and B, showing the lack of co-localization of the green fluorescence labeled truncated protein and nuclear stain.*

### **KHIII domain also involved in directing the nuclear localization of PCBP**

To further examine if the KHIII domain is also required for directing its nuclear localization in neuronal cells, we performed the 3'-deletion analysis. The deletion construct, pGFP-K12v, devoid of the KHIII domain and fused with GFP, was generated, and then transfected into neuronal cells. As seen in Fig. 6A, deletion of the KHIII domain resulted in a stronger staining seen in the cytoplasm than that in the nucleus. However, the decrease of distribution in nucleus was not as drastic as that seen in the nuclear distribution of  $\Delta$ V protein (Fig. 5A). This observation is further confirmed in Fig. 6C by merging the green fluorescence signals (Fig. 6A) with the nuclear stain propidium iodide signals (Fig. 6B). The yellow color (the co-localization signal) is visible, but is not as strong as in the full length PCBP protein (Fig. 3C), indicating that this mutant protein is able to enter the nucleus but is not as highly expressed in the nucleus as the PCBP protein or KHI domain deletion protein, K2v3.

In summary, these results demonstrated that the KHIII domain also contains the NLS signal and also plays a role in directing nuclear localization, but not as strongly as

the variable domain, indicating that it may function to enhance the effects of the variable domain on directing the nuclear localization of PCBP in the neuronal cell.



**Figure 6:** Neuronal cells were transiently transfected with pGFP-K12v plasmid. After two days of transfection, the cells were fixed and viewed using a laser scanning confocal microscope at 40X magnification. A, Cellular expression (indicated in the green color) of the truncated PCBP protein, devoid of the KHIII domain. B, Nuclear staining using propidium iodide. C, The merged image of A and B, showing co-localization (yellow color) of the green fluorescence labeled truncated protein and nuclear stain.

#### **Double Domain Deletions:**

**Both variable domain and KHIII domain are required for directing the nuclear localization.**

In order to define the functional roles of the NLS related domains, double domain truncation constructs were created. Based on the above results, we then generated a GFP-fusion construct containing only two regions involved in directing nuclear localization. The construct containing the variable domain and the KHIII domain, pGFP-K3v, was prepared, and then transfected into neuronal cells.

As seen in Fig. 7A, the K3v mutant protein is most highly expressed in the nucleus, with a low level of expression seen in the cytoplasm. This expression pattern was further verified in Fig. 7C, showing that the high degree of co-localization (yellow

signal) between the fluorescent tagged protein (Fig. 7A) and the nuclear stain (Fig. 7B) was observed in the nucleus region.

Taken together, these results clearly demonstrated that the variable domain and the KHIII domain do contain the NLS for directing its nuclear location, and the presence of both domains is required for the stronger nuclear expression of PCBP in neuronal cells.



**Figure 7:** Cells were transiently transfected with pGFP-K3v plasmid. After two days of transfection, the cells were fixed and viewed using a laser scanning confocal microscope at 40X magnification. A, Cellular expression (indicated in the green color) of the truncated PCBP protein, containing only the variable and KHIII domains. B, Nuclear staining using propidium iodide. C, The merged image of A and B, showing co-localization (yellow color) of the green fluorescence labeled truncated protein and nucleus.

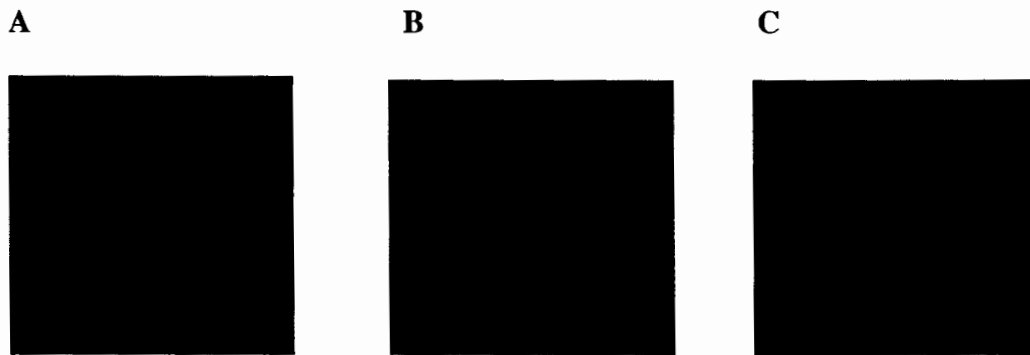
#### **KHI and KHII domains are not required for directing PCBP nuclear distribution. .**

In order to examine whether KHI and KHII domains contained any NLS for directing its nuclear localization, and to further verify the functional role of the KHIII and the variable domain, a GFP-fusion construct devoid of the variable and KHIII domains, pGFP-K12, was made. This mutant plasmid was then transfected into neuronal cells. As seen in Fig. 8A, the nuclear expression was drastically reduced, when compared to the protein that contained both the variable and the KHIII domain (Fig. 7A). The lack of yellow color in Fig. 8C demonstrates that there is little co-localization of the green



fluorescence labeled truncated protein (Fig. 8A) and the nuclear staining (Fig. 8B) in the cell.

These results confirmed that KHI and KHII domains are not critical for directing the nuclear localization, whereas the variable domain and the KHIII domain function together to confer nuclear localization of PCBP in neuronal cells.

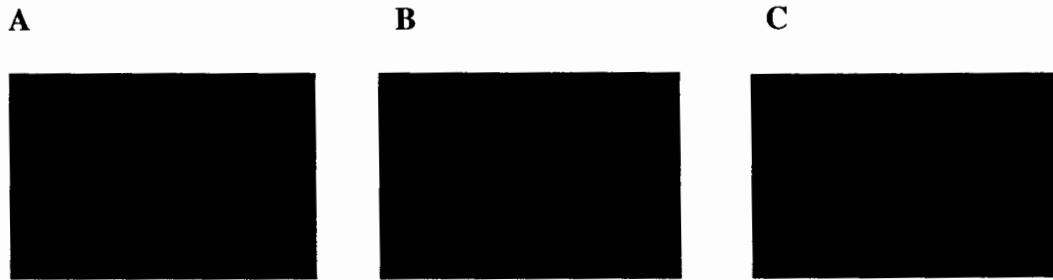


**Figure 8:** Cells were transiently transfected with pGFP-K12 plasmid. After two days of transfection, the cells were fixed and viewed using a laser scanning confocal microscope at 40X magnification. A, Cellular expression (indicated in the green color) of the truncated PCBP protein, containing only the KHI and KHII domains. B, Nuclear staining using propidium iodide. C, The merged image of A and B, showing co-localization (yellow color) of the green fluorescence labeled truncated protein and nucleus.

#### **The variable domain does not exclusively direct nuclear localization of PCBP.**

To further examine the functional role of the variable domain we therefore generated a GFP-fusion construct, pGFP-K2v, containing only the variable domains. This mutant plasmid was then transfected into neuronal cells. As seen in Fig. 9A, the expression pattern of this truncated protein was found evenly throughout the cell. No discrepancy between the nuclear or cytosolic morphology was observed, which was further confirmed in Fig. 9C (merged image of Fig. 9A and Fig. 9B, with nuclear staining using propidium iodide).

These results suggested that the variable region alone is not sufficient to optimally direct the nuclear localization of PCBP in neuronal cells.

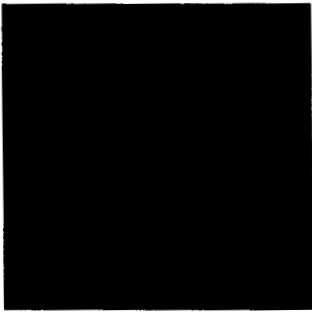
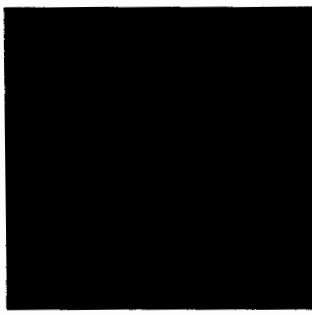
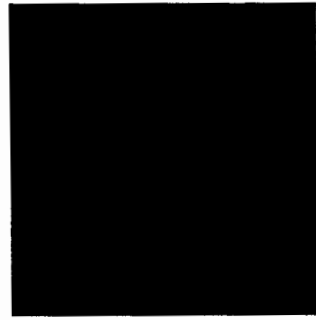


**Figure 9:** Cells were transiently transfected with pGFP-K2v plasmid. After two days of transfection, the cells were fixed and viewed using a laser scanning confocal microscope at 40X magnification. A, Cellular expression (indicated in the green color) of the truncated PCBP protein, containing only variable and KHII domains. B, Nuclear staining using propidium iodide. C, The merged image of A. and B, showing co-localization (yellow color) of the green fluorescence labeled truncated protein and nucleus.

#### **The KHIII domain directs some degree of nuclear localization of PCBP.**

In order to examine the functional role of KHIII domain, a GFP-fusion construct (pGFP-K23), containing only the KHIII and KHII domains, was made and this mutant plasmid was transfected into neuronal cells. As seen in Fig. 10A, the nuclear and cytosolic distribution of this truncated protein is even throughout the cell, with no clear preference toward nuclear or cytosolic distribution, which resembles the expression pattern of the GFP-K2v protein (Fig. 9). The results of Fig. 10B and 10C further supported this conclusion.

In summary, these results demonstrated that the KHIII domain is able to restore some nuclear stain but not as strongly as was seen with the presence of both the variable domain and the KHIII domain (Fig.7).

**A****B****C**

**Figure 10:** Cells were transiently transfected with pGFP-K23 plasmid. After two days of transfection, the cells were fixed and viewed using a laser scanning confocal microscope at 40X magnification. A, Cellular expression (indicated in the green color) of the truncated PCBP protein, containing KHIII and KHII domains. B, Nuclear staining using propidium iodide. C, The merged image of A and B, showing co-localization (yellow color) of the green fluorescence labeled truncated protein and nuclear stain.

## Discussion

Previous studies have demonstrated that the poly-C binding protein (PCBP) can serve as a transcription regulator and is involved in the regulation of mouse  $\mu$ -opioid receptor (MOR) gene expression (Ko and Loh 2005). MOR, a seven-transmembrane G-protein coupled receptor, is mainly expressed in the central nervous system and mediates the effects of the major clinically used analgesics, such as morphine. In order to regulate the MOR gene expression, the PCBP regulator protein must be able to enter the nucleus to exert its effects on the target gene. Therefore, we first examined the subcellular distribution pattern of the full length PCBP in the neuronal N2A cells. Our results demonstrated that PCBP was distributed throughout the nucleus and cytoplasm; however, it displayed a higher nuclear localization with some hot spots (punctuated stainings) in the nucleus of neuronal cells. (Fig. 3)

PCBP contains four domains (Fig. 1); a variable region and three K homology domains (KHI, KHII and KHIII), first studied in the heteroribonuclear protein K (hnRNP K) of the hnRNP superfamily (Adinolfi et al, 1999). The nuclear localization signals (NLS) of other members of the hnRNP family have been elucidated, with the NLS of the hnRNP K serving as a model for the entire family (Michael et al. 1997). However, no such consensus NLS is found for PCBP. We therefore examined the functional role of each domain of PCBP in directing its nuclear trafficking in neuronal cells.

Based on one-domain deletion studies, our results showed that deleting the PCBP KHI domain yielded no dramatic difference in the protein subcellular distribution pattern when compared to the full-length protein (Fig. 4). In contrast, deletion of the variable domain resulted in protein expression with drastically reduced nuclear staining, when

compared to the full length protein (Fig. 5). These results suggested that the variable domain, but not the KHI domain, played a critical role in determining the nuclear localization. In addition, these results also indicated that KHII domain contains no essential NLS. Furthermore, deletion of the KHIII domain resulted in the decrease of nuclear localization when compared to the subcellular expression pattern of the full length protein, but not as drastic a change as when the variable domain was deleted (Fig. 6). Taken together, these results suggested the importance of the physiological role of variable domain in directing the nuclear localization, and the functional role of KHIII domain being able to confer some degree of nuclear localization, but not as strongly as the variable domain.

This notion is further supported by our two-domain deletion studies. When both the variable domain and the KHIII domain were present, the nuclear distribution was dominant (Fig. 7), whereas when both of these essential domains were deleted, the nuclear distribution was drastically reduced (Fig. 8). Furthermore, when just one essential domain was included, some degree of nuclear distribution was restored, but not as strongly as when both domains were present (Figs. 9 and 10). In conclusion, these results further confirmed the critical roles of both variable region and KHIII domain in directing PCBP nuclear trafficking in neuronal cells.

One interesting thing to note when considering the double domain deletion results is the generation of a reduced size of the truncated protein. When two of the four domains of PCBP are deleted, the protein drops below the size required for recognition in order to cross the nuclear membrane. Therefore, these two-domain truncated proteins may be able to pass freely through the nuclear membrane and may not require recognition

by the transport receptors and the nuclear pore complex as compared to the one-domain deletion protein. Although the double-domain deleted proteins have the possibility of entering the nucleus without the inclusion of the NLS sequence, our results showed that these proteins still will only express dominantly in the nucleus while the regions of the variable domain and KHIII domain are present (Fig. 7), suggesting that the variable domain and the KHIII domain must be present for optimal nuclear distribution of PCBP, regardless of the overall size of the protein.

Based on this study, we concluded that two different regions related to NLS of PCBP, residing in the variable domain and KHIII domain, were involved in directing nuclear localization of PCBP in neuronal cells. We hypothesize that the NLS of the variable domain is essential for the nuclear localization of PCBP, while the NLS related region located in the KHIII domain enhances its nuclear distribution. There are a few possible mechanisms. One possibility is that the NLS of the KHIII domain may interact directly with the NLS of the variable domain, and serves to enhance the effects of the variable domain on directing the nuclear trafficking of PCBP in neuronal cells, because the KHIII domain alone is not able to confer the dominant nuclear distribution of PCBP without the presence of the variable domain. This notion can be supported by the example of hnRNP type I protein. It has been shown in the hnRNP type I protein that importin- $\alpha$  binding is drastically reduced when either one of the two domains responsible for this binding is mutated (Romanelli and Morandi, 2002). The PCBP belongs to the same superfamily as hnRNP type I protein, and it is possible that there is an interaction between the variable and KHIII domains of PCBP to allow nuclear localization of the protein.

A second possibility is that the NLS residing in the KHIII domain contains a weak NLS signal, which may not be able to interact directly with the NLS of the variable domain, but it may interact with other nuclear transport related proteins and therefore enhance the PCBP nuclear localization. In the PDZ-Domain protein, L-periaxin, there are three domains that are essential for nuclear distribution (Sherman and Brophy 2000). The first domain is essential for nuclear localization. Addition of one or both of the remaining domains enhanced the observed nuclear staining. This data suggests that the second and third domains involved in the nuclear localization of this protein contain a weaker signal that serves to enhance the nuclear localization signal of the first, essential domain.

The third possibility is that the KHIII domain may interact with other proteins which can be translocated into nucleus, and therefore enhance the PCBP nuclear localization. This notion can be supported by the example of the  $\text{I}\kappa\text{B}\alpha$  protein. One domain of  $\text{I}\kappa\text{B}\alpha$  protein serves as the dominant NLS of the protein, but two other domains contain weak NLS signals that are able to direct some proteins into the nucleus (Sachdev et al.1998), and it has been hypothesized that the second and third domains control nuclear import by interactions with different cytoplasmic proteins, including hnRNPA1 (Hay et al. 2001). It is possible that the KHIII domain of PCBP contains a weaker signal, similar to those domains observed in  $\text{I}\kappa\text{B}\alpha$ , that interacts with proteins such as hnRNPA1 to enhance the effects of the variable domain on the nuclear localization of PCBP.

The punctated nuclear staining observed in the full length PCBP (Figure 3) is another point of interest. Punctated nuclear staining often indicates that the protein is a

component of a nuclear complex, involved in transcriptional control. This idea has been observed in proteins such as Ikaros that contributes to a repressor complex (Cobb et al. 2000), the AIRE protein, a transcription factor that serves as a component of a protein complex on the target site of chromatin (Riderle et al. 1998), the ATRX protein, associated with the nuclear matrix during mitosis (Berube et al. 2000), and proliferating cell nuclear antigen, a protein that helps make up a DNA replisome complex (Leonhardt et al. 2000). Since PCBP has been shown to be involved in  $\mu$ -opioid receptor gene expression, it would follow logically that the protein would be a component of nuclear complexes involved in transcriptional regulation and RNA splicing, explaining the presence of the punctated nuclear staining seen in the full length PCBP expression.

Future studies will determine the exact amino acid that serves as the NLS in PCBP. We will also elucidate the role that the KHIII domain plays in the nuclear localization of the PCBP, and additional studies will also be conducted to investigate the pathway of import that allows PCBP to enter the nucleus in neuronal cells.



## Conclusion

In summary, we have explored the regions of the Poly C Binding Protein that are necessary for its nuclear localization in neuronal cells. We successfully created single and double domain deletion proteins and fused them with GFP to allow visualization of their distributions in N2A cells. A laser scanning confocal microscope was used to allow visualization of the subcellular distribution of the green fluorescent tagged proteins.

In this study, we were able to show that the variable domain of PCBP is directly responsible for its nuclear localization in the neuronal cells. Furthermore, the KHIII domain is able to direct some nuclear distribution, albeit not as strongly as the variable domain. We were also able to show that the KHI and KHII domains are not essential for nuclear distribution of the protein.

These data indicated that the functional interaction of the variable domain with the KHIII domain serves to direct optimal nuclear distribution of PCBP. However, the exact mechanism underlying this functional interaction is still unknown, and will be further investigated. In addition, determination of the exact pathway of protein import into the nucleus will also be useful in further understanding the functional role that PCBP in regulating the MOR gene expression.

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