CHAPTER 3

IDENTIFICATION OF GENES INVOLVED IN MYELINATION FOR WHICH MECP2 ACT AS TRANSCRIPTIONAL REPRESSOR OR ACTIVATOR IN THE DEVELOPING CENTRAL NERVOUS SYSTEM

3.1. INTRODUCTION

Methyl CpG Binding protein 2 (MeCP2) is known to bind the methylated CpG dinucleotides inDNA. MeCP2 is believed to function as transcriptional repressor by recruiting the co-repressor Sin 3A and histone deacetylase (HDAC) to methylated promoter (Bienvenu and Chelly, 2006). Recent genome wide studies demonstrated that MeCP2 can repress or activate its target gene expression and bind to both methylated and unmethylated regions of genome in *vivo* (Hansen et al., 2010). Epigenetic evaluations has established that MeCP2 act as transcriptional repressor, but later studies found that MeCP2 also activates expression of target genes in the hypothalamus and cerebellum in mice over expressing MeCP2. MeCP2 is associated with transcriptional activator CREB1 (cAMP responsive element binding protein 1) at the promoter region of an activated targets but not at a repressed targets (Ben-Shachar et al., 2009; Chahrour et al., 2008). Intrinsic highly disordered nature of MeCP2 allows it to plethora of post translational modifications and to interact with large number of protein partners. MeCP2 was found to interact with HP1 (heterochromatin protein 1), transcriptional co-repressors cSki (first isolated at Sloan Kettering Institute) and N-CoR (nuclear receptor co repressor 1), Sin3A (Switch-independent 3A), and transcriptional co-activators CREB (cAMP response element binding protein), YB1 (Y box-binding protein), TDP-43(TAR DNA-binding protein 43), and Dnmt1 [DNA (Cytosine-5)-methyltransferases 1](Ausio et al., 2014). BDNF was one of the first target genes of MeCP2, involved in neuronal survival, maturation and synaptic plasticity. Previous works have shown that MeCP2 represses the expression of BDNF by binding to the promoter IV and de-represses after neuronal membrane depolarization.Neuronal membrane depolarization phosphorylates MeCP2 causing its dissociation from BDNF promoter (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006). Other MeCP2 target genes includes Dlx5, Dlx6 (Horike et al., 2005), Fxyd1, Reln, and Gtl2 (Jordan et al., 2007).

Methyl-CpG binding protein 2 is required for correct brain function and involved in neuronal maturation and synaptogenesis. Loss of MeCP2 in neurons cause neurodevelopmental disorder Rett syndrome (RTT) (Amir et al., 1999). In addition to MeCP2 role in neurons, it is also expressed in and regulates the glial cells (astrocytes, oligodendrocytes and microglia) functions. Several MeCP2 target genes have been identified in glial cells whose dysregulation impair the glial and neuronal functions (Cronk et al., 2015; Yamamuro et al., 2015).

In the present study, we analyzed the MeCP2 recruitment to myelin genes and neurotrophin (BDNF) promoters in oligodendrocytes. In the earlier chapter, we discussed that MeCP2 regulates the expression of the major the myelin genes (MBP & PLP) and BDNF in cultured rat oligodendrocytes. Further to confirm that MeCP2 regulates the major myelin genes (MBP&PLP) and BDNF by direct binding to their promoter regions, ChIP (Chromatin immunoprecipitation) assay was carried out using anti-MeCP2 antibody.

3.2. MATERIALS AND METHODS

3.2.1 Oligodendrocytes culture and isolation

Oligodendrocytes were isolated from mixed glial culture generated from cerebral cortex of 1 to 2 day old rat pups (Charles Foster) as described in Chapter-2 (Material and Methods section).

3.2.2 Isolation of genomic DNA and PCR

Cells were lysed in lysis buffer (100mM NaCl,10mM Tris pH8.0, 25mM EDTA pH8.0, 0.5% SDS, Proteinase K) for overnight at 56 ºC, followed by phenol: chloroform: isoamyl alcohol added for phase separation (Sambrook and Russell, 2001). After centrifugation, clear top phase was collected avoiding lower organic phase and DNA was precipitated with isopropanol. DNA pellet was washed with 70% ethanol and re-suspended in distilled water after air dry. 1 µl of DNA was used in PCR for standardization of promoter specific primers. Following condition was used for PCR: initial denaturation at 95ºC for 3 min. After this initial step, 30 cycles of PCR (heating at 95ºC for 30 sec, annealing at 58ºC to 60ºC for 30 sec and elongation at 72ºC for 45sec), and final elongation at 72 º C for 10 min. PCR product was analyzed by agarose gel electrophoresis.

3.2.3 Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed using EZ-Magna ChIP™ HiSens kit (Millipore) following the manufacturer instructions. OPCs (oligodendrocytes precursor cells) were allowed to proliferate for 24 hrs in Sato medium (Bottenstein and Sato, 1979) containing 10 ng/ml PDGF-A followed by differentiation in Sato medium for 48 hrs. Briefly, oligodendrocytes cells were cross-linked with 1% formaldehyde at 37 °C for 10 min and then rinsed with ice-cold PBS twice. Cells were lysed in nuclei isolation buffer and sonicated (20 sec on and 20 sec off cycles for 10 min in

sonics Vibra cell) to produce chromatin with average length of 200–1000 base pairs. Chromatin was precleared by adding 1µg of rabbit IgG for 2-4 hrs at 4°C. Precleared chromatin was subjected to immunoprecipitation using anti-MeCP2 antibodies 07-013(Millipore) & Ab2828 (Abcam). The DNA was analyzed by PCR using the MBP, PLP and BDNF gene promoter primers (Table-1) The PCR products were analyzed by 1.5% agarose gel electrophoresis.

3.3. RESULTS

3.3.1 *In silico* analysis of CpG island in myelin genes (MBP and PLP), Primer designing and optimization

MBP and PLP promoter region sequences were retrieved from NCBI and UCSC genome browser. Approximately 1000 bp sequences upstream of transcription start site (TSS) were used for designing primers for ChIP PCR. In silico identification of CpG sites and islands in promoter region were carried out online by DataBase of CpG islands and Analytical Tool (DBCAT) (http://dbcat.cgm.ntu.edu.tw/). There were one CpG island and many CpG sites were predicted in MBP promoter (Figure 3.1) while in PLP promoter region only 3 CpG sites were present (Figure 3.2). Primer pairs span + 27 to -690 bp region of MBP promoter and -211 to -1026 bp region of PLP promoter. BDNF Promoter IV primer spans -56 to-201 bp and contains 6 CpG sites (Figure 3.3). PCR conditions were optimized with promoter region primers using genomic DNA isolated from oligodendrocytes (Figure 3.4).

3.3.2 MeCP2 binds to promoter regions of MBP and PLP genes in oligodendrocytes

In earlier chapter, we observed that MeCP2 suppress the expression of major myelin proteins MBP and PLP in differentiating oligodendrocytes, suggesting that MeCP2 remain possibly bound to regulatory region of these genes. Chromatin immunoprecipitation (ChIP) assay was carried out to test this possibility. Before ChIP assay, sonication conditions were optimized with primary oligodendrocytes to produce the chromatin with average length of 200–1000 base pairs (Figure 3.5). Chromatin immunoprecipitaion was performed with anti-MeCP2 antibody from two different suppliers, Milipore and Abcam. After, immunoprecipitation with antibodies, the promoter regions of MBP and PLP were analyzed by semi-quantitative PCR. Anti-MeCP2 antibodies precipitate MBP and PLP promoter DNA, whereas Negative control immunoglobulin G (IgG) yields no MBP and PLP Promoter DNA. MBP and PLP promoter DNA is more

enriched in immunoprecipitation performed with MeCP2 antibody from Abcam compared to Millipore (Figure 3.6 and 3.7).

3.3.3 MeCP2 also binds to BDNF promoter in oligodendrocytes

BDNF is one of first gene found to be the target of MeCP2 in neurons. In neurons, MeCP2 binds to the BDNF promoter IV and represses the transcription of BDNF. We found in our study (discussed in earlier chapter) that MeCP2 also suppresses the expression of BDNF in oligodendrocytes. Next, to investigate that like MBP and PLP, MeCP2 also binds to the promoter of BDNF gene in oligodendrocytes, ChIP assay was performed. ChIP assay was carried out using anti-MeCP2 antibody from Millipore and Abcam. MeCP2 binding to BDNF promoter IV was confirmed by semi-quantitative PCR of immunoprecipitated DNA. MeCP2 binding to BDNF PIV was observed only in DNA immunoprecipitaed with MeCP2 antibodies and no binding were observed in DNA immunoprecipitated with IgG (Figure 3.8).

3.4. DISCUSSION

In the present study we investigated that MeCP2 regulates the expression of myelin proteins MBP, PLP and neurotrophin BDNF through binding to their promoter regions. MeCP2 is binding to MBP, PLP and BNDF promoter regions were confirmed by Chromatin Immunoprecipitation using anti MeCP2 antibody. MeCP2 binds preferentially to methylated CpG sites in promoter region of target genes and form transcription repression complex by recruiting corepressor mSin3a and histone deacetylases (HDACs) (Nan et al., 1998). In silico analysis predicted one CpG island and many CpG sites in MBP promoter region. Methylation of CpG sites may increase the possibility of MeCP2 binding, which can recruit HDACs to mediate gene silencing (Georgel et al., 2003). In contrast to MBP promoter, there were only 3 CpG sites that were predicted in PLP promoter region and still MePC2 binds to it, suggesting there is possibility of MeCP2 to bind to these sites or unmethylated promoter DNA. In addition to classical methylation dependent gene repression, MeCP2 can also repress the gene expression by binding to unmethylated DNA (Yakabe et al., 2008; Hansen et al., 2010). MeCP2 also regulate gene expression through its binding to 5-hydroxymethyl-CpG (5hmC) and non-CG methylated (Shin et al., 2013; Chen et al., 2015). Recent studies have shown that MeCP2 can also activate its target gene by recruiting activator CREB1 (cAMP responsive element binding protein 1) at the promoter region of an activated targets and promoter region of activated gene were not heavily

methylated compared to those repressed genes (Chahrour et al., 2008; Ben-Shachar et al., 2009). Current study and other studies have shown recently that MeCP2 was found to regulate CNS myelin proteins MBP and PLP expression (Vora et al., 2010; Nuyen et al., 2013; Sharma et al., 2015), but MeCP2 binding to promoter region were not shown. In the current study, it was confirmed by ChIP assay that MBP and PLP are direct targets of MeCP2. Myelin-associated oligodendrocytic basic protein (MOBP) is one of the myelin proteins which may be involved in the compaction or stabilization of myelin (Montague et al., 2006). MOBP was found to be up regulated in *Mecp2*- null mice and directly regulated by MeCP2 binding through its promoter (Urdinguio et al., 2008). BDNF promoter IV predicted to contain 10 CpG sites, suggesting possibility of methylation of these sites which may further facilitate the MeCP2 binding. BDNF (brain derived neurotrophic factor) is one the most studied target gene of MeCP2 and have been shown to be involved in the neuronal survival, differentiation and synaptic plasticity. MeCP2 represses the expression of BDNF and is de-repressed after neuronal membrane depolarization. Neuronal membrane depolarization phosphorylates MeCP2 causing its dissociation from methtylated BDNF promoter (Chen et al., 2003; Martinowich et al., 2003; Zhou et al., 2006). In the earlier chapter, we showed that MeCP2 also regulate the BDNF gene expression in oligodendrocyte. In the current study, we observed that MeCP2 binds to BDNF PIV in oligodendrocytes is consistent with findings of past studies in neurons.

3.5. CONCLUSION

The present study investigated the recruitment of MeCP2 to the promoter of myelin proteins (MBP and PLP) and neurotrophin (BDNF) genes in rat cultured oligodendrocytes. The results of this study indicate that MeCP2 acts as transcriptional regulator of MBP, PLP and BNDF gene expression in oligodendrocytes by directly binding to their promoter regions.

Rat MBP Gene Promoter Sequence

(GenBank: AY208921.1)

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   1 ctgcaggatt gcacccacca tactacccat gagccgcgca tcttcacgcg atcacttctg
  61 caaagactct atttccagtc aaagtcacag tcacaggcag agagagggct gggatgtgaa
 121 aacatcaatt gggggagggg cgctcaattc ttagcataca ttttcttcat ctgatccccc
 181 ccccccagta gaatgtaagt ttcgtgagaa aagggaccag gccttgttca tcactatggc
 241 tctaacattt agagaaatgc gttcctccta ttcaacaagt aacgcacagt gggtagatag
 301 aggaccagca ctgcatagtc ccatgcatgg gtgcatagtc ccatgcatgg gttcatgtgt
 361 gcatggattg ttgggcagaa tagataaaga agtggataga tgaataatgt caggtaaata
 421 ctgctgggtg gacagatgga tgggtaagtg ggtagatgga tggacagaca gacagatggt
 481 aggtggatgg atgaatagaa tagacagaat aaggatgcat ggataaggtg aacagatgat
 541 gggtagactg gaaggatggt cagtagggag agggttaaat aaacggacaa aatattatag
 601 gaaagtgtga gctaaccaaa tttctacagg tcagtaatag agttgggaga ggaggttaaa
 661 ttacattcgt ttaaacctca cactaagttg agagggaatg gacttgcggc cgtccgtgag
 721 aacattgcat tttccatgtg ctcggtagtg aagtgcccag gtcccacgtg aagcaatctg
 781 gttaccatga cttgcaaggg agccagatcc aaatgccctg aataaacagt tccgggagcc
 841 agagcctcca gcggaatccg ggagagagga atgctcagtg ccctgcttcc ctggactgta
 901 agttgcagaa agatgtggga agttctgttc tccactgaga acactaaaag caccttttgt
 961 caaacaacgg ctccacatct ggggcttgtg caccagtggt cttttagaca gtgagcagcc
1021 cacaagatac ctaacctgcg gggctctctg gtacagtgag caactcagga aatgtttttg
1081 cttcattgct gccggctctc aggtcatcgc tctctggagt ggttcttttg ataaggatct
1141 gaagacagcc cctgagccac gtataccaag caagctcaat acgggttagc tccctttggt
1201 tgaggctatt caaagtgcta cgaggctcct tgggccctgc gcatatctgt gcgttttata
1261 ggagacagct agttcaagac cccaggaaga aagccggctttt gtccctctcg aggcctccca
1321 caggcccaca ttcatatctc attgttgttg caggggaggc agatgccgatc cagaacaatg
1381 ggacctcggc cgaggacaca gccgtgacag actccaagca cacagcagac ccaaagaata
1441 actggccggg tgcccaccca gctgacccag ggaaccgccc ccacttgatc cgcctctttt
1501 cccgagatgc cccggaagg gaggacaaca ccttcaaaga caggccctca gagtccgacc
1561 agcttcAgac catccaagaa gatcccacag cagcttccga aggcctggat gtgatggcat
1621 cacagaagag accctcacag cg
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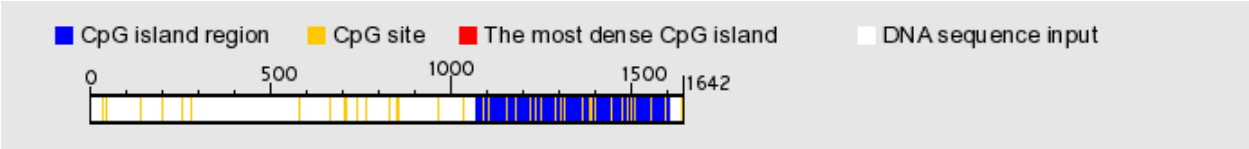
Transcription start site (TSS) - 1567 (highlighted in uppercase yellow)

CpG sites are highlighted in blue

There are 1 possible CpG island(s) found. Possible CpG island is shown in graph

start site:1072 end site:1602 length:531 GC content:55%



http://dbcat.cgm.ntu.edu.tw/

Figure 3.1: Rat MBP Gene promoter sequence and CpG island prediction.

Rat PLP Gene Promoter sequence
(-1000 bp to + 50 bp)

http://genome.ucsc.edu/cgibin/hgc?hgsid=358874461&c=chrX&o=107379830&t=107394880&g=refGene&i=NM_030990

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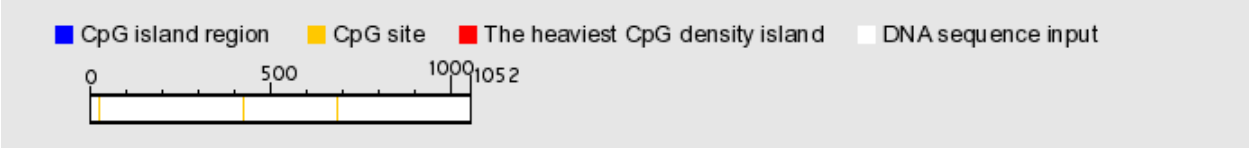
1    tataaagata atcccacagc tgcactttcg taacaggcag aatctgtgtc
51   ttgagggcag tcctaggaag gcatgggtct tagatcctca acagtcagtc
101  atggccttag tctggtatac cctgggaagc tactacctta ctgttcctca
151  agaggcaggt ataaggatac cttacctttc atcttcactt cctgggaaga
201  gttttgactg gctgatttcc agtttgtgat aatgttttgt ctcaaagtac
251  attttccttg gaaatcacct acatgttcca ctcttgccat ctttttttcct
301  tgtgaccttg gcacaggctt cagcctctcc tttctttctg ttctcccagc
351  tatactatca ttatacttct ggctctcttg agcctggtca cacacagtct
401  gttcagagac ttagctgctt gttttatcgt cattcttctg aaagcaaagg
451  ttttgaagaa attattttaa atgacaactt ttccttccct attgtgtttc
501  cagggcaaga gaaaaaatgc ttttttgctt gaagagggaa ggaaagattc
551  catggtcaag gccaactaac agtgagtgtt gggtggggca tgtttggtaa
601  tatagcaagt agggtttatg atccagatcc cctcctcacc agggctacca
651  tttcacatga cttcacatgc tcaggctgag gtacgcataca tttaaatgga
701  cccaaggaca atttgggagg atttaaggac ccctccactt aatttccacc
751  cacaatttac atttcatgat tcatttatat caaaatgaaa ttctagagaa
801  gctttggtgg ggggagatga gagagaaaga aaaaaaacaa ttggaagtga
851  aaagacagaa agagaagatg gagtccttaa agaagggagt atcccaaagg
901  aggtggggac aaggggagga gaaggggagg aggagaggag gaggaaagtg
951  agcctgtctc tttaaggggg ttggctgtca atcagaaagc ccttttcatt
1001Gcaggagaag aggacaaaga tactcagaga gaaaaagtaa aggacagaag
```

Transcription start site (TSS) - 1567 (highlighted in uppercase yellow)

CpG sites are highlighted in blue

There is No possible CpG island(s) found



http://dbcat.cgm.ntu.edu.tw/

Figure 3.2: Rat PLP Gene promoter sequence and CpG island prediction.

Rat BDNF Gene promoter IV (originally termed promoter III) sequence

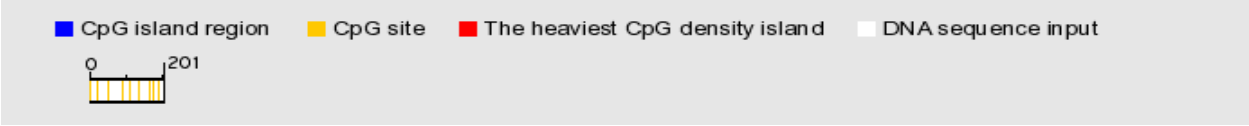
GenBank: X67107.1

```
1   gttcgctagg actggaagtg gaaacgtcta caaagcatgc aatgccctgg
51  aacggaattc ttctaataaa aagtagtatc attttaaatg cgcggaattc
101 tgattctggt aattcgtgca ctagagtgtc tatttcggagg cagaggaggt
151 atcatatgac agctcacgtc aaggcagcgt ggagccctct cgtggactcc
 201 C
```

Transcription start site (TSS) - 201 (highlighted in uppercase yellow)

CpG sites are highlighted in blue

There is No possible CpG island(s) is found.



http://dbcat.cgm.ntu.edu.tw/

Figure 3.3: Rat BDNF Gene promoter IV sequence and CpG island prediction

Table-3.1 Sequence of primers used for ChIP-PCR

Symbol	**Primer sequence (location from transcription start site)**	**Gene promoter**
MBPa F MBPa R MBPb F MBPb R MBPc F MBPc R	5'-AGGCAGATGCGATCCAGAAC-3' 5'- CTGCTGTGGGATCTTCTTGGA- 3' (+ 27 ~ -210) 5'- GGAGTGGTTCTT TTG ATAAGG- 3' 5'- CCCCTGCAACAACAATGAGA- 3' (- 211 ~ -450) 5'-CAG TGC CCT GCT TCC CTG GA- 3' 5'-AGAGAG CGATGACCTGAGAG -3' (- 451 ~ -690)	MBP promoter
PLPa F PLPa R PLPb F PLPb R PLPc F PLPc R	5'-AGGGTT TAT GATCCAGATCCC-3' 5,- CCACCAAAGCTTCTCTAGAAT-3' (- 211 ~ -410) 5'-TTAGCTGCTTGTTTTATCGTCA-3' 5'-ACTTGCTATATTACCAAACATGC-3' (- 411 ~ -610) 5'- AGATAATCCCACAGCTGCACT-3' 5'- CAGTCAAAACTCTTCCCAGG -3' (- 821 ~ -1026)	PLP promoter
BDNFPIV F BDNFPIV R	5'-GTTCGCTAGGACTGGAAGTGG-3' 5'-CCTCTGCCTCGAAATAGACAC-3' (- 56 ~ -201)	BDNF Promoter IV (He et al., 2010)

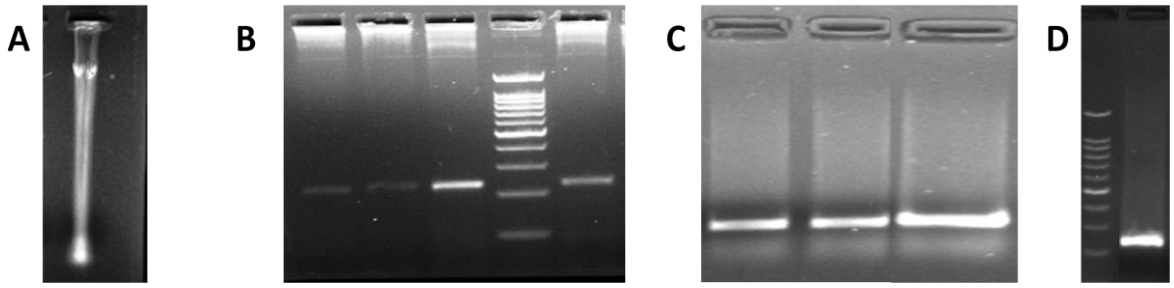


Figure 3.4: Genomic DNA isolation and Optimization of PCR for Promoter region primers. **(A)** Genomic DNA isolation from oligodendrocytes**.** PCR amplification of **(B)** MBP promoter regions MBPa, MBPb and MBPc **(**left to right) and **(C)** PLP promoter regions PLPa, PLPb and PLPc **(**left to right) and **(D)** BDNF promoter PIV region.

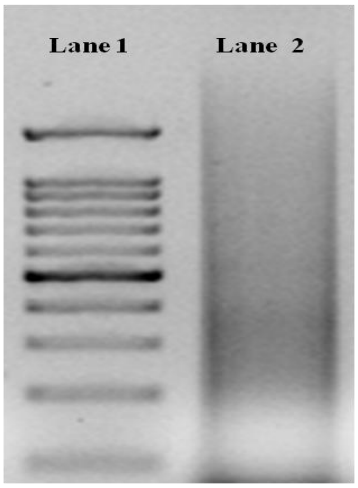


Figure 3.5: Optimization of sonication condition for oligodendrocytes chromatin shearing. Agarose gel analysis of chromatin following sonication for 10 min at 25% amplitude with pulse of 20 sec ON and 30 sec OFF (Lane 1: Marker and Lane 2: Sheared chromatin).

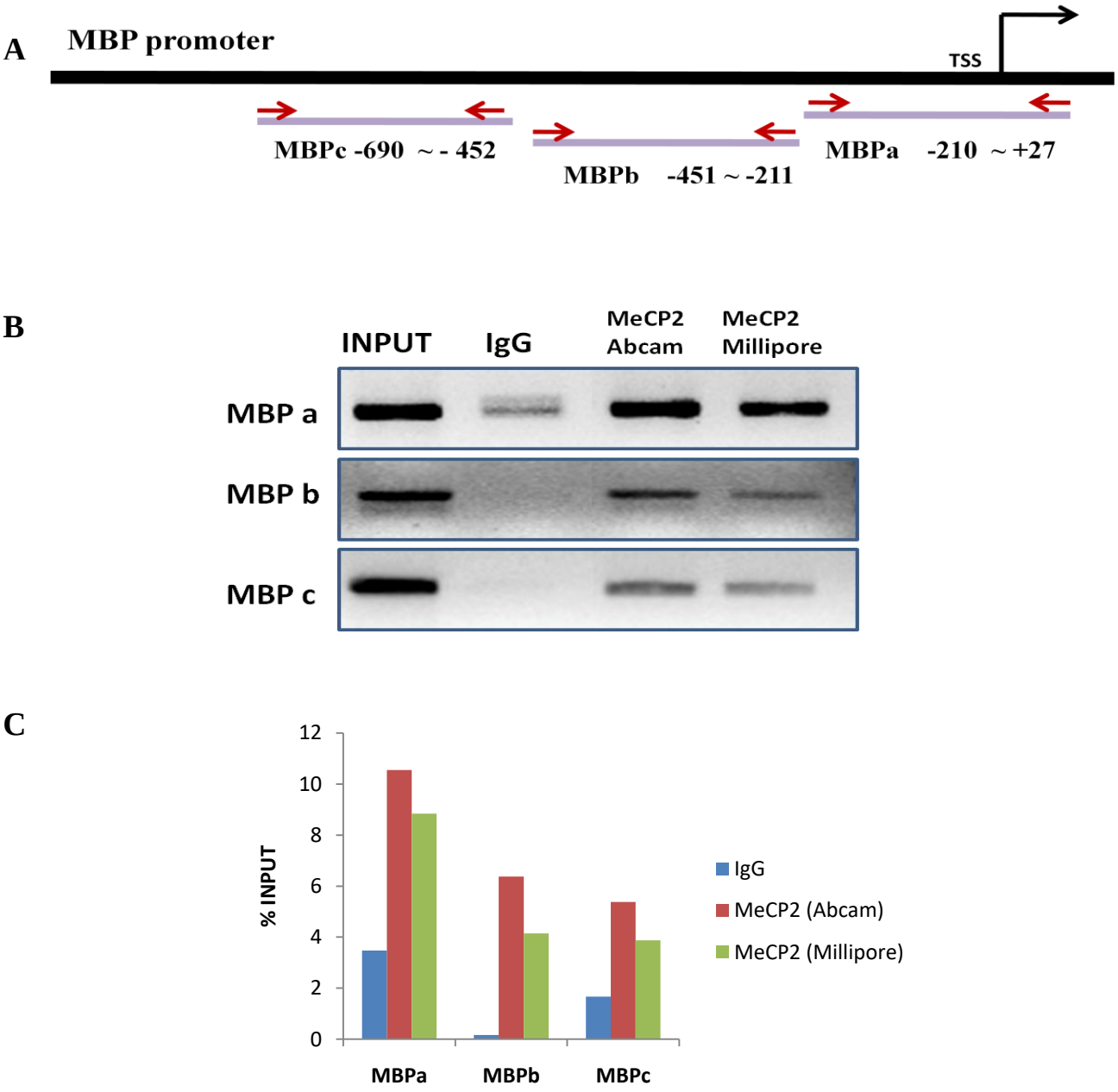


Figure 3.6: MeCP2 binds to promoter regions of MBP gene. (A) Schematic representation of 5' promoter region of MBP, horizontal lines indicate the relative locations for primers used for ChIP assay, MBP transcription start site (TSS) **(B)** Anti-MeCP2 antibody (MeCP2 antibody from two different suppliers Millipore and Abcam were used) directly pulls down the MBP promoter sequence using MBPa, MBPb and MBPc primers. Anti-immunoglobulin G (IgG) antibody was used as negative control, the input sample as positive control. **(c)** Semi-quantitative PCR analysis of ChIP by MeCP2 of the MBP promoter sequence.

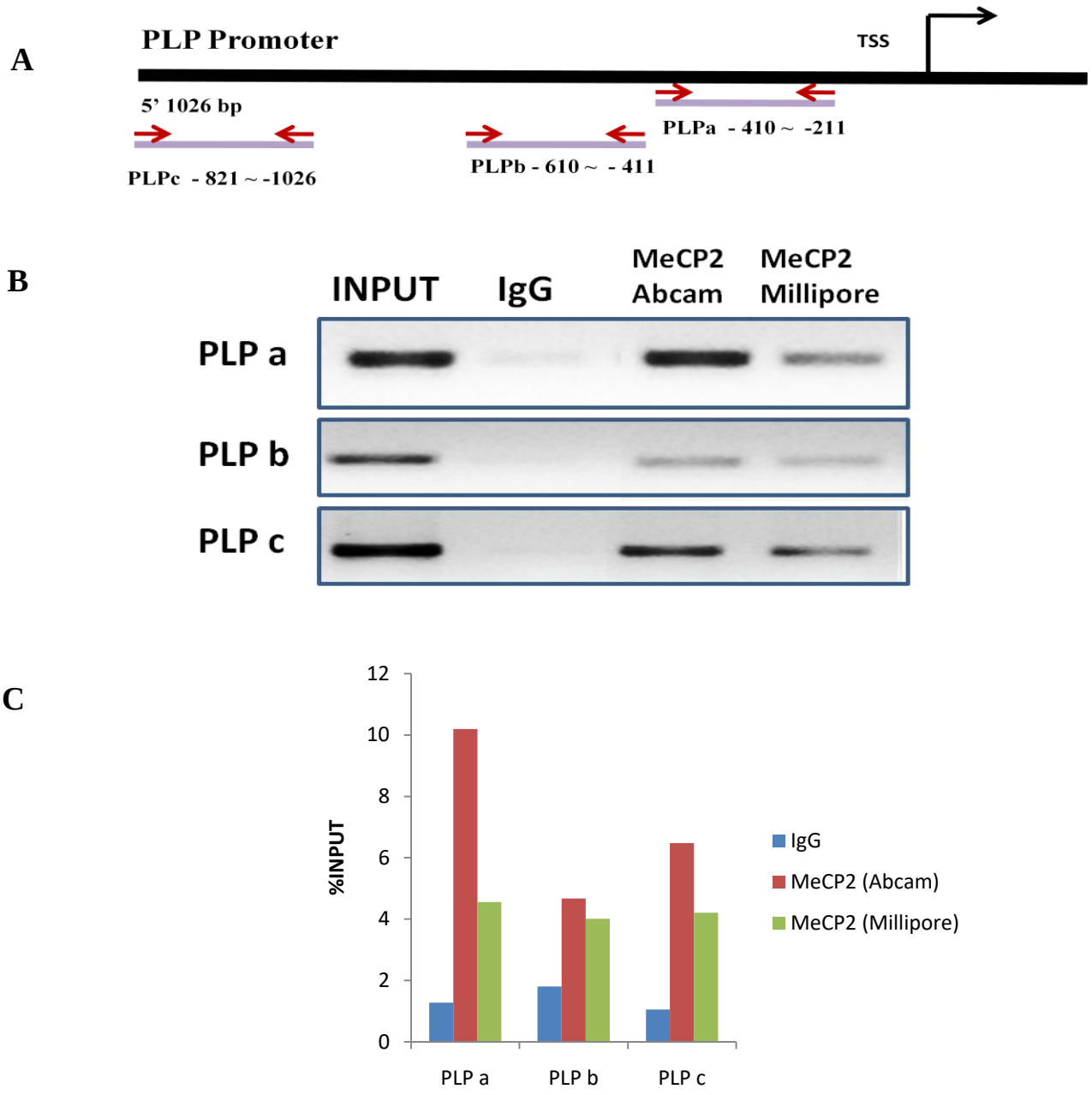


Figure 3.7: MeCP2 binds to promoter regions of PLP gene. (A) Schematic representation of 5' promoter region of PLP, horizontal lines indicate the relative locations for primers used for ChIP assay, PLP transcription start site (TSS) **(B)** Anti-MeCP2 antibody (MeCP2 antibody from two different suppliers Millipore and Abcam were used) directly pulls down the PLP promoter sequence using PLPa, PLPb and PLPc primers. Anti-immunoglobulin G (IgG) antibody was used as negative control, the input sample as positive control. **(c)** Semi-quantitative PCR analysis of ChIP by MeCP2 of the PLP promoter sequence.

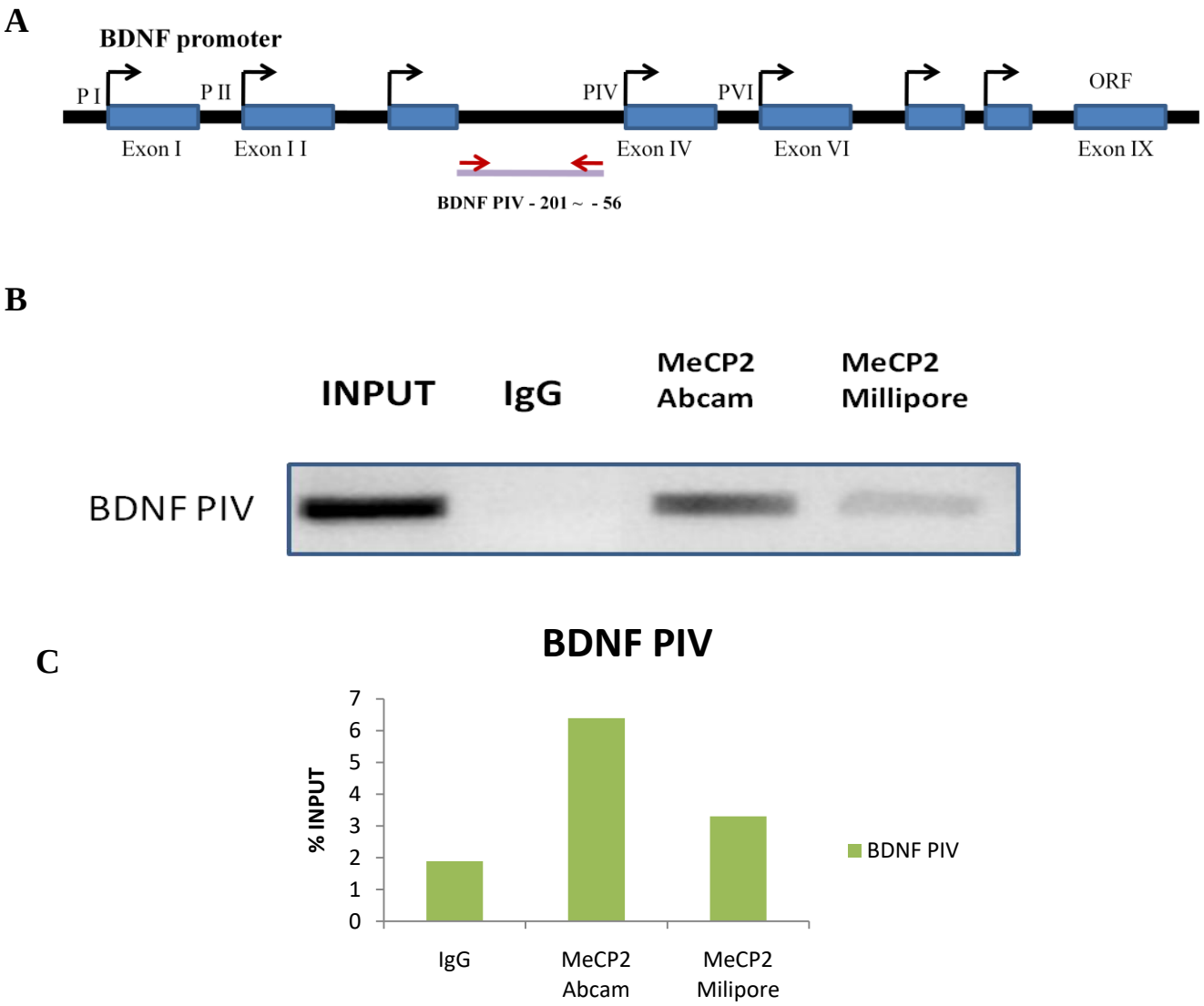


Figure 3.8: MeCP2 binds to BDNF Promoter IV. (A) Schematic representation of Rat BDNF gene structure consisting of nine exons, in which each of eight 5'-exons is spliced into the shared 3'-exon (IX) that contains the open reading frame (ORF), horizontal line indicate the relative location of BDNF PIV primers used for ChIP assay **(B)** Anti-MeCP2 antibody (MeCP2 antibody from two different suppliers Millipore and Abcam were used) directly pulls down the BDNF P IV promoter sequence using primers. Anti-immunoglobulin G (IgG) antibody was used as negative control, the input sample as positive control. **(c)** Semi-quantitative PCR analysis of ChIP by MeCP2 of the BDNF PIV sequence.