Differential Promoter Methylation and Histone Modification Contribute to the Brain Specific Expression of the Mouse Mbu-1 Gene

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Mbu-1 (Csrnp-3) is a mouse gene that was identified in our previous study as showing highly restricted expression to the central nervous system. In this study, to elucidate the regulatory mechanism for tissue specificity of the gene, epigenetic approaches that identify the profiles of CpG methylation, as well as histone modifications at the promoter region were conducted. Methylation-specific PCR revealed that the CpG sites in brain tissues from embryo to adult stages showed virtually no methylation (0.052-0.67%). Lung (9.0%) and pancreas (3.0%) also showed lower levels. Other tissues such as liver, kidney, and heart showed much higher methylation levels ranging from approximately 39-93%. Treatment of 5-aza-2'-deoxycytidine (5-Aza-dC) significantly decreased promoter methylation, reactivating Mbu-1 expression in NG108-15 and Neuro-2a neuronal cells. Chromatin immunoprecipitation assay revealed that 5-Aza-dC decreased levels of acetylated H3K9 and methylated H3K4, and increased methylated H3K9. This result indicates that CpG methylation converses with histone modifications in an opposing sense of regulating Mbu-1 expression.

INTRODUCTION

Mbu-1 (also known as Csrnp-3; GenBank accession no., NM-178634) is a mouse gene that was first identified as a brain-specific unigene by using the differential digital display program (DDD) (Yang et al., 2007). The expression of Mbu-1 is strictly confined to the brain and spinal cord during development from embryo to adult. Mbu-1 (Csrnp-3) is a member of cysteine-serine-rich nuclear protein family that also includes Csrnp-1 and Csrnp-2. Deletion of the individual genes had no obvious consequences on normal mouse development. However, combined deficiencies caused partial neonatal lethality suggesting that the genes have redundant functions (Jensen Pena et al., 2012). The 480 bp of the 5′-flanking sequence was enough to show the highest expression in neuronal cell cultures. To identify transcriptional factors acting on the promoter and leading to the neuron-specific expression, an extensive search within data-bases of transcriptional factors was carried out on the 480 bp as well as upstream sequences extending up to 2 kb. However the work has failed to identify any neuron-specific activators or silencers, such as REST, that are known to suppress expression of neuron-specific genes in non-neuronal cells (Chong et al., 1995; Nakatani et al., 2005).

Methylation of the CpG sites at the promoter has emerged as an alternative explanation for the neuron-specific expression of brain genes (Davies et al., 2012; Furuya et al., 2012; Jensen Pena et al., 2012). In a comprehensive DNA analysis of neuronal and non-neuronal nuclei obtained from the human prefrontal cortex, neuronal nuclei manifested qualitatively and quantitatively distinctive DNA methylation patterns, including relative global hypomethylation, differential enrichment of transcription-factor binding sites, and higher methylation of genes expressed in astrocytes (Iwamoto et al., 2011). In a further study, DNA methylation was found to be dynamically regulated in the human cerebral cortex throughout the entire lifespan, involving differentiated neurons, and affecting a substantial portion of genes, increasing with age, predominantly (Siegmund et al., 2007).

Epigenetic modification of genomic DNA and histones has been tightly linked to chromatic organization and transcriptional regulation (Fuks, 2005; Lee and Lee, 2012; Majid et al., 2009). Histone acetylation in gene promoter/enhancer regions is generally correlated with transcriptional activation (Hattori et al., 2004; Tomikawa et al., 2006). Methylation of DNA is essential for mammalian development and is associated with gene silencing in conjunction with histone core modifications, probably through chromatin remodeling (Jones et al., 1998; Li et al., 1992). For example, histone acetylation in the Kiss1 gene, that is upregulated in the anteroventral periventricular nucleus of brain, enhanced chromatic loop formation of Kiss1 promoter and Kiss1 gene enhancer, resulting in an increase in Kiss1 gene-specific expression (Tomikawa et al., 2012).

The present study aims to determine the epigenetic regulatory mechanism underlying the neuron-specific expression of Mbu-1. We first identified the methylation level at the Mbu-1 promoter in various mouse tissues. Methylation-specific PCR (MSP) and sequence analysis of the bisulfite-treated DNA were...
carried out for DNAs from various mouse tissues. Next, a chromatin immunoprecipitation (ChIP) assay with histone antibodies was performed to determine whether the methylation is linked to histone modifications such as acetylation and methylation for the CNS-specific expression of Mbu-1.

MATERIALS AND METHODS

Cell culture and 5-Aza-2'-deoxycytidine treatment
A mouse neuroblastoma, Neuro-2a and a mouse neuroblastoma x rat glioma hybrid cell line, NG108-15, were purchased from the American Type Culture Collection (ATCC; Manassas, USA), and grown in Dulbecco’s modified Eagle’s medium (DMEM) 10% fetal bovine serum (FBS). The 1× HAT was supplemented (Invitrogen, USA) to the NG108-15 cell. Demethylation of the cytosine residues was achieved by exposing the cells to culture media containing a methyltransferase inhibitor, 5-Aza-dC (Sigma, USA), at 5 μM for 48 h and 72 h. Culture medium with or without treatment was changed every 24 h.

Bisulfite sequencing
Chromosomal DNA extracted from the cultured cell was subjected to bisulfite treatment using an Epitext Bisulfite kit (Qiagen, USA). The bisulfite-treated DNA was subjected to polymerase chain reaction (PCR) to amplify the 174-bp promoter region of Mbu-1 (nucleotides -402 to -228 of GenBank accession no. EF210820; transcriptional start site, +1) containing four CpG sites. The PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 5 min. The resulting products were purified using a Qiagen II gel extraction kit (Qiagen) and were then sub-cloned into the pGEM-T vector. The DNA sequences were confirmed by analyzing each plasmid clone in both directions.

Methylation-specific PCR (MSP)
Chromosomal DNA was isolated from mouse tissues and cell culture in a 75 cm² culture flask using a genomic DNA purification kit (Promega, USA) according to the manufacturer's protocol. The extracted DNA was then eluted with 250 μl of distilled water. Sodium bisulfite modification of genomic DNA was conducted using an Epitext Bisulfite kit (Qiagen) according to the manufacturer's protocol using 0.1 mg of purified DNA. Design of PCR primers (sequences are in Supplementary Table S1) and reaction conditions were described previously (Kim et al., 2011). To assign a quantitative measure to the level of methylation, a methylation index was calculated for each sample using the following formula: methylation index = [1 / (1 + 2^{ΔCt})] × 100%, as previously described (Kron et al., 2009), where ΔCt is the cycle threshold (CT) obtained from quantitative PCR analyses using the unmethylated primer set, and CTm is the average CT obtained using the methylated primer pair.

Quantitative real-time RT-PCR analysis
Total RNA from cell culture was prepared using Trizol reagent according to the manufacturer's protocols (GibcoBRL, USA). Reverse transcription was conducted using 10 μg of total RNA with a reverse transcription kit (Promega). One microliter of cDNA was used for the PCR, and duplicate reactions were performed for each sample using a Kapa SYBR Fast qPCR Kit (Kapa Biosystems, USA) with Mbu-1-specific primers (Supplementary Table S1) on an ABI 7300 instrument (Applied Biosystems). RNA quantity was normalized to GAPDH content, and gene expression was quantified according to the 2⁻ΔCT method.

Fig. 1. Hypomethylation of Mbu-1 in the mouse brain. Methylation level of the Mbu-1 promoter was examined in various mouse tissues by real-time MSP analysis. A. Nucleotide sequence of the Mbu-1 promoter. Nucleotide sequence spanning from -400 to -161 (relative to the transcription start site) is shown. The four CpG sites are numbered on its sequence. The potential cis-regulatory elements in the promoter region are underlined and labeled. B. Relative methylation level of Mbu-1 in tissues. E12 and P5 are brain at embryo day12 and postnatal day5, respectively. Each sample was examined in duplicate and the average relative methylation level is presented. The inset is a result of RT-PCR for mouse tissues referred in the study by Yang et al. (2007), indicating brain-specific expression of Mbu-1.

Chromatin immunoprecipitation-PCR (ChIP-PCR)
ChIP assays were performed using the EZ ChIP™ Chromatin Immunoprecipitation kit (Millipore, USA) as described in the supplier’s protocol. Briefly, after cell lysis, the cross-linked chromatin was sonicated and was then incubated with antibodies against modified histones at 4°C overnight. Antibodies against methylated H3 at K4 (ab8580), dimethylated H3 at K9 (ab8898), and acetylated H3 at K9 (ab10812) were purchased from Abcam (UK). The immunocomplex was precipitated with protein A-agarose (Abcam) and the beads were washed and sequentially treated with 10 μl of RNase A (37°C for 30 min) and 75 μl of proteinase K (45°C for 4 h), after which they were incubated at 65°C overnight to reverse the cross-link of the chromatin. The DNA was recovered by phenol-chloroform extractions and