A segment of the \textit{Mecp2} promoter is sufficient to drive expression in neurons

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Rett syndrome (RTT) is caused by mutations in the gene encoding methyl CpG-binding protein 2 (MeCP2). Although MeCP2 shows widespread expression in both neuronal and non-neuronal tissues, the symptoms of RTT are largely neurological. Herein, we have identified the regulatory region of the mouse \textit{Mecp2} gene that is sufficient for its restricted expression in neurons. A segment of the \textit{Mecp2} gene (−677/+56) exhibited strong promoter activity in neuronal cell lines and cortical neurons, but was inactive in non-neuronal cells and glia. The region necessary for neuronal-specific promoter activity was located within a 19 bp region (−63/+45). Several nuclear factors were found to bind to this region and some of these factors were enriched in nuclear extracts prepared from the brain. To examine the activity of the \textit{Mecp2} promoter \textit{in vivo}, we generated transgenic mice expressing the LacZ reporter driven by the −677/+56 region of the \textit{Mecp2} gene. The transgene was expressed in the mesencephalon as early as embryonic day 10 and in the hindbrain and spinal cord by E12. Interestingly, a marked induction of transgene expression was observed postnatally throughout the brain, similar to that of endogenous MeCP2. However, expression of the transgene was absent in non-neuronal tissues that are known to express \textit{Mecp2}. Taken together, these data indicate that the −677/+56 region of the \textit{Mecp2} promoter partially recapitulates the native expression pattern of the \textit{Mecp2} gene, which possesses restricted expression in neurons of the central nervous system.

INTRODUCTION

Rett syndrome (RTT) is a neurological disorder involving cognitive and motor dysfunction in young females. Symptoms of RTT appear after an initial period of normal development between 6 and 18 months of age and include loss of purposeful hand movement and speech, microcephaly, seizures, autism and hyperventilation (1). The majority of children affected by RTT have mutations in the gene encoding methyl CpG-binding protein 2 (MECP2) on the X-chromosome (2). MeCP2 is a transcriptional repressor and has been proposed to globally silence the transcription of genes by binding to methylated cytosines within the promoters of these genes and subsequently recruiting the co-repressor protein complexes containing Sin3A and histone deacetylases (3,4). Additionally, MeCP2 binds to DNA methyltransferase 1 as well as regulates histone methyltransferase activity, which serves to maintain DNA methylation and facilitates methylation of Lys9 in histone H3 (5,6). Thus, by binding to methylated DNA, MeCP2 reinforces its repressive function through multiple epigenetic modifications, such as the maintenance of DNA methylation and the deacetylation and methylation of histones.

Despite the widespread expression of MeCP2 in both neuronal and non-neuronal tissues, symptoms of RTT are largely neurological. MeCP2 is highly expressed in the brain, lung and spleen and moderately in the heart and kidney (7). In the central nervous system (CNS), MeCP2 is expressed exclusively in neurons (7–9). Moreover, targeted deletion of the \textit{Mecp2} gene in the CNS is sufficient to produce the symptoms of RTT, suggesting that neuronal expression of \textit{Mecp2} is crucial for normal development and function of the brain (10,11). Other studies have also revealed the necessity of precisely regulating the level of MeCP2 protein expression \textit{in vivo}. For instance, overexpression of MeCP2 in postmitotic neurons by replacing the \textit{Tau} locus with \textit{Mecp2} cDNA caused profound motor dysfunction in mice (12). Similarly, transgenic mice engineered to mildly overexpress MeCP2 (2-fold over the levels normally observed in wild-type mice) displayed progressive neurological disorders and premature death (13). These abnormalities appeared to result from twice normal levels of MeCP2, but not from ectopic
expression. The findings described earlier prompt the conclusion that the \textit{Mecp2} gene expression is tightly regulated in the CNS and that this regulation is necessary to sustain normal neurological function.

On the basis of these findings, it is important to identify DNA elements in the \textit{Mecp2} promoter sufficient to drive expression in neurons as well as to regulate the level of its expression \textit{in vivo} throughout development. At present, little is known about mechanisms that control \textit{MECP2} expression during neuronal development and maturation. In the present study, we identified a segment of mouse \textit{Mecp2} gene promoter ($-677/+56$) which shows restricted expression in neurons during embryonic and postnatal development.

**RESULTS**

**Preferential activity of $-677/+56$ \textit{Mecp2} gene promoter in neuronal cells**

In order to map the transcription initiation sites within the mouse \textit{Mecp2} gene, we carried out RNA ligase-mediated rapid amplification of 5' cDNA ends (RLM-RACE). RLM-RACE analysis has been shown to be a sensitive and efficient method to eliminate truncated products and to amplify only full-length cDNAs (14,15). RACE products were obtained from E14 mouse cortex and 23 clones were sequenced. For these experiments, we utilized a primer located on exon 4 (Fig. 1A). As shown in Figure 1B, 91% of the clones obtained by RLM-RACE encoded the isoform of MeCP2 which contains exons 1, 3 and 4, but lacks exon 2 (called MeCP2e1). The rest of the clones encoded MeCP2e2, the isoform that contains exons 1, 2, 3 and 4. These results indicate that MeCP2e1 is the major isoform expressed in the brain, consistent with the findings by Kriaucionis and Bird and Mnatzakanian et al. (16,17). We also found that the 5' ends of RACE products varied in size, suggesting that there are multiple start sites for transcription in the \textit{Mecp2} gene. The locations of the 5' end nucleotides from these RACE products are summarized in Figure 1B and C. The most frequently obtained RACE products contained a segment of 47 nucleotides upstream of exon 1. Therefore, we designated this nucleotide '$+1'$—the major transcription start site.

To examine the activity of the mouse \textit{Mecp2} promoter, we prepared a luciferase reporter construct containing a 733 bp fragment ($-677/+56$) of the mouse \textit{Mecp2} gene and examined its activity in two neuronal (PC12, phenochromocytoma, and SK-N-MC, neuroblastoma) and two non-neuronal (NIH3T3, fibroblast, and HepG2, hepatoma) cell lines. In all four of these cell lines, expression of \textit{MECP2}/\textit{Mecp2} mRNAs was detected at nearly equal levels by reverse transcription (RT)–PCR analyses (Fig. 2A). In transfection experiments of PC12 and SK-N-MC cells, we observed that MeCP2–Luc reporter construct produced luciferase activities similar to or greater than that produced by the RSV promoter (Fig. 2B). We also examined the activity of MeCP2–Luc in PC12 cells that were treated with NGF, which extended...
numerous neurite processes and resembled cells of a neuronal phenotype. After transfection with MeCP2–Luc, PC12 cells were partially differentiated by treatment of NGF (50 ng/ml) for 2 days. This NGF treatment resulted in a 2.4-fold increase in the activity of MeCP2–Luc (data not shown), which is similar to the increase in endogenous MeCP2 expression shown previously in PC12 cells (18). In contrast, the promoter showed much weaker activity when compared with that of the RSV promoter in the non-neuronal cell lines, NIH3T3 and HepG2. This weak activity of the MeCP2 promoter is not due to the absence of (or less) expression of MECP2/MeCP2 transcripts in non-neuronal cells. These experiments demonstrate that the −677/+56 promoter has considerably more activity in the neuronal cell lines.

Next, we examined the activity of MeCP2–Luc in primary mouse cortical cultures, which contain both neurons and glia. As shown in Figure 3A, MeCP2 protein is expressed only in β-tubulin positive neurons in cortical cultures. Cortical cultures that were transfected with the MeCP2–Luc reporter together with the CMV–β-Gal expression construct were subjected to immunohistochemistry to examine expression of luciferase. About 50% of transfected cells that expressed β-galactosidase (β-Gal) were also stained with anti-β-tubulin antibody, indicating that these cells were indeed neurons (data not shown). In accord with levels of the Mecp2 promoter activities in cell lines (Fig. 2B), we found that MeCP2–Luc construct was preferentially expressed in cortical neurons, but not in glia (Fig. 3B). By double staining of the cultures transfected with MeCP2–Luc, we confirmed that the luciferase-expressing cells were indeed neurons, as they were found to also express the neuronal marker, β-tubulin (Fig. 3C). In transfected cortical cultures, 92% of luciferase positive cells also expressed β-tubulin (data not shown). Taken together, these results indicate that the −677/+56 region of the Mecp2 promoter is active in neurons, but not in glia.

DNA elements that regulate activity of the −677/+56 Mecp2 promoter

To identify regions within the Mecp2 promoter that control its expression in neuronal cells, a number of serially truncated and deletion constructs were generated (Fig. 4A) and tested for their activities in PC12 and SK-N-MC cell lines as well as in primary cortical cultures. The construct designated Apa–Luc showed a 60–90% increase in promoter activity when compared with that of MeCP2–Luc, indicating that a region that represses Mecp2 promoter activity resides between −677 and −322 (Fig. 4B). A positive regulatory region was also identified in the region between −322 and −27, as both Pvu– and Aat–Luc constructs exhibited progressive reductions in activity when compared with that of MeCP2–Luc (Fig. 4B). Notably, deletion of the −77/+2 segment (ΔBssH–Luc) resulted in a 70–80% reduction in Mecp2 promoter activity when compared with that of MeCP2–Luc, suggesting that this region contains a major positive regulatory element. Within the −77 to +2 region, putative binding motifs for SP1 and bHLH/E-box transcription factors were identified. Mutation of either or both of these binding motifs within the full-length MeCP2–Luc construct showed minimal effects on Mecp2 promoter activity of MeCP2–Luc construct in neuronal cells (Fig. 4C, data not shown). However, deletion of the region between the E-box motif and the SP1 site (−64/−46) eliminated 70% of Mecp2 promoter activity in both PC12 and SK-N-MC cells (Fig. 4C). These results therefore indicate that the −64 to −46 segment is responsible for most of the activity in the proximal Mecp2 promoter and that reduction in Mecp2 promoter activity produced by ΔBssH–Luc construct is not due to the absence of the major transcription start site. As this element is clearly important for Mecp2 gene regulation, we have named it the MR (MeCP2 regulatory) element.

To examine the role of the MR element in controlling Mecp2 gene expression in neurons, primary cortical cultures were transfected with a construct that lacks the MR element (ΔMR–Luc). As the transfection efficiency of reporter constructs in primary cortical cultures was very low, we measured directly levels of luciferase expression in individual neurons by fluorescent immunohistochemistry using an antibody against luciferase. The level of fluorescence from each neuron was normalized by the expression level of β-Gal in
the same neuron. As shown in Figure 4D, luciferase expression produced by ΔMR–Luc was significantly less than that of the native MeCP2–Luc construct. Measurements of fluorescent intensity detected in transfected neurons revealed that deletion of the MR element led to a 70% decrease in Mecp2 promoter activity when compared with that of MeCP2–Luc, which is comparable to the activity observed in PC12 and SK-N-MC cells (Fig. 4E).

The −677/+56 Mecp2 promoter shows restricted expression in CNS neurons

To examine whether the −677/+56 segment is sufficient to drive Mecp2 expression during development and maturation of neurons in the brain, we generated transgenic mice expressing a LacZ reporter driven by the −677/+56 promoter (MeCP2–LacZ). In this reporter construct, sequences encoding a nuclear localization signal from SV40 large T antigen were cloned inframe to the 5′ end of the LacZ expression cassette to localize its expression in the nucleus (19). After microinjection of the transgene, we obtained five founders carrying the transgene. Two of the founders showed no expression of the transgene. Three remaining lines were positive for LacZ expression and showed nearly identical patterns of the MeCP2–LacZ transgene expression throughout development. One of these lines (designated Tg−677/+56) was selected for comprehensive analyses of MeCP2–LacZ transgene expression patterns at different stages of development.

As shown in Figure 5A–D, expression of the transgene appeared as early as embryonic day (E) 10 in cells at the base of the mesencephalon and became prominent in the hindbrain and spinal cord by E13 (Fig. 5E–N). However, between E10 and E13, we did not observe any obvious expression of the transgene in the forebrain, where endogenous Mecp2 mRNA transcripts are abundantly expressed using in situ hybridization analyses of the same embryonic stages.
Notably, MeCP2 mRNA was also detected in limbs and craniofacial tissues in E13 embryos, in which expression of the transgene was absent in Tg^{2677/+56} embryos. These expression patterns of MeCP2 mRNA in the embryos are very similar to those reported previously (18,20). Overall, the results indicate that the 2677/+56 region of the MeCP2 promoter partially recapitulates the endogenous expression patterns of MeCP2 in the CNS and possesses promoter activity that restricts expression of the reporter to neuronal tissues during embryogenesis.

As the expression of MeCP2 in the CNS shows robustly enhanced expression after birth (7,21), we examined the expression of the MeCP2–LacZ transgene during postnatal development. We observed very faint LacZ expression in ventral parts of brainstem in newborn pups (data not shown). However, later at P7, expression of the transgene became prominent in the superior colliculus and inferior colliculus (Fig. 6A and D), as well as in the olfactory bulb, hypothalamus, midbrain, pons and medulla (Fig. 6A). In contrast, at the same age in the cortex (Fig. 6C) and cerebellum (Fig. 6E), expression of the transgene remained relatively weak. After P7, however, postnatal expression of the transgene became more intense. For instance, at P24, most of cortical layers displayed moderate expression of the transgene (Fig. 6B). Intense expression of the transgene was observed in the inferior colliculus, cerebellum, hippocampus, caudate putamen, thalamus, hypothalamus and pons (Fig. 6G–M). In the midbrain and medulla, expression of the transgene was moderate.

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The most significant induction of transgene expression was observed in Purkinje cells of cerebellum between P7 and P24 (Fig. 6E and H). In addition, transgene expression was initiated in the granule cell layer at P24 (Fig. 6K). The dorsal column of the spinal cord also exhibited transgene expression (Fig. 6N). This expression faded near the roof of the central canal (Fig. 6O).

To examine cell-type specificity of transgene expression in the CNS, X-Gal stained brain sections (Fig. 6P) were subjected to fluorescent Nissl staining (Fig. 6Q). Most of X-Gal stained cells were Nissl positive; however, not all Nissl positive cells were X-Gal positive (Fig. 6R). These results indicate that expression of the transgene is restricted to a subpopulation of neurons in the brain. Overall, these developmental expression patterns of the transgene in the postnatal brain partially recapitulate temporal and spatial expression patterns of endogenous MeCP2 in the CNS, suggesting that the promoter region contains some of the information necessary to respond to developmental stimuli that control MeCP2 gene expression postnatally.

To further evaluate the neuronal specificity of the −677/+56 promoter in Tg−677/+56 mice, we compared relative levels of mRNA that were produced by the MeCP2–LacZ transgene in several regions of the brain (olfactory bulb, cortex, striatum, cerebellum and brainstem) as well as in non-neuronal tissues (liver, lung, heart and spleen) using QRT-PCR. In general, at P7, Mecp2 transcripts were abundant in all regions of the brain examined in control and Tg−677/+56 mice (Fig. 7A). Although levels of endogenous Mecp2 transcript in the olfactory bulb, cortex and striatum of Tg−677/+56 mice were lower by 36–48% than those of control mice, Tg−677/+56 mice did not demonstrate any abnormal phenotype throughout and after postnatal development. This reduction in Mecp2 expression could arise from a squelching effect, i.e. a limiting factor that normally binds to the endogenous MeCP2 promoter might be displaced by binding to the −677/+56 Mecp2 promoter within the integrated MeCP2–LacZ transgene. In non-neuronal tissues, levels of Mecp2 mRNA varied. For example, Mecp2 mRNA transcripts were highly expressed in lung and heart, whereas low in liver and spleen. QRT-PCR analyses in Tg−677/+56 revealed that LacZ mRNA driven by Mecp2 promoter was found only in the brain, but not in non-neuronal tissues (Fig. 7B). The level of LacZ mRNA in the cerebellum appeared to be less than those in other regions of the brain. This would reflect the observation that expression of the transgene in the cerebellum at P7 is lower than those in other regions of the brain and that its expression was localized only in Purkinje cells, which constitute small number of cells in the cerebellum (Fig. 6E). In the lung and heart, where endogenous Mecp2 is expressed, very small levels of LacZ mRNA were detected. These levels were between 49- and 80-fold less than those found in the cortex. Consistently, X-gal staining of all the non-neuronal tissues in Tg−677/+56 mice were absent at P7 (data not shown). Overall, these expression analyses indicate that the −677/+56 MeCP2 promoter contains neuronal-specific elements.

**Identification of nuclear factors that bind to the MR element**

To identify nuclear factors that bind to and may regulate the −677/+56 MeCP2 promoter, we conducted electrophoretic mobility-shift assay (EMSA) using nuclear extracts prepared from several tissues of P7 mice. As the MR element mediates the majority of −677/+56 MeCP2 promoter activity, we utilized a probe called MRSP that contains both the MR and...
As shown in Figure 8B, the MRSP probe was bound by several proteins in the nuclear extracts from the cortex, striatum, cerebellum and brainstem. The binding patterns of the extracts to the probe were nearly identical among the brain regions. Similarly, binding experiments using lung and heart nuclear extracts also revealed several DNA–protein complexes. Interestingly, extracts from liver and spleen, which expressed low levels of MeCP2 transcript (Fig. 7A), showed binding patterns that were distinct from those observed using the brain, lung and heart extracts.

Figure 6. The $-677^{+56}$ MeCP2 promoter is active in a subpopulation of neurons in the brain and shows enhanced activity during postnatal development. X-gal staining of brain sections from Tg $-677^{+56}$ mice are shown. (A and C–E) and (B and F–M) are sagittal sections of P7 and P24, respectively, of Tg $-677^{+56}$. The regions of the brain shown are as follows: (C and F) cortex; (D and G) superior colliculus (SC) and/or inferior colliculus (IC); (E, H and K) (higher magnification of H) cerebellum (Cbl); (I) hippocampus; (J) caudate putamen (CPu); (L) thalamus (tha) and hypothalamus (hyp); (M) dorsal pons (pn) and medulla (mdl). Coronal sections of rostral spinal cord from P24 Tg $-677^{+56}$ are shown in (N and O) (higher magnification of N). Sections from P7 SC were stained with X-gal (P) and neuro trace fluorescent Nissl (Q). (R) is an overlay of (P and Q). Cells that are both X-gal and Nissl positive were indicated by red circles in (Q) and (R). Scale bars in (A–B, C–K, L–O and P) indicate 1 mm, 0.1 mm, 0.5 mm and 50 μm respectively. Abbreviations are as follows: PGL, Purkinje cell layer; GCL, granule cell layer; Py, pyramidal cell layer; DG, dentate gyrus; mid, midbrain; DC, dorsal column; cc, central canal.
In EMSA involving the MRSP probe and the liver nuclear extract, DNA–protein complex formation was almost absent, whereas the spleen extract produced DNA–protein complexes that were different from those found in the brain extracts. As the MRSP probe contains a putative binding site for proteins of the SP1 transcription factor family, we carried out EMSA using cortical nuclear extracts in the presence of antibodies against SP1 and SP4. However, addition of these antibodies to the binding reactions showed neither supershift nor disruption of any of DNA–protein complexes (data not shown). These results further support the transfection results shown in Figure 4C that deletion of SP1 site has little effect on the nucleotides in the 3’ and 5’ ends of the MR and SP1 sites, respectively, play essential roles in binding of nuclear factors to the MRSP probe. In summary, EMSA and southwestern-blot analyses demonstrated differential binding of nuclear factors to the MRSP probe depending on the amounts of MeCP2 transcripts in the tissue, further supporting the conclusion that the region spanning the MR and SP sites is crucial for regulating the activity of the MeCP2 promoter.

**DISCUSSION**

In the present study, we have identified a minimal promoter region in the mouse MeCP2 gene sufficient to recapitulate the dynamic expression of MeCP2 gene in neurons of the CNS. In transfection experiments, we defined an MeCP2 promoter fragment (−677/+56) that shows preferential activity in neuronal cell lines and cortical neurons. We also located a 19 bp segment (MR element) within this promoter region, which is responsible for the majority of −677/+56 MeCP2 promoter activity. EMSA and southwestern-blot analyses...
demonstrated that several proteins bound to the region containing the MR element. These proteins were enriched in tissues that also express MeCP2 mRNA transcripts. Furthermore, to examine the activity of the −677/+56 fragment in vivo, we engineered transgenic mice carrying a LacZ reporter driven by the −677/+56 MeCP2 promoter. The transgene was expressed in a subpopulation of neurons and also mirrored the endogenous expression of MeCP2 in the brain throughout embryonic and postnatal development. Taken together, our findings indicate that the −677/+56 MeCP2 promoter is sufficient to drive neuronal expression of MeCP2 in the brain in a pattern that closely resembles that of the native gene.

RLM-RACE experiments revealed that the MeCP2 gene contains multiple transcription start sites. These start sites are embedded in a region that is GC rich and contains CpG islands. The MeCP2 promoter does not contain either a canonical TATA or CAAT box. The sequences surrounding the transcription start sites also differ from the initiator sequence, which is often found in TATA-less promoters (22,23). These features of the MeCP2 gene promoter resemble those of other housekeeping gene promoters. Transcription of the MeCP2 gene generates two distinct mRNAs, MeCP2e1 and e2 (16,17). MeCP2e1 is the dominant isoform in the brain, thymus and lung, whereas MeCP2e1 and e2 are equally

Figure 8. Nuclear factors from neuronal and non-neuronal tissues bind differentially to the MR element. (A) The sequences corresponding to the wild-type (WT) MRSP and the four mutant probes used in (B–E) are depicted. The MRSP probe is a segment of the MeCP2 promoter (−66 to −34), which contains both the MR element and the adjacent SP1 site. (B) Nuclear extracts from neuronal (left panel) and non-neuronal (right panel) tissues were subject to EMSA with 32P-labeled MRSP probe. Abbreviations are as follows: Ctx, cortex; Str, striatum; Cbl, cerebellum; Brs, brainstem; Li, liver; Lu, lung; Ht, heart; Sp, spleen. Open arrowheads indicate DNA–protein complexes that were competed off by addition of an excess of unlabeled MRSP probe. A closed arrowhead indicates free probe. (C) EMSA was carried out with nuclear extracts from Ctx and 32P-labeled WT or each of the four mutant probes, m1–m4. Lanes marked ‘+’ indicate binding reactions in which an excess of the unlabeled competitor with the same sequence as the radioactive probe was included. (D) and (E) Fifteen micrograms of nuclear extracts used in (B) and (C) were separated on SDS–PAGE, transferred to nitrocellulose membranes and incubated with 32P-labeled MRSP probe either in the absence (D) or in the presence (E) of unlabeled MRSP probe. As a control for gel loading, the same membranes were subjected to western-blot analyses using anti-histone1 antibody (αH1). As indicated by open circles, at least three proteins were identified that specifically bind to the MRSP probe.
expressed in liver and testis. In the brain, the −677/+56 promoter most likely regulates transcription of both MeCP2e1 and e2 isoforms, as one of the transcription start sites for MeCP2e2 is common to that for MeCP2e1. In tissues where both isoforms are equally present, however, it is tenable that different transcription start sites are used to transcribe e1 and e2 isoforms. In such tissues, alternative promoters might be utilized to initiate transcription of MeCP2e1 and e2.

The sequence of the mouse −677/+56 MeCP2 promoter exhibits a high degree of similarity (68%) to the corresponding promoter sequence from the human MECP2 gene. In particular, 92% of the nucleotides are identical to the human sequence between −87 and +56, which contains the MR element. However, the sequence similarity is reduced to 38% in the 1 kb region upstream of the −677/+56 promoter (Megumi Adachi and Frederick S. Jones, unpublished data). The extensive sequence similarity between the mouse and human MeCP2/MECP2 genes supports the idea that the −677/+56 region contains the DNA elements sufficient to drive expression of MeCP2 in vivo.

Indeed, mice carrying a LacZ transgene driven by the −677/+56 promoter recapitulated many of the dynamic features of MeCP2 expression in the CNS. Notably, expression of the transgene was restricted to a subpopulation of neurons and overlapped with the embryonic and postnatal expression pattern of MeCP2 in the CNS. These data indicate that the −677/+56 promoter possesses cognate regulatory elements that are sufficient for neuronal expression of the MeCP2 gene. This feature of the MeCP2 promoter is distinct from those that drive the expression of neuronal genes, such as synapsin I and type II sodium channel. These genes are expressed preferentially in neurons (24). Their restricted expression in neurons is accomplished by a negative regulatory mechanism via binding of repressor element 1 silencing transcription factor (REST). Removal of the REST binding site in these promoters results in expression of these neuronal genes in non-neuronal tissues (25–27). Thus, the REST binding site prevents ectopic expression of neuronal genes in non-neuronal tissues. In contrast, MeCP2 is not a neurally restricted gene and is widely expressed outside the nervous system. MeCP2 transcripts are present in most of tissues in mice and human (7,9). However, our analyses of MeCP2–LacZ transgenic mice demonstrated expression of the transgene in neurons, but not in non-neuronal tissues during embryonic and postnatal development. Furthermore, robust expression of the transgene during postnatal development nearly paralleled that of native MeCP2 in most regions of the CNS. This feature of the transgene expression was particularly evident in the cerebellum: transgene expression was observed minimally in Purkinje cells at early postnatal age (P7), but showed a dramatic increase by P24, when the transgene also exhibited expression in a small population of cells within the granule cell layer. In the cerebellum of adult Tg−677/+56 mice, many but not all of the cells in the molecular cell layer expressed the transgene (data not shown). This spatial expression pattern of the transgene is strikingly similar to that of endogenous MeCP2 in the cerebellum (21). However, temporal expression pattern of the transgene appeared to be delayed in comparison to that of endogenous MeCP2. In order to fully recapitulate spatiotemporal expression pattern of MeCP2, the −677/+56 promoter perhaps requires additional enhancer elements. Overall, the expression patterns of the transgene in the CNS imply that −677/+56 promoter contains DNA elements, which not only restrict the expression in neuronal cells, but also are responsive to physiological stimuli that neurons receive during postnatal development.

Although the −677/+56 promoter was sufficient to drive neuronal expression of MeCP2, this promoter does not appear to contain DNA elements necessary for non-neuronal expression of MeCP2 during embryonic and postnatal development. Such elements are likely to reside upstream of the −677/+56 region and/or in intron 1 of the MeCP2 gene. As the first intron from mouse and human MeCP2/MECP2 genes display extensive sequence similarity (28), it is tenable that this region may contain elements that regulate MeCP2 expression in non-neuronal tissues. In support of this notion, a reporter construct containing intron 1 and exon 2 produced more luciferase activity in non-neuronal than in neuronal cells. However, the overall activity of this reporter construct was weak, most likely as the construct lacks any of the native transcription start sites (Megumi Adachi and Frederick S. Jones, unpublished data). These observations suggest that intron 1 and/or exon 2 may contain DNA elements that control non-neuronal expression of MeCP2.

Our studies also identified a critical regulatory element (the MR element) that regulates the majority of MeCP2 promoter activity. In both neuronal and non-neuronal cell lines, deletion of the MR site in the MeCP2 promoter resulted in 70 and 60% reduction, respectively, in the promoter activity (data not shown). Consistently, the ΔMR–Luc reporter construct also exhibited a 70% reduction in MeCP2 promoter activity in primary cortical cultures containing glia. However, we did not observe any ectopic expression of the reporter gene in glia. Collectively, these results suggest that the MR site serves as a positive regulatory element for MeCP2 expression in both neuronal and non-neuronal tissues. Supporting this notion, results from EMSA experiments showed similar binding occupancy of the MRSP probe by nuclear proteins in the brain, lung and heart that highly express MeCP2 mRNA. In contrast, extracts from liver and spleen (tissues that express lower levels of MeCP2 mRNA) showed little or no binding to the MRSP probe. Southwestern-blot analyses, however, demonstrated different amounts of proteins that bound to the MRSP probe among tissues that express MeCP2 mRNA. For instance, fewer proteins were detected in extracts from the lung and heart than in those from the brain, although the molecular weight of these proteins was identical. These data indicate that the extent of protein binding to the MRSP probe is correlated with the overall MeCP2 promoter activity in neuronal and non-neuronal tissues.

Although expression of MeCP2 is robustly enhanced during postnatal development, little is known about developmental factors that influence expression of MeCP2 in vivo. However, MeCP2 expression has been found to be induced by NGF in PC12 cells (18), providing evidence that neurotrophic factors enhance the expression of MeCP2 in neurons. We also observed a similar extent of upregulation of the MeCP2 promoter in PC12 cells upon NGF induction (data not shown). Our finding that the MeCP2–LacZ transgene expression is
dynamically upregulated in the postnatal CNS supports the idea that the −677/+56 promoter contains DNA elements that respond to neurotrophic factors. In this regard, the MR element resembles a binding site for the early growth response (Egr) transcription factor. Egr transcription factors are known to be induced rapidly by a variety of extracellular stimuli in the CNS such as NGF, excitatory amino acids, and also by seizures (reviewed in 29). A hypothesis to examine in future studies is that neurotrophic factors released during postnatal development induce Egr expression, leading to the induction of Mecp2 expression. Of particular interest will be to identify which region of the −677/+56 promoter is responsible for induction of Mecp2 gene expression observed during postnatal CNS development.

In light of the observations that precise levels of MeCP2 expression are critical for normal development and function of the brain (13) and that duplication of the −677/+56 promoter contains DNA elements that respond to neurotrophic factors, it will be important in future studies to examine whether the −677/+56 promoter fragment produces levels of MeCP2 protein equivalent to that of native protein. Although further characterization of the −677/+56 Mecp2 promoter is necessary, identification of the −677/+56 promoter region provides an important first step in designing vectors for gene therapy applications in which exogenous MeCP2 is introduced in RTT patients.

MATERIALS AND METHODS

Cell culture

PC12 and SK-N-MC cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% horse and 10% fetal bovine sera (FBS). NIH3T3 cells were cultured in DMEM with 10% FBS. HepG2 cells were cultured in minimum Eagle’s medium supplemented with 10% FBS, 1% non-essential amino acids and 1 mm sodium pyruvate. All cultures were maintained at 37°C in an atmosphere of humidified air containing 5% CO2. For primary cortical cultures, E14 mouse embryos were collected from pregnant mice by Caesarian section. Cortices were dissected out in ice-cold dissection buffer (9.8 mM HEPES pH 7, 135 mM NaCl, 5 mM KCl, 0.3 mM Na2HPO4, 0.2 mM KH2PO4, 22 mM sucrose and 16.5 mM glucose), cut into pieces, placed in culture medium (Neurobasal supplemented with B27, Glutamax-I and 2% FBS), and incubated with DNase I at 50 mg/ml for 15 min at 37°C, followed by trituration using a pipette. Cells were plated at a density of 150 × 10^3 on a circular cover slip (12 mm diameter) pre-coated with poly-l-ornithine and laminin (Sigma, St Louis, MO, USA). All media and supplements used for cell cultures were purchased from Invitrogen (Carlsbad, CA, USA).

RNA ligase-mediated rapid amplification of cDNA ends

RLM-RACE was carried out using GeneRacer kit according to the instruction provided by the manufacturer. Total RNA was extracted from E14 mouse cortex using Trizol as described subsequently. The RNA was then decapped with tobacco phosphatase and ligated with a GeneRacer RNA oligo to pool full-length mRNA. After these modifications, the mRNA was subjected to an RT reaction with random hexomers. The resulting cDNA was used to amplify 5’ cDNA ends of the Mecp2 gene sequence using a GeneRacer 5’ nested primer and a primer located within exon 4 of Mecp2. The Mecp2 primer used in these experiments was 5’-CACCTGAACACCTTTCTGATGCTGCTGCC-3’. Amplified RACE products were cloned into the pCR4-TOPO vector for sequencing. All reagents used for RLM-RACE were obtained from Invitrogen.

RT–PCR

Total RNA was extracted with Trizol according to the method provided by the manufacturer. Extracted RNA was treated with DNase I for 30 min at 37°C and reverse transcribed at 42°C for 50 min by using random hexamers and reverse transcriptase II (Invitrogen) to synthesize single stranded cDNA. Using the cDNA as a template, PCR reactions were carried out at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min to detect mRNA levels for MECP2/Mecp2 and β-actin. This cycle was repeated 30 times followed by an extension cycle at 72°C for 5 min. PCR products were separated on ethidium bromide containing 2% agarose gels. Primers used for PCR were: 5’-GGAAAGTGTGCGATCTGCTGAAAGTA-3’ and 5’-CACCTGAACACCTTTCTGATGCTGCTGCC-3’ for MECP2/Mecp2; 5’-TGATACAGCAACGTACATGGC-3’ and 5’-ATTCCTATATGCGGGGACGAG-3’ for β-actin. To validate specificity of PCR products for Mecp2 and β-actin, RT reactions were carried out without reverse transcriptase. Resultant reactions were then subject to PCR reactions under the same conditions as described earlier and no PCR products were detected (data not shown). For quantitative (Q) RT–PCR analyses, transcripts for MECP2/Mecp2, LacZ and cyclophilin A were amplified using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were consisted of 1 cycle of 50°C for 2 min and 95°C for 10 min and 40 cycles of PCR amplification at 95°C for 15 s and 60°C for 1 min. The primers used for QPCR were: 5’-GGCGGATGACGAGCCC-3’ and 5’-GGCGGATGACGAGCCC-3’ for cyclophilin A; 5’-CAGAGGAGGACCCCTGGA-3’ and 5’-TTTATTATCTGTTTGAGAAGCTTG-3’ for Mecp2; 5’-GACCTCTCGGTGGCAGCACT-3’ and 5’-CAGGCGCAGACCATTTCCAA-3’ for β-galactosidase (β-Gal).

Plasmid constructs

The Mecp2 promoter region was amplified from mouse genomic DNA by PCR using the following set of primers: 5’-TACCTGAGAAGTTTTTCCCGGACGGGTGTTTACCAAGC-3’ and 5’-CGACCGCTCAAAACCATTGTTGCAACCAGAATGCGAAGCAGAT-3’. The amplified product (750 bp) was then subcloned into the pGL3basic luciferase reporter vector (Promega, Madison, WI, USA) to create the MeCP2–Luc construct. Serially truncated reporter constructs were generated by utilizing unique restriction enzymes as indicated in Figure 4A. For the ΔMR–Luc construct, the MeCP2–Luc plasmid was used as a template for PCR using the following primer sets: 5’-GACCTCTGCGGAGGCGGCCGCCCCACCCCTT-3’ and 5’-GAGGGCGGGGGCGACGTCG-3’.
Transfection experiments

The DNA used in transfection experiments was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. For PC12 and SK-N-MC cells, 1 × 10⁶ and 0.5 × 10⁶ cells, respectively, were plated in each well of six-well plate 1 day prior to transfection. Cells were then transfected with 1 μg of a specific MeCP2 luciferase reporter construct together with 0.5 μg of CMV–β-galactosidase expression vector (Clontech, Mountain View, CA, USA) using Lipofecta-
mine 2000 (Invitrogen) at a ratio of 1 : 3 for DNA:lipid. For transfections involving NIH3T3 and HepG2 cell lines, the cells were plated at a density of 0.15 × 10⁶ and 1 × 10⁶, respectively, in each well of six-well plate 1 day before transfection, and then transfected with the same amounts of DNA as described for PC12 cells using calcium phosphate precipitation. Twenty-four hours after transfection, the cells were harvested and extracts were prepared by using Reporter Lysis Buffer (Promega). Aliquots of the extracts were then assayed for luciferase and β-Gal activities using Luciferase Assay Reagent (Promega) and Fluoro Reporter (Molecular Probes, Eugene, OR, USA), respectively. The values presented for reporter gene activities were standardized to β-Gal activity within each extract. For primary cortical cultures, 7 days in vitro (DIV) cultures were transfected with 5 μg of the MeCP2–Luc construct and 1 μg of CMV-β-Gal using calcium phosphate precipitation. Cells were incubated with DNA–calcium phosphate precipitates for 2 h at 37°C, washed with PBS twice, and replaced with fresh culture media for an additional 24 h. The cells were then subjected to immunohistochemical analyses. For double staining of transfected cortical cultures with anti-luciferase and anti-
βIII-tubulin antibodies, experiments were repeated three times and 119 cells were counted in total. Similarly, for the cultures co-stained with anti-βIII-tubulin and anti β-Gal antibo-
dies, the experiments were repeated three times and 260 cells were counted in total.

Immunohistochemistry

Primary cortical cultures were fixed in 4% paraformaldehyde for 10 min, blocked in 5% normal donkey serum for 1 h at room temperature and then incubated with a primary antibody at 4°C overnight. The dilutions of primary antibodies were as follows: anti-MeCP2 antibody (Upstate, Charlo
tesville, VA, USA) 1 : 200; anti-βIII-tubulin antibody (Promega) 1 : 1000; anti-luciferase antibody (Promega) 1 : 300; anti-β-Gal antibody (Promega) 1 : 200. Cells were then incubated with secondary antibodies conjugated with Alexa Fluor 488 or 594 (Molecular Probes) at 1 : 200 dilution for 45 min at room temperature. Nuclei were stained with a solution containing 300 nM of 4',6-diamidino-2-phenylindole (DAPI) for 20 min at room temperature. The cells on cover slips were mounted in ProLong Antifade (Molecular Probes). For image capture, 12-bit fluorescent images were acquired with a Cooke CCD Sensicam mounted on a Leica DMIRBE microscope using a 20 × 0.7 NA air and a 63 × 1.30 NA oil-immersion objective. Illumination was provided by a Lambda DG-4 175-W Xenon light source (Sutter Instrument Co. Novato, CA, USA). The system was controlled by Slidebook (Intelligent Imaging Inc., Denver, CA, USA) imaging software. Postprocessing was performed with the deconvolution module in Slidebook.

Generation of transgenic mice

To generate the transgene, a segment of mouse MeCP2 pro-
moter (−677/+56) was subcloned into the pLacF vector (19). The transgene (MeCP2–LacZ) was isolated from vector sequences by restriction enzyme digestion, purified and microinjected into fertilized oocytes of FVB mice at Xenogen Biosciences (Cranbury, NJ, USA). Founder animals were genotyped by PCR with the following primer set: 5′-TTCCGGTACTGGTTGGTAAAG-3′ and 5′-AATGTGAGCGAGTAAACACC-3′.

β-Galactosidase staining

Embryos and organs were fixed in 0.2% glutaraldehyde, 1% formaldehyde, 2 mM MgCl₂, 5 mM EGTA and 0.02% NP-40 in PBS at 4°C for 20 min. The embryos were then stained at room temperature overnight in PBS containing 5 mM potassium hexacyanoferrate (II) trihydrate, 2 mM potassium hexacyanoferrate (III), 2 mM MgCl₂, 0.1% deoxycholate, 0.02% NP-40 and 50 ng/ml 4-chloro-5-bromo-3-indolyl-
β-galactoside (X-Gal). Brains that had been stained with X-Gal were protected through a step sucrose/PBS gradient (5, 10, 20 and 30%), embedded in OCT (Tissue-Tek) and sectioned at 50 μm. Some of the sections were subject to immunohistochemistry for deep-red fluorescent Nissl staining (Molecular Probes), which does not interfere with autofluores-
cence from fixation.

In situ hybridization

Whole-mount in situ hybridization was carried out as previously described (31). The riboprobe for in situ hybridization encompassed the entire region of the methyl DNA binding and transrepression domains; thus, the riboprobe used in the experi-
ments is capable of detecting all forms of Mecep2 transcripts, splice variant e1 and e2 isoforms, and the transcripts with different lengths of 3'-UTR. Primers used to make the ribo-
probe are 5′-GATGGTACAGGCTCAGGG-3′ and 5′-CAG GTCTTCAGTCCCTC-3′.

Electrophoretic mobility-shift assays

Nuclear extracts were prepared by using CelLytic NuCLEAR extraction kit, according to the protocol provided by the manu-
facturer (Sigma). For probes, synthetic sense and antisense oligonucleotides were 5' end-labeled with [γ-32P]ATP (Perkin-
Elmer, Boston, MA, USA) using T4 polynucleotide kinase and then annealed. EMSAs were carried out as previously described using 2.5 μg of nuclear extracts and 25 000 c.p.m. of labeled probe in each binding reaction (32). The probes used for EMSAs are shown in Figure 8A.

Southwestern-blot analyses

Southwestern-blot analyses were carried out as described by Kim et al. (33). Briefly, 15 μg of nuclear extracts were
separated on SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in a solution containing 5% non-fat dry milk, 10 mM Tris pH 7.5, 10% glycerol, 2.5% NP-40, 0.1 mM DTT and 150 mM NaCl at room temperature for 1 h. The membranes were then incubated with 32P-labeled probe (500 000 c.p.m./ml) in a binding buffer consisting of 10 mM Tris pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 8% glycerol, 10 μg/ml poly(dI–dC) and 0.125% non-fat dry milk at room temperature overnight. After incubation, the membranes were washed three times in a buffer consisting of 10 mM Tris pH 7.5 and 50 mM NaCl, followed by autoradiography.

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Conflict of Interest statement. None declared.

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