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DNA Binding Selectivity of MeCP2 Due to a Requirement for A/T Sequences Adjacent to Methyl-CpG

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Summary

DNA methylation is interpreted by a family of methyl-CpG binding domain (MBD) proteins that repress transcription through recruitment of corepressors that modify chromatin. To compare in vivo binding of MeCP2 and MBD2, we analyzed immunoprecipitated chromatin from primary human cells. Genomic sites occupied by the two MBD proteins were mutually exclusive. As MeCP2 was unable to colonize sites vacated by depletion of MBD2, we tested the hypothesis that methyl-CpG alone is insufficient to direct MeCP2 binding. In vitro selection for MeCP2 bound DNA-enriched fragments containing A/T bases ($[A/T]_{\geq 4}$) adjacent to methyl-CpG. $[A/T]_{\geq 4}$ was found to be essential for high-affinity binding at selected sites and at known MeCP2 target regions in the *Bdnf* and *Dlx6* genes. MBD2 binding, however, did not require an A/T run. The unexpected restriction of MeCP2 to a defined subset of methyl-CpG sites will facilitate identification of genomic targets that are relevant to Rett Syndrome.

Introduction

About 70% of CpG dinucleotides in the mammalian genome are methylated at position five of the cytosine ring (Bird, 2002). This epigenetic DNA modification affects gene expression and genome integrity (Eden et al., 2003) and is required for normal mouse development (Jackson-Grusby et al., 2001; Li et al., 1992). Mechanistically, DNA methylation is thought to elicit its effects by interfering with binding of proteins to their cognate binding sites (Watt and Molloy, 1988) or by creating a binding site for proteins that recognize methyl-CpG (Boyes and Bird, 1991; Lewis et al., 1992; Meehan et al., 1989). A family of methyl-CpG binding proteins has been characterized comprising MBD1, MBD2, MBD3, MeCP2, and MBD4, each of which contains a conserved methyl-CpG binding domain or MBD (Hendrich and Bird, 1998; Nan et al., 1993). MBD1, MBD2, and MeCP2 are associated with transcriptional repression and chromatin remodeling activities (Feng and Zhang, 2001; Jones et al., 1998; Klose and Bird, 2004; Nan et al., 1998; Ng et al., 1999; Sarraf and Stancheva, 2004). Mammalian MBD3 does not bind specifically to methylated DNA, and MBD4 is a DNA repair protein (Hendrich et al., 1999; Millar et al., 2002), although recent evidence suggests that MBD4 may also act as a transcriptional repressor (Kondo et al., 2005).

The biomedical relevance of MBD proteins became apparent with the discovery that the human neurodevelopmental disorder Rett syndrome is caused by mutations in the *MECP2* gene (Amir et al., 1999). Mutational profiling of Rett syndrome patients identified a significant fraction of point mutations that inactivated the MBD itself (Kriaucionis and Bird, 2003). The DNA and protein features that determine the specificity of the MBD for methyl-CpG sites have been examined (Free et al., 2001; Meehan et al., 1992; Nan et al., 1993; Yusufzai and Wolffe, 2000), and the three-dimensional structure of the domain, both alone (Ohki et al., 1999; Wakefield et al., 1999) and in complex with methylated DNA (Ohki et al., 2001), has been solved by NMR analysis. The MBD core consists of an α/β sandwich, with specific residue clusters mediating contacts with each 5-methylcytosine moiety in the major groove (Ohki et al., 2001).

Evidence that nuclear factors associate with methylated DNA in vivo came initially from studies examining the accessibility of CpG dinucleotides to cleavage by restriction endonucleases (Antequera et al., 1989). Methylated CpG dinucleotides were refractory to digestion, whereas nonmethylated sites were relatively accessible. Immunofluorescence confirmed that MBD proteins colocalize with sites of dense DNA methylation in living cells, in particular mouse satellite DNA, which is concentrated at pericentromeric heterochromatin (Hendrich and Bird, 1998; Nan et al., 1996). Several studies have since shown that MeCP2 associates with a variety of methylated gene sequences in vivo, but not with the same sequences when they are nonmethylated (El-Osta et al., 2002; Ghoshal et al., 2002; Gregory et al., 2001; Nan et al., 1996; Nguyen et al., 2001; Rietveld et al., 2002). Likely MBD protein target sites in human cancer cells have been identified by characterizing DNA fragments that were immunoprecipitated with MBD protein-specific antibodies (Ballestar et al., 2003; Koch and Stratling, 2004; Sarraf and Stancheva, 2004). A similar study of mouse brain chromatin using an anti-MeCP2 antibody identified the mouse *Dlx5*/*Dlx6* genes as targets for MeCP2-mediated repression (Horike et al., 2005).

Given their common DNA binding domains, different MBD proteins might be expected to compete for binding to methyl-CpG sites in the genome. About 9% of MeCP2 sites in a cancer cell line, however, were not immunoprecipitated by antibodies against MBD1 or MBD2 (Ballestar et al., 2003). In the present study using primary human cells, we find that the great majority of MeCP2 bound genomic fragments do not bind MBD1 or MBD2. Moreover, MeCP2 is unable to colonize most sites that are vacated by depletion of MBD2. To explain this specificity, we entertained the hypothesis that methyl-CpG is necessary, but not sufficient, for MeCP2

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binding. Artificial selection of MeCP2 binding sites in vitro using Methyl-SELEX demonstrated that MeCP2 requires an A/T run of four or more base pairs ($[A/T]_{\geq 4}$) adjacent to the methyl-CpG for efficient DNA binding. We propose that the presence of an A/T run is essential at biologically relevant MeCP2 binding sites and may help to predict MeCP2 interaction sites.

Results

MeCP2 Occupies Unique Sites In Vivo

In order to identify genomic loci occupied by MeCP2 in vivo, we performed chromatin immunoprecipitation (ChIP) with MeCP2-specific antibodies. We chose to study the MRC-5 human lung embryonic fibroblast cell line, as it displays characteristics of untransformed cells and has a normal diploid karyotype. At least three methyl-CpG binding proteins, MeCP2, MBD2, and MBD1, are detectable in nuclear extracts of MRC-5 cells (data not shown). To identify specific DNA sites bound by MeCP2, we used a "ChIP-and-clone" approach. Growing MRC-5 cells were formaldehyde fixed, and isolated chromatin was sonicated to generate DNA fragments of 250–750 bp. MeCP2-associated chromatin was then immunoprecipitated with MeCP2-specific antibodies, recovered, and adaptor sequences added. Primers complementary to the adaptor sequences were used to amplify DNA fragments, which were then cloned, and 98 plasmids were sequenced. As a control for random effects, sonicated genomic DNA was also cloned and sequenced (Tables S1 and S3 available in the Supplemental Data with this article online). Blast analysis indicated that 57% of MeCP2 binding sites mapped to intergenic sequences and 28% to introns and exons within annotated genes (Tables S1 and S3).

To ensure that the recovered loci were genuine MeCP2 targets, we repeated the ChIP but assayed with primers that were specific for each of 24 representative single-copy sequences from the first experiment (Figure 1A and Figure S1C). MeCP2 was reproducibly immunoprecipitated at loci identified in the initial ChIP-and-clone screen but rarely (one out of 18 fragments tested) at loci recovered from cloning of random genomic DNA (Figure S1B). When cells were pretreated with 5-azacytidine, the MeCP2 ChIP signal was lost, demonstrating that binding is dependent on DNA methylation (Figure 1A, lanes MeCP2*). Bisulfite sequencing of eight of these loci independently confirmed that all these sites contain highly methylated CpGs in MRC-5 cells (Figure S2). Surprisingly, ChIP of the same fixed chromatin sample with antibodies against MBD1 and MBD2 (Figure 1A) showed that only one of 12 MeCP2 bound fragments was immunoprecipitated by the anti-MBD2 antibody, and none were precipitated by anti-MBD1 antibody. We conclude that in these primary human cells, MeCP2 binds to unique loci that, for the most part, do not attract the MBD2 and MBD1.

MBD2 Can Colonize Sites Vacated by MeCP2, but Not Vice Versa

Given that MeCP2 and MBD2 each recognize methyl-CpG, we wondered if MBD2 would take over these sequences in the absence of MeCP2. To investigate this possibility, we first depleted MeCP2 in MRC-5 cells by transfection with a morpholino oligonucleotide (MeCP2-MO) directed against its translational initiation codon (Stancheva et al., 2003). A related oligonucleotide with five mismatches (MeCP2-mis-MO) served as a control. MeCP2 was efficiently depleted after two sequential MeCP2-MO transfections, as indicated by the loss of MeCP2 protein from the nuclear extracts, but treatment with MeCP2-mis-MO had no effect (Figure 1B). Levels of MBD2 or the unrelated protein PCNA were unaffected by either morpholino. Depletion of MeCP2 led to loss of DNA bound MeCP2, as anti-MeCP2 antibodies now weakly precipitated only five of the original 24 loci, the other 19 loci appearing unbound by this assay. Treatment with the control MeCP2-mis-MO allowed normal recovery of all 24 sites by ChIP. To find out if MBD2 could occupy vacated MeCP2 binding sites, chromatin from MeCP2-MO-treated cells was immunoprecipitated with anti-MBD2 antibodies, and the loci were tested for altered occupancy (Figure 1C and Figure S1C). In MeCP2-deficient cells, 13 of the 24 tested loci were now bound by MBD2.

Having established that MBD2 could colonize methylated sites vacated by MeCP2, we next asked the converse question: could MeCP2 move into sites vacated by MBD2? To address this, we isolated loci from the MRC-5 genome that were bound by MBD2 using the ChIP-and-clone methodology. Cloned loci were sequenced, located on the human genome sequence assembly (Tables S2 and S3), and verified by PCR amplification of an independent MBD2 ChIP fraction using locus-specific primers (Figure S1A). To determine if these loci could be colonized by MeCP2, we depleted MBD2 in MRC-5 cells, again using a morpholino approach (Figure 1B). After two sequential transfections with MBD2 morpholino (MBD2-MO), MBD2 protein was undetectable in nuclear extracts from these cells. The effect was specific, as levels of MeCP2 or the unrelated protein PCNA were unaltered. As expected, depletion of MBD2 greatly reduced the recovery of MBD2-associated loci by ChIP with anti-MBD2 antibodies (Figure 1D and Figure S1D). Only five of the 25 loci gave a residual signal, whereas fragments were recovered normally after treatment with a mismatched morpholino (MBD2-mis-MO) that had no effect on MBD2 abundance. To assess whether MeCP2 was able to take over MBD2 sites, ChIP experiments were carried out in parallel on the MBD2-MO-treated samples by using MeCP2-specific antibodies. Depletion of MBD2 resulted in MeCP2 occupancy at only three of the 25 sites vacated by MBD2. These data indicate that MeCP2 is reluctant to replace MBD2.

Enrichment of MeCP2 Binding Sites by Methyl-SELEX

The specificity of MeCP2 for a subset of methylated sites in the genome could be due to a requirement for additional DNA sequences at its genomic binding sites. According to this hypothesis, methyl-CpG would be necessary, but not sufficient, for DNA binding of MeCP2. To establish the molecular determinants for methyl-CpG-dependent DNA recognition by MeCP2, we employed a modified form of the in vitro DNA binding site selection technique SELEX (He et al., 1996;

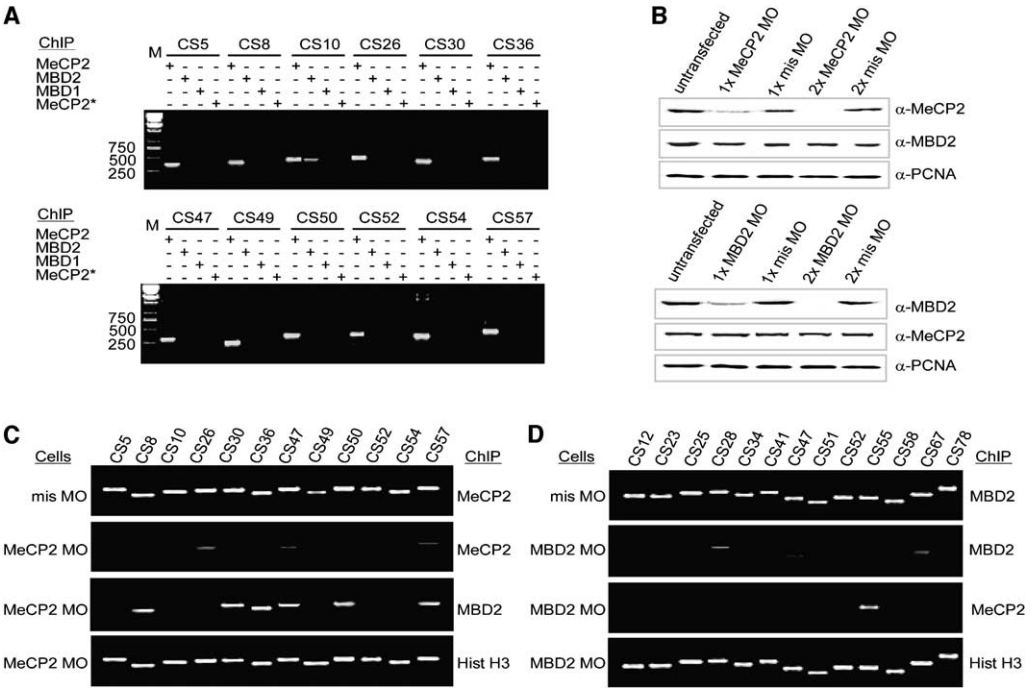

Figure 1. MeCP2 Primarily Binds to Loci Not Occupied by Other MBD Proteins

(A) MeCP2 ChIP-and-clone loci were verified by site-specific PCR on independently precipitated chromatin and shown to be occupied by MeCP2. ChIP sequences (CS), as numbered, were tested for occupancy with antibodies against MeCP2, MBD2, and MBD1 as indicated above each PCR lane with a plus symbol (+). The asterisk indicates ChIP for MeCP2 on cells grown with 5-azacytidine and demonstrates that MeCP2 binding is dependent on DNA methylation.

(B) Efficient morpholino depletion of MeCP2 and MBD2 was achieved with two sequential transfections (2 X) in MRC-5 cells. MO indicates use of a morpholino against target mRNA, and mis-MO is the control morpholino with five mismatched bases. MeCP2 and MBD2 were efficiently and specifically depleted as indicated by loss of Western signal for each protein. The unrelated control protein PCNA was unaffected and serves as a loading control.

(C) Depletion of MeCP2 by morpholino results in colonization of several vacant MeCP2 sites by MBD2, as tested using site-specific ChIP primers. Indicated above are CS number, type of morpholino treatment (left), and ChIP antibody (right).

(D) Vacant MBD2 loci are not widely occupied by MeCP2 upon MBD2 depletion.

Klug and Famulok, 1994). Methyl-SELEX involved generating double-stranded DNA fragments that have a fixed central CpG in the context of an HpaII methyltransferase/restriction endonuclease site flanked by random DNA sequence (Figure 2A). The initial pool of DNA fragments was methylated by using M.HpaII methyltransferase and tested for complete methylation by loss of sensitivity to cleavage by HpaII restriction endonuclease. We used the N-terminal half of human MeCP2 (amino acids 1–205) for DNA binding, as it forms a discrete DNA-protein complex more reproducibly than the full-length protein (1–486) in our hands. The protein was mixed with the starting DNA, and DNA-protein complex was recovered after an electrophoretic mobility shift assay (EMSA). DNA from the complex was amplified, remethylated, and once more bound to MeCP2 (1–205). After eight cycles of binding and amplification, we observed that the efficiency of complex formation increased ~5-fold compared with the random starting DNA (Figure 2B).

MeCP2 Requires an A/T-Rich Sequence Adjacent to Methyl-CpG for Efficient DNA Binding

The sequence of 88 selected fragments and 86 unselected fragments was determined (see Unselected Methyl-SELEX Fragments and Mecp2 Methyl-SELEX Fragments in the Supplemental Data). Analysis of these sequences revealed the frequent presence of an A/T run of four or more bases ($[A/T]_{\geq 4}$) close to the methyl-CpG site in the enriched sample. When sequences were aligned with the A/T run to the right of the methyl-CpG (Figure 3A), an $[A/T]_{\geq 4}$ motif appeared to cluster in two vertical stripes located one to three base pairs or six to nine base pairs from the methylated CCGG site. In contrast, when the MBD of MBD2 was used in the same Methyl-SELEX experiment, there was no enrichment of an $[A/T]_{\geq 4}$ motif, but instead, sequences containing more than one methylated CCGG site were recovered (Figure S3 and MBD2 Methyl-SELEX Fragments in the Supplemental Data). To determine whether the DNA fragments in the MeCP2-selected pool had an increased affinity for MeCP2, we analyzed five of the selected fragments (S1–S5) and two unselected fragments (U6 and U7) by EMSA (Figures 3B and 3C). Fragments with an A/T run within three bases (S1 and S2) or eight bases (S3 and S4) of the methyl-CpG bound efficiently to MeCP2 (1–205), whereas two unselected fragments that lacked an $[A/T]_{\geq 4}$ motif did not show detectable complexes. Within the selected pool, six out of 88 fragments contained no A/T run. EMSA analysis

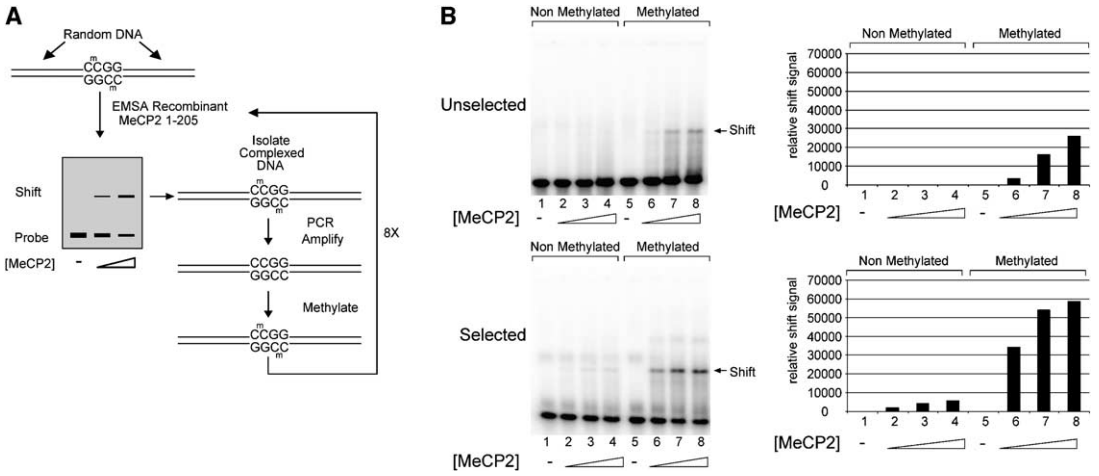

Figure 2. Methyl-SELEX Selects for High-Affinity MeCP2 Binding Sequences

(A) A four-step Methyl-SELEX enrichment involves evolution of high-affinity MeCP2 binding sites by multiple EMSA enrichment steps.

(B) Unselected and selected DNA sequences from Methyl-SELEX were end labeled with ^{32}P and used as probes for EMSA. Methyl-SELEX-selected material showed an increase in the amount of DNA-protein complex in comparison to unselected material. The methylation status of each probe is indicated above, and MeCP2 protein concentration is shown below. (–) indicates free probe. The intensity of each signal was quantified and plotted as relative shift signal in the right-hand panel.

using one of these sequences (S5) showed low affinity for MeCP2, suggesting that this was a contaminant (Figure 3C).

To directly test the importance of the A/T-rich sequences for MeCP2 binding, we placed C/G pairs within the A/T runs of fragments S1 and S3 by mutagenesis (Figure 4A). In each case, the mutations drastically reduced the MeCP2 binding affinity of these probes to levels seen in unselected DNA (Figure 4B). As all probes were methylated at a fixed central CCGG, we

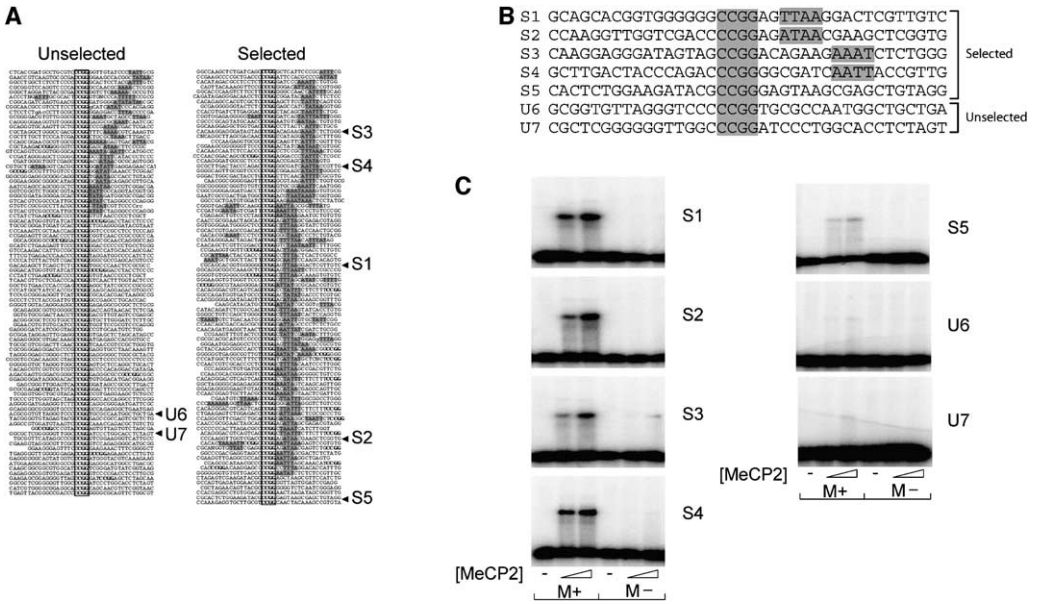

Figure 3. High-Affinity MeCP2 Binding Sites Isolated by Methyl-SELEX Contain Methyl-CpG with an Adjacent Run of A/T DNA

(A) A representative sample of the random unselected and MeCP2-selected DNA sequences from the Methyl-SELEX experiment was cloned, sequenced, and aligned. The central fixed CCGG site is indicated by an open box, and runs of four or more A/T's are highlighted with a shaded box.

(B) Five selected (S1–S5) and two unselected sequences (U6 and U7; see arrows in [A]) were chosen for further analysis.

(C) EMSA analysis of MeCP2-selected probes S1–S4 show efficient binding, but selected probe S5 lacking $[A/T]_{\geq 4}$ binds poorly. No detectable binding was observed to unselected probes U6 and U7. M+ and M– indicate presence or absence of methyl-CpG in the EMSA fragment. The concentration of MeCP2 is indicated below with (–) indicating free probe.

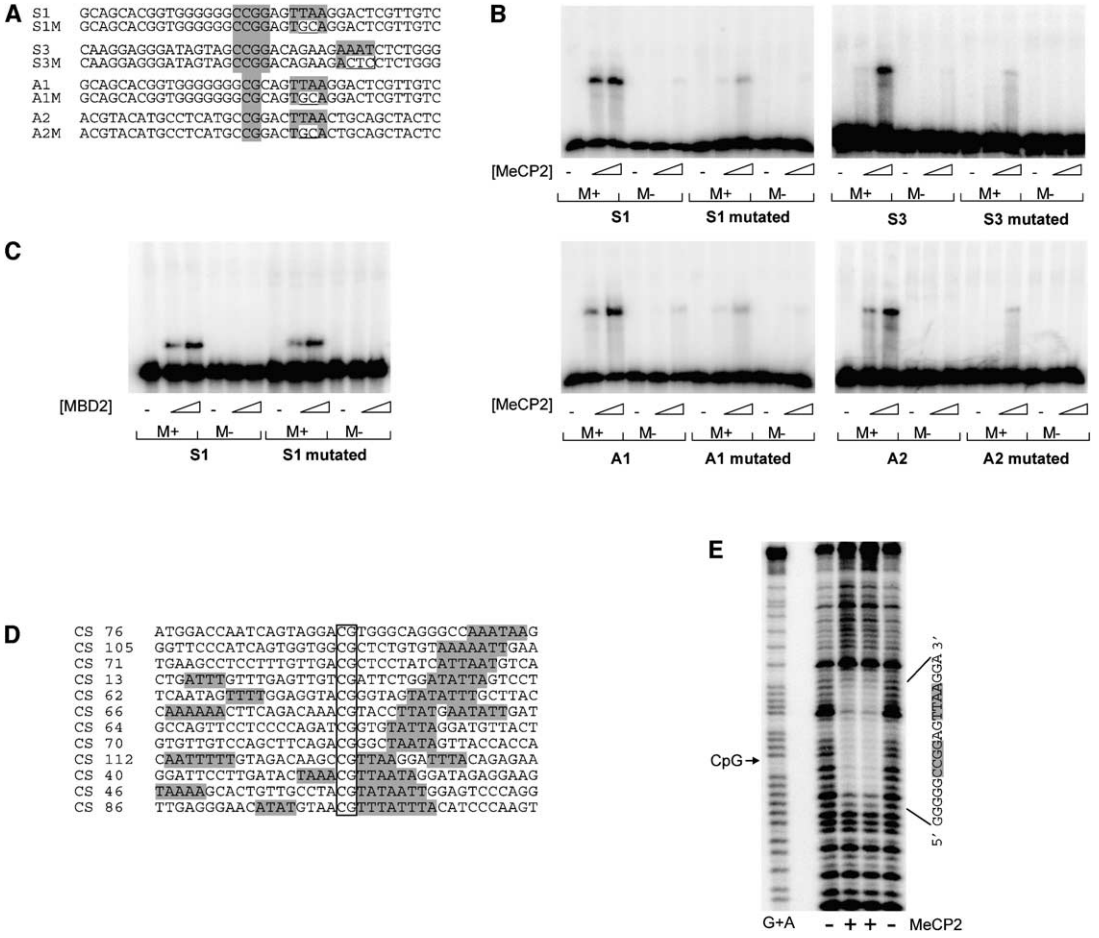

Figure 4. Requirement for an A/T Run at Natural and In Vitro-Selected MeCP2 Binding Sites

(A) Selected fragments S1 and S3 were mutated in the A/T run as indicated by the shading (S1M and S3M). In fragment A1, the HpaII site has been altered to an HhaI site (GCGC), and A1M combines this with a mutated A/T run. Fragments A2 and A2M are artificially engineered MeCP2 binding sites consisting of a random DNA sequence in which a methyl-CpG with an adjacent A/T run (A2) or mutated A/T run (A2M) is embedded.

(B) EMSA analysis shows that mutation of the A/T run (S1M and S3M) reduces the affinity of MeCP2 for S1 and S3, as indicated by reduction of DNA-protein complex in S1M and S3M lanes. A1 and A2 both bind tightly to MeCP2, and binding is dependent on an adjacent A/T run (A1M and A2M).

(C) A polypeptide comprising amino acids 145–218 of MBD2a showed equivalent binding to both the S1 and S1-mutated probes, indicating MBD2 does not require an A/T run for efficient binding.

(D) Alignment of DNA sequences recovered by ChIP and clone that represent in vivo MeCP2 binding sites in MRC5 cells. Of 12 inserts with only one CpG, all have $[AT]_{\geq 4}$ runs (shading) nearby.

(E) DNase I footprinting analysis on the ^{32}P end-labeled S1 fragment demonstrates asymmetrical protection of the methyl-CpG toward the A/T run (large bracket with protected DNA sequence to the right). Location of the methyl-CpG in the footprinting probe is indicated by an arrow to the left. G+A indicates the Maxam/Gilbert sequencing reaction on the same DNA fragment as size marker. Samples were incubated with (+) or without (–) MeCP2 protein.

tested whether the two nucleotides flanking the CpG contributed to the observed binding. Changing CCGG in probe A1 to GCGC (methylated by M.HhaI methyltransferase) did not affect the high-affinity binding to MeCP2 (Figure 4B). If an A/T-rich run adjacent to methyl-CpG is sufficient for efficient MeCP2 binding, it should be possible to engineer a high-affinity MeCP2 binding site by using these criteria alone. Therefore, probe A2 was constructed in which an $[A/T]_{\geq 4}$ run was placed three base pairs away from the central CpG within the context of a random flanking nucleotide sequence. The engineered DNA sequence bound MeCP2 as efficiently as the positively selected Methyl-SELEX fragments, and mutation of the A/T run again abolished high-affinity binding (Figure 4B). An $[A/T]_{\geq 4}$ sequence adjacent to a methyl-CpG is therefore necessary and sufficient for high-affinity MeCP2 binding.

The unexpected DNA sequence specificity of MeCP2 raises the possibility that MBD2, whose binding sites in the genome are largely distinct from those of MeCP2, might exhibit comparable selectivity. Full-length MBD2 did not form complexes with DNA under conditions used in our EMSA assay, but a fragment corresponding to the MBD bound equally to sites with and without the

adjacent A/T run (Figure 4C). MBD2 is therefore indifferent to the presence or absence of $[A/T]_{\geq 4}$, in agreement with results obtained in Methyl-SELEX using MBD2 (Figure S3 and MBD2 Methyl-SELEX Fragments in the Supplemental Data).

Examination of DNA fragments obtained from MRC-5 cells by ChIP and clone showed that CpGs followed by $[A/T]_{\geq 4}$ were somewhat enriched among the MeCP2 ChIP sequences. Within MeCP2 ChIP fragments, most of which contained multiple CpGs, 50% of CpGs (523 in total) were followed by an A/T run compared to 33.5% of CpGs (435) in MBD2 ChIP fragments. Considering only sequences that contained a single CpG, 11 out of 12 MeCP2 bound loci contained an $[A/T]_{\geq 4}$ motif within eight base pairs of the CpG (Figure 4D). In one MeCP2 bound fragment (CS76), an $[A/T]_6$ motif was 11 base pairs away from the CpG (Figure 4D). Independent ChIP and PCR demonstrated that CS76 is weakly precipitated by anti-MeCP2 antibodies compared with the other fragments (data not shown) and thus may represent a relatively low-affinity binding site in vivo. These findings confirm that the DNA binding requirements for MeCP2 that were established by Methyl-SELEX are relevant in vivo.

Methylated CpG sites are symmetrical, and therefore, MeCP2 can theoretically bind in either of two orientations. The presence of an adjacent A/T run, however, potentially confers asymmetry to the MeCP2 binding site. To test for directional binding of MeCP2, we bound MeCP2 to the S1 probe and performed DNase I footprinting analysis. The zone of protection was not centered on the methyl-CpG but extended to cover the adjacent A/T run (Figure 4E). The A/T run therefore confers directional binding of MeCP2 at a methyl-CpG site.

The Specificity for Methyl-CpG Flanked by $[A/T]_4$ Resides in the MBD and Is Independent of the AT Hook

It was noted in early studies that MeCP2 contains a domain that is shared with proteins that bind the minor groove of A/T-rich DNA (Lewis et al., 1992; Nan et al., 1993). The effect of this domain on MeCP2 function remains unknown, but close sequence similarity with an AT hook domain of HMGA1 (formerly HMG I/Y) and across MeCP2 orthologs is striking (Aravind and Landsman, 1998) (Figure 5A). We asked whether the AT hook might be responsible for the A/T run-dependent binding of MeCP2 by mutating two conserved arginine amino acids within the MeCP2 AT hook to glycine (Figure 5A). Equivalent mutations within the AT hook of HMGA1 significantly inhibited the recovery of binding to heterochromatin in photobleaching experiments (Harrer et al., 2004). When wt and mutant MeCP2 proteins were assayed by EMSA for binding to the S1 and S1-mutated DNA fragments, however, we were surprised to find that loss of the AT hook domain had no effect on the affinity or specificity of MeCP2 for probes that contained an A/T run (Figure 5B).

It seemed possible that the AT hook affects MeCP2 binding in vivo but escapes detection by in vitro EMSA. To examine the in vivo dynamics of the association between MeCP2 and mouse heterochromatin, we employed fluorescence recovery after photobleaching (FRAP). In mouse cells, MeCP2 has a distinct subnuclear localization profile at pericentric heterochromatin, which contains the major satellite DNA (Figure 5C). Mouse satellite DNA accounts for about 10% of the mouse genome and comprises an A/T-rich repeated sequence in which CpG dinucleotides are highly methylated (Hörz and Altenburger, 1981; Manuelidis, 1981). Based on the observations reported above, the combination of concentrated methyl-CpGs adjacent to runs of A/T should provide an optimal binding location for MeCP2. For FRAP analysis, wt and AT hook mutant MeCP2 were fused to GFP and transfected into mouse cells. Both the wt and the AT hook mutant protein localized normally to DAPI bright spots, which correspond to heterochromatic foci (Figure 5C).

To test the kinetics of MeCP2 and the MeCP2 AT hook mutant in heterochromatic foci, $p53^{-/-}$ mouse fibroblasts were photobleached and the recovery time measured. Wt MeCP2 had a recovery half time of about 25 s, with no significant difference ($p > 0.0001$) between wt and AT hook mutant in both cell lines tested (Figure 5D). This recovery time is comparable to that of linker histone H1 (Lever et al., 2000) but is almost ten times slower than other structural nonhistone chromatin proteins, for example HP1 (Cheutin et al., 2003, 2004; Schmiedeberg et al., 2004). From both the in vitro EMSA and in vivo FRAP analysis, we conclude that the AT hook is not essential for the recognition of high-affinity MeCP2 binding sites in which methyl-CpG is followed by an A/T run.

To map the MeCP2 domain that is responsible for high-affinity binding, a series of deletion mutants were expressed as recombinant proteins (Figure 6) and used in EMSA on the S1 and S1-mutated probes. MeCP2 1–167, which lacks the AT hook domain, has the same selectivity for a methyl-CpG with an A/T run as fragment 1–205 that contains the AT hook domain. This observation conclusively demonstrates that the AT hook is dispensable for the observed binding specificity of MeCP2. Further deletion from both the N and C terminus of MeCP2 toward the MBD (fragments 77–167 and 78–161) allowed retention of high-affinity binding for the A/T run-containing probe, but additional N-terminal deletion (fragment 90–161) abolished DNA binding altogether. This suggests that high-affinity MeCP2 binding requires the 13 amino acids between residues 78 and 90 in addition to the conserved MBD domain itself.

MeCP2 Binds to the *Dlx6* and *Bdnf* Target Genes at High-Affinity MeCP2 Binding Sites

MeCP2 contributes to the silencing of the *Dlx5* and *Dlx6* genes in mouse brain (Horike et al., 2005). High-resolution ChIP analysis across the Dlx5/6 locus identified a region near the third exon of the *Dlx6* gene that is enriched for bound MeCP2 and contains two CpGs that are consistently methylated in mouse brain (see Figure 7A). One of the two methyl-CpG dinucleotides in the *Dlx6* MeCP2 binding region has adjacent A/T runs and is a predicted high-affinity MeCP2 binding site (ds1), whereas the second site lacks A/T runs (ds4) (Figure 7B). To test their MeCP2 binding efficiencies, probes corresponding to each methyl-CpG were assayed by EMSA. MeCP2 bound efficiently to the predicted high-

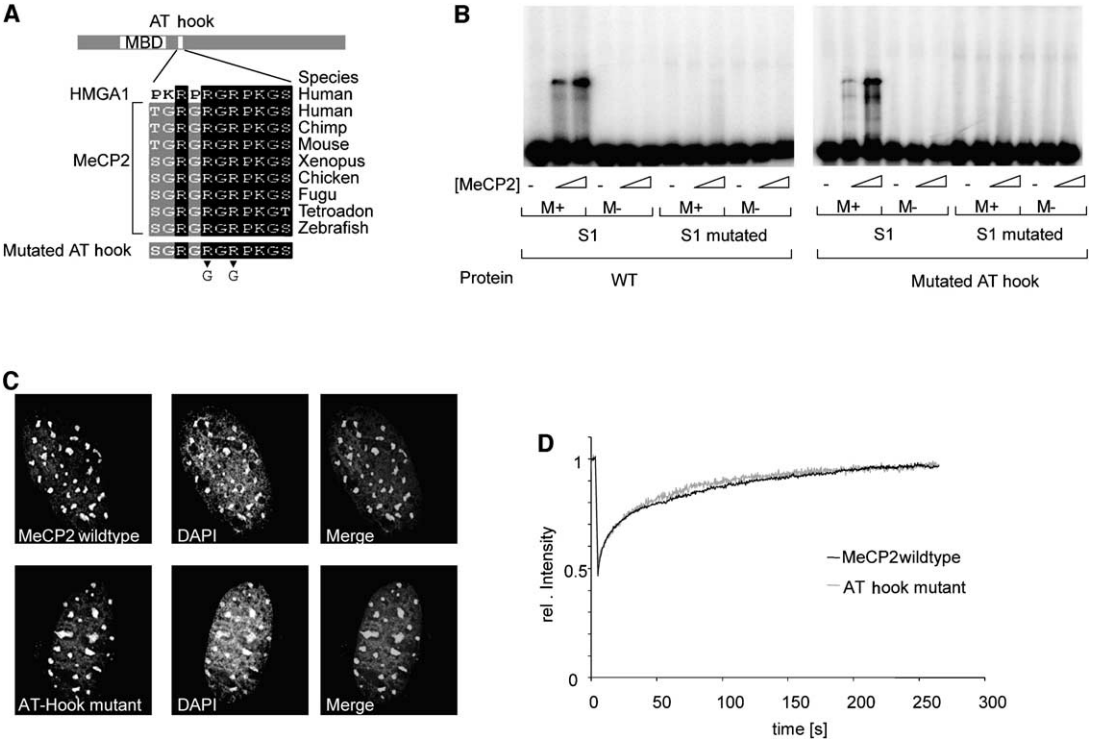

Figure 5. The Conserved MeCP2 AT Hook Domain Is Not Required for A/T Run-Dependent Binding of MeCP2 In Vitro or In Vivo

(A) Alignment of the conserved AT hook domains of MeCP2 with an AT hook found in HMGA1. A mutant MeCP2 protein was generated by replacing two essential arginines in the MeCP2 AT hook with glycine.

(B) Mutation of the AT hook of MeCP2 does not affect methyl-CpG A/T run-dependent binding to the S1 probe, as indicated by EMSA using ^{32}P-labeled probes.

(C) GFP-MeCP2 and GFP-MeCP2 (AT hook mutant) both localized to DAPI-stained foci in living cells.

(D) Fluorescence recovery after photobleaching at heterochromatic sites is indistinguishable between wild-type and mutant MeCP2. The half-time of recovery in both cases is ~25 s.

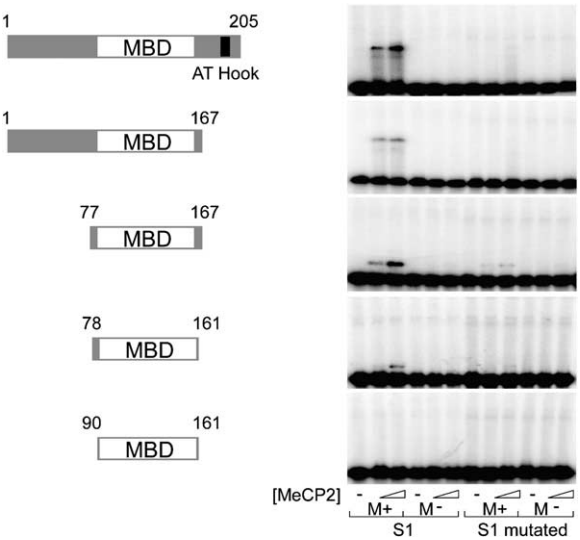

Figure 6. A Polypeptide Containing Amino Acids 78–161 of MeCP2 Is Sufficient for Methyl-CpG and A/T Run-Dependent DNA Binding

Deletion fragments of MeCP2 were expressed in bacteria and used in EMSA with ^{32}P-labeled S1 probe. Methyl-CpG A/T run-dependent binding was localized to amino acids 78–161.

affinity *Dlx6* site (ds1) when methylated, and binding was drastically reduced by mutagenesis of the A/T runs (Figure 7C). The ds4 methyl-CpG site, by contrast, showed little detectable MeCP2 binding by EMSA (Figure 7C). We conclude that MeCP2 most likely binds to the ds1 site within the *Dlx6* binding region that was mapped by ChIP in vivo (Horike et al., 2005).

MeCP2 has been shown to bind close to an inducible promoter of the rodent *Bdnf* gene and contribute to maintenance of the silenced state (Chen et al., 2003; Martinowich et al., 2003). Bisulfite genomic sequencing and competitive EMSA analysis suggested that MeCP2 bound preferentially to a methyl-CpG at –148 base pairs from the transcription start site (Figure 7D) (Chen et al., 2003; Martinowich et al., 2003). Analysis of the sequence surrounding –148 indeed revealed downstream A/T runs (Figure 7E). To test the efficiency of MeCP2 binding to this sequence, we generated a probe (–148) corresponding to the MeCP2 binding site and a control probe containing one CpG from within *Bdnf* exon 1 (Figures 7D and 7E). The –148 sequence bound MeCP2 by EMSA as predicted, and the control fragment showed no obvious binding (Figure 7F). Mutation of the A/T runs adjacent to –148 greatly reduced binding to MeCP2. It is notable that the sequence corresponding to the *Bdnf* site (5′-CGGAATT-3′) occurred

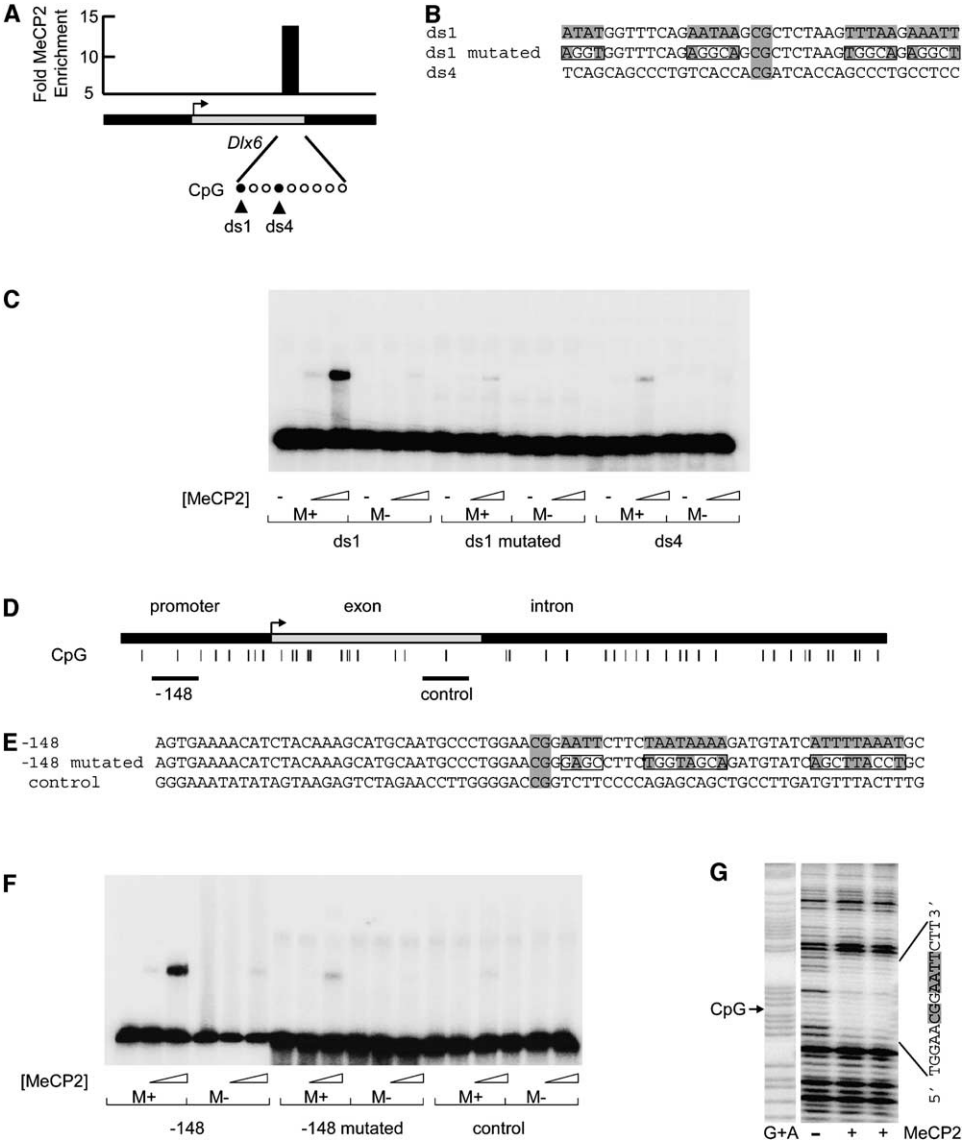

Figure 7. Two Natural MeCP2 Target Genes Have High-Affinity MeCP2 Binding Sites

(A) A schematic illustrating the MeCP2 binding region in the *Dlx6* target gene (Horike et al., 2005). Two methyl-CpG dinucleotides that were identified in brain tissue (ds1 and ds4) are indicated by closed black circles; unmethylated CpG's are indicated with open circles.

(B) Probes corresponding to ds1, ds1 mutated, and ds4 are illustrated with differences between the wt and mutated ds1 probe indicated by shading.

(C) EMSA shows that efficient MeCP2 binding to the ds1 probe is reduced when the A/T runs are mutated and the ds4 probe shows weak binding.

(D) Analysis of the mouse *Bdnf* promoter IV region showing the MeCP2 binding site at –148 bases from the transcription start.

(E) Probes are shown corresponding to the –148 sequence, –148 with a mutated A/T run, and a CpG-containing fragment from exon 1 of the *Bdnf* gene.

(F) EMSA shows that efficient MeCP2 binding to the –148 probe is reduced when the A/T runs are mutated. The exon 1 control probe shows little detectable binding.

(G) DNase I footprinting analysis on a ^{32}P-labeled –148 *Bdnf* fragment in the presence (+) or absence (–) of MeCP2. The footprint due to the addition of MeCP2 is bracketed, with the protected DNA sequence to the right. G+A indicates the Maxam/Gilbert sequencing reaction on the same DNA fragment as a size marker. Methyl-CpG is indicated by an arrow (left).

six times in the Methyl-SELEX sample of 88 sequences (see Selected Methyl-SELEX Fragments in the Supplemental Data). To determine if MeCP2 binds directionally to the –148 *Bdnf* site, a DNase I footprinting analysis was performed. As was observed with artificially selected S1 fragment (Figure 4D), MeCP2 binding was not centered on the methyl-CpG site but extended asymmetrically to cover the adjacent AATT run (Figure 7G). These data suggest that MeCP2 binding at this regulatory region is orientated with respect to the methyl-CpG through interaction with the adjacent AT-run.

Both the *Dlx6* and *Bdnf* binding sites have more than one A/T run flanking the methyl-CpG. Mutation of only the closest A/T run in each probe resulted in reduced MeCP2 binding, but the effect was less drastic than mutating all A/T runs together (Figure S4). These data indicated that the closest A/T run contributes most significantly to MeCP2 binding and that other nearby A/T runs also enhance binding affinity.

Discussion

Exclusive Binding Sites for MBD Proteins

Methyl-CpG binding proteins MeCP2, MBD1, and MBD2 share a methyl-CpG binding domain, but there is little evidence for functional redundancy. Although the phenotypic consequences of the genetic deficiency of each protein are primarily manifested in the brain, the processes that are affected appear to be physiologically distinct. Loss of MeCP2 in mice affects motor functions and leads to death at ~10 weeks of age (Chen et al., 2001; Guy et al., 2001), whereas deficiencies of MBD1 (Zhao et al., 2003) and MBD2 (Hendrich et al., 2001) affect neuronal development and maternal behavior, respectively, but not survival. The combination of *Mbd2* and *Mecp2* mutations does not enhance the neurological phenotype or the limited viability of *Mecp2* single mutant mice, suggesting that these gene products do not share substantial functions (Guy et al., 2001). The present results offer an explanation for some of these differences, as MeCP2 and MBD2 for the most part occupy distinct and nonoverlapping sites in the genome. The existence of differential binding sites was first suggested by a ChIP-and-array experiment with breast cancer cell lines in which a small fraction of MeCP2 sites (about 9%) were not immunoprecipitated with either anti-MBD2 or anti-MBD1 antibodies (Ballestar et al., 2003). Most fragments, however, were precipitated by more than one of the anti-MBD protein antibodies, with MBD2 having the widest binding profile. The much greater distinction between MeCP2 sites and those for MBD1 and MBD2 that was seen in our experiments may be attributable to the use of primary human cells rather than tumor cell lines, as the latter are known to be aberrantly methylated (Klug and Famulok, 1994; Paz et al., 2003).

Focusing on MBD2 and MeCP2, we asked whether removal of one protein would allow the other protein to take over the vacant sites. We found that the answer varied according to which protein was depleted. MBD2 was able to occupy many of the sites vacated by MeCP2 (13 out of 24), but MeCP2 was markedly less promiscuous, occupying only three out of 24 ex-MBD2 sites. This difference could potentially be explained in several ways, but we followed up the simple hypothesis that MeCP2 is tied to a subset of methyl-CpGs through a requirement for additional DNA sequence at its binding site. The speculation proved correct, as high-affinity binding sites required an adjacent $[A/T]_{\geq 4}$ motif. The distance of the $[A/T]_{\geq 4}$ from the CpG site could vary but occurred in two clusters that may represent preferred distances. MBD2, on the other hand, did not discriminate between sites with or without the $[A/T]_{\geq 4}$ flanking sequence. We suggest that MBD2 can bind to methyl-CpG sites regardless of flanking sequence. If so, all MeCP2 sites would be potential MBD2 sites, and this could account for the ability of MBD2 to colonize many vacant MeCP2 sites in the genome.

MBD2 appears to be the ancestral MBD protein, as it is the only member of the family to occur in invertebrate organisms (Hendrich and Tweedie, 2003). Previous work has not identified any sequence preference for MBD2 binding beyond a single symmetrically methylated CpG dinucleotide, and our study sustains this view (Figure S3). It is possible that expansion of the MBD protein family in vertebrates created specialized MBD proteins that acquired additional DNA sequence specificity, as seen for MeCP2. Specialization of this kind may also affect MBD1, as a major isoform contains a second DNA binding domain, the CxxC3 motif, which specifically recognizes nonmethylated CpG (Jorgensen et al., 2004). Conceivably, this form of MBD1 requires the simultaneous presence of a methyl-CpG and a nonmethylated CpG at its favored binding sites.

High-Affinity DNA Binding by MeCP2 Requires an Adjacent AT Run

Sequence analysis of the ChIP DNA fragments (250–750 bp) revealed an $[A/T]_{\geq 4}$ motif adjacent to CpG in all 12 MeCP2 ChIP DNA fragments that contained only a single CpG. This finding, together with the analyses of known MeCP2 binding sites in the *Dlx5* and *Bdnf* genes, supports the conclusion that the DNA sequence requirements that were established in vitro have in vivo relevance. Knowing DNA sequence requirements for MeCP2 does not allow us to predict with certainty occupied sites in the genome, as not all potential MeCP2 binding sites are precipitated by anti-MeCP2 antibodies. This suggests that there are other as yet undefined constraints on site occupancy by methyl-CpG binding proteins. For example, access to eligible CpG dinucleotides may be sterically hindered due to their association with phased nucleosomes or other DNA binding factors, including histone H1. Alternatively, MeCP2 may require interaction with other DNA binding proteins to form a stable complex with a genomic site. As is the case with other transcription factors, occupancy of potential MeCP2 binding sites will require experimental verification.

The structural basis of the $[A/T]_{\geq 4}$ requirement is currently unknown. We have established that the AT hook motif does not play a major part in $[A/T]_{\geq 4}$ recognition, as its mutation or deletion had no discernable effect in vivo or in vitro on targeting of MeCP2 to methylated sites. Moreover, the in vivo rate of exchange between chromosomally bound and unbound MeCP2 was not altered by mutation of the AT hook. It is possible that a nearby $[A/T]_{\geq 4}$ motif alters the three-dimensional configuration of a methyl-CpG site, although MBD2 bound equally well regardless of the presence of $[A/T]_{\geq 4}$, suggesting that the structure of the methyl-CpG site is not profoundly affected. Alternatively, part of the MBD domain or its immediate flanking sequences may extend to contact either the major or minor groove. Structural analysis is required to adequately test these possibilities. Interestingly, binding of the transcription factors E2F4 (Zheng et al., 1999) and SKN-1 (Kophengnavong

et al., 1999) to a minimal cognate sequence is also significantly enhanced by the presence of an adjacent run of AT-rich DNA.

As methyl-CpG sites are symmetrical when methylated on both DNA strands, a protein that recognizes methyl-CpG could potentially bind in either of two orientations. The addition of flanking A/T DNA confers asymmetrical binding to methyl-CpG, which probably reflects association of MeCP2 with the DNA in one orientation only. Directional MeCP2 binding and $[A/T]_{\geq 4}$ protection were seen for both artificial binding sites and the natural MeCP2 binding site in the promoter region of its known target gene *Bdnf* (Chen et al., 2003; Klose and Bird, 2003; Martinowich et al., 2003). It is significant that the observed DNase I footprint does not extend to cover the distal AT runs. This suggests that these sequences exert their effect through conformational changes in the DNA that influence MeCP2 binding remotely. The functional consequences of binding site orientation are not yet known, but it is attractive to hypothesize that the direction in which MeCP2 binds a regulatory region may influence how cofactors are recruited with respect to protein architectures required for gene regulation.

MeCP2 Is Primarily a Methyl-CpG Binding Protein

Is MeCP2 primarily targeted to the sites identified here or is it potentially able to bind other sequences, including some that are nonmethylated? The evidence that binding of MeCP2 is dependent on DNA methylation is based on multiple independent studies. In addition to many in vitro studies that define its preference for methylated CpG sites (Fraga et al., 2003; Meehan et al., 1992; Nan et al., 1993), ChIP analyses have consistently shown that MeCP2 associates with a specific methylated DNA sequence in vivo, but not with the same sequence at the same genetic locus when it is unmethylated (El-Osta et al., 2002; Ghoshal et al., 2002; Gregory et al., 2001; Lorincz et al., 2001; Nan et al., 1996; Nguyen et al., 2001; Rietveld et al., 2002). In the present study, we show that 12 randomly selected MeCP2 binding sites, eight of which were independently verified as methylated by bisulfite DNA sequencing, all lose their association with MeCP2 when DNA methylation is reduced by 5-azacytidine treatment. Taken together, these studies leave little doubt that MeCP2 functions as a methyl-CpG binding protein in vivo.

In vitro studies showed that MeCP2 can associate with nonmethylated fragments of genomic DNA and mouse satellite DNA, although DNA methylation, when present, significantly altered the pattern and increased the affinity of these interactions (Lewis et al., 1992; Weitzel et al., 1997). Analysis of binding sites for chicken MeCP2 (originally designated ARBP; von Kries et al., 1991) identified a common GGTGT motif, mutation of which reduced the affinity for MeCP2. Our in vitro binding site selection with mammalian MeCP2 did not lead to enrichment of the GGTGT motif in the tight binding population of DNA fragments. Interestingly, earlier studies proposed that MeCP2 binding to GGTGT is enhanced by a flanking A/T run, as two molecules that intercalate with A/T DNA interfered with binding to MeCP2 (Buhrmester et al., 1995). These findings might be explained if GGTGT represents a weak mimic of methyl-CpG, whose affinity for MeCP2 can be strengthened by a flanking run of A/T DNA. Given the current lack of evidence that MeCP2 binds nonmethylated DNA in vivo, the biological relevance of its putative DNA methylation-independent binding remains uncertain.

Experimental Procedures

Cell Culture

MRC-5 cells were cultured in MEM supplemented with 10% fetal calf serum, nonessential amino acids, sodium pyruvate, and antibiotics (Gibco). NIH3T3 cells were cultured in DMEM with 10% newborn calf serum, and $p53^{-/-}$ cells were cultured in DMEM supplemented with 10% newborn calf serum, nonessential amino acids, sodium pyruvate, and antibiotics (Gibco).

Chip and Clone

Chip-and-clone experiments were carried out as described previously (Sarraf and Stancheva, 2004) except that 3×10^8 MRC-5 cells were used as starting material. Amplified ChIP DNA was cloned into pGEM-T easy plasmid (Promega) and sequenced. The sequence information for MeCP2 and MBD2 bound loci is available upon request.

ChIP

The cells were crosslinked and chromatin was sonicated and immunoprecipitated as above. Histone H3 antibody (Upstate) was used as a positive control. The linear range of ChIP-PCR reactions was determined by amplifying serial dilutions of ChIP DNA. Typically 0.5 μl of MeCP2 and MBD2 ChIP DNA and 0.1 μl of H3 ChIP DNA were used per PCR reaction. Primer sequences for MeCP2 and MBD2 bound loci are available upon request.

Morpholino Transfections

MeCP2 and MBD2 morpholinos and control five-mismatch morpholino oligos were designed by and purchased from Gene Tools LLC (sequences available upon request). MRC-5 cells were transfected in T25 flasks according to the manufacturer's instructions, grown for 3 days, transferred into T75 flasks, transfected again, and left to grow for an additional 3 days. The cells from the first and the second round of transfection were collected, and nuclear extracts prepared according to the standard protocols and analyzed by Western blotting.

Expression of Recombinant MeCP2 and MBD2

Recombinant proteins were expressed in bacteria as described previously (Klose and Bird, 2004). All MeCP2 and MBD2 recombinant protein expression vectors were constructed in the same manner containing a C-terminal histidine tag to facilitate purification.

Methyl-SELEX, EMSA, and DNase I Footprinting

Detailed protocols for Methyl-SELEX, EMSA, and DNase I footprinting are available in the Supplemental Data online.

Microscopy and FRAP Experiments

Microscopy and FRAP analysis was carried out as reported previously (Schmiedeberg et al., 2004) and is described in detail in the Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, four figures, and three tables and are available with this article online at http://www.molecule.org/cgi/content/full/19/5/*bxs/DC1/.

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