Methyl CpG-binding proteins and transcriptional repression*

Paul A. Wade

Summary

Since its discovery, methylation of DNA in mammalian cells has been correlated with transcriptional repression and with specialized chromatin structures. Recently, considerable progress has been reported in the identification of protein factors with a highly conserved DNA interaction surface, termed the methyl CpG-binding domain or MBD. A subset has been biochemically linked to histone deacetylases, suggesting a molecular mechanism for the functional properties of methylated DNA. Despite several obvious attractions, the connection between MBD proteins and histone deacetylases fails to explain all the existing data. In fact, the biochemistry and DNA-binding properties of most MBD family members have not been adequately described and considerable evidence exists for alternative mechanisms in the repression of methylated loci. Null mutations have been generated in mice for several MBD family members, the phenotypes of the mutant animals raise important questions regarding the functions of the MBD family. Here, I review the biochemistry, DNA-binding properties, and genetics of the MBD proteins that are linked to transcriptional repression, namely, MeCP2, MBD1, MBD2, and MBD3. Several models to account for the functional properties of methylated DNA are presented. *BioEssays* 23:1131–1137, 2001. © 2001 John Wiley & Sons, Inc.

Introduction

The information content of mammalian DNA is not limited strictly to a linear sequence of bases. Like many other animals and plants, mammals modify their DNA through methylation of cytosine residues at the 5 position of the pyrimidine ring. In mammals, this modification occurs almost exclusively within the context of a simple dinucleotide site—CpG. Roughly 70% of all CpG dinucleotides in the mammalian genome are methylated, the majority of these sites occur in repetitive DNA elements. An amazing 45% of the human genome is composed of transposon-derived repeats;[1] these sequences are relatively CpG rich and are uniformly methylated. Cytosine methylation confers information at these loci; they are maintained in an irreversibly silent state, providing host defense against damage. In addition to transposon-derived repeats, high levels of cytosine methylation are observed on the inactive X-chromosome and at imprinted loci.[2] The majority of unmethylated CpG sites are located within the roughly 29,000 CpG islands found frequently near the promoter and first exon regions of protein coding genes.[1,3] The precise mechanisms by which these regions remain free of methylation remains a subject of controversy[4,5] but the consequence is a partitioning of the genome into active and inactive functional fractions.

In theory, there are several potential mechanisms that might lead to transcriptional repression at methylated loci. Certain transcription factors are unable to bind to their recognition sites within DNA when 5-methyl cytosine occurs within a critical base. DNA methylation might conceivably result in structural effects on local chromatin architecture through influencing nucleosome position or stability, or on higher order chromatin structure. While these are likely to be biologically significant regulatory mechanisms in some cases, it is widely believed that the repressive effects of DNA methylation result from selective recognition of the 5-methyl CpG dinucleotide by a conserved family of proteins, the methyl CpG-binding domain (MBD) family.

MeCP2—the founding member of the MBD family

The first member of the MBD family to be described at the molecular level was MeCP2.[6] It is a multidomain protein, containing the first methyl CpG-binding domain to be defined.[7] The protein is chromatin associated[8] and localizes to densely methylated regions (major satellite DNA) of the mouse genome.[9] In MeCP2, only a small portion of the protein is devoted to selective recognition of methyl CpG. It also contains a transcriptional repression domain (TRD) that overlaps a nuclear localization signal.[10] A region amino terminal to the MBD has no known function (see Fig. 1).

Emory University, Department of Pathology and Laboratory Medicine, Woodruff Memorial Research Building Room 7105B, 1639 Pierce Drive, Atlanta, GA 30322. E-mail: pwade@emory.edu

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Abbreviations: DNMT, DNA methyltransferase; HDAC, histone deacetylase; MAR, matrix attachment region; MBD, methyl CpG-binding domain; MeCP1, methyl CpG-binding protein 1; MeCP2, methyl CpG-binding protein 2; TF, transcription factor; TRD, transcriptional repression domain

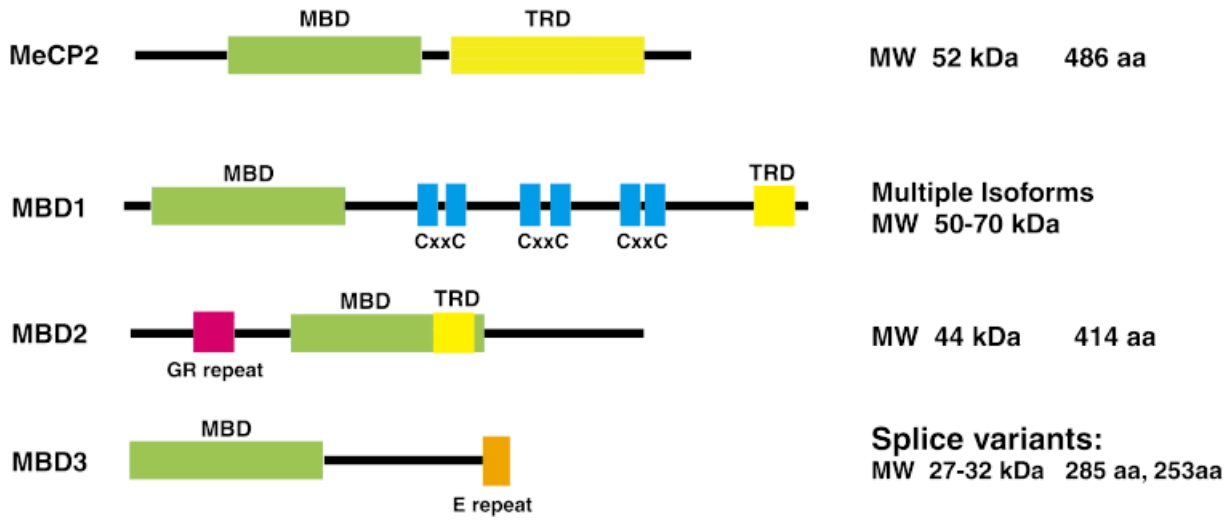

Figure 1. Domain organization of MBD family members. The cartoon depicts the organization of domains in MBD proteins. The various members of the MBD family implicated in transcriptional repression are depicted. Descriptions of the domain structure of each family member are found in the text. For each protein, the methyl CpG-binding domain is depicted as a green box, the transcriptional repression domain (where identified) as a yellow box. The CXXCXXC motifs of MBD1 are depicted as blue boxes (see text) and the GR repeats of MBD2 are depicted as a purple box. The acidic repeat at the carboxyl terminus is an orange box.

The carboxyl terminus of MeCP2 has unusual, repetitive sequences that are similar at the amino acid level to members of the fork head family.[11]

The DNA-binding properties of MeCP2 from mammals, birds, and amphibians have been analyzed. In a Southwestern assay, where the protein is immobilized on a membrane, full-length mammalian MeCP2 binds a single methylated CpG dinucleotide selectively regardless of sequence context.[6] The MBD domain itself also selectively binds a DNA fragment in solution containing a single methyl CpG dinucleotide.[7] The assignment of key residues within the MeCP2 MBD as crucial for either structural integrity or DNA interaction represents an important contribution to understanding the properties of the MBD.[12,13] Somewhat surprisingly, a chicken protein purified and cloned based on its ability to bind a specific sequence from a matrix attachment region of the lysozyme gene is chicken MeCP2.[14] The domain of the chicken protein required for specific binding to the MAR element overlaps the MBD defined in mammalian MeCP2.[14] In solution assays, chicken MeCP2 binds the lysozyme MAR element as well as repetitive DNA fragments from chicken and mouse satellite DNA. CpG methylation has at best two-fold stimulatory effects on binding.[14] Recombinant *Xenopus* MeCP2 binds selectively to methylated nucleosomal DNA in instances where the methyl CpG is solvent exposed.[15] Deletion of 60 residues from the carboxyl terminus, which removes a portion of the region with similarity to fork head proteins, compromises the ability to recognize methyl CpG on the nucleosome surface.[15] To date, there is no published report of a systematic study of the solution binding properties, including measurement of rate constants and preference for methylation density, of full-length MeCP2 from any species.

Transcriptional repression mediated by MeCP2 has been demonstrated both in vivo and in vitro. In vivo, tethering assays (see Fig. 2) have been the predominant tool utilized to localize the transcriptional repression functions of MeCP2. In mammalian cells, the minimal transcriptional repression domain of MeCP2 was localized to a region encompassing amino acids 207 to 310.[10] In addition, repression was observed even when the tethering sequence in the reporter construct was distant from the transcription start site.[10] Experiments using a heterologous system, *Drosophila* SL2 cells, however, came to a somewhat different conclusion. In transient transfection assays, MeCP2 repressed transcriptional activation by exogenous SP1 in a methylation-dependent manner, but the repressive function was mapped to a different region, namely amino acids 1–193.[16] A major breakthrough in the study of MeCP2-dependent repression came with the finding that MeCP2 was associated in cell extracts with histone deacetylases.[17,18] Solution interactions between MeCP2 and the transcriptional corepressor Sin3 were documented in both mammals and *Xenopus*.[17,18] Furthermore, the region of interaction with Sin3 on MeCP2 significantly overlapped the previously defined transcriptional repression domain[18] and tethering of the TRD resulted in repression that is sensitive to inhibitors of histone deacetylase.[17,18] These observations led

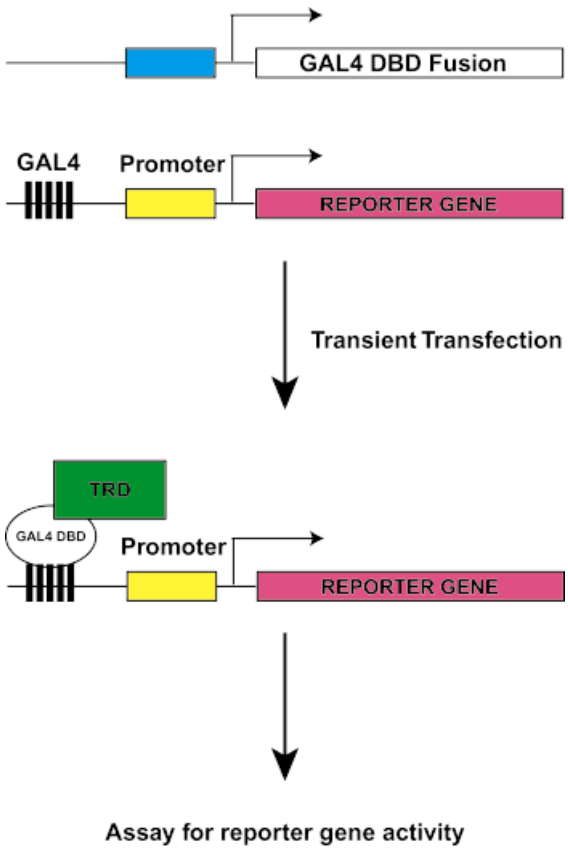

Figure 2. Schematic of a tethering assay. Two plasmids are cotransfected into a host cell. One plasmid serves as a reporter, it contains a core promoter with Gal4 sites upstream. The second plasmid encodes the Gal4-MBD protein fusion. Synthesis of this protein in the host cell leads to 'tethering' of the protein fragment following recognition of the Gal4-binding site by the Gal4 DNA-binding domain (GAL4 DBD).

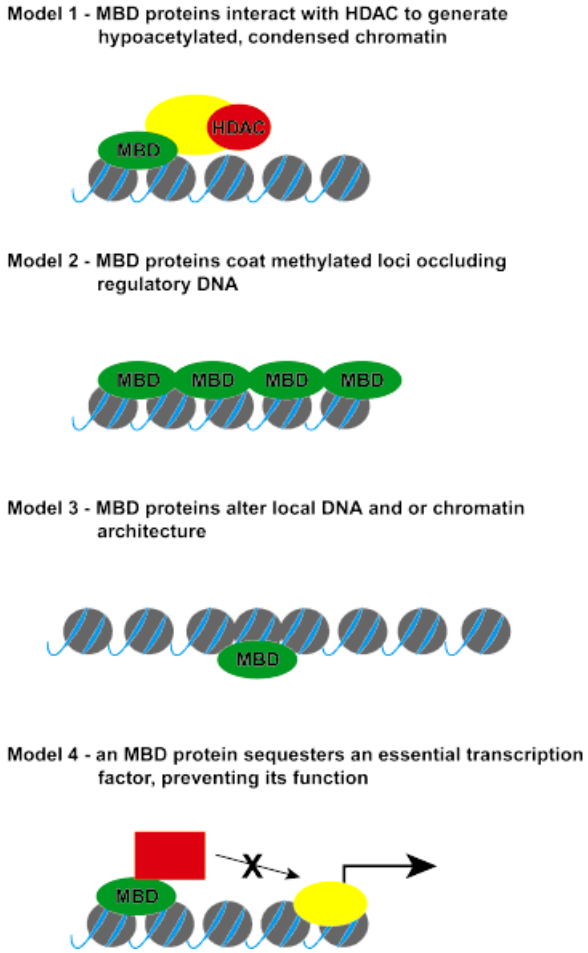

Figure 3. Potential mechanisms for repression mediated by MBD proteins. The figure depicts several mechanisms potentially utilized by MBD proteins to achieve transcriptional repression. In the cartoons, histone octamers are represented by gray balls and DNA is in blue. In Model 1, binding of an MBD protein to methylated sequences leads to local recruitment of histone deacetylase. The action of the deacetylase leads to hypoacetylation of histones and chromatin condensation. In Model 2, an MBD protein binds to methylated sequences and physically prevents access to this sequence by transcription factors. In Model 3, an MBD protein binds to methylated DNA and locally perturbs chromatin (or DNA) structure. In Model 4, an MBD protein directly interacts with an essential transcription factor, preventing its normal function.

to a currently popular model (see Fig. 3) that predicts that MeCP2 is recruited to methylated regions of the genome where its interactions with a protein complex containing Sin3 and histone deacetylases lead to the establishment and maintenance of repressive chromatin architecture.[2] In spite of the attractive features of this model, it is prudent to recall that, to date, MeCP2 has not been purified to homogeneity in its native state from any mammalian cell type and that the relative affinity of MeCP2 for Sin3 has not been accurately determined.

While this model is certainly an attractive one, it cannot explain all the data on transcriptional repression by MeCP2. While repression due to tethering of the TRD is sensitive to inhibitors of histone deacetylase, there is still considerable repression in the presence of these drugs.[17,18] In fact, the mechanism utilized for repression in the tethering assay may differ depending on the promoter and cell type used. Deacetylase-independent repression has been demonstrated in human and mouse cells on the SV40 promoter by tethering the minimal TRD element.[19] The molecular mechanism of this HDAC-independent repression is not clearly defined at this point but several potential mechanisms exist, including promoter occlusion by an overexpressed DNA-binding protein (see Fig. 3). Additional mechanisms have been suggested by

in vitro transcription experiments. In experiments with a highly purified system, full-length MeCP2 represses transcription in a methylation-dependent manner and tethering the TRD also leads to profound transcriptional repression on naked DNA. MeCP2 was found to interfere with preinitiation complex assembly, possibly through direct interaction of the TRD with TFIIB.[20] Additionally, both full-length MeCP2 and Gal4–TRD fusions were shown to have the capacity to organize DNA into large nucleoprotein complexes.[20] If we consider all the data, it seems likely that MeCP2 can utilize multiple pathways to achieve a repressed state (Fig. 3). Presumably, the choice of mechanism is influenced by cell type, by DNA sequence, and by local chromatin architecture.

MBD1

MBD1–MBD4 were all discovered as EST clones with sequence similarity to the MBD motif of MeCP2.[24,25] MBD1 was initially named PCM1, for Protein Containing MBD.[24] The cDNA sequence is characterized by the presence of multiple CXXCXXC sequences (see Fig. 1), similar to sequence motifs found in DNA methyltransferase I and human ALL1/HRX, a homolog of the *Drosophila* Trithorax protein.[24] The cDNA is alternatively spliced, with variations in the number of CXXC motifs as well as differences at the carboxyl terminus.[25,26] The MBD motif is located at the extreme amino terminus of the protein.[24] Like MeCP2, MBD1 has a region that represses transcription in tethering assays; it is located at the C terminus of the protein.[27,28] Full-length MBD1 binds methylated DNA in solution and exhibits a preference for densely methylated sequences.[27,28] One of the CXXC motifs also interacts with DNA, although in a methylation-independent manner.[28] When expressed as a GFP fusion, MBD1 localizes to major satellite DNA in mouse cells[25] and in human cells is dispersed throughout the euchromatin with concentration at the pericentromeric region of chromosome 1.[26] The endogenous protein is found concentrated at several pericentromeric regions as well as dispersed along the chromosome arms in metaphase chromosome spreads from diploid human cells.[27]

Transcriptional repression mediated by MBD1 has been assessed using several different approaches. In vitro transcription assays on naked DNA templates demonstrated that preincubation of methylated, but not unmethylated DNA, with MBD1 results in repression in a HeLa nuclear extract.[26] In transient transfection assays in mouse cells, MBD1 repressed transcription of methylated reporter constructs, repression required both the TRD and MBD motifs and was sensitive to HDAC inhibitors.[27] Mitsuyoshi Nakao and colleagues have undertaken a comprehensive study of transcriptional repression by MBD1 in various cell types. They have reported that some isoforms repress transcription from only methylated reporter constructs while others repress regardless of methylation status.[26,28] Further, these workers have identified the third CXXC motif as important for binding to and repressing transcription from unmethylated DNA.[28] Point mutations in conserved residues in the MBD motif compromise both nuclear localization and transcriptional repression.[28]

The MBD1 protein itself has not been purified from a native source, although it elutes from gel filtration consistent with a molecular mass of 200 to 400 kDa suggesting it may be a component of a multiprotein complex.[27] While repression mediated by MBD1 has been reported to be HDAC dependent,[27] the identity of the deacetylase is unknown. Whether HDAC-independent mechanisms of repression exist for MBD1 is currently unclear, although the in vitro repression data suggest that other mechanisms may be utilized by MBD1.

MBD2 and MBD3

MBD2 and MBD3 are the only members of the MBD family to share extensive sequence similarity outside the MBD motif.[25] Interestingly, mammalian MBD3 is also the only member of this protein family that lacks the capacity to selectively recognize methylated DNA.[25] The ability of both mammalian MBD2[25] and *Xenopus* MBD3[29] to bind methylated DNA, however, suggests that the ancestral form of this protein was a bona fide methyl CpG-binding protein, a function lost during the evolution of mammals but retained in amphibians.

MBD3 is biochemically the best-characterized member of the MBD family. Multiple groups have reported it as a component of a multiprotein complex containing a chromatin remodeling ATPase, a histone deacetylase and other proteins.[29–31] This complex is termed the Mi-2 complex,[32] the NuRD complex,[31] or HDAC 1 cII.[30] Each version of this complex differs slightly from the others, although the methodologies used for purification differ as well. Despite minor differences in polypeptide composition, it seems clear that this MBD complex functions as a transcriptional corepressor. Genetic evidence from *Drosophila* and *C. elegans* implicates it in repression of specific genes and suggests recruitment by DNA-binding transcription factors.[33] Presumably, the enzymatic functions of the Mi-2 ATPase and HDAC1/HDAC2 deacetylases are integral to the ability of this complex to repress target loci. Clearly, MBD3 is crucial to normal mammalian development as MBD3 knockout mice fail to develop to term.[34]

The biochemistry of MBD2 is less well understood. The mammalian protein can bind a single methyl CpG dinucleotide in solution binding assays and localizes to major satellite DNA when transfected into mouse cells as a GFP fusion.[25] Like MBD1 and MeCP2, MBD2 has a transcriptional repression domain identified in a tethering assay.[35] Repression mediated by tethering MBD2 is sensitive to HDAC inhibitors.[36] In MBD2, however, the TRD overlaps substantially with the MBD domain.[35] The amino terminus of MBD2 contains an extensive stretch of alternating glycine and

arginine residues.[25] There is a testis-specific form of MBD2 in the mouse;[25] its functional differentiation from the major somatic form of the protein is not understood.

In1999, MBD2 was reported to be a component of the long-sought MeCP1 complex[36] first described a decade previously and defined functionally as an activity that could bind methylated DNA in solution.[37] It was distinct from MeCP2 in that it required a densely methylated DNA fragment (11 or more methyl CpG sites) for productive binding.[37] An earlier report describing MBD1 as the MBD component of MeCP1[24] was ascribed to cross reaction of MBD1 antisera with other MBD proteins unknown at the time.[36] A question regarding MBD2 and the MeCP1 complex remains unanswered, however, namely why the MeCP1-binding activity should require multiple methyl CpG sites when MBD2 can selectively recognize a single methyl CpG in solution. Recently, it has been reported that MBD2 copurifies with a subset of the NuRD complex, which has been renamed MeCP1.[38] It is likewise unexplained why MBD2 was not observed in the initial purification of this complex[31,39] and why antisera specific for a component of NuRD fail to immunoprecipitate MBD2 from nuclear extracts.[27,31] The size discrepancy between the NuRD complex (1 MDa, Ref. 39) and MeCP1 (400 and 800 kDa, Ref. 37) is also unexplained. Whatever the eventual explanations for these discrepancies, it is clear that solution DNA-binding (gel shift) assays using methylated probes and crude extracts reveal different protein–DNA complexes depending on cell type.[34] Cells mutant for MBD2 also are clearly defective in their ability to generate a subset of these protein–DNA complexes, consistent with at least some of the characteristics of MeCP1.[34]

Genetic analysis of MBD family members

Genetics represents an invaluable tool for testing hypotheses based on biochemical and molecular experiments. Recently, two groups independently reported the phenotypes of MeCP2 null mutant mice.[21,22] Surprisingly, the mice are viable and fertile.[21,22] Males mutant for the X-linked MeCP2 gene, however, develop neurological symptoms and die prior to their wild-type littermates.[21,22] Both homozygous and heterozygous females also develop similar neurological symptoms although onset is delayed in heterozygous animals.[21,22] The phenotypic consequences of MeCP2 deletion in mice are strikingly similar to the symptoms of Rett Syndrome, a neurological disorder of human females caused by mutation of MeCP2.[21–23] Surprisingly, repression of methylated reporters in transfection assays in MeCP2 null fibroblasts was not severely affected.[22] The fact that these animals develop to term and are viable strongly suggests that MeCP2 has no essential role in dosage compensation, an essential function correlated with DNA methylation.[2]

In contrast to MeCP2, mutations in MBD2 have minimal phenotypes.[34] These mice have normal methylation patterns and exhibit no defects in genomic imprinting (including the *Xist* gene involved in X-inactivation), or silencing of endogenous transposable elements.[34] Despite the apparent lack of any defect at endogenous methylated loci, fibroblast lines derived from MBD2 null animals exhibit drastic defects in the ability to repress methylated reporter genes in transfection assays.[34] This finding is in sharp contrast to MeCP2 null mutant fibroblasts, which exhibit minimal defects in this assay.[22] The closely related MBD3 gene is essential for mouse development, as predicted from the phenotypes of Mi2/NuRD/HDAC1 cII complex subunits in other systems.[33,34]

The striking difference in the phenotypes observed between animals with null mutations in MBD family members as compared to DNA methyltransferases bears careful consideration. DNMT mutant mice fail to develop to term and mutant cell lines exhibit multiple defects in biology classically associated with DNA methylation, such as genomic imprinting, X-inactivation, and silencing of endogenous retrotransposons.[4] The sole MBD mutant required for mouse development is MBD3, a protein that fails to bind to methylated DNA.[25] How can we rationalize this discrepancy? At least two alternative explanations are currently viable. The formal possibility exists that the MBD family is not involved in transcriptional repression in vivo at all. There are several problems with this model including the probability that the neurological phenotypes exhibited by MeCP2 null animals result from alterations in patterns of gene expression. The lack of any molecular defect in events such as silencing of transposable elements or X-inactivation coupled with the inability (thusfar) to demonstrate any alterations in gene expression in the MeCP2 and MBD2 null animals, however, provide support for this hypothesis. The alternative view, consistent with the biochemical and molecular evidence summarized here, predicts that the MBD family does play a role in transcriptional repression but that the phenotypic consequences of mutations are clouded by functional redundancy. Examples exist in several systems for gene products to compensate for loss of function of another member of a gene family and the MBD family could well be one of these cases. Importantly, it is currently unclear whether MBD family members themselves represent the sole means of establishment of repression at methylated loci. Functional redundancy with other factors or roles in maintenance of repression established by other means might not be evident under standard laboratory conditions. The solution to this question will likely be answered with the generation of mutants in MBD1 and the eventual analysis of double and triple mutant animals.

What have we learned?

The past decade has seen the discovery of a family of proteins with the capacity to bind to methylated CpG sites. A great deal of work has led to a model ascribing the functional properties of methylated DNA to the biological action of the MBD protein

family. One facet of this model proposes that these proteins participate in the assembly of repressive local chromatin architecture at methylated regions of the genome through their interactions with histone deacetylases.[2] This model is certainly attractive and there is considerable biological evidence to support it. There is currently no reason, however, to preclude alternative mechanisms as a subset, perhaps even a majority, of methylated loci within the genome. The ability of MeCP2 to assemble naked methylated DNA into nucleoprotein complexes[20] suggests that at least this MBD family member has the capacity to organize DNA and potentially affect local chromatin architecture in the absence of any effect on the histones themselves. Likewise, the finding that human MeCP2 repressed transcription in heterologous cells and that repressive activity colocalized with the MBD motif[16] suggests that promoter occlusion or perturbation of local DNA or chromatin organization remains a viable mechanism for repression.

An important issue that remains to be addressed is the distribution of the different MBD family members in the genome. These proteins are fairly ubiquitous[25] and many cell and tissue types express multiple MBD proteins. Their distribution pattern will likely be dictated in large part by the binding properties of the proteins themselves. The definition of solution binding preferences of the various MBD family members on naked and nucleosomal DNA will be important in predicting their localization in the genome. However, these binding properties will undoubtedly be influenced both by association with other proteins and by local chromatin architecture.

It is currently unclear whether the MBD proteins are the primary means for establishment of repressive chromatin states at methylated loci. The relative lack of phenotype outside the central nervous system in mice lacking functional MeCP2[21,22] and MBD2[34] suggests that other factors may compensate for their absence. An obvious candidate is MBD1 and generation of mutations in this factor will be crucial to sorting out the relative importance of the MBD family to repression at methylated loci. Of course, it is by no means clear that every protein with the capacity to selectively recognize methylated DNA has been identified or even whether the MBD proteins are responsible for the establishment of repression. Recent reports have described the association of the mammalian DNA methyltransferases with enzymatic machinery capable of establishing a repressed state (reviewed in Ref. 40). These findings suggest that repression might be established during S phase concurrent with methylation and chromatin maturation, with the MBD proteins primarily responsible for maintenance.

Finally the association of specific MBD proteins with individual loci remains enigmatic. In a cell population that expresses multiple MBD family members, there could be a strict correspondence of a given MBD with a given locus or distribution could be random within the population. The phenotypes of the MeCP2[21,22] and MBD2[34] knockout animals suggest that any strict one-to-one correspondence is readily compensated on the loss of MeCP2 or MBD2. Supporting this notion, exogenous MBD2 and MeCP2 can restore repression in transfection assays in MBD2 mutant cell lines.[34] In contrast, a recent biochemical analysis of MBD occupancy on a methylated promoter in the context of human cancer cell lines concluded that there was a strong association of a single MBD protein with a specific locus.[41] This issue clearly requires further investigation and its resolution will provide many answers to the enduring questions of how the information content of DNA methylation is translated into functional states.

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