Promoter-specific Activation and Demethylation by MBD2/Demethylase*

MBD2 is the only member of a family of methyl-CpG-binding proteins that has been reported to be both a transcriptional repressor and a DNA demethylase (dMTase). To understand the apparently contradictory function of MBD2/dMTase, we studied the effects of dMTase overexpression on the activity of various in vitro methylated promoters transiently transfected into HEK293 cells. We found that forced expression of a MBD2/dMTase expression vector (His-dMTase) differentially activated two methylated reporters, pSV40-CAT (the SV40 enhancerless promoter adjacent to the chloramphenicol acetyltransferase (CAT) reporter gene) and pGL2T+I4xTBRE (a region of the p21 promoter next to the luciferase reporter gene), in a time- and dose-dependent manner. His-dMTase increased pSV40-CAT expression by 3-10-fold after 96 h, while pGL2T+I4xTBRE expression was increased by 2-3-fold after only 48 h and did not further increase at 96 h. Gene activation was not universal because no effect was seen with the p19-ARF promoter. We then assessed whether activation might be due to demethylation within the promoter region. Using bisulfite mapping, we found that exogenous expression of His-dMTase induced demethylation at 8 of the 10 CpG sites within the SV40 promoter. The observation that His-dMTase increases the demethylase activity in the cells was also confirmed using an in vitro CpG demethylase assay with a mC32pG oligonucleotide substrate and purified Q-Sepharose fractions from HEK293 cells transfected with His-dMTase or empty pcDNA3.1His vector. We propose that a single protein possessing both repressor and demethylase functions has evolved to coordinate a program that requires suppression of some methylated genes and activation of others.

The epigenome consists of an additional component that is part of the coherent structure of the genome, a coating of methyl groups. In vertebrates, 80% of cytosine residues within the dinucleotide sequence CpG are modified by methylation in a pattern that is tissue-specific and that is formed during development and maintained in somatic cells (1). It has been well established that the DNA methylation pattern is maintained exclusively by DNA methyltransferase activities, but we have recently proposed that DNA demethylation activities might also participate in the process (2-4) and that the methylation pattern is a steady state balance of reversible methylation-demethylation reactions (5, 6). We have shown that histone acetylation promotes active demethylation of ectopically methylated genes (3) and that inhibitors of histone acetylation inhibit demethylation (4).

It is well documented that the state of activity of a gene, the chromatin structure, and DNA methylation are correlated (7) such that areas of the genome that are methylated are usually less expressed. One molecular mechanism that explains this relationship has recently been elucidated. Repressor complexes are recruited to methylated DNA via the binding of methyl-CpG binding domain proteins (MBDs). These complexes contain proteins that have histone deacetylase and chromatin remodeling activities, leading to the formation of a more compact and transcriptionally inactive chromatin (8). The earliest discovered MBD, MeCP2, has been found to associate with the Sin3a co-repressor complex (8) and can also repress transcription in a histone deacetylase-independent manner (9). MBD1, MBD2, and MBD3 were later discovered and were also shown to be involved in transcriptional repression (for review, see Ref. 10).

In contrast, we have reported that MBD2 is an enzyme (dMTase) capable of actively demethylating DNA (2). This activity was shown both in vitro (2) and in vivo (3). A demethylase is expected to activate genes by removing the repressive methyl residues. The assignment of a demethylase function to a protein that was independently discovered as a recruiter of repressor complexes has triggered obvious controversy in the field (11), and several groups have reported that they failed to confirm the demethylase activity of MBD2 (11-13).

In this study we tested the hypothesis that MBD2 is a multifunctional protein and that its activity might depend on the context of the promoter with which it interacts. By examining the effects of MBD2/dMTase expression on the activity of various reporter constructs methylated in vitro and transfected into HEK293 cells, we found that MBD2/dMTase differentially activated some but not all promoters in a time- and concentration-dependent manner. Using bisulfite mapping, we found that exogenous expression of MBD2/dMTase induced demethylation within the SV40 promoter, and we also confirmed the demethylase activity of MBD2/dMTase in vitro. These data support our hypothesis that the complex functional role of this protein depends on the promoter context.

**EXPERIMENTAL PROCEDURES**

**In Vitro Methylation of Substrates—Enhancerless pSV40-CAT (GenBank™ accession no. X65320), pGL2T+I4xTBRE (14), or p19-ARF-LUC (kindly provided by Dr. V. Lobanenkov) were methylated in vitro by incubating 10 μg of plasmid DNA with 12 units of SssI CpG meth-
ytransferase (New England Biolabs) in the recommended buffer containing 800 μM S-adenosylmethionine for 3 h at 37 °C. Another 12 units of SsI and 0.16 μmol of S-adenosylmethionine were then added, and the reaction was further incubated another 3 h. The methylated plasmid was recovered by phenol/chloroform extraction and ethanol precipitation and complete methylation was confirmed by observing full protection from HpaII digestion.

Transient Transfections and Reporter Assays—HEK293 cells were plated at a density of 7 × 10⁴/well in a six-well dish and transiently transfected with 100 ng of reporter plasmid (methylated or mock-methylated) and 1.2 μg of one of the following plasmids: pcedNA3.1HisB vector (Invitrogen), pDNA-His-dMTase (2), which contains a Histag-human MBD2/demethylase cDNA as described in the Ref. 2, AdTrack-McPC2 (constructed from GST-McPC2 kindly provided by Dr. X. Nan; Ref. 15), or pcedNA3.1-Sp1 (16) using the calcium phosphate precipitation method as described previously (17). 0.3 μM trichostatin A (TSA) was added 24 h post-transfection, and cells were harvested after 48 or 96 h. Chloramphenicol acetyltransferase (CAT) assays were performed as described previously (17), and luciferase activity was assessed using the Promega luciferase assay system. The activity of each extract was measured in triplicate and then normalized to the protein concentration. Fold induction was calculated relative to the activity observed with the HisB vector alone. Experiments were performed several times using different cultures of HEK293 cells and different preparations of plasmids. For dose curve experiments, transfections were performed in triplicate using increasing amounts of HisB or His-dMTase vectors (0.05, 0.1, 0.6, 1.2, and 3 μg). Cells were then harvested after 96 h for CAT or luciferase assays. Fold induction was calculated relative to the activity observed with the HisB vector alone at each concentration.

Bisulfite Mapping—Bisulfite mapping was performed as described previously (18) with minor modifications. The SV40 promoter sequence was amplified from 5 μg of sodium bisulfite-treated DNA using the following primers: 5'-AAGGGGGATGTGTTGTAAG-3' (sense) and 5'-CTAAAATACCTCAAAATATTCTT-3' (antisense). PCR products were then used as templates for subsequent nested PCRs using the primers 5'-'GGTTAGTGAATTTTAGATTTGT-3' (sense) and 5'-TAATC-CAATAATTATTTTCTCC-3' (antisense). PCR products were then cloned using the TA cloning kit (Invitrogen), and clones were then sequenced using the T7 sequencing kit (Amersham Biosciences).

Western Blot Analysis—Whole cell extracts were prepared using radioimmune precipitation assay buffer according to the protocol from Santa Cruz Biotechnology. 50 μg of extract were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Transfected His-dMTase protein was detected either by anti-MBD2 sheep polyclonal IgG (Upstate Biotechnologies no. 07198) according to the manufacturer’s protocol or by anti-Xpress mouse monoclonal IgG antibody (Invitrogen R910-25), which recognizes the Xpress epitope within the pcDNA3.1His vector) at 1:5000 followed by peroxidase-conjugated antiserum R910-25, which recognizes the Xpress epitope within the pcDNA3.1His vector) at 1:5000 followed by peroxidase-conjugated antiserum (New England Biolabs) in the recommended buffer containing 8 μl of micrococcal nuclease were added followed by an overnight incubation at 37 °C. 2 μl of each sample were then resolved by thin layer chromatography and visualized by autoradiography.

RESULTS

Fig. 1. Recombinant MBD2/dMTase can activate certain promoters in a time-dependent manner. A, structure of the SV40 promoter-CAT and pGL2+14XTBRE constructs. B and C, HEK293 cells were transiently transfected with either SV40-CAT, pGL2+14XTBRE, or p19-ARF-LUC reporter plasmids (m, methylated in vitro with SsI) and either empty HisB vector or plasmids expressing His-dMTase, McPC2, or Sp1 using the calcium phosphate precipitation method. 0.3 μM TSA was added 24 h post-transfection. Cells were harvested after 48 or 96 h, and each extract was assayed for CAT or luciferase activity and then normalized to the protein concentration. Fold induction was calculated relative to the activity observed with the HisB vector alone. Experiments were performed several times using different cultures of HEK293 cells and different preparations of plasmids with similar results.

Determination of Demethylase Activity—HEK293 cells were transfected with 10 μg of His-dMTase/10-cm plate (10) or left untransfected, and nuclear extracts were prepared 48 h later as described previously (19). Approximately 8.5 mg of extract (1.4 ml) was diluted to 50 mM NaCl with 10 μl of buffer L (10 mM Tris-HCl, 10 mM MgCl₂, pH 7.8) containing a 1 μg/ml concentration of each of the following protease inhibitors: Pefabloc, aprotinin, and leupeptin. 2 ml of Q-Sepharose beads (Amersham Biosciences) were washed three times with 8 μl of buffer L with 50 mM NaCl and then pre-equilibrated for 30 min in the same buffer. Each extract (HEK or HEK + dMTase) was then subjected to 4 × 10 min washings, each for 0.25 ml of Q-Sepharose, with rotating at 4 °C. The beads were then pooled (1 ml/sample) and washed 5 × 10 min, each with 4 ml of buffer L with 50 mM NaCl. Batch elution was then performed with 5 × 1 ml of buffer L, each containing the following concentrations of NaCl: 0.2, 0.4, 0.6, 0.8, and 1.0 M for 10 min each. The different fractions, flow through, and washes were then assayed for demethylase activity.

Demethylation Assay—Demethylation activity was measured using a methyl-[3H]dG oligonucleotide as described previously (20) with minor modifications. 1 μl of a McPG oligonucleotide substrate (5′-[3H]dGTP-labeled) was incubated with 30 μl of buffer L and 20 μl of each of the purification samples for 48 h at 37 °C. Samples were then subjected to a phenol/chloroform extraction followed by ethan-ol precipitation and resuspension in 8 μl of double distilled H₂O. 1 μl of 10× micrococcal nuclease buffer (250 mM Tris-HCl, 10 mM CaCl₂) and 1 μl of micrococcal nuclease were added followed by an overnight incubation at 37 °C. 2 μl of each sample were then resolved by thin layer chromatography and visualized by autoradiography.

Recombinant MBD2/dMTase Can Activate Certain Promoters in a Time-dependent Manner—Since MBD2b was found to act as a DNA demethylase (2, 3) and since promoter demethylation is associated with gene activation (21), we first wanted to determine whether ectopic expression of MBD2/dMTase would lead to promoter activation. We performed various co-transfection experiments using several in vitro methylated reporter constructs. A long line of data has established that in vitro methylation can suppress genes when these genes are ectopically introduced into vertebrate cells (22). As expected, both the SV40 and 4XTBRE (a 4X repeat of the transforming growth factor-β response element of the p21 promoter) reporter constructs (Fig. 1A) were inhibited considerably by in vitro methylation (Fig. 1B). Co-transfection of His-dMTase led to activation of the SV40 promoter, but only after 96 h, while the 4XTBRE promoter was activated almost equally at both 48 and 96 h post-transfection. Similar results were obtained with TSA, which activates transcription by inhibiting histone deacetylases and which has also been found to induce DNA demethylation (3). Sp1 was used as a positive control for activation since both the SV40 and 4XTBRE promoters are highly enriched in Sp1 sites (Fig. 1C). Thus, MBD2/dMTase activates transcription seen with MBD2/dMTase is not universal since expression of MBD2/ dMTase was not able to activate the p19-ARF promoter even after 96 h (Fig. 1C). Thus MBD2/dMTase acts differently on different CG-rich ubiquitous promoters. If the co-repressors required for suppressing methylated promoters by MBDS are saturated in HEK293 cells, then this might explain why MBD2
Purified His-dMTase from HEK293 cells exhibits in vitro demethylase activity. A, HEK293 cells were transfected with His-dMTase (+) or harvested untransfected (−). Nuclear extracts were subjected to chromatography on Q-Sepharose, and the active demethylase fractions were eluted with a step gradient of NaCl. His-dMTase elutes at 0.2 and 0.4 M NaCl steps. The fractions were tested by a subjecting the DNA to a step gradient of NaCl, tested for the presence of His-dMTase in the active fractions (indicated by the arrow). B, the fractions were assayed for demethylase activity using a deoxycytidine 3'-monophosphate. C, the 0.2 M NaCl fraction was assayed in triplicate, and the degree of demethylation was quantified by densitometry.

Dose-dependent activation by MBD2/dMTase. A and B, transfections were performed as in Fig. 1 using increasing amounts of HisB or His-dMTase vector. Cells were harvested after 96 h for CAT or luciferase assays. Fold induction was calculated relative to the activity observed with the HisB vector alone at each concentration. C, cells were transfected as in A, and radioimmune precipitation assay extracts were subjected to Western blotting using anti-MBD2 antibody (Upstate Biotechnologies).

Expression of His-dMTase increases demethylation in the SV40 promoter. A, nucleotide sequence of SV40 promoter region analyzed by bisulfite mapping. CpG dinucleotides whose methylation status was determined are numbered (1–10), and Sp1 sites are marked A–F. B, the SV40 construct in Fig. 1A was methylated in vitro and transfected into HEK293 cells with either His-dMTase (+dMTase) or HisB vector (control). Cells were harvested 96 h post-transfection, and DNA was isolated and treated with sodium bisulfite. The region in A was amplified by PCR, subcloned, and sequenced. The graph indicates the average percentage of demethylation at each CpG site. Results are an average of three independent experiments, where n is the total number of clones sequenced. C, representative CAT assay from one of the three experiments analyzed by bisulfite mapping in B, a.u., arbitrary units.

MBD2 confirms that the His-dMTase construct is expressed in HEK293 cells (Fig. 2C) and demonstrates that the protein levels correlate with the transcriptional activation observed. These results, together with those in Fig. 1, indicate that MBD2/dMTase can function as a dose-dependent activator of gene transcription, which is both time- and promoter-dependent.

Promoter-specific Activation by MBD2/Demethylase

Dose-dependent activation by MBD2/dMTase—to provide further evidence that MBD2/dMTase can act as a transcriptional activator, we transfected the methylated SV40 or 4xTBRE reporters along with different amounts of pcDNA3.1-His-dMTase. As seen in Fig. 2, A and B, while we observed a general dose-dependent increase in activation of both promoters, these promoters also varied slightly in their sensitivity to the amount of transfected His-dMTase as well as in the magnitude of their response, similar to the observations presented in Fig. 1. A Western blot using an antibody against MBD2 confirms that the His-dMTase construct is expressed in HEK293 cells (Fig. 2C) and demonstrates that the protein levels correlate with the transcriptional activation observed. These results, together with those in Fig. 1, indicate that MBD2/dMTase can function as a dose-dependent activator of gene transcription, which is both time- and promoter-dependent.

Expression of His-dMTase Increases Demethylation of the SV40 Promoter Region—Since demethylation within a pro-
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Motor is associated with transcriptional activation (21), we next wanted to determine whether this was a possible mechanism by which MBD2/dMTase overexpression led to the activation of the SV40 promoter. Following transfection of methylated SV40-CAT along with His-dMTase or empty vector as a control, we used bisulfite mapping to examine the 10 different CpG sites within the SV40 promoter (Fig. 4A). As shown in Fig. 4B, expression of dMTase increased the level of demethylation at 8 of the 10 CpG sites. Remarkably, most of the CpG sites remained fully or almost (<5%) fully methylated in the control clones with the exception of site 6. Since between 20 and 30 clones were sequenced from three independent experiments, it is highly unlikely that this is a random event. These results are also consistent with previous studies demonstrating that exogenous expression of MBD2/dMTase leads to increased demethylation within a promoter (3) and that expression of MBD2/dMTase is correlated with demethylation within the promoters of c-erbB-2 and survivin genes (23). We also assessed the CAT activity from the same transfections used for bisulfite mapping and found that, as in our previous experiments, dMTase overexpression led to transcriptional activation of the SV40 promoter (Fig. 4C). This supports the hypothesis that the likely mechanism by which dMTase causes transcriptional activation is by demethylating the promoter and thus allowing an open chromatin configuration free of transcriptional repressor complexes.

DISCUSSION

Our study provides evidence that MBD2/dMTase can act as a transcriptional activator, consistent with its role as a DNA demethylase. However, the activation observed is critically dependent on several parameters, including promoter identity and length of transcription time (Fig. 1). Our results are consistent with previous data, demonstrating that MBD2/dMTase is an active demethylase in vitro (2) and that transfection of exogenous dMTase can lead to demethylation in living cells with a concomitant increase in gene expression (3).

The activation of methylated promoters by MBD2/dMTase is partial as is the demethylation. This suggests the presence of other factors that protect the DNA from complete demethylation and activation of transcription.

Although it is accepted in recent publications that MBD2 acts as a transcriptional repressor (11, 12, 24, 25), the data presented here do not necessarily contradict previously published data once the experimental conditions stated in these reports are carefully considered. First, in all the studies demonstrating repression by MBD2/dMTase, transcriptional assays were performed anywhere from 24 to 48 h post-transfection. Since we do not see activation of the SV40 promoter until 96 h post-transfection, it is possible that some of the promoters in these reports could be activated by MBD2/dMTase provided that the transfection time is extended. The fact that a longer time is necessary for activation by MBD2/demethylase may be explained by previous data showing that demethylation of ectopically methylated DNA in living cells is a slow process (3). Second, previous studies (11, 25) used the Gal4 DNA binding domain to bind MBD2/GAL4 chimeras to their promoters rather that looking at the effect of cognate MBD2 on methylated DNA as done in this study, which may also account for some of the discrepancies. Third, not all promoters are repressed by MBD2, and not all concentrations of transfected MBD2 bring about repression. For example, Boeke et al. (12) demonstrated that although the TK promoter was repressed by methylase, transfection of MBD2 had no effect; and in a study by Sekimata et al. (25), certain concentrations of transfected Gal4-MBD2 alone did not repress their reporter construct. The data presented above are consistent with our findings that MBD2 had no effect on the p19-ARF promoter (Fig. 1C) and that the effect on transcription we observed is dose-dependent, where the dose causing activation varies with the promoter type (Fig. 2, A and B). Last, although MBD2 has been found to associate with the NuRD repressor complex (24), it was not originally purified as part of this complex (26), and there is evidence that the NuRD complex may associate with different DNA-binding proteins, not only MBD2, depending on the physiological state of the cell (24). Thus, it is possible that under certain cellular conditions and within certain promoters MBD2/dMTase may act as a transcriptional repressor by recruiting the NuRD complex. However, it is equally possible that in a different cell environment and within different promoters MBD2 may act independently of NuRD as a demethylase and activator. In support of the latter, expression of dMTase is correlated with demethylation within the promoter of c-erbB-2 and survivin genes (23). In addition, a recent report has demonstrated that the Drosophila homolog of MBD2, dMBD2/3, formed foci that associated with DNA at the cellular blastoderm stage, concurrent with the activation of the embryonic genome, and also associated with the active Y chromosome (27).

Several proteins have been found to possess dual repressor and activator functions, such as E2F (28), ATF4 (29), and the Sp family of proteins (30), so it is similarly feasible that MBD2 is a protein with dual functions. It is possible that both repressor and demethylase functions reside in one protein to coordinate a program of gene expression that requires suppression of some methylated genes and activation of others. Further studies will be necessary to determine what are the key factors involved in determining the role of MBD2/dMTase in transcription.

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