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Impact of Base Analogues within a CpG Dinucleotide on the Binding of DNA by the Methyl-Binding Domain of MeCP2 and Methylation by DNMT1[†]

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ABSTRACT: The epigenetic control of transcription requires the selective recognition of methylated CpG dinucleotides by methylation-sensitive sequence-specific DNA binding proteins. In order to probe the mechanism of selective interaction of the methyl-binding protein with methylated DNA, we have prepared a series of oligonucleotides containing modified purines and pyrimidines at the recognition site, and we have examined the binding of these oligonucleotides to the methyl-binding domain (MBD) of the methyl-CpG-binding protein 2 (MeCP2). Our results suggest that pyrimidine 5-substituents similar in size to a methyl group facilitate protein binding; however, binding affinity does not correlate with the hydrophobicity of the substituent, and neither the 4-amino group of 5-methylcytosine (mC) nor Watson−Crick base pair geometry is essential for MBD binding. However, 5-substituted uracil analogues in one strand do not direct human DNA methyltransferase 1 (DNMT1) methylation of the opposing strand, as does mC. Important recognition elements do include the guanine O6 and N7 atoms present in the major groove. Unexpectedly, removal of the guanine 2-amino group from the minor groove substantially enhances MBD binding, likely resulting from DNA bending at the substitution site. The enhanced binding of the MBD to oligonucleotides containing several cytosine analogues observed here is better explained by a DNA−protein interface mediated by structured water as opposed to hydrophobic interactions.

The control of gene expression in higher organisms requires an array of interacting DNA sequence and protein partners functioning at multiple levels. At the DNA level it is now established that enzymatic methylation of specific cytosine residues, generally within promoter regions, is associated with gene silencing (*1*−*3*). Cytosine methylation at the CpG dinucleotide substantially increases the binding of a group of proteins that share a conserved methyl-binding domain (MBD)[1] (*4*−*6*). Proteins containing the MBD then recruit histone-modifying enzymes, resulting in the formation of a compact chromatin structure less accessible for transcription (*7*). As the methylation-dependent binding of the MBDs would initiate the cascade of events resulting in chromatin condensation and gene silencing, it is important to understand the features of the methylated CpG dinucleotide that facilitate the initial binding and to understand how DNA damage and nucleoside analogues might interfere with or augment the binding of MBD-containing proteins.

In this paper, we have constructed a series of oligonucleotides (ODNs) containing both normal and modified bases. The binding of purified MBD to these analogues has been measured in quantitative electrophoretic mobility shift assays (EMSAs). We have investigated the impact of several 5-substituted pyrimidine analogues on MBD binding, including the 5-halogenated pyrimidine analogues, oxidation damage products, and nucleoside analogues known to perturb methylation patterns in cells (*6*, *8*). We have also investigated the impact of several purine analogues paired opposite cytosine and 5-methylcytosine (mC) on MBD binding. Our results are compared with the findings of a recently published crystal structure of a DNA−MeCP2 complex (*9*). Collectively, these results reveal that substituents close in size to a methyl group facilitate binding for both cytosine and uracil analogues paired with guanine. However, substituent hydrophobicity does not appear to be a significant factor. The impact of several purines on binding was also studied. It was found that the O6 and N7 positions of guanine are critical for MBD binding and that substitution of hypoxanthine surprisingly increases the binding in unmethylated sequences. Results presented here demonstrate that the mechanism of sequence recognition by the MBD is distinct from that of the methyltransferase DNMT1 in that DNMT1 does not methylate a CpG dinucleotide containing a T-G mispair. The results of the studies reported here will facilitate an understanding of the binding of proteins containing the MBD to the CpG dinucleotide and suggest mechanisms by which DNA modification or damage might decrease or enhance protein binding at this early step of epigenetic programming.

MATERIALS AND METHODS

Oligonucleotide Synthesis. The majority of the ODNs used in this study were prepared by standard solid-phase synthesis using either the Gene Assembler Plus (Pharmacia, now part of

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[1]Abbreviations: DNMT1, DNA methyltransferase 1; MBD, methyl-binding domain; MeCP2, methyl-CpG-binding protein 2; ODNs, oligonucleotides; mC, 5-methylcytosine; HmC, 5-hydroxymethylcytosine; ClC, 5-chlorocytosine; FC, 5-fluorocytosine; 2AP, 2-aminopurine; FU, 5-fluorouracil; ClU, 5-chlorouracil; BrU, 5-bromouracil; IU, 5-iodouracil; dzG, 7-deazaguanine; oxoG, 8-oxoguanine; Hx, hypoxanthine; Neb, nebularine; 26Di, 2,6-diaminopurine; Xan, xanthine; pdC, 5-propynylcytosine; pyC, pyrrolo-dC; HoC, 5-hydroxycytosine.

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GE Healthcare, Piscataway, NJ) or Expedite Nucleic Acid Synthesis System (Applied Biosystems, Foster City, CA) automated DNA synthesizers. The ODN 27-mers containing a central CpG dinucleotide were synthesized with systematic replacement of the cytosine within the central CpG with pyrimidine analogues, as well as systematic replacement of guanine in the central CpG with purine analogues (Figure 1). The sequence used in this study was chosen based upon binding experiments previously conducted with the methyl-binding domain (MBD) of MeCP2 (*5*, *6*). A "Y" in the sequence (Figure 1) indicates the position where C in the normal sequence is substituted with a pyrimidine derivative. The "P" symbol indicates the position where G is substituted with a purine derivative.

The preparation and synthesis of ODNs containing 5-hydroxymethylcytosine (HmC) (*10*), 5-chlorocytosine (ClC) (*11*), 5-fluorocytosine (FC) (*12*), and 2-aminopurine (2AP) (*13*) have been previously reported by this laboratory. ODNs containing normal bases and analogues, including 5-fluorouracil (FU), 5-bromouracil (BrU), 5-iodouracil (IU), 5-methylcytosine (mC), 7-deazaguanine (dzG), 8-oxoguanine (oxoG), hypoxanthine (Hx), nebularine (Neb), 2,6-diaminopurine (26Di), and xanthine (Xan), were prepared with phosphoramidites from Glenn Research (Sterling, VA). ODNs containing zebularine (*14*), 5-methylzebularine (*15*), 5-azacytosine (*16*), and 5-chlorouracil (ClU) (*17*) were prepared by published procedures. ODNs containing 5-propynylcytosine (pdC), pyrrolo-dC (pyC), and BrU were obtained from TriLink Biotechnologies (San Diego, CA).

In general, ODNs were removed from the solid support and deprotected in aqueous ammonia (Aldrich) at 60 °C overnight. The deprotected ODNs were purified with Poly-Pak II cartridges (Glen Research). The base composition of the ODNs was confirmed via HPLC analysis following enzymatic digest of the oligonucleotides with nuclease P1 (Sigma, St. Louis, MO) at 37 °C for 1 h and bacterial alkaline phosphatase (Sigma) at 37 °C overnight.

Protein Expression and Purification. The pAFB105 construct encoding 6× His-tagged MBD of mouse MeCP2, residues 77−165, in a pET6H vector, was overexpressed in *Escherichia coli* BL21 (DE3)/pLysS and purified as previously described, according to the protocol of Free et al. (*5*, *6*).

Electrophoretic Mobility Shift Assay. The 27-mer ODNs were 5′-^{32}P-end labeled by T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) with [γ-^{32}P]ATP (MP Biomedicals, Costa Mesa, CA) under conditions recommended by the enzyme supplier and purified using G50 Sephadex columns (Roche, Indianapolis, IN). The labeled strand was annealed with a 1.5-fold excess of the complementary unlabeled strand in 20 mM HEPES, pH 7.3, and 1 mM EDTA at 95 °C for 5 min and then allowed to slowly cool to room temperature. Previous data indicate that placement of the base lesion in either the top or bottom strand yields the same binding results (*6*). To confirm duplex formation and the presence of cytosine modifications for 5-halocytosine containing duplexes, the annealed ODNs were digested with *Msp*I and *Hpa*II (New England Biolabs) according to conditions recommended by the enzyme supplier, and the products were visualized on denaturing polyacrylamide gels [20% (20 g/100 mL of H_2O) acrylamide, acrylamide:bisacrylamide, 19:1] containing 8 M urea (*18*) . *Msp*I was able to cleave duplexes in which the cytosine was modified, both symmetrically and asymmetrically, in the CpG site (data not shown). However, *Hpa*II was only able to cleave the unmodified cytosine-containing duplexes (data not shown).

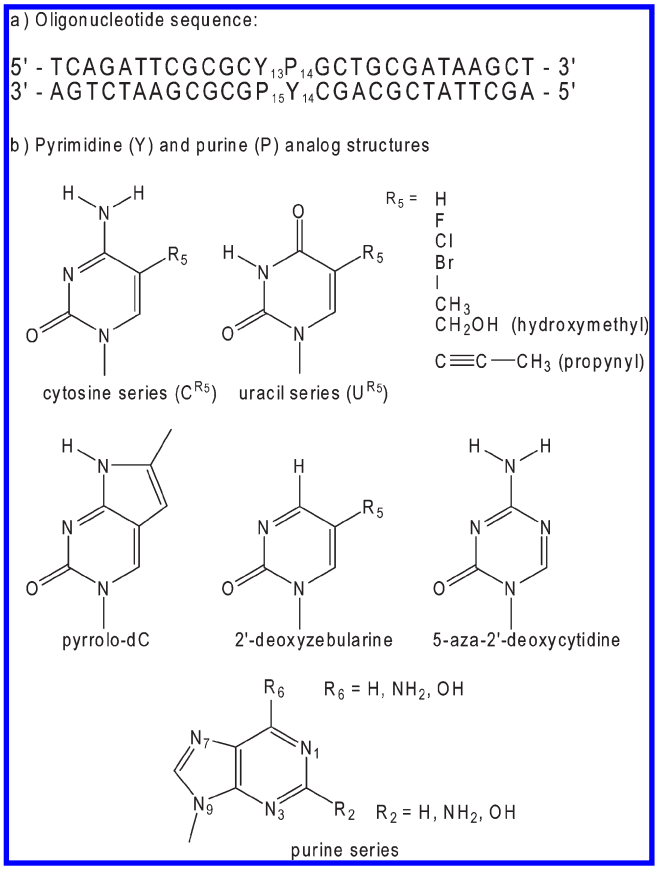

FIGURE 1: Oligonucleotide sequence and analogues used in quantitative electrophoretic mobility shift assays with the methyl-binding domain of MeCP2. (a) Oligonucleotide 27-mer with a central CpG dinucleotide sequence. Y denotes location of pyrimidine analogue substitutions. P denotes location of purine analogue substitutions. (b) Structure of purine and pyrimidine analogues. R_5 represents the 5-substituent of the pyrimidine ring. For the cytosine series, when R_5 is H, the structure is cytosine. When R_5 is F, the structure is 5-fluorocytosine. When R_5 is Cl, the structure is 5-chlorocytosine. When R_5 is Br, the structure is 5-bromocytosine. When R_5 is I, the structure is 5-iodocytosine. When R_5 is CH_3, the structure is 5-methylcytosine. When R_5 is CH_2OH, the structure is 5-hydroxymethylcytosine. When R_5 is a propynyl group as illustrated, the structure is 5-propynylcytosine. For the uracil series, when R_5 is H, the structure is uracil. When R_5 is F, the structure is 5-fluorouracil. When R_5 is Cl, the structure is 5-chlorouracil. When R_5 is Br, the structure is 5-bromouracil. When R_5 is I, the structure is 5-iodoracil. When R_5 is CH_3, the structure is thymine. The structures for pyrrolo-dC, 2′-deoxyzebularine, and 5-aza-2′-deoxycytidine are as shown. For the purine series, when R_6 is OH and R_2 is NH_2, the structure is guanine. When R_6 is OH, R_2 is NH_2, and N_7 is replaced with a carbon, the structure is 7-deazaguanine. When R_6 is OH, R_2 is NH_2, and OH is present as a 8-substituent, the structure is 8-oxoguanine. When R_6 is OH and R_2 is H, the structure is hypoxanthine. When R_6 is H and R_2 is H, the structure is nebularine. When R_6 is NH_2 and R_2 is H, the structure is adenine. When R_6 is H and R_2 is NH_2, the structure is 2-aminopurine. When R_6 is NH_2 and R_2 is NH_2, the structure is 2,6-diaminoadenine. When R_6 is OH and R_2 is OH, the structure is xanthine.

Purified MBD (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 nM) was incubated with 2 nM labeled duplex and 50 ng/μL poly[dA-dT]·poly[dA-dT] (Sigma) in 20 mM HEPES, pH 7.3, 1 mM EDTA, 10 nM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 0.2% Tween 20, and 30 mM KCl for 15 min at room temperature in a 30 μL reaction volume, prior to the addition of 7.5 μL of loading buffer (60% 0.25× TBE, 40% glycerol) (*5*, *6*). Note that for zebularine- and 5-azacytosine-containing ODNs, the poor binding with MBD required higher concentrations of MBD, and

concentrations up to 2000 nM were required to obtain sufficient binding for regression analysis. The binding reactions were then electrophoresed on 10% nondenaturing polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels at 250 V for 2.5 h at 4 °C after prerunning the gel at 200 V for 1.5 h. Visualization and quantification of the gels were carried out using a phosphorimager and the ImageQuant 5.0 software (Amersham Biosciences, now part of GE Healthcare).

Binding Model and Data Analysis. The noncooperative, single-site binding scheme previously used to describe the monomeric binding of the MBD of MeCP2 to its symmetrically methylated DNA substrate was used in this study (*6*). The fraction of duplex bound was determined from the EMSA data at each protein concentration in the following manner, where [E] is the concentration of unbound MBD, [O] is the concentration of unbound ODN, and [EO] is the MBD−ODN complex:

$$\text{fraction of duplex bound} = [EO]/([EO] + [O])$$

When $[E] \gg [O]$, then $[E]_{total} - [EO] = [E] \approx [E]_{total}$, where $[E]_{total}$ is the total concentration of MBD (bound and unbound), the following equation can be used to determine the dissociation constant (K_d, nM) for the ODN duplexes used in the study:

$$\text{fraction of duplex bound} = [E]_{total}/([E]_{total} + K_d)$$

A minimum of three data sets for each duplex were averaged and subsequently used to fit the above equation by nonlinear regression using SigmaPlot 10.0 software (SPSS Science).

DNMT1 Methylation-Restriction Endonuclease Protection Assay. DNMT1 methylation protection assays were performed as previously described (*19*). DNMT1 methyltransferase and cofactors were obtained from New England Biolabs. The 27-mer oligonucleotide containing cytosine within the central CpG (Figure 1a, bottom strand) was 5′-^{32}P-end labeled by T4 polynucleotide kinase (New England Biolabs) with [γ-^{32}P]ATP (MP Biomedicals) under conditions recommended by the enzyme supplier and subsequently purified using G50 Sephadex columns (Roche). Labeled strands were mixed with a 2-fold excess of the complementary strand containing the specific cytosine modifications (Figure 1a, top strand) in 10 mM Tris-HCl (pH 7.0), incubated at 95 °C for 5 min, and allowed to cool slowly to room temperature. The labeled duplex (1 pmol) was incubated with 12 units of DNMT1 (~8 pmol), 1.6 mM *S*-adenosylmethionine, and 1 μg/mL bovine serum albumin in 1× DNMT1 reaction buffer [50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 5% glycerol (pH 7.8)] in a final volume of 25 μL for 4 h at 37 °C. One unit of DNMT1 is defined as the amount of enzyme required to catalyze the transfer of 1 pmol of methyl group to poly(deoxyinosinic-deoxycytidylic acid) substrate in a total reaction volume of 25 μL in 30 min at 37 °C. The reactions were stopped by incubating at 65 °C for 20 min. Oligonucleotides were then purified through G50 Sephadex columns and reannealed in 10 mM Tris-HCl with a 250-fold excess of the unmodified oligonucleotide. The unmodified competitor oligonucleotide contained only normal nucleotides and corresponds to the upper strand in Figure 1a, where Y = cytosine and P = guanine. After reannealing, the duplex was incubated with 20 units of *Hpa*II restriction endonuclease (New England Biolabs) in a total volume of 25 μL with 1X NEB 1 buffer [10 mM Bis-Tris-propane-HCl, 10 mM $MgCl_2$, 1 mM DTT (pH 7.0); New England Biolabs]. The reactions were incubated for 2 h at 37 °C and stopped using equal volumes of Maxam−Gilbert loading buffer (98% formamide, 0.01 mol/L EDTA, 1 mg/mL xylene cyanole, 1 mg/mL bromophenol blue). The reaction products were electrophoresed on denaturing polyacrylamide gels [20% (20 g/100 mL of H_2O) acrylamide, acrylamide:bisacrylamide, 19:1] containing 8 M urea and visualized and quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant 5.0 software (Molecular Dynamics).

Table 1: Dissociation Constants of the MBD of MeCP2 Binding to 27-mer Duplexes Containing the Series of 5-Halogenated Cytosine Analogues, 5-Halogenated Uracil Analogues, and Methyltransferase Inhibitors within a CpG or MethylCpG Sequence Context[a]

X	CG GC^X	R_2	^{m}CG GC^X	R_2	^{m}CG GU^X	R_2
H	1030 ± 20	0.99	127 ± 3	0.99	92.6 ± 4.7	0.99
F	393 ± 5.3	0.99	38.2 ± 4.3	0.96	73.8 ± 4.6	0.99
Cl	131 ± 4	0.99	11 ± 0.9	0.99	36 ± 4	0.97
Br	102 ± 3	0.99	10 ± 1	0.99	29.0* ± 1.8	0.99
I	96.8 ± 3.9	0.99	11.5 ± 1.2	0.98	23.9 ± 1.9	0.98
CH_3	152 ± 4	0.99	14.7 ± 1.0	0.99	17.5 ± 2.0	0.97
OH	418 ± 2.8	0.99	45.1 ± 3.3	0.98		
CH_2OH	953 ± 11	0.99	151 ± 7	0.98		
pyC	758 ± 15	0.99	115 ± 5.9	0.99		
pdC	1220 ± 17	0.99	171 ± 7.2	0.99		
mZeb	718 ± 23	0.99	126 ± 7.6	0.98		
Zeb	889 ± 40	0.99	234 ± 22	0.94		
DAC	640 ± 46	0.97	147 ± 19	0.90		

[a]The K_d (nM) for MBD binding to each of the duplexes were obtained via nonlinear regression of three or more data sets obtained from EMSA. The plot of the average percent binding of each duplex against the concentration of MBD was fitted to the equation for simple, noncooperative monomeric binding (see Materials and Methods). The ranges seen in the K_d values are the standard error for the regression analysis.

RESULTS

A series of ODNs containing purine and pyrimidine analogues within a central CpG dinucleotide was prepared by solid-phase synthesis. The sequence of the ODNs in this series is shown in Figure 1, along with the structures of the base analogues examined. The binding constants of the ODNs containing the base analogues with the MBD of MeCP2 were measured quantitatively using EMSA, as previously described (*6*). Dissociation constants are presented in Tables 1, 2, and 3.

In this study, the 5-fluoro, 5-chloro, 5-bromo, and 5-iodo analogues of both cytosine and uracil were examined when paired opposite guanine. The magnitude of the binding of the MBD to the oligonucleotide increased, as indicated by decreasing K_d (nM), as the size of the 5-substituent increased in both the cytosine and uracil series (Table 1). The binding to the cytosine series was stronger than the binding to the uracil series; however, the observed difference between the two series was less than a factor of 3.

Additional analogues were also examined to gauge the impact of steric bulk on the interaction of the MBD with DNA. The pdC analogue pushes the methyl group further away from the base pairs into the major groove and results in decreased binding. Decreased binding is also observed with the bulky pyC analogue (see Figure 1 for structures). The oxidation damage product, HmC, shows decreased binding relative to 5mC (*6*), but surprisingly, increased binding relative to C is observed for the oxidation damage product 5-hydroxycytosine (HoC) (Figure 2).

A pyrimidine with a 5-substituent similar in size to a methyl group, when paired with guanine in a CpG dinucleotide, promotes MBD binding (Table 1). We previously demonstrated that a cytosine residue in a CpG dinucleotide with a 5-substituent similar in size to a methyl group also promotes DNMT1-directed

Table 2: Dissociation Constants of the MBD of MeCP2 Binding to 27-mer Duplexes Containing the Series of Purine Analogues within a CpG or MethylCpG Sequence Context[a]

X	^{m}CG XC^{m}	R_2	^{m}CG XC	R_2	CG XC^{m}	R_2	CG XC	R_2
G	14.7 ± 1.0	0.99	127 ± 3	0.99	152 ± 4	0.99	1030 ± 20	0.90
dzG	116 ± 1.6	0.99	729 ± 20	0.99	478 ± 9.7	0.99	1920 ± 110	0.97
oxoG	292 ± 11	0.99	1110 ± 41	0.98	1930 ± 130	0.96	2130 ± 49	0.99
Hx	15.4 ± 2.2	0.95	33.8 ± 2.3	0.99	41.2 ± 1.8	0.99	149 ± 3.5	0.99
Neb	523 ± 6.3	0.99	1250 ± 11	0.99	1020 ± 6.9	0.99	1420 ± 15	0.99
2AP	235 ± 8.8	0.99	1480 ± 29	0.99	318 ± 7.4	0.99	2400 ± 35	0.99
26Di	181 ± 3.6	0.99	2360 ± 560	0.68	218 ± 4.8	0.99	2190 ± 190	0.93
Xan	102 ± 11	0.95	448 ± 9.6	0.99	134 ± 2.8	0.99	444 ± 3.3	0.99

[a]The K_d (nM) for MBD binding to each of the duplexes were obtained via nonlinear regression of three or more data sets obtained from EMSA. The plot of the average percent binding of each duplex against the concentration of MBD was fitted to the equation for simple, noncooperative monomeric binding (see Materials and Methods). The ranges seen in the K_d values are the standard error for the regression analysis.

Table 3: Dissociation Constants of the MBD of MeCP2 Binding to 27-mer Duplexes Containing the Series of Purine Analogues within a CpG or MethylCpG Sequence Context with a T-G Mispair[a]

X	TG XC^{m}	R_2	TG XC	R_2
A	309 ± 5.3	0.99	1920 ± 18	0.99
G	17.5 ± 2.0	0.97	123 ± 5.9	0.99
Hx	22.8 ± 3.4	0.94	146.5 ± 10	0.98
Neb	78.5 ± 2.0	0.99	679 ± 6.9	0.99
2AP	251 ± 10	0.99	412 ± 12	0.99
26Di	309 ± 5.5	0.99	1580 ± 45	0.99
Xan	90.2 ± 12	0.92	216 ± 8.4	0.99

[a]The K_d (nM) for MBD binding to each of the duplexes were obtained via nonlinear regression of three or more data sets obtained from EMSA. The plot of the average percent binding of each duplex against the concentration of MBD was fitted to the equation for simple, noncooperative monomeric binding (see Materials and Methods). The ranges seen in the K_d values are the standard error for the regression analysis.

methylation of cytosine in the opposing strand (*19*). We therefore sought to determine if TpG opposite CpG could similarly promote DNMT1-directed cytosine methylation. The results of this study are shown in Figure 3. Under conditions where mC in one strand directs methylation of the opposing strand, as indicated by protection from *Hpa*II-mediated strand cleavage, neither cytosine nor any of the 5-substituted uracil analogues including thymine directs DNMT1 methylation. The impact of TpG on MBD binding is therefore opposite from its impact on DNMT1-mediated methylation.

We also assayed the binding of MBD to ODNs containing the base analogue methyltransferase inhibitors, 5-fluorocytosine, zebularine, and 5-azacytosine. Incorporation of these analogues has only modest impact on the binding of the MBD relative to cytosine. Surprisingly, addition of a methyl group to zebularine does not increase MBD binding (Table 1).

Purine analogues were also examined in this study to elucidate critical MBD contacts with guanine residues of the symmetric CpG dinucleotide. The replacement of guanine by dzG diminished MBD binding by an order of magnitude. The replacement of guanine by 2AP also diminished MBD binding. Surprisingly, the replacement of guanine by Hx increased MBD binding within an unmethylated sequence and did not diminish binding in a fully methylated sequence (Figure 4).

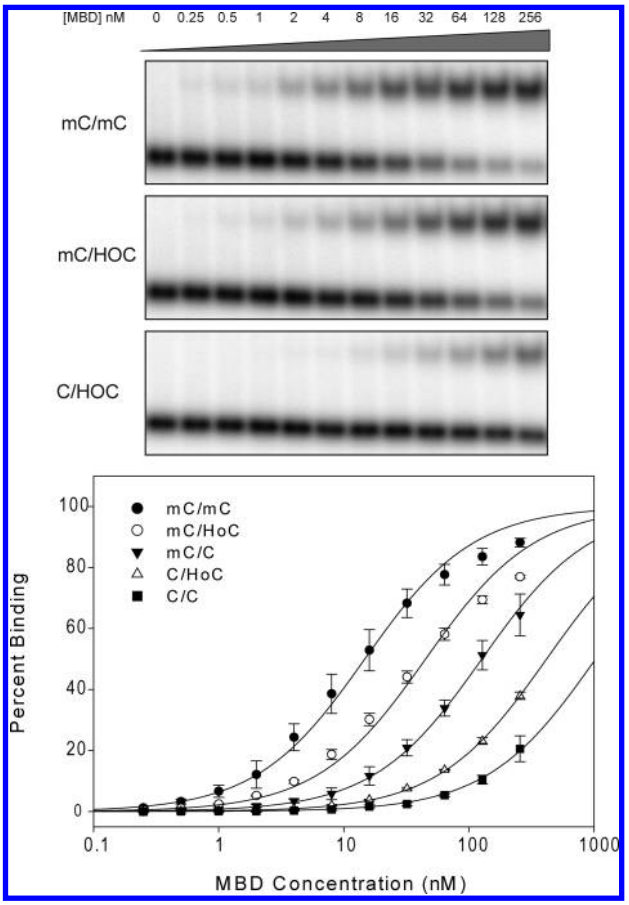

FIGURE 2: Electrophoretic mobility shift binding assays of duplexes containing a central CpG with 5-methylcytosine (mC) in both strands, mC in one strand and 5-hydroxycytosine (HoC) in the opposite strand, and cytosine in one strand with HoC in the opposite strand. The duplexes were bound to varying concentrations (0−256 nM) of the methyl-binding domain of MeCP2. Curves represent the nonlinear regression analysis of the plot of average percent binding (determined from three or more sets of titrations per duplex) and MBD concentration of fully methylated (filled circle), hemimethylated (filled triangle), and unmethylated (filled square) duplexes as well as mC across from HoC (open circle) and cytosine across from HoC (open triangle). The damage product HoC binds with higher affinity than cytosine. The *x*-axis represents MBD concentration (nM), logarithmic scale. The *y*-axis represents percent binding.

DISCUSSION

Enzymatic methylation of cytosine residues in DNA has long been associated with gene silencing. It has been demonstrated that the methylation of both cytosine residues in a CpG dinucleotide increases the binding affinity of the MBD to a CpG dinucleotide by 2 orders of magnitude (*6*), providing a mechanistic link between cytosine methylation and changes in chromatin structure. The DNA methylation-directed binding of

MBDs results in the recruitment of histone-modifying enzymes that covalently alter local histone proteins, driving a cascade of events that results in chromatin condensation and transcriptional silencing (*1*−*3*, *7*). These events are components of the epigenetic programming of gene activity in higher organisms. The proper control of the epigenetic program is essential for cellular differentiation and development and is often deregulated in human tumors (*7*).

As the initial binding of an MBD-containing protein is a critical event in epigenetic programming, we sought to examine the mechanisms by which the methyl-binding proteins recognize and bind with high affinity to DNA containing methylated CpG dinucleotides. Conversely, DNA damage or the incorporation of nucleoside analogues into DNA could potentially corrupt the integrity of the methylation imprint and disrupt epigenetic programming.

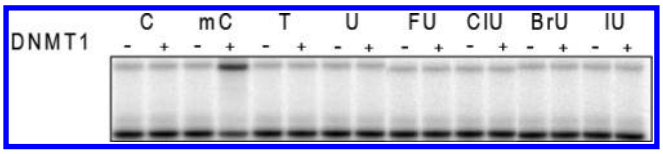

FIGURE 3: DNMT1 methylation direction properties of uracil analogues. Top band, oligonucleotide was methylated by DNMT1 methylase and protected from *Hpa*II cleavage; bottom band, oligonucleotide was not methylated by DNMT1 methylase and therefore was susceptible to *Hpa*II cleavage. Oligonucleotide duplexes were incubated with either DNMT1 (+) or buffer alone (−) as a negative control.

MBD Binding and 5-Substituent Size. In previous studies, we demonstrated that the 5-halogenated cytosine analogues BrC and ClC could mimic mC by enhancing MBD binding (*8*). In the current study, we expanded this set to additionally include FC and IC (Table 1). Dissociation constants are plotted as a function of substituent size (*19*) in Figure 5. Dissociation constants are observed to decrease (increased binding) with increasing substituent size for the 5-halocytosine series and increase with increasing size for the bulkier substituents.

The 5-methyl and 5-chloro substituents are of similar size, and the oligonucleotides containing these analogues have essentially the same dissociation constants. Initial structural studies suggested that the MBD binding to methylated DNA was facilitated by a hydrophobic patch on the MBD that would favorably interact with the mC methyl groups but not with the 5-hydrogen substituent of cytosine (*20*, *21*). The similar binding constant observed with IC as compared to mC indicates that the MBD−DNA interface can accommodate substituents larger

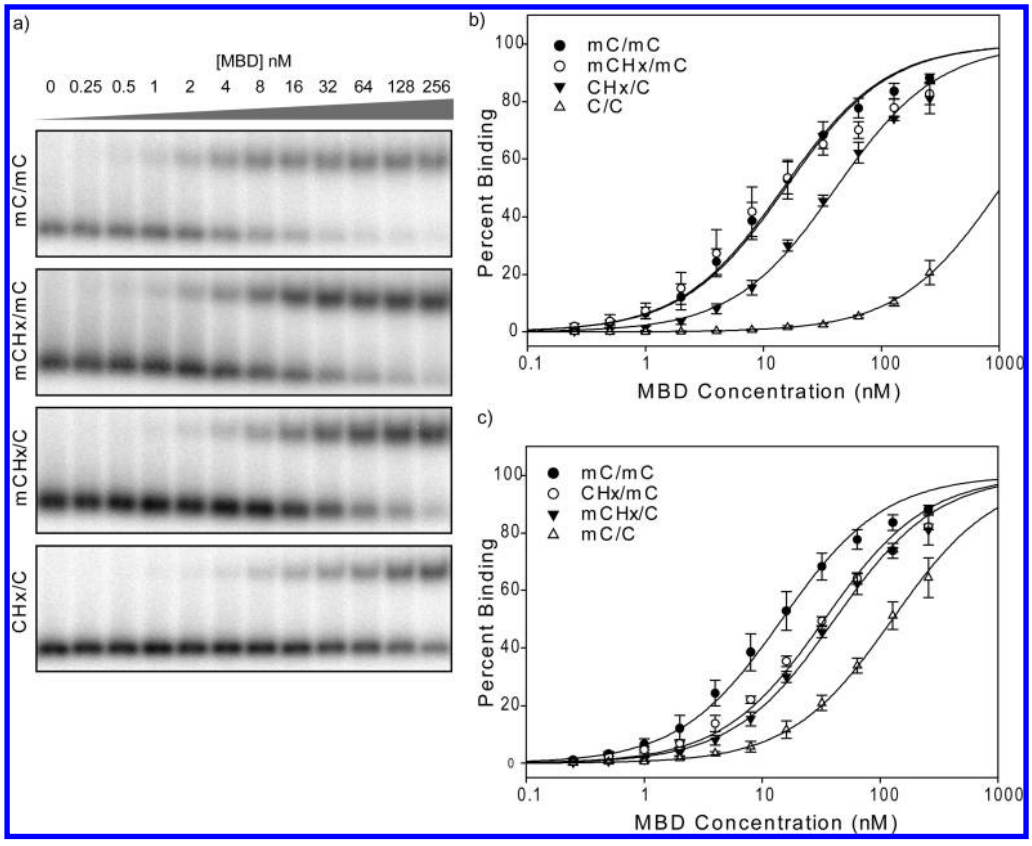

FIGURE 4: Binding of 5-methylcytosine (mC) and hypoxanthine (Hx) containing duplexes to varying concentrations of the methyl-binding domain of MeCP2 from 0 to 256 nM; sequence of duplex is seen in Figure 1. (a) Electrophoretic mobility shift binding assays of duplexes containing a central CpG that is fully methylated, fully methylated with Hx replacing a guanine, hemimethylated with Hx replacing a guanine, and unmethylated with Hx replacing a guanine. Hypoxanthine promotes MBD binding even in the absence of cytosine methylation of the central CpG. (b) Curves represent the nonlinear regression analysis of the plot of average percent binding (determined from three or more sets of titrations per duplex) and MBD concentration of fully methylated with mC at Y_{12} and Y_{14} (filled circle), fully methylated with Hx replacing a guanine residue at P_{15} (open circle), unmethylated with cytosine at Y_{12} and Y_{14} and Hx replacing a guanine residue at P_{15} (filled triangle), and unmethylated (open triangle) duplexes. (c) Curves represent the nonlinear regression analysis of the plot of average percent binding (determined from three or more sets of titrations per duplex) and MBD concentration of fully methylated with mC at Y_{12} and Y_{14} (filled circle), hemimethylated with mC at Y_{12} with Hx replacing a guanine residue at P_{15} in the opposite strand (open circle), hemimethylated with mC at Y_{14} with Hx replacing a guanine residue at P_{15} in the same strand (filled triangle), and hemimethylated with mC at Y_{12} (open triangle) duplexes. The *x*-axis represents MBD concentration (nM), logarithmic scale. The *y*-axis represents percent binding.

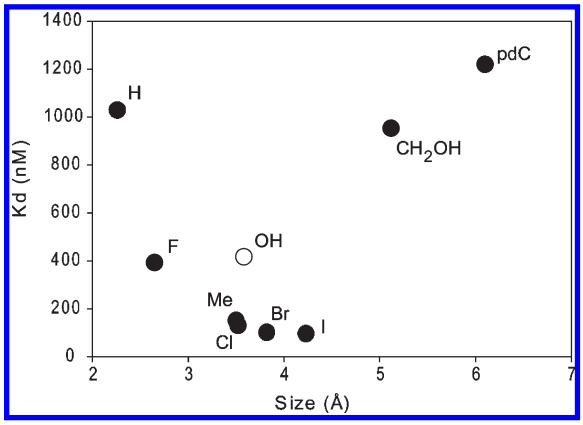

FIGURE 5: Plot of size of 5-substituent in angstroms versus binding constant for cytosine analogues assayed for MBD binding. The *x*-axis is the size of the 5-substituent in angstroms (*19*). The *y*-axis is the K_d (nM) for the oligonucleotide 27-mer containing the 5-substituted cytosine analogue. As the size of the 5-substituent increases, the binding affinity for MBD increases; however, once substituents bulkier than iodine are placed at the 5-position, binding affinity decreases dramatically, indicating an upper size limit to what MBD can accommodate at the 5-position. HoC can bind with better affinity than cytosine, likely due to the ability of OH to better form hydrogen bonds with interfacial water in the binding pocket as compared to H. However, given its size, one would expect better binding than what is observed; this is likely due to the interaction of OH with neighboring phosphates.

than the methyl group. Decreased binding upon substitution with the bulkier analogues pyC and pdC indicates there are upper limits to the size of the binding pocket (Figure 5).

The analogue, FC, could have potentially opposing epigenetic effects, depending upon which protein interacts with the analogue. When located within a CpG dinucleotide, a FC residue could increase MBD binding, facilitating chromatin condensation. Alternatively, DNA methyltransferases could bind covalently to the FC residue (*22*, *23*), resulting in decreased methylation of cytosine in the vicinity. Methyltransferases do not form covalent complexes with 5-substituted cytosine residues containing the larger halogens (*18*).

The interaction of sequence-specific DNA binding proteins with pyrimidine methyl groups has been described as driven by hydrophobic forces (*24*). Goddard and co-workers have previously examined the impact of pyrimidine methyl groups on the binding of sequence-specific DNA binding proteins (*25*). Contributions of both hydrophobic and solvation effects were considered important with solvation effects accounting for the greater part of the binding affinity (*25*). However, contacts observed within a recent crystal structure of MBD bound to an oligonucleotide duplex containing a CpG dinucleotide (*9*) suggest that the MBD−DNA interface might use a different approach. The presence of water molecules within the MBD−DNA interface, as well as apparent interactions between the methyl group protons of mC and the water interface, suggests that the interaction in this case is not hydrophobic. If the mC methyl protons do form hydrogen bonds with the water interface, how do we account for the apparent high affinity of the 5-halopyrimidines? Recently, Ho and co-workers (*26*) reported close contacts with halogen atoms of other nucleic acid structures, suggesting that the pyrimidine 5-halogens examined here might also form "halogen bonds" analogous to methyl hydrogen bonds. The data reported here are consistent with a role for halogen bonding in the increased affinity of the MBD for DNA containing halogenated cytosine analogues.

Previously, we observed that the replacement of mC by HmC resulted in decreased MBD binding, effectively reversing the affinity provided by cytosine methylation (*6*). The decreased affinity for HmC was previously attributed to the conversion of a hydrophobic methyl group to a hydrophilic hydroxymethyl group. However, in light of the recent crystallographic data discussed above, this might not be the best answer because the hydroxymethyl group of HmC could readily interact with water molecules in the DNA−protein interface. Alternatively, as shown in Figure 5, the increased size of the HmC substituent alone might be sufficient to explain the decreased binding. Recently, HmC has been identified in the DNA of higher organisms, suggesting that the oxidation of mC to HmC might be part of an as yet unidentified pathway for modulating epigenetic patterns (*27*−*29*).

In the current study, we also examined the cytosine oxidation damage product, HoC, which is an extremely mutagenic base analogue (*30*). The mutagenicity of HoC has been attributed to an enhanced tendency to form the rare tautomer during DNA replication (*31*). If the MBD recognized a hydrophobic patch in the major groove of a DNA duplex, conversion from C to HoC would be expected to interfere with MBD binding. In contrast, we observe that MBD binds more tightly to the HoC-containing oligonucleotide relative to the C-containing control (Figure 2). Again, this difference could potentially be attributed to the capacity of the HoC hydroxyl substituent to form hydrogen bonds with the interfacial water. Previous studies have shown the hydroxyl group of 5-hydroxypyrimidines can also hydrogen bond with an adjacent phosphate group (*32*), potentially disrupting the organization of interfacial water molecules and perhaps explaining why the magnitude of the binding to the HoC-containing ODN is not as great as might be expected on the basis of substituent size alone.

MBD Binding with Uracil Analogues. Previously, Bird and co-workers demonstrated that thymine, the hydrolytic deamination product of mC in a mCpG dinucleotide, could promote the binding of the MBD (*33*). Our group repeated this study and similarly measured the binding affinity of the MBD to the T·G base pair at a CpG dinucleotide to being nearly identical with that of the normal mC·G base pair. As shown in Table 3, increasing size of a uracil 5-substituent beyond hydrogen increases the affinity of the MBD binding. However, within the halogen series, increasing halogen size does not promote MBD−DNA binding to the same degree as observed for the corresponding cytosine analogues (Table 1).

The deamination of a cytosine analogue to the corresponding uracil analogue converts the geometry of the corresponding base pair with guanine from Watson−Crick to wobble (*34*, *35*). One might expect that formation of the wobble mispair would significantly decrease binding affinity; however, in the wobble configuration, the position of the pyrimidine group within the helix changes only slightly. The deamination of mC to T is thought to drive the most frequent single-base change observed in human cancer cells (*36*). The repair of T by the BER pathway is substantially less efficient than the repair of the cytosine deamination product, uracil (*37*, *38*). The low efficiency of glycosylase repair of thymine versus uracil has been proposed to explain why transition mutations occur with hot-spot frequency at methylated cytosine sites; however, the tight binding of the MBD to T·G at a CpG dinucleotide could shield and further diminish repair of this lesion.

The substitution of uracil with 5-halogens increases relative binding with MBDs as with the thymine methyl group.

The FU·G (*39*), BrU·G (*40*), and ClU·G (*41*) base pairs like T·G are also in a predominantly wobble geometry. The relative binding affinity within this group appears to be related to the substituent size, as with the cytosine series. We note that the binding constant reported in the current study for the BrU-containing ODN is lower than our previously reported value (*8*). Significant effort was made in the current study to protect the halogenated oligonucleotides from light. The lower binding constant reported here was observed reproducibly with newly synthesized BrU-containing ODNs made in our laboratory, as well as those obtained from a commercial source.

The similar affinity of the MBD for mC and T paired with G suggests that the cytosine 4-amino group is not an essential contact point. To further probe the importance of the pyrimidine 4-position, the base analogue and methyltransferase inhibitor 4-pyrimidinone (zebularine), which carries a hydrogen atom at C4, was examined. We observed substantially reduced binding for zebularine, and the reduced binding could not be recovered by introduction of a 5-methyl group by substitution with 5-methylzebularine (Table 1). These data, in conjunction with data discussed above, suggest that a hydrogen-bonding substituent is needed in the pyrimidine 4-position, but it can be a hydrogen bond donor or acceptor. In the crystal structure (*9*) the MBD does not directly interact with the 4-amino group but interacts indirectly through a series of water molecules, perhaps explaining the apparent lack of specificity for the cytosine amino group.

The increased binding of the MBD for BrU described here might potentially explain the observation from Thilly (*42*) and co-workers that BrU can alter gene expression by epigenetic changes, as well as through genetic mutation (*43*). In that study, it was demonstrated that BrU treatment of human cells could result in the loss of the HPRT gene product and that the observed change was not the result of a BrU-induced transition mutation because the apparent loss of the HPRT gene could be reversed following treatment with the methyltransferase inhibitor, 5-azacytidine. The base analogue, BrU, is a well-known mutagenic base analogue due to its greater tendency to form mispairs with guanine (*44*). If the BrU was incorporated into a CpG dinucleotide opposite guanine, it could enhance the binding of an MBD and promote epigenetic silencing.

5-Substituted Uracil Analogues and DNMT1 Activity. In a previous study, we showed that 5-halocytosine analogues could mimic mC in directing DNMT1 methylation of the CpG dinucleotide in the complementary strand (*19*). As demonstrated here with 5-halouracil analogues and previously with the T-G mispair (*30*), the corresponding uracil analogues also promote MBD−DNA interaction. These findings suggest that perhaps the 5-substituent of thymine or 5-halouracil analogues could similarly direct DNMT1 to methylate the CpG in the opposing strand. Using a restriction endonuclease protection assay previously developed (*19*), we confirmed the ability of mC to direct DNMT1 methylation. However, under identical experimental conditions, no methylation was observed with cytosine or any of the 5-substituted uracil analogues including thymine (Figure 3). These data establish that the interactions of the MBD are different from those of DNMT1 with respect to distinguishing cytosine and mC. Further, if BrU and similar analogues can alter epigenetic patterns in a reversible manner, it is more likely that it results from enhanced MBD binding as opposed to misdirected DNMT1 methylation.

MBD Binding and Methyltransferase Inhibitors. Currently, three nucleoside analogues are known to result in reduced methylation in eukaryotic cells: 5-fluoro-2′-deoxycytidine, 5-azacytidine, and zebularine (*45*−*47*). All three analogues are metabolized to the corresponding deoxynucleoside 5′-triphosphate and are incorporated opposite template guanine residues. If incorporated into a CpG dinucleotide that is a target of DNA methyltransferase, the analogues form a covalent, irreversible suicide complex with the methyltransferase, resulting in decreased genomic methylation. Previous studies from Ghoshal et al. (*48*) have also demonstrated that 5-aza-2′-deoxycytidine could bind directly to DNMT1 and induce its degradation as an alternative mechanism of analogue-induced demethylation. In this study, we wished to examine the impact of these analogues on MBD binding, which could independently alter epigenetic patterns. The substitution of cytosine with 5-azacytosine or 2-pyrimidinone (zebularine) modestly increases the binding of the MBD relative to unsubstituted cytosine, but this effect would not likely have a significant impact on epigenetics. 5-Azacytosine within a mCpG in place of mC does show increased binding to MBD as compared to cytosine. This result would not be anticipated if the interaction between DNA and the MBD was mediated by a hydrophobic patch. However, the model supported by recent structural studies (*9*) demonstrates the importance of bridging water molecules, and the lone-pair electrons on N5 of 5-azacytosine would be expected to interact well with these water molecules.

MBD Binding and Purine Substitutions. In a previous study, we demonstrated that the replacement of guanine with the oxidation damage product 8-oxoguanine (oxoG) substantially decreased MBD affinity (*6*). That result was attributed to potential steric interference caused by the 8-oxo atom or by conversion of the N7 position from a hydrogen bond acceptor to a hydrogen bond donor. In this study, we have replaced guanine with dzG, replacing the N7 hydrogen bond acceptor with a CH group. The substitution diminishes the binding of the MBD significantly, indicating the importance of the N7 guanine position as a hydrogen bond acceptor.

The MBD−DNA crystal structure reveals that arginines 111 and 133 interact with the N7 and O6 positions of symmetric guanines within the CpG dinucleotide. Previous data have shown that enzymatic methylation of either arginine inhibits MBD2 binding to a methylCpG (*49*). Furthermore, Free et al. (*5*) showed that mutation of an arginine residue eliminates high-affinity MBD binding. Our data are consistent with these previous findings as we show that MBD has decreased affinity to both oxoG and dzG in place of guanine. Replacing guanine by oxoG converts the N7 position from a hydrogen bond acceptor to a hydrogen bond donor, which can no longer interact with the hydrogen-bonding amino group of arginine (*6*).

In order to probe the importance of the O6 oxygen of guanine, we replaced guanine with adenine paired with T and observed the binding affinity to decrease significantly. This result suggests that mC deamination to thymine in a mCpG dinucleotide would not impact MBD binding initially. However, the subsequent pairing of thymine with adenine in the next round of DNA replication would result in a transition mutation that would eliminate that site as a potential MBD binding location.

Guanine was also replaced with 2AP and paired with either thymine or 5mC. The replacement of the O6 oxygen with a hydrogen atom substantially decreased MBD binding although the 2-amino group was maintained. To further probe the importance of the 2-amino group of guanine, guanine was replaced with Hx (inosine), which lacks the 2-amino group. Both guanine and

Hx base pairs with cytosine are in Watson−Crick geometry (*50*). The replacement of guanine by Hx did not diminish binding in a hemimethylated site and surprisingly increased binding with an unmethylated CpG site. The increased binding afforded by Hx substitution is remarkably similar in magnitude to the increased binding provided by cytosine methylation.

The replacement of guanine with Hx decreases ODN thermal and thermodynamic stability but increases flexibility by decreasing the magnitude of base-stacking interactions (*51*, *52*). The recent MBD−DNA crystal structure reveals a bend in the DNA at the CpG site likely induced by MBD binding. The absence of the 2-amino group would result in increased DNA flexibility and narrowing of the minor groove, which would promote MBD binding as it would require less energy to bend the DNA. Under physiological conditions, dITP could arise from the spontaneous deamination of dATP (*53*). However, the incorporation of dITP into CpG sites would be countered by the action of dITPase (*54*).

The presence of the guanine residue is very important for MBD binding, as well. The oxidation of guanine to oxoG interferes with MBD binding (*6*). The N7 position and the O6 position appear to be critical for high-affinity binding whereas the 2-amino group is dispensable. Surprisingly, the decreased helix stability resulting from the placement of guanine by Hx can increase binding affinity to the same degree as cytosine methylation.

The replacement of cytosine with base analogues known to form inhibitory complexes with methyltransferases only modestly alters MBD binding affinity, suggesting that these analogues perturb epigenetic patterns primarily by direct inhibition of the methyltransferase rather than by perturbing MBD−DNA binding. Alternatively, base analogues with an increased tendency to form base mispairs, such as BrU, could cause epigenetic changes by fraudulently enhancing MBD binding, but not through any direct effect on a methyltransferase. The results of the studies reported here provide new insight into critical contacts between MBD and the target CpG, as well as into the mechanisms by which DNA modification and damage might perturb epigenetic patterns in human cells.

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