Methyl binding domain protein 2 mediates γ-globin gene silencing in adult human βYAC transgenic mice

Jeremy W. Rupon*†, Shou Zhen Wang*, Karin Gaensler‡, Joyce Lloyd*§, and Gordon D. Ginder*†§¶‖

*Massey Cancer Center and Departments of ¶Internal Medicine, †Microbiology and Immunology, and §Human Genetics, Virginia Commonwealth University, Richmond, VA 23298; and ‡Department of Medicine, University of California, San Francisco, CA 94143

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The genes of the vertebrate β-globin locus undergo a switch in expression during erythroid development whereby embryonic/fetal genes of the cluster are sequentially silenced and adult genes are activated. We describe here a role for DNA methylation and MBD2 in the silencing of the human fetal γ-globin gene. The γ-globin gene is reactivated upon treatment with the DNA methyltransferase inhibitor 5-azacytidine in the context of a mouse containing the entire human β-globin locus as a yeast artificial chromosome (βYAC) transgene. To elucidate the mechanism through which DNA methylation represses the γ-globin gene in adult erythroid cells, βYAC/MBD2−/− mice were generated by breeding βYAC mice with MBD2−/− mice. Adult βYAC/MBD2−/− mice continue to express the γ-globin gene at a level commensurate with 5-azacytidine treatment, 10- to 20-fold over that observed with 1-acetyl-2-phenylhydrazine treatment alone. In addition, the level of γ-globin expression is consistently higher in MBD2−/− mice in 14.5- and 16.5-days postcoitus fetal liver erythroblasts suggesting a role for MBD2 in embryonic/fetal erythroid development. DNA methylation levels are modestly decreased in MBD2−/− mice. MBD2 does not bind to the γ-globin promoter region to maintain γ-globin silencing. Finally, treatment of MBD2-null mice with 5-azacytidine induces only a small, nonadditive induction of γ-globin mRNA, signifying that DNA methylation acts primarily through MBD2 to maintain γ-globin suppression in adult erythroid cells.

DNA methylation | epigenetics | transcription

Methylation of the 5′ position of cytosine in the CpG dinucleotide in vertebrates is associated with transcriptional repression. A group of proteins, the methyl-CpG binding proteins (MCBP), specifically recognizes and binds to DNA sequences containing methylated cytosines. Most of these proteins belong to a subgroup based on the common features of a characteristic domain, the methyl-CpG binding domain (MBD), which is necessary and sufficient for binding of the proteins to methylated DNA (1). The MBD protein family consists of MeCP2, MBD1, MBD2, MBD3, and MBD4. MBD2 and MeCP2 have been found to bind methylated DNA as large multiprotein complexes (2–4). MBD4 differs from the other MBD proteins in that its primary function is as a DNA repair enzyme (5). DNA methylation-mediated transcriptional repression is thought to be due to the recruitment of MCBPs and repressive protein complexes to methylated DNA. These complexes are thought to mediate transcriptional repression by recruiting histone deacetylases and transcriptional repressor proteins to methylated DNA (6–10). MBD2 is part of the methyl-CpG binding protein complex 1 (MeCP1), which contains Mi-2, MTA1, MTA2, MBD3, HDAC1, HDAC2, RbAp46, and RbAp48 (4). Early studies showed an interaction between MeCP2 and the SIN3A transcriptional repression complex. However, recent evidence suggests that this interaction may not be stable (11). Brahma, a component of the SWI/SNF nucleosome remodeling complex, does form a stable complex with MeCP2 (12). The Kaiso factor represents another type of MCBP that contains a transcriptional repressor domain as well as a zinc finger domain that confers sequence specificity, but lacks an MBD (13). The zinc fingers of Kaiso can bind methylated CGCGs or the Kaiso binding site TCCTGCNA (14).

Targeted deletions of MCBPs have brought insight into the functions of these proteins *in vivo*. Mice null for MBD1 have deficits in adult neurogenesis and hippocampal function (15). Mice lacking functional MeCP2 display a phenotype similar to Rett syndrome, a human neurodevelopmental disorder leading to loss of voluntary movements (16). Loss of MBD2 has a milder phenotype with the chief manifestation being decreased maternal nurturing of pups (17). Loss of MBD2 specifically interferes with the regulation of the IL-4 gene in mouse Th1 and Th2 lymphocytes indicating that MBD2 can contribute to the regulation of genes in a tissue-specific manner (9). However, no other mammalian genes have been reported to be primarily regulated by MCBPs in noncancerous cells. Unlike the embryonic lethal phenotype of DNA methyltransferase (DNMT) knockout mice, loss of individual MBDs is not catastrophic in mammals, indicating that MBDs may compensate for each or that only a small number of genes required for normal cell function are regulated by any specific MBD (18, 19). Interestingly, Kaiso, a MCBP not in the family of MBD-containing genes, has been shown to be essential for *Xenopus* development but not for mouse development (20, 21).

The genes of the human β-globin locus are expressed sequentially during development in the order that they appear on chromosome 11: 5′ ε-, ^{G}γ-, ^{A}γ-, and β-3′. The individual genes have been shown to be regulated by a complex interplay between cis elements, trans factors, competition for an upstream enhancer, and epigenetics (22). Despite a tremendous amount of progress in this field, the exact mechanism(s) of globin gene silencing is still not completely understood. Among the initial observations in vertebrates that DNA methylation is inversely related to polymerase type II gene expression were those noted in the globin locus (23, 52, 53). Furthermore, the globin genes were the first group of genes for which the DNA methyltransferase inhibitor 5-azacytidine was shown to activate silenced embryonic and fetal genes *in vivo* in both animal models and in a therapeutic setting (24–27). Previously, we have shown a role for DNA methylation and MBD2 in the normal developmental regulation of the chicken embryonic ρ-globin gene (25, 28–30). The ρ-globin gene becomes highly methylated at the same time that the gene becomes transcriptionally silent. Furthermore, the ρ-globin gene is enriched for MBD2 in adult erythrocytes when the gene is silent but not when it is actively transcribed. Finally, a protein complex containing MBD2 purified from primary chicken erythrocytes binds to a methylated ρ-globin gene 5′

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Abbreviations: βYAC, human β-globin locus yeast artificial chromosome; ChIP, chromatin immunoprecipitation; dpc, days postcoitus.

‖To whom correspondence should be addressed at: Departments of Internal Medicine, Human Genetics, and Microbiology and Immunology, Massey Cancer Center, Virginia Commonwealth University, 401 College Street, P.O. Box 980037, Richmond, VA 23298-0037. E-mail: gdginder@vcu.edu.

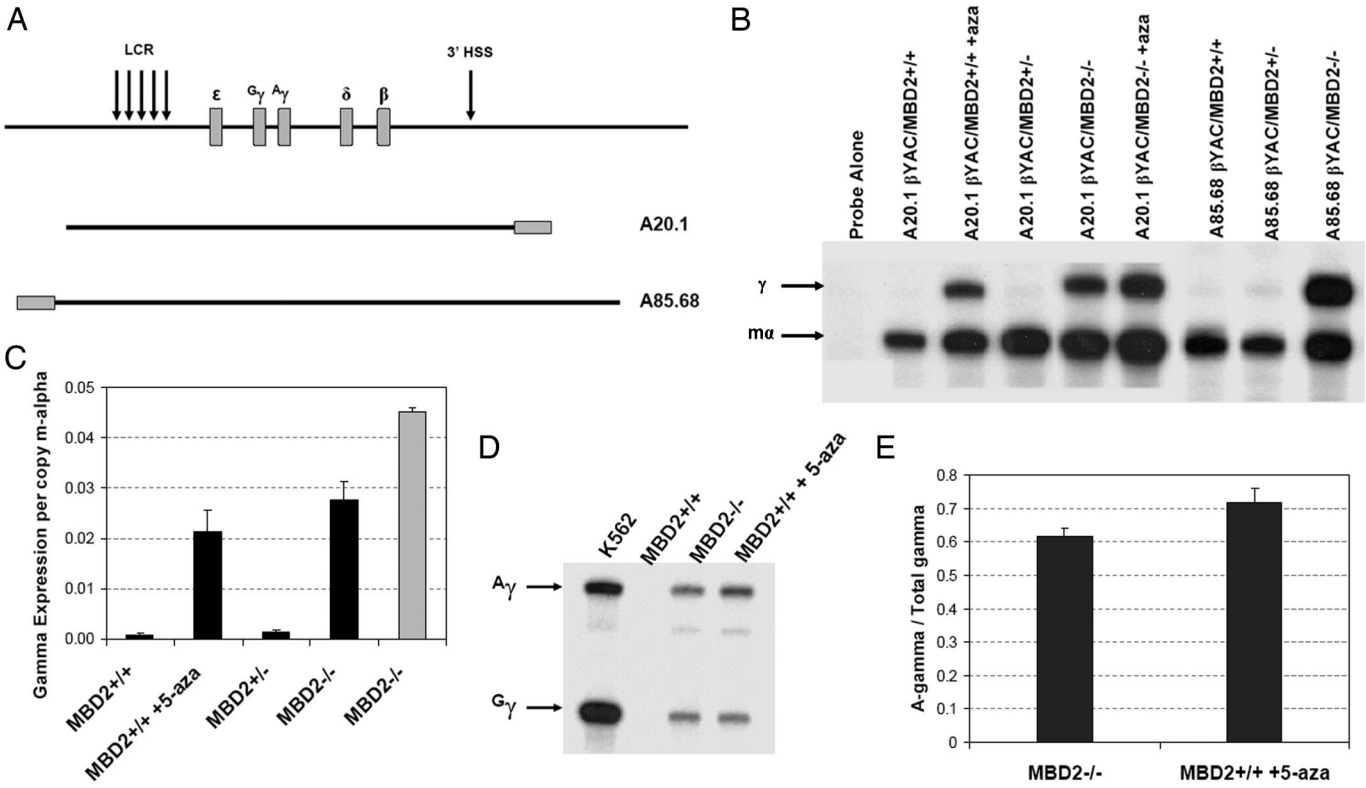

Fig. 1. Transcription of the γ-globin gene in wild-type and MBD2−/− βYAC transgenic mice. (*A*) The A20.1 and A85.68 βYAC constructs were used to generate the transgenic mice used in this study. Expression analysis was performed with both lines; the rest of the experiments were performed with the A20.1 line. (*B*) RNA was analyzed by RNase protection assay. Fifty times more RNA was analyzed by using the γ-probe than mouse α-globin. Samples were mixed after hybridization for RNase digestion. ϕ, treatment with 1-acetyl-2-phenylhydrzine; 5-aza, treatment with 5-azacytidine. (*C*) Quantitation of RNase protection assay (RPA) results. The level of γ-globin mRNA is expressed per copy of the endogenous mouse α-globin. Bands were quantitated by using a phosphorimager. The results shown are the average of three separate RPAs from at least two mice per genotype or treatment. The black bars represent the A20.1 line, and the gray bar represents the A85.68 line. (*D*) RPA showing levels of ^{G}γ- and ^{A}γ-globin expression in βYAC mice treated with 5-azacytidine and βYAC/MBD2−/− mice. The probe used in this RPA is able to distinguish between the ^{G}γ- and ^{A}γ-globin genes. (*E*) Quantitation of RPA results showing the level of ^{A}γ-globin expression relative to total γ-globin expression.

sequence and differs in biochemical make-up from MeCP1 (unpublished data). These findings are evidence that MCBPs may form tissue-restricted complexes that are targeted to specific sets of genes. Taken together, this evidence suggests MBD2 may be involved in the process of globin gene silencing by DNA methylation.

To determine the role of MBD2 in mammalian globin gene silencing, mice containing the entire human globin locus as a yeast artificial chromosome (βYAC) were bred with mice containing a targeted deletion of MBD2. Adult βYAC/MBD2−/− mice inappropriately express the γ-globin gene at a level commensurate to mice treated with 5-azacytidine, whereas adult wild-type βYAC mice do not express the γ-globin gene. This is, to our knowledge, the first evidence of a specific MCBP regulating γ-globin expression. In addition, loss of MBD2 delays the silencing of the γ-globin gene during embryonic development. Loss of MBD2 slightly decreases DNA methylation levels around the γ-globin promoter, and inhibiting DNA methylation does not greatly enhance the expression of the γ-globin gene, indicating that the inductive effect of 5-azacytidine seen in knockout mice does not largely depend on DNA methylation or is mediated by a different MCBP.

Results

γ-Globin Expression Is Increased in Adult βYAC/MBD2−/− Mice. βYAC/MBD2−/− mice were generated by breeding βYAC (lines A20.1 and A85.68; Fig. 1*A*) mice with MBD2−/− mice and breeding the subsequent transgenic hemizygotes with MBD2−/− mice (17, 31). Mice were treated with 1-acetyl-2-phenylhydrazine to induce hemolytic anemia for the purpose of increasing the number of reticulocytes for RNA analysis and converting the spleen primarily to a site of erythropoiesis (32). Peripheral blood was collected on the third day after treatment. For 5-azacytidine treatment, βYAC/MBD2+/+ mice were treated for 2 days with 1-acetyl-2-phenylhydrazine followed by 5 days of 5-azacytidine treatment. Peripheral blood was collected the day after the final treatment. Globin gene expression was analyzed by using RNase protection assay. As seen in Fig. 1*B*, γ-globin mRNA was barely detectable by this assay in adult βYAC/MBD2+/+ mice and βYAC/MBD2+/− mice. However, both βYAC mice treated with 5-azacytidine and βYAC/MBD2−/− mice express the γ-globin gene at increased levels, ≈2% per copy of mouse α-globin (Fig. 1*C*). Similar results were obtained when using nonanemic animals (data not shown). Only trace levels of endogenous mouse embryonic globin genes or human ε-globin gene transcripts were detectable by RNase protection assay or real-time PCR (data not shown). However, we have observed that a human epsilon gene transgene containing a full LCR and 12 kb of downstream flanking sequence but no γ- or β-globin gene is partially activated in the MBD2−/− background. Thus, MBD2 is necessary for maintaining complete γ-globin silencing in adult mice, and loss of MBD2 results in markedly increased γ-globin expression but not expression of mouse or human embryonic β-type globin genes. This result is likely due to the ability of the fetal γ-globin gene but not the embryonic β-type globin genes to partially compete with the

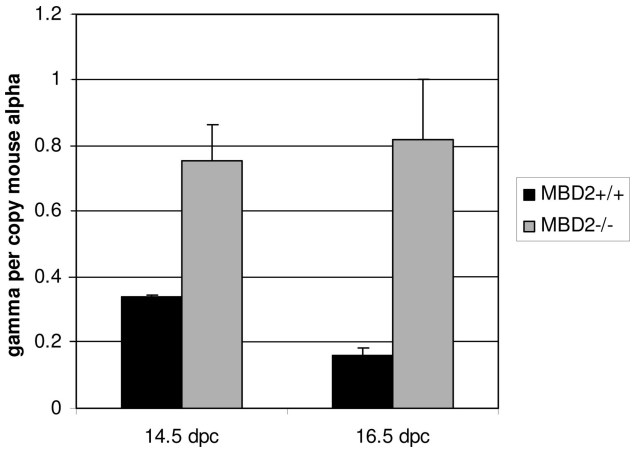

Fig. 2. Level of expression of the γ-globin gene per copy of mouse α-globin gene during development in wild-type and MBD2−/− βYAC transgenic mice. Fetal livers were dissected from 14.5- and 16.5-dpc embryos, and RNA was isolated. The level of expression of the γ-globin relative to the endogenous mouse α-globin was determined by using RPA and quantitated by using a phosphorimager. The results shown are the average of at least three livers from a total of at least two litters.

adult β-globin gene for the LCR because of more dominant autonomous silencing of the embryonic genes. In addition, the level of induction seen is similar to the level achieved after treatment with 5-azacytidine, indicating that MBD2 and 5-azacytidine possibly work through the same pathway. The percentage of $^{G}\gamma$- vs. $^{A}\gamma$-globin mRNA was determined by using an RNase protection assay probe that discriminates between the two genes (33). The percentage of $^{A}\gamma$-globin mRNA was 60% for βYAC/MBD2−/− mice and 70% for βYAC/MBD2+/+ mice treated with 5-azacytidine (Fig. 1 *D* and *E*). These values are very similar to the levels seen in adult human F-cells and opposite to the $^{A}\gamma$- to $^{G}\gamma$-globin ratio in K562 cells or fetal human erythroid cells, suggesting that the γ-globin mRNA is from definitive cells rather than from a reactivation of primitive erythropoiesis.

The γ-globin gene is also expressed in adult transgenic knockout mice derived from a different βYAC transgene (A85.68; Fig. 1 *B* and *C*). Thus, the effect seen is not an isolated position effect, because each single copy βYAC transgene is located in a different region of the mouse genome. In addition, each line is derived from a different genetic background, and through multiple rounds of breeding, heterogeneous backgrounds were generated, indicating that the effect seen is not via an unknown genetic modifier.

Loss of MBD2 Delays γ-Globin Gene Silencing During Development. Adult erythroid cells from mice lacking MBD2 inappropriately express the γ-globin gene. We next wanted to determine the impact of MBD2 on globin gene expression during development. Timed matings were performed and embryos were harvested 14.5 and 16.5 days postcoitus (dpc). At 14.5 dpc, embryos lacking MBD2 expressed 2-fold more γ-globin mRNA than did wild-type embryos (Fig. 2). This difference increased to 3.5-fold in 16.5-dpc fetal livers (Fig. 2). In addition, no human ε-globin transcripts are detectable by RNase protection assay after 16.5 dpc in wild-type or knockout mice (data not shown). The effect seen in adult knockout mice appears to be at least in part due to an inability to fully silence the γ-globin gene during the switch to definitive erythropoiesis. The effect is specific for the γ-globin genes and not the embryonic β-type globin genes.

DNA Methylation Around the γ-Globin Promoter Is Largely Unaffected in MBD2−/− Erythroblasts. Lack of MBD2 results in expression of the γ-globin gene in adult βYAC transgenic mice. MCBPs bind

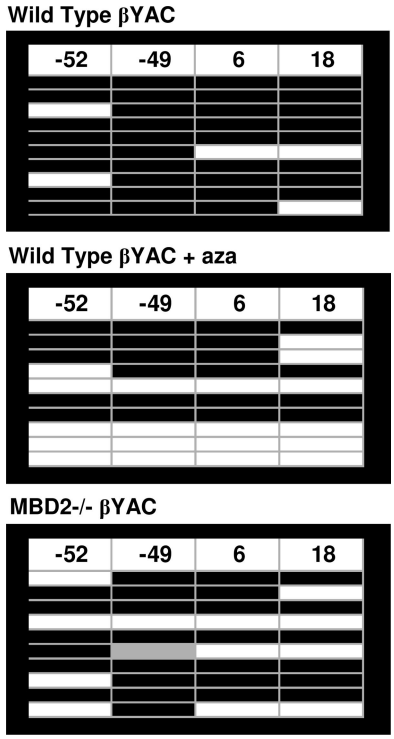

Fig. 3. Bisulfite analysis of 1-acetyl-2-phenylhydrazine-treated spleens. The number on top shows the position of the CpG relative to the transcription start site. Each row represents one sequenced clone. A black bar indicates a methylated CpG, a white bar indicates an unmethylated CpG, and a gray bar indicates an undetermined CpG.

to methylated DNA and recruit factors that lead to transcriptional repression. In addition, MBD2 has been shown to colocalize with DNA methyl transferase 1 (DNMT1) (34). We therefore postulated that if MBD2 binds at or near the γ-globin gene promoter, then the effect seen in adult βYAC/MBD2−/− mice may be accompanied by a loss of DNA methylation around the γ-globin promoter. Bisulfite sequencing was performed to determine the methylation status in βYAC/MBD2+/+ mice, βYAC/MBD2+/+ mice treated with 5-azacytidine, and βYAC/MBD2−/− mice. A 70-bp region containing four CpGs around the γ-globin promoter was analyzed. As shown in Fig. 3, wild-type adult erythroblasts show a high level of methylation (75–100%) at each CpG tested. Furthermore, no individual clone (represented as a row in Fig. 3) was either completely or ≥75% unmethylated, indicating that all cells are highly methylated around the γ-globin promoter. Treatment with 5-azacytidine led to a decrease in methylation at all sites (40–60% methylated) with four clones having no methylation at any site tested. This result is similar to results seen in MEL cells containing human chromosome 11 and primary erythroid cells of patients treated with 5-azacytidine (35, 36). MBD2−/− mice show an intermediate level of DNA methylation at each site (60–90% methylated). In addition, one clone was fully demethylated, and another was 75% demethylated. DNA methylation levels were modestly decreased in the absence of MBD2 relative to the results seen in animals treated with 5-azacytidine. The observed partial demethylation is more consistent with increased transcription of the gene preventing DNA methylation by DNMT rather than MBD2 recruiting DNMT activity to the γ-globin promoter.

MBD2 Does Not Bind Near the γ-Globin Promoter. To determine directly whether MBD2 was mediating its effect at or near the γ-globin promoter, chromatin immunoprecipitation (ChIP) as-

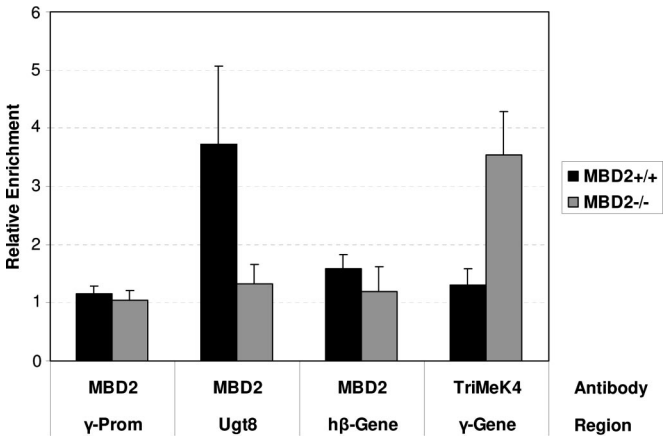

Fig. 4. Results from ChIP assay. Chromatin was immunoprecipitated with anti-MBD2, anti-TriMeK4, and control antibodies (IgG or normal serum) from MBD2+/+ and MBD2−/− splenic erythroblasts. The sequence-specific enrichment was determined by real-time PCR and calculated by using the following formula: (experimental antibody-gene/amylase)/(control antibody-gene/amylase). MBD2 and MBD2-control IPs were performed on three mice. TriMeK4 and control IPs were performed on two mice.

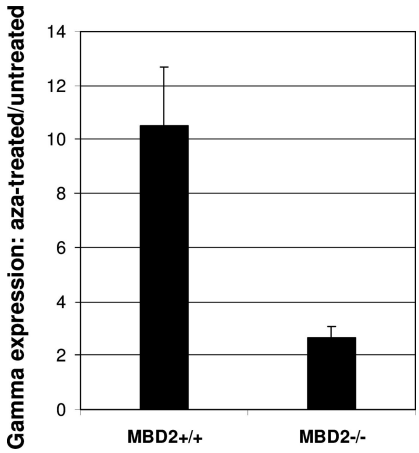

Fig. 5. Real-time PCR analysis of γ-globin induction by 5-azacytidine in wild-type and MBD2−/− βYAC transgenic mice. Data are shown as the level of γ-globin expression after 5-azacytidine treatment over γ-globin expression before 5-azacytidine treatment. Mice were treated for 2 days with 1-acetyl-2-phenylhydrazine, after which peripheral blood was collected and 5-azacytidine treatment begun for 5 days. Peripheral blood was collect after 5-azacytidine treatment. The level of γ-globin RNA expression was compared before and after 5-azacytidine treatment by using real-time PCR and SYBR green chemistry.

says were performed. Both βYAC/MBD2+/+ and βYAC/MBD2−/− mice were treated with 1-acetyl-2-phenylhydrazine to promote conversion of the spleen to a primarily erythroid organ (32). Splenic erythroblasts were used for ChIP with antibody directed against MBD2. As shown in Fig. 4, no enrichment is seen for MBD2 near the γ-globin promoter in wild-type βYAC transgenic mice. However, MBD2 does bind *in vivo* to the CpG island region of the Ugt8 gene. This gene is induced ≈4-fold in splenic erythroblasts in the absence of MBD2 (data not shown). In addition, only baselines levels of enrichment are seen at the Ugt8 CpG island region and the γ-globin promoter region in MBD2-null mice, indicating that the MBD2 antibody is very specific. As expected, no MBD2 enrichment is seen near the highly transcribed human β-globin gene. These results indicate MBD2 is not acting directly at the γ-globin promoter region to mediate γ-globin silencing in adult βYAC transgenic mice.

Treatment of βYAC/MBD2−/− Mice with 5-Azacytidine Results in only a Small Further Increase in γ-Globin Gene Expression. Treatment of βYAC/MBD2+/+ mice with 5-azacytidine leads to a 10- to 15-fold induction of the γ-globin gene versus mice treated with 1-acetyl-2-phenylhydrazine only (Fig. 5), which is similar to the level of activation seen in βYAC/MBD2−/− mice and as reported by Pace *et al.* (37) in a different βYAC line treated with 5-azacytidine. To determine whether 5-azacytidine and loss of MBD2 induce γ-globin via the same mechanism through overcoming DNA methylation, βYAC/MBD2−/− mice were treated with 5-azacytidine. Blood was collected before 5-azacytidine treatment and again 5 days after treatment and analyzed by both RNase protection assay and quantitative real-time PCR (Figs. 1 and 5, respectively). 5-Azacytidine only induces the γ-globin by 2.5-fold in MBD2−/− mice. The lack of an additive effect of MBD2 deficiency upon 5-azacytidine-mediated induction strongly suggests that both are largely working through the same pathway, which is mediated by DNA methylation. This pathway regulates the γ-globin gene at the transcriptional level because loss of MBD2 leads to a ≈4-fold enrichment of TriMeK4, a mark of active transcription, at the γ-globin gene region (Fig. 4) (38). To confirm that mice deficient for MBD2 do not display the hematological parameters observed in erythropoietic stress, red blood cell indices and reticulocyte counts were determined. As shown in Table 1, the red cell indices of wild-type and MBD2−/− mice are not statistically different except for a slight microcytosis seen in MBD2-null mice. However, the MBD2−/− mice used in this study were all females, whereas the wild-type group consisted of more male mice that were younger in age. This age and sex difference may account for the slight difference in red cell mean corpuscular volume observed. Regardless, the reticulocyte counts did not differ significantly, indicating that MBD2 loss does not result in erythropoietic stress.

Discussion

MBD2 is needed for complete suppression of γ-globin gene expression in βYAC transgenic mice. Loss of the protein leads to a level of expression from a single copy of the γ-globin gene commensurate with βYAC mice treated with 5-azacytidine, ≈2–4% of the endogenous mouse α-globin RNA per gene copy representing a >10-fold increase. In addition, developmental silencing of the transgene is delayed in the absence of MBD2 because these mice express the γ-globin gene at higher levels in

Table 1. Red cell indices and reticulocyte counts

	RBC, $10^6/\mu$l	Hb, g/dl	Hct	MCV, fl	MCH, pg per cell	MCHC, g/dl	Retic, %
MBD2+/+	7.6 ± 1.1	13.2 ± 1.2	37.2 ± 5.1	49.3 ± 1.5	17.5 ± 0.9	35.8 ± 1.8	6.2 ± 0.9
MBD2−/−	6.7 ± 1.6	11.7 ± 2.7	30.3 ± 8.1	44.6 ± 0.3	17.2 ± 0.4	38.6 ± 1.2	5.9 ± 1.5
P value	0.42	0.41	0.28	0.01	0.64	0.08	0.73

Red cell indices were determined using blood from three nonanemic mice of each genotype. Reticulocyte count was performed on two MBD2−/− mice. *P* values were determined by using a Student *t* test.

14.5- and 16.5-dpc fetal livers. Studies in human–mouse erythroid cell hybrids indicated that the γ-globin gene was methylated after it was silenced (39). The results in this transgenic model show that silencing is at least delayed in the absence of the methyl-CpG binding protein MBD2, suggesting that DNA methylation may contribute to γ- to β-globin gene switching. The results shown here are consistent with the requirement for DNA methylation and MBD2-mediated silencing at a site other than the γ-globin gene for complete switching to occur. In this case, methylation at the γ-globin promoter might be expected to lag behind silencing, thus reconciling the present results with those in human–mouse erythroid cell hybrids.

DNA methylation levels are modestly reduced whereas TriMeK4 levels are modestly increased around the γ-globin gene in adult βYAC/MBD2−/− mice. Treatment of βYAC/MBD2−/− mice with 5-azacytidine leads to only a 2.5-fold induction of γ-globin mRNA relative to βYAC mice treated with 5-azacytidine. Together these results indicate that MBD2 and 5-azacytidine both are influencing the same pathway to induce γ-globin expression. In as much as the regulation of the γ-globin gene in this transgenic model reflects the situation in human erythroid cells, the results support the notion that most of the effect of 5-azacytidine on stimulating γ-globin gene expression in humans is through its inhibitory effects on DNA methylation (27, 40, 41). The additional stimulation of γ-globin gene expression in βYAC/MBD2−/− mice may be due to cytotoxic or cell cycle effects, such as those observed in primates treated with S-phase active cytotoxic drugs that do not affect DNA methylation (42–44).

Most MCBPs are thought to inhibit transcription by binding near promoters and recruiting transcriptional repression complexes. We have shown here that MBD2 does not bind near the γ-globin promoter and that only minor changes in DNA methylation status are present in βYAC/MBD2−/− mice. This is not completely surprising because the γ-globin promoter does not possess a CpG island, a region of 200 bp or more that has a CpG:GpC ratio of >0.5 and that is required for MeCP1 complex formation *in vitro* (45, 46). In addition, there are no significant changes in global DNA methylation levels in MBD2−/− mice (17). While MBD2 has been shown to bind to a sequence containing as few as three CpG *in vitro*, MBD2 has only been shown to bind *in vivo* to methylated CpG island sequences (7, 9, 47). The lack of major enrichment in posttranslational histone modifications in the absence of MBD2 may be due to the relatively low level of γ-globin expression. The human β-globin gene is highly expressed in both MBD2+/+ and MBD2−/− βYAC transgenic mice and shows a much higher enrichment for H3 trimethyl K4 (data not shown) (31). The γ-globin gene may not be expressed highly enough to impart large changes in histone modifications that occur secondary to recruitment of activating modifier enzymes by the RNA polymerase II complex. Similar results have been seen in transgenic mice carrying a mutant βYAC expressing the human β-globin gene at low levels (48). Taken together, the results here show that the γ-globin gene becomes actively transcribed either in the absence of MBD2 or upon treatment with 5-azacytidine with definite but not major changes in epigenetic modifications that most likely reflect changes induced by transcription rather than by loss of inactivating histone modifier enzymes recruited directly by MBD2. In addition, because erythropoiesis in MBD2−/− mice is not globally perturbed, the effect is quite specific. Because there are no CpG islands within 6 kb of the γ-globin gene, these results are most consistent with a model in which loss of MBD2 results in transcriptional activation of a gene or genes that are normally silent in adult erythroid cells. The products of this gene(s) would, in turn, results in transcriptional activation of the γ-globin gene. Microarray analyses have identified numerous genes that are activated in splenic erythroblasts of MBD2−/− mice, including genes that reside in hematopoietic pathways (data not shown).

Many compounds currently available to treat patients with hemoglobinopathies carry short- or long-term potential risks of toxicity, and, in addition, the response to the agents is variable. Here, we show that loss of MBD2 leads to expression of the normally silent γ-globin gene in adult βYAC transgenic mice. Given the mild phenotype of MBD2-null mice, MBD2 may represent an attractive potential target for the treatment of β-thalassemia or sickle cell anemia, conditions in which increased γ-globin gene expression has an ameliorating effect.

Methods

Generation of βYAC/MBD2−/− Mice. MBD2−/− mice (a generous gift from Adrian Bird, University of Edinburgh, Edinburgh, U.K.) were mated with βYAC mice. The subsequent βYAC/MBD2+/− mice were bred with MBD2−/− to generate βYAC/MBD2−/− mice. To maintain the line, βYAC/MBD2−/− were bred with MBD2−/− mice. All progeny were screened for the presence of the transgene and absence of MBD2 by using PCR of DNA from tail snips. Two lines of βYAC mice were used to generate transgenic knockout mice, the A20.1 and A85.68 lines (Fig. 1*A*). Both contain a single copy of the βYAC transgene in the C57 BL/6 background (A20.1) or the FVB/N background (A85.68). The MBD2−/− mice were in the BALB/C background. Through multiple rounds of breeding to establish and maintain lines, the final transgenic knockout mice were in a heterogeneous genetic background.

Timed Matings. For MBD2 wild-type embryos, βYAC males were bred with nontransgenic females. For MBD2−/− embryos, βYAC/MBD2−/− males were bred with nontransgenic MBD2−/− females. The presence of a vaginal plug was designated day 0.5. Fetal livers were dissected from day-14.5 and day-16.5 embryos.

Treatments. Mice were treated for 2 days with i.p. injection of 1-acetyl-2-phenylhydrazine (10 mg/ml; Sigma) at a dose of 0.4 mg/10 g. On the third day, mice were treated with 5-azacytidine (0.5 mg/ml; Sigma) at a dose of 2 mg/kg for 5 days via i.p. injection.

RNA Isolation. RNA was extracted from fetal livers or peripheral blood by using TRIzol (Invitrogen) according to the manufacturer's protocol. Fetal liver RNA was further purified by LiCl precipitation.

RNase Protection Assay. The assay was performed as described in ref. 49. The probe for mouse α-globin has been described (50). Probes for γ-globin analysis were from a 1.7-kb fragment of the ^{A}γ-globin gene protecting two bands (205 and 144 bp) when cut with EcoRI or one band when cut with NcoI (188 bp). To discriminate between ^{G}γ- and ^{A}γ-globin mRNA, a probe identical to one used previously was generated by PCR (33).

Bisulfite Conversion. Mice were treated with 1-acetyl-2-phenylhydrazine for 2 days and harvested on the fifth or seventh day. Genomic DNA was isolated from spleens by using the Zymo Research genomic isolation kit. The DNA was bisulfite-converted by using the Zymo Research EZ methylation kit. Bisulfite converted DNA was amplified by using nested PCR with primers specific for the γ-globin promoter region. PCR products were cloned, screened, and sequenced.

ChIP. Mice were treated for 2 days with 1-acetyl-2-phenylhydrazine. On the fifth day, spleens were harvested and gently brushed into single-cell suspension in ice-cold RPMI containing 2% FBS and 5 mM butyrate, PMSF, aprotinin, leupeptin, and

pepstatin. Protease inhibitors were included in all steps until the final wash. Cells were then passaged through a 70 μM nylon filter to remove debris. Cells were spun and washed with ice-cold PBS containing 2% FBS. Cells were then resuspended in room-temperature PBS/FBS. Formaldehyde was added dropwise to a final concentration of 0.4% (TriMeK4) or 1% (MBD2), and cells were crosslinked for 10 min at room temperature. The reaction was terminated by adding glycine to 0.125 M and incubating at room temperature for 5 min. Cells were washed twice with PBS/FBS. Cells were resuspended in SDS/lysis buffer at a concentration of 25–30 mg/ml and incubated on ice for 10 min. Chromatin was sonicated six times (TriMeK4) or eight times (MBD2) for 20 s. Twenty-five milligrams of spleen was used per IP with anti-trimethyl H3 K4 (Abcam or Upstate), rabbit IgG (Upstate), normal rabbit serum (Santa Cruz Biotechnology), anti-MBD2 (Upstate), and sheep IgG (Upstate). The rest of the procedure was performed as described previously with slight modifications for MBD2 ChIPs (51). For MBD2 ChIP, protein G salmon sperm agarose beads (Upstate) were used and were washed three times with low-salt buffer and one time with medium-salt buffer (250 mM NaCl).

Real-Time PCR. For expression analysis, RNA was reverse-transcribed by using iScript (Bio-Rad) and quantitated by using SYBR green chemistry on an Applied Biosystems 7900HT PCR system. For ChIP, real-time PCR was performed as described in ref. 51. Primer sequences are available upon request.

Red Cell Indices. Peripheral blood was collected into citrate-coated tubes from nonanemic mice. Red cell indices were measured on a Coulter MicroDiff 1600. Reticulocyte counts were determined by manually counting methylene blue-stained cells.

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