DNA methylation plays an important role in mammalian development and correlates with chromatin-associated gene silencing. The recruitment of MeCP2 to methylated CpG dinucleotides represents a major mechanism by which DNA methylation can repress transcription. MeCP2 silences gene expression partly by recruiting histone deacetylase (HDAC) activity, resulting in chromatin remodeling. Here, we show that MeCP2 associates with histone methyltransferase activity in vivo and that this activity is directed against Lys\(^9\) of histone H3. Two characterized repression domains of MeCP2 are involved in tethering the histone methyltransferase to MeCP2. We asked if MeCP2 can deliver Lys\(^9\) H3 methylation to the H19 gene, whose activity it represses. We show that the presence of MeCP2 on nucleosomes within the repressor region of the H19 gene (the differentially methylated domain) coincides with an increase in H3 Lys\(^9\) methylation. Our data provide evidence that MeCP2 reinforces a repressive chromatin state by acting as a bridge between two global epigenetic modifications, DNA methylation and histone methylation.

Methylation of cytosines is essential for mammalian development and is associated with gene silencing (1). DNA methylation represses genes partly by recruitment of methyl-CpG-binding domain proteins, which selectively recognize methylated CpG dinucleotides. MeCP2 is the founder member of the methyl-CpG-binding domain proteins, which consists of a single polypeptide that contains a methyl-CpG-binding domain and a transcriptional repression domain. MeCP2 is capable of binding to a single symmetrically methylated CpG both in naked DNA and within chromatin (2, 3).

It is now well established that MeCP2 silences transcription by recruiting the histone deacetylase (HDAC)\(^1\) repressive machinery, which removes acetyl groups from histones resulting in gene silencing (4, 5). However, the inhibition of histone deacetylase activity using drugs such as Trichostatin A only partially relieves MeCP2-mediated transcriptional repression. This partial relief indicates that additional mechanisms of repression by MeCP2 likely exist aside from the recruitment of histone deacetylase.

Beside histone deacetylation, histone methylation is emerging as another key post-translational modification of histones and represents an important epigenetic mechanism for the organization of chromatin structure and the regulation of gene expression. In particular, methylation at lysine 9 of histone H3 is associated with gene silencing, and several enzymes that catalyze the addition of methyl groups to lysine 9 have recently been identified (6). Interestingly, recent data have shown that the retinoblastoma protein represses transcription through the recruitment of HDAC activity, but in a second step it recruits histone methylation activity specific for lysine 9 of histone H3 (7). By analogy to retinoblastoma, we considered in the present work whether MeCP2-mediated repression might also include, besides histone deacetylation, a second stage involving histone methylation.

Here, we show that MeCP2 associates with histone methylation in vitro as well as in vivo. The MeCP2-associated methylation activity is found to be specific for lysine 9 of histone H3. By means of chromatin immunoprecipitation (ChIP) experiments, we show that MeCP2 facilitates H3 Lys\(^9\) methylation of the H19 gene, a bona fide MeCP2-regulated gene. Our results indicate that MeCP2 acts as a mechanistic bridge between DNA methylation and histone methylation and thus reinforce the repressive function of these two distinct methylation events.

**EXPERIMENTAL PROCEDURES**

Plasmids—We cloned MeCP2 1–77 into the pGEX vector (Pharmacia) by PCR using the appropriate sets of primers. The other pGEX expression vectors for full-length and deletion mutants of MeCP2 have been described previously (8). The HA-tagged mammalian expression construct of MeCP2 (HA-MeCP2) was generated by insertion of a synthetic oligonucleotide encoding the HA epitope sequence into the 5’ end of MeCP2 cDNA in frame in pBluescriptSK\(^-\) based vector p65Δ (8). pCMV-HA-MeCP2 was constructed by insertion of the NotI/EcoRI-restricted fragment encoding HA-MeCP2 into the BamHI site of pCMV-Bam-Neo vector such that the CMV promoter drives HA-MeCP2 expression. pTRE-MeCP2 was constructed by insertion of the EcoRI-restricted HA-MeCP2 fragment from pCMV-HA-MeCP2 into the EcoRI site of pTRE (Clontech). pTRE-HA-MeCP2/Zeo was constructed by insertion of a BamHI/BglII-restricted Zeocin resistance cassette from pZeoSV (Invitrogen) into the XhoI site of pTRE-HA-MeCP2.

Generation of MeCP2 Tet-Off Cell Line, Cell Culture, and Transfections—L292 mouse fibroblast cells (L cells) were co-transfected with pTet-Off and pTRE-HA-MeCP2/Zeo by electroporation. The cells were plated under selection for G418-resistance and Zeocin-resistance after 24 h following transfection. G418/Zeocin-resistant cell lines were tested.
for background and induced expression of HA-MeCP2 in the presence and absence of tetracycline, respectively. Expression was detected by anti-HA and anti-MeCP2 antibodies on Western blots. Clone L-4 did not express detectable HA-MeCP2 background but was strongly inducible by tetracycline withdrawal. Mouse MeCP2 Tet-Off fibroblasts (L cells) were grown to 75% confluence in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% tetracycline-free fetal calf serum (Clontech) at 37°C for 12 h. The beads were then washed four times in 1 ml of radioimmune precipitation assay buffer, and assayed for histone methyltransferase activity. The reaction products were then analyzed by SDS-PAGE followed by Western blotting and autoradiography. The radiolabeled H3 is indicated by an arrow on the left and was identified through its molecular size as well as by Ponceau staining. B, MeCP2-associated activity methylates recombinant histone H3. Histone methyltransferase assays were performed as in A, this time with recombinant H3 as a substrate instead of bulk histones. C, a region in the N terminus of MeCP2, which overlaps with the methyl-CpG-binding domain and to a lesser extent the transcriptional repression domain, mediates the association with histone H3 methyltransferase activity. The indicated GST MeCP2 fusion fragments were tested for their association with histone methyltransferase activity. Residues 1–77 (lane 8) were tested in a separate experiment than the other constructs (lane 1–7). Below is shown a schematic representation of MeCP2 with its methyl-CpG-binding domain and transcriptional repression domain. Also shown are the GST fusion proteins of MeCP2 tested in the histone methyltransferase assay, with the results summarized on the right (++, indicates strong MeCP2-associated methyltransferase activity; --, indicates no associated activity).

Fig. 1. MeCP2 binds in vitro to a histone methyltransferase activity specific for H3. A, GST MeCP2 binds histone H3 methyltransferase activity from nuclear extracts. Histone methyltransferase assays using bulk histones as substrate incubated with HeLa nuclear extracts and equivalent amounts of GST or GST MeCP2 bound to Sepharose beads. After incubation, the beads were washed and assayed for histone methyltransferase activity. The reaction products were then analyzed by SDS-PAGE followed by Western blotting and autoradiography. The radiolabeled H3 is indicated by an arrow on the left and was identified through its molecular size as well as by Ponceau staining. B, MeCP2-associated activity methylates recombinant histone H3. Histone methyltransferase assays were performed as in A, this time with recombinant H3 as a substrate instead of bulk histones. C, a region in the N terminus of MeCP2, which overlaps with the methyl-CpG-binding domain and to a lesser extent the transcriptional repression domain, mediates the association with histone H3 methyltransferase activity. The indicated GST MeCP2 fusion fragments were tested for their association with histone methyltransferase activity. Residues 1–77 (lane 8) were tested in a separate experiment than the other constructs (lane 1–7). Below is shown a schematic representation of MeCP2 with its methyl-CpG-binding domain and transcriptional repression domain. Also shown are the GST fusion proteins of MeCP2 tested in the histone methyltransferase assay, with the results summarized on the right (++, indicates strong MeCP2-associated methyltransferase activity; --, indicates no associated activity).

Histone Methyltransferase Assays and Protein Sequencing—Precipitations from pull downs or immunoprecipitations from HeLa nuclear extract or 293T transfected extracts were incubated with either 10 μg of histones (Sigma) or 5 μg of recombinant H3 (gift from Karl Nightingale) and 2 μl [3H-Me]-S-adenosyl methionine (Amersham Biosciences, 67 Ci mmol⁻¹) in buffer MAB (50 mM Tris, pH 8.5, 20 mM KCl, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, and 250 mM w/v sucrose) at 30°C for 4 h. The reaction products were then resolved by SDS-PAGE, Western blotted, and autoradiography was performed. The position of radiolabeled H3 was identified by its size using radiolabeled molecular weight markers (Amersham Biosciences) as well as by comparing the autoradiography with the gel stained with Ponceau. For N-terminal sequencing, radiolabeled histone H3 was blotted to polyvinylidene difluoride (Millipore) and sequenced by Edman degradation (Protein Sequencing Facility, University of Cambridge, Cambridge, UK). Amino acid fractions were analyzed for the presence of tritium by scintillation counting.

Western Blot Analysis—Western blotting from MeCP2 Tet-Off cells were done as described (11) using MeCP2 antibody (07–013, Upstate Biotechnology).
RNA Purification and RT-PCR Analysis—Total RNA was isolated from wild type and MeCP2−/− mouse cells (9) following the Qiagen RNeasy Midi protocol. Purified RNA (0.5 μg) was used for quantitative RT-PCR, following the Qiagen One Step protocol, for 28 PCR cycles.

ChIP—MeCP2 Tet-Off cells treated or not with tetracycline were cross-linked with 0.75% formaldehyde (Sigma) at room temperature for 10 min. Cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and collected and counted using a liquid scintillation counter.

FIG. 2. MeCP2 associates with histone H3 methyltransferase activity in vivo. A, MeCP2 enriches histone H3 methyltransferase activity from transfected cells. 293T cells were transiently transfected with 20 μg of either pCMV-HA-MeCP2 (expressing HA-tagged full-length MeCP2, HA-MeCP2, lane 1) or empty pCMV vector (−, lane 2). Whole-cell extracts were precipitated with anti-HA antibody (12CA5) followed by histone methyltransferase assay. The reaction products were then analyzed by SDS-PAGE followed by Western blotting and autoradiography. The radiolabeled H3 is indicated by an arrow on the right. IP ab, immunoprecipitation antibody. B, endogenous MeCP2 associates with H3 methyltransferase activity. HeLa nuclear extracts were immunoprecipitated with either an antibody against MeCP2 (07–013, lane 1) or an irrelevant antibody for green fluorescence protein (ab290, lane 2). After washing, the immune complexes were tested for histone methyltransferase activity. IP ab, immunoprecipitation antibody.

RESULTS

Silencing of transcription by MeCP2 involves the recruitment of HDAC activity (4, 5). However, additional pathways by which MeCP2 represses transcription probably exist because HDAC inhibitors do not completely relieve MeCP2-mediated silencing (4, 5). The retinoblastoma protein represses transcription not only through recruitment of HDAC activity but also through recruitment of histone methylation activity specific for lysine 9 of histone H3 (7). These recent observations prompted us to ask whether histone methylation might represent another pathway by which MeCP2 silences transcription. To test this possibility we first asked if MeCP2 associates with histone methyltransferase activity. Fig. 1A shows that MeCP2 fused to GST is able to purify from nuclear extracts, an activity that specifically methylates histone H3 from a mixture of bulk histones. Recombinant histone H3 can also be used as the substrate for the methyltransferase associated with GST MeCP2 (Fig. 1B). Deletion analysis of MeCP2 indicates that the association of MeCP2 with histone H3 methyltransferase activity is primarily mediated by its N-terminal portion, which overlaps with its methyl-CpG-binding domain (Fig. 1C). In addition, the more C-terminal region of MeCP2, 108–392, that contains the transcriptional repression domain also contributes to the binding of enzymatic activity (Fig. 1C).

To further verify the association of MeCP2 with a methyltransferase, we used a co-immunoprecipitation approach. We transfected mammalian cells with an HA-tagged, full-length MeCP2 (HA-MeCP2), lysed the cells, and carried out precipitation with anti-HA antibody. The resulting immunoprecipitate was assayed for the presence of methyltransferase activity on histones. Fig. 2A (lane 1) indicates that HA-MeCP2 purifies activity specific for histone H3. When endogenous MeCP2 was immunoprecipitated from HeLa nuclear extracts with a MeCP2-specific antibody, the association with the histone H3
methyltransferase was also detected (Fig. 2A, lane 1). Control immunoprecipitation with an irrelevant antibody (green fluorescence protein) gave background activity (Fig. 2B, lane 2). Together, these results demonstrate that MeCP2 is associated with a histone methyltransferase in vivo and that this enzyme specifically methylates histone H3.

We next wanted to identify the residue(s) of histone H3 that are modified by the MeCP2-interacting methyltransferase. Given that a tailless recombinant H3 did not act as a substrate (data not shown), histone H3 radioactively methylated by the MeCP2-interacting methyltransferase was put through Edman degradation to identify the methylation site...
in the H3 tail. Protein sequence analysis revealed that MeCP2 binds histone methyltransferase activity that is specific for lysine 9 of histone H3 (H3 Lys9; Fig. 3). The nature of the MeCP2-interacting histone Lys9 methyltransferase is currently unknown. So far five mammalian H3 Lys9 methyltransferases have been identified: SuvarH1 (14), SuvarH2 (15), G9a (16), ESET (17), and Eu-HMTase1 (18), and several others are predicted to exist (6).

We next wanted to assess whether MeCP2 can facilitate H3 Lys9 methylation of a DNA-methylated gene that it regulates. We chose to investigate the H19 gene because it contains a characterized repressor domain in its upstream regulatory region, (the DMD), which is known to be DNA-methylated (19) and associated with MeCP2 (20). To establish whether MeCP2 represses transcription of H19 in vivo, we monitored the expression of H19 in wild type and knock-out MeCP2−/− cells (9). RNA isolated from each cell type was reverse transcribed and amplified by polymerase chain reaction (RT-PCR). Fig. 4A shows that H19 messenger RNA levels are elevated in the MeCP2 knock-out cells compared with wild type cells. In contrast, the expression of an unrelated house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase, is unchanged (Fig. 4A). Thus, H19 is a bona fide MeCP2-regulated gene that can be used to analyze MeCP2 functions.

To establish if MeCP2 delivers H3 methylation to the H19 gene we next examined the wild type and knock-out MeCP2−/− cells for differential histone methylation at the H19 DMD. An antibody that recognizes histone H3 when methylated at lysine 9 was used in ChIPs. The outcome of these experiments was not conclusive because the chromatin isolated from the MeCP2−/− knock-out cells was qualitatively different from wild type controls. Indeed, purification followed by fragmentation (by sonication or nuclease digestion) of the chromatin from the MeCP2 null and wild type cells consistently resulted in different size fragments upon analysis on agarose gel electrophoresis. So direct quantification and comparison between the two chromatin sets was not possible. We therefore turned to an inducible Tet-Off cell line, where the levels of MeCP2 could be manipulated by the addition of tetracycline (Tc). In this Tet-Off cell line, the presence of tetracycline reduces MeCP2 expression down to endogenous levels (Fig. 4B).

Using this Tet-Off MeCP2 cell line, we performed ChIPs analysis on the H19 DMD using the MeCP2 antibody and the methyl lysine 9 H3-specific antibody. Fig. 4C shows that in the absence of Tc, when MeCP2 expression is high, MeCP2 binds to nucleosomes associated with the DMD (Fig. 4C, lane 1). This MeCP2 binding is specific because immunoprecipitations of the MeCP2−/− knock-out cells was qualitatively different from wild type controls. Indeed, purification followed by fragmentation (by sonication or nuclease digestion) of the chromatin from the MeCP2 null and wild type cells consistently resulted in different size fragments upon analysis on agarose gel electrophoresis. So direct quantification and comparison between the two chromatin sets was not possible. We therefore turned to an inducible Tet-Off cell line, where the levels of MeCP2 could be manipulated by the addition of tetracycline (Tc). In this Tet-Off cell line, the presence of tetracycline reduces MeCP2 expression down to endogenous levels (Fig. 4B).

We then monitored the levels of Me-Lys9 H3 on the nucleosomes associated with the H19 DMD. As shown in Fig. 4D (lane 1), it is clear that the DMD is methylated at Lys9 when MeCP2 levels are high. If MeCP2 was required for H3 Lys9 methylation on the H19 DMD, then down-regulation of MeCP2 cellular level should result in a reduced histone H3 methylation. Fig. 4D shows that under conditions in which the MeCP2 level is low (i.e. in the presence of Tc), nucleosomes within the H19 DMD show a significant reduction in histone H3 Lys9 methylation (Fig. 4D, compare lane 4 with lane 1). These results indicate that the presence of MeCP2 on the H19 gene correlates with the appearance of histone H3 Lys9 methylation at the differentially DNA-methylated and repressive DMD region of the H19 gene.

**DISCUSSION**

The present study provides evidence that the targeting of histone methylation to DNA methylated regulatory regions is part of the mechanism by which MeCP2 functions. To date, MeCP2 action is known to involve the delivery of histone deacetylase activity. However, this mechanism cannot account for the full repressive potential of MeCP2 because inhibitors of histone deacetylase activity only relieve repression partially (4, 5). Thus the delivery of histone methylating activity by MeCP2 may represent a repressive event that follows the targeting of deacetylases. This order of events is suggested by the fact that deacetylation of histone H3 at lysine 9 is necessary for methylation to take place on this residue (21). In this way, deacetylation of histone 3 at lysine 9 would be followed by methylation, which in turn may result in the recruitment of proteins such as HP1 (14, 22). The identity of the lysine methyltransferase associated with MeCP2 is unknown, but members of a family of lysine 9-methylating proteins can be considered candidates (6).

The data presented here identify MeCP2 as a protein that can connect a repressive modification on DNA to a repressive modification on histones. Recent evidence from *Neurospora crassa* and *Arabidopsis thaliana* indicate that the reverse is also possible: methylation of histone H3 at lysine 9 leads to methylation of DNA (23, 24). If we assume that this latter mechanism is also operational in mammalian cells, our data suggest that MeCP2 may set up a self-reinforcing cycle of repression, which may be necessary for the maintenance and heritability of the repressed state. In other words, by promoting further rounds of DNA methylation following histone methylation, MeCP2 would maintain the methylation status at the DMD and thus distinguish the “permanent” repressive state of imprinted promoters from the “regulated” repressive state of other promoters.

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MeCP2 Facilitates H3 Lys\(^9\) Methylation of a Target Gene

The Methyl-CpG-binding Protein MeCP2 Links DNA Methylation to Histone Methylation
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