0888-8809/05/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 19(7):1740–1751

doi: 10.1210/me.2004-0011

Release of Methyl CpG Binding Proteins and Histone Deacetylase 1 from the Estrogen Receptor α (ER) Promoter upon Reactivation in ER-Negative Human Breast Cancer Cells

Dipali Sharma, Julie Blum, Xiaowei Yang, Normand Beaulieu, A. Robert Macleod, and Nancy E. Davidson

The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (D.S., J.B., X.Y., N.E.D.), Baltimore, Maryland 21231; and MethylGene, Inc. (N.B., A.R.M.), Montreal, Canada H45 2A1

Estrogen receptor α (ER) is an epigenetically regulated gene. Inhibitors of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) synergistically activate the methylated ER gene promoter in ER-negative MDA-MB-231 human breast cancer cells. Chromatin immunoprecipitation was used to examine the chromatin status and repressor complex associated with silenced ER and changes in the key regulatory factors during reactivation by inhibitors of DNMT (5-aza-2′-deoxycytidine) and HDAC (trichostatin A). The silencing of ER due to CpG hypermethylation correlates with binding of specific methyl-binding proteins, DNMTs, and HDAC proteins. Inhibition of HDAC activity by trichostatin A results in the accumulation of hyperacetylated core histones. The activation of ER gene expression by 5-aza-2′-deoxycytidine also involves the release of the repressor complex involving various methyl-binding proteins, DNMTs, and HDAC1. HDAC and DNMT inhibitors modulate histone methylation at H3-K9 and H3-K4 to form a more open chromatin structure necessary for reactivation of silenced ER transcription. Together these results impart a better understanding of molecular mechanisms of chromatin remodeling during ER reactivation by DNMT and HDAC inhibitors. These findings will aid in the application of agents targeting epigenetic changes in the treatment of breast cancer. (*Molecular Endocrinology* 19: 1740–1751, 2005)

NORMAL MAMMARY DEVELOPMENT, as well as the initiation and progression of breast cancer, are intensely influenced by hormonal factors. Breast tumors expressing estrogen receptor-α (ER) respond well to therapeutic strategies directed at the ER or its ligands, whereas those that lack ER do not (1, 2). It is imperative to understand what factors determine ER expression in breast cancer. Earlier studies have shown that the ER promoter is hypermethylated and ER mRNA is absent in some ER-negative breast cancer cells. Treatment of ER-negative breast cancer cells with DNA methyltransferase (DNMT) and/or histone deacetylase (HDAC) inhibitors leads to the reactivation of expression of ER mRNA and functional protein, underscoring the importance of DNMTs and HDACs in maintaining the repressive environment at target genes such as ER (3, 4).

First Published Online March 3, 2005

Abbreviations: 5-aza-dC, 5-aza-2′-Deoxycytidine; ChIP, chromatin immunoprecipitation; DNMT, DNA methyltransferase; ER, estrogen receptor; GSTP1, glutathione *S*-transferase P1; HDAC, histone deacetylase; MBD, methyl-CpG binding domain; MDR, multidrug resistance; MSP, methylation-specific PCR; TSA, trichostatin A

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

The mechanisms involved in suppression of transcription of genes via hypermethylation at CpG islands and histone modifications are an area of active research (5). CpG island hypermethylation may inhibit transcription by interfering with the recruitment and function of basal transcription factors or transcriptional coactivators. Also, hypermethylation of CpG dinucleotides near the transcriptional regulatory region may initiate the recruitment of the methyl-CpG binding domain (MBD) family proteins that mediate silencing of genes via facilitation of a repressive chromatin environment (6, 7). At least five methyl-CpG binding proteins, including MeCP2, MBD1, MBD2, MBD3, and MBD4, have been identified in vertebrates (8–13). Although MeCP2, MBD1, MBD2, and MBD3 can all recruit HDAC-containing repressor complexes, distinctive features of each of these proteins have been reported (10, 14–23). Gel shift assays showed that MBD1, MBD2, and MBD4 bind specifically to a variety of DNA sequences containing methyl CpG, whereas MBD3 does not either *in vitro* or *in vivo* (19–21).

Several DNMTs that initiate methylation at position 5 of cytosines of CpG dinucleotides have been identified (24). DNMT1, the chief enzyme responsible for maintenance of mammalian DNA methylation during DNA replication using hemimethylated DNA, can also bind HDAC2 and DNMT-associated protein 1 (DMAP1) to

mediate transcriptional repression (25). The *de novo* methylases, DNMT3a and DNMT3b, which are encoded by different genes (26, 27), can act as transcriptional repressors by using their ATRX domain to recruit HDAC1 (28, 29). Heterochromatic structure characterized by differential modifications of histones is another facet of the complex machinery influencing repression. Amino-terminal tails of the core histones undergo modifications such as acetylation at lysine, methylation at lysine or arginine, and phosphorylation at serine to evolve a histone code for transcriptional activation and repression (30, 31). These posttranslational modifications modulate the chromatin structure by altering the electrostatic interactions between histone proteins and DNA and also by modifying the recruitment of various nonhistone proteins such as coactivators and corepressors to chromatin.

The findings that CpG methylation of the ER promoter results in transcriptional silencing (32) and inhibition of HDAC and/or DNMT activity reactivates ER (3, 4) support a model in which methyl-CpG binding proteins, DNMTs, and HDACs might be involved in transcriptional control of ER. Chromatin immunoprecipitation (ChIP) was used to monitor the epigenetic determinants of ER chromatin remodeling associated with ER expression.

RESULTS

ER Promoter Chromatin Is Associated with Different Proteins in Active *vs.* Repressed State

The ER-negative MDA-MB-231 cells with a hypermethylated ER promoter and the ER-positive MCF-7 cells with an unmethylated ER promoter were used as our model system. Using antibodies against acetylated H4, acetylated H3, methylated H3-K4, methylated H3-K9, HDAC1, various MBD proteins, or DNMT isoforms, formaldehyde cross-linked protein-chromatin complexes were immunoprecipitated from MCF-7 cells or MDA-MB-231 cells. Precipitated genomic DNA was analyzed by PCR using primers encompassing the *Not*I site in the ER gene, the region where methylation is most closely associated with ER gene expression in our earlier work (Fig. 1A). As shown in Fig. 1B, ChIP analysis with anti-MeCP2, anti-MBD1, and anti-MBD2 antibodies revealed that these proteins are associated with the silenced ER promoter in MDA-MB-231 breast cancer cells, whereas the active ER promoter in MCF-7 cells shows little association of these proteins. Consistent with earlier reports that MBD3 does not form specific complexes with methylated DNA (19–21), we found that MBD3 is not associated with the silenced or active ER promoter. Moreover, DNMT1 and DNMT3b were associated with ER promoter in MDA-MB-231 but not MCF-7 cells, whereas DNMT3a showed little association with either promoter. A significant amount of acetylated H3 and H4 was observed at the ER promoter in MCF-7 cells, as evidenced by ChIP analyses using antiacetylated histone H3 and H4 antibodies, whereas the silenced ER promoter in MDA-MB-231 cells showed little or no association of acetylated histones. HDAC1 recruitment was observed only at the silenced chromatin in MDA-MB-231 cells. In ER-negative MDA-MB-231 cells, the promoter region, which has a high level of cytosine methylation and methyl binding protein occupancy, also displayed a high level of core histone H3-K9 methylation, whereas ER-positive MCF-7 cells showed a higher level of H3-K4 methylation.

Efficient Dissociation of Methyl-Binding Proteins from the Silenced ER Promoter Is Observed after Treatment with 5-aza-2′-Deoxycytidine (5-aza-dC) and Trichostatin A (TSA)

As shown in Fig. 2A, treatment of MDA-MB-231 cells with 5-aza-dC or TSA results in reexpression of ER mRNA as detected by RT-PCR; combination therapy is more effective than either agent alone as previously described (4). Methylation-specific PCR (MSP) analysis also confirmed our previous findings (4) that the ER CpG island remains methylated in MDA-MB-231 cells treated with TSA, whereas treatment with 5-aza-dC alone or in combination with TSA partially demethylates the ER CpG island (Fig. 2B). As our findings in Fig. 1 clearly demonstrate specific differential recruitment of various MBD and DNMT proteins to silenced and active ER promoters in ER-negative and -positive human breast cancer cells, respectively, we examined the effects of inhibitors of DNMT and HDAC alone and together on proteins associated with ER promoter.

Treatment of MDA-MB-231 cells with 5-aza-dC or TSA alone leads to a significant reduction in MeCP2 occupancy of the ER promoter by ChIP assay (Fig. 3A). The combination of 5-aza-dC and TSA further reduces MeCP2 binding in conjunction with demethylation of the ER promoter and enhanced ER expression as shown in Fig. 2A. Treatment with 5-aza-dC was associated with a similar pattern of release of MBD1 (Fig. 3B) and MBD2 (Fig. 3C), and this was enhanced by cotreatment with TSA. Unlike MeCP2, treatment with TSA alone did not affect association of MBD1 or MBD2 with the ER promoter in MDA-MB-231 cells. In striking contrast, there was little association of MBD3 with the ER promoter under any circumstances (Fig. 3D). Taken together, these results indicate that MeCP2, MBD1, and MBD2 (but not MBD3) are indeed associated with the methylated inactive ER promoter in MDA-MB-231 cells, and this association is dramatically reduced when the ER gene is partially demethylated and reexpressed.

To ascertain whether these changes are simply the result of altered levels of global methyl-binding protein expression in treated *vs.* untreated cells, immunoblot analyses were performed with antibodies specific for MeCP2, MBD1, MBD2, and MBD3 using protein lysates from MDA-MB-231 cells after treatment with 5-aza-dC, TSA, or the combination (Fig. 3E). None of

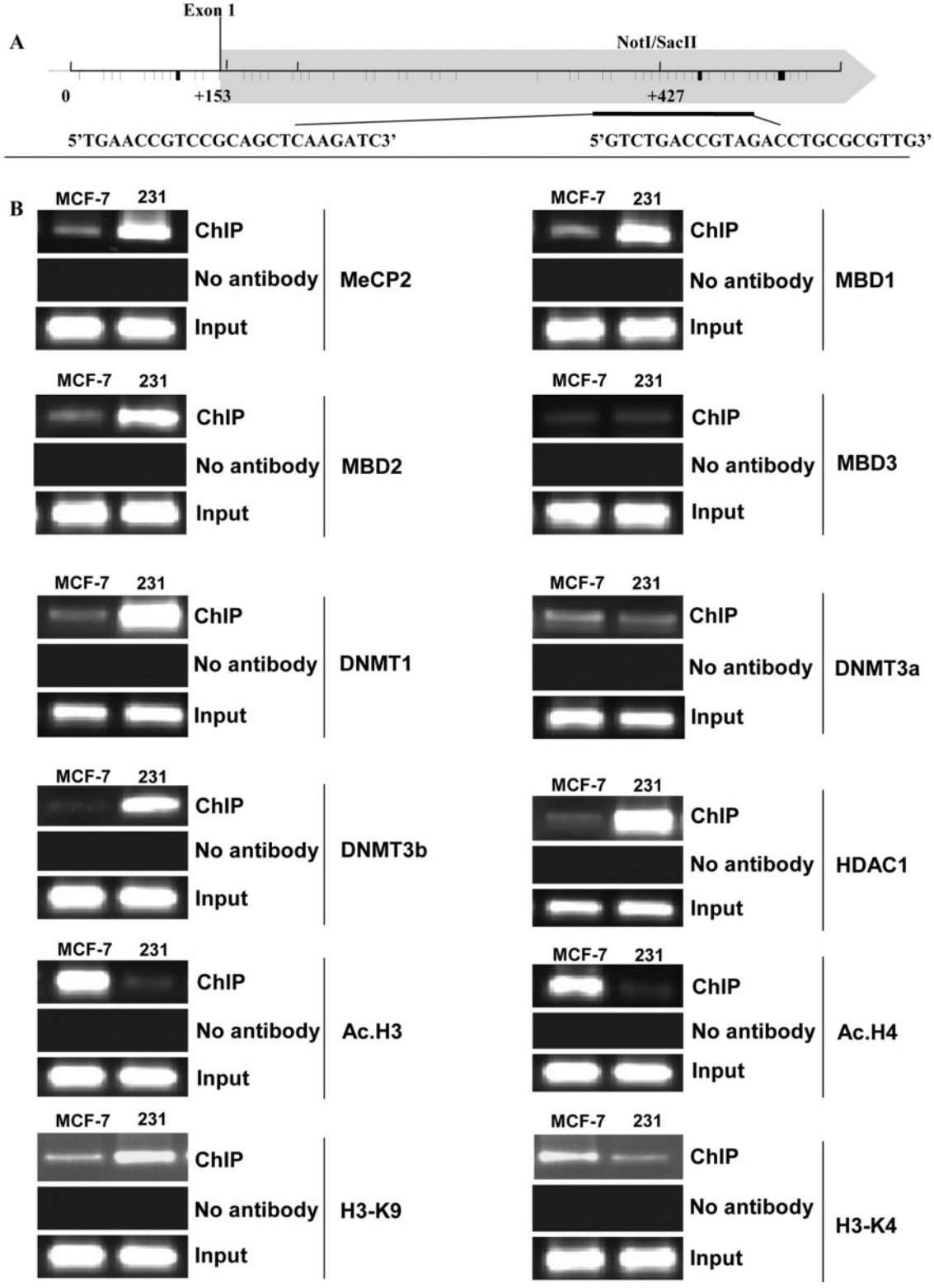

Fig. 1. Recruitment of MBDs, DNMTs, HDAC1, and Histone Acetylation and Methylation Status at the Human ER CpG Island A, ER CpG island and primer sets used for ChIP assay. B, Cross-linked chromatin prepared from ER-positive MCF-7 and ER-negative MDA-MB-231 human breast cancer cells was immunoprecipitated with antibodies indicated on the *right*. The immunoprecipitates were subjected to PCR analysis using primer pairs spanning the *Not*I site on the ERα promoter CpG island (depicted schematically in panel A). Aliquots of chromatin taken before immunoprecipitation were used as input controls, whereas chromatin aliquots eluted from immunoprecipitations lacking antibody were used as no antibody controls. MeCP2, Methyl-CpG protein 2; Ac.H3, acetylated H3 histone; Ac.H4, acetylated H4 histone.

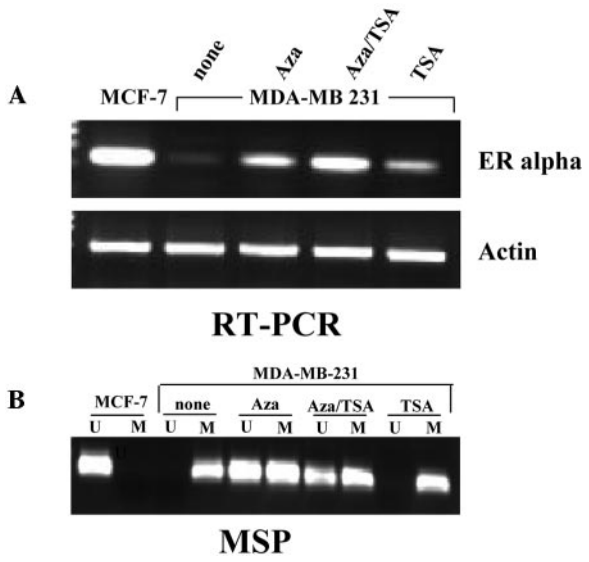

Fig. 2. ER mRNA Expression and ER Gene Methylation Status after Aza and/or TSA Treatment

A, ER reexpression is induced by the HDAC and DNMT inhibitors. RT-PCR analysis shows ER mRNA reexpression after treatment of MDA-MB-231 cells with 5-aza-dC (2.5 μM for 96 h) (Aza), TSA (100 ng/ml for 12 h), or 5-aza-dC and TSA (Aza/TSA). The ER-positive prototype, MCF-7, was used as an RT-PCR-positive control. The RT-PCR product for β-actin provides a control for the amount of intact RNA used in the reactions. B, MSP analysis of ER CpG island. Methylation pattern was analyzed using a previously reported primer set ER5 (32) after 5-aza-dC and TSA treatments in MDA-MB-231 cells. ER-positive MCF-7 cells were used as an unmethylated control, whereas untreated ER-negative MDA-MB-231 cells were used as a methylated control. U, Unmethylated; M, methylated.

these treatments changed global protein levels of any of the methyl-binding proteins, indicating that these drugs do not modulate the expression of these proteins in a substantive way.

Treatment of MDA-MB-231 cells with 5-aza-dC and TSA Alters the Association of DNMT1 and DNMT3b with ER Promoter

We next determined the effects of the HDAC inhibitor, TSA, and the DNMT inhibitor, 5-aza-dC, on the association of DNMT1, DNMT3a, and DNMT3b with the ER promoter using ChIP assays with specific DNMT antibodies. Treatment with 5-aza-dC, but not TSA, reduced the level of association of DNMT1 (Fig. 4A) and DNMT3b (Fig. 4C) with the ER promoter in MDA-MB-231 cells; cotreatment led to synergistic dissociation of DNMT1 and DNMT3b from the ER promoter. There was little association of DNMT3a with the ER promoter in untreated or treated cells (Fig. 4B). Thus, recruitment of DNMT1 and DNMT3b to methylated ER promoter decreases upon demethylation of the promoter.

5-aza-dC inactivates DNMTs by forming an irreversible protein-DNA complex that leads to demethylation of DNA strands followed by transcriptional activation of methylated promoter. We investigated whether treatment with DNMT or HDAC inhibitors alters the level of DNMT protein expression. Treatment of MDA-MB-231 cells with 5-aza-dC for 96 h strikingly depleted DNMT1 and DNMT3a protein levels by Western blot (Fig. 4D). Combined treatment with 5-aza-dC and TSA resulted in further depletion of both DNMT1 and DNMT3a, whereas TSA alone also down-regulated DNMT1 and DNMT3a slightly. The level of DNMT3b in nuclear extracts was unaltered after 5-aza-dC and/or TSA treatments. Thus, reduction of global protein expression may account, in part, for the dissociation of DNMT1 but not DNMT3b from the ER promoter in 5-aza-dC-treated cells. These results suggest that the three DNMT isoforms might have differential sensitivity to 5-aza-dC *in vivo*, a finding previously reported in HCT116 colon cancer cells (36).

Association of Acetylated Histone H3 and H4 with the ER Promoter Increases and HDAC1 Decreases after Treatment with HDAC and DNMT Inhibitor

We investigated whether the observed differential recruitment of MBD and DNMT proteins to the ER promoter and their modulation by 5-aza-dC and TSA are related to altered chromatin structure. Generally, transcriptionally inactive methylated promoters are associated with hypoacetylated histones, whereas active promoters are associated with hyperacetylated histones. We wished to determine whether the enhanced reexpression of ER in MDA-MB-231 cells after treatment with 5-aza-dC and TSA is due to localized hyperacetylation of histones at ER promoter. We performed ChIP assays using an antiacetylated histone H3 antibody (that recognizes histone H3 acetylated at K9 and K14) and an antiacetylated histone H4 antibody (that recognizes histone H4 acetylated at K5, K8, K12, and K16) using MDA-MB-231 cells treated with 5-aza-dC, TSA, or the combination. A very low level of association of acetylated histone H3 (Fig. 5A) and acetylated histone H4 (Fig. 5B) with the ER promoter was seen in MDA-MB-231 cells in contrast with MCF-7 cells. Treatment with TSA significantly elevated the levels of association of acetylated histone H3 and acetylated histone H4 with the ER promoter in MDA-MB-231 cells, whereas treatment with 5-aza-dC had much less effect either alone or when used in combination with TSA (Fig. 5, A and B).

Not only do the MBD family proteins (MeCP2, MBD1, MBD2, and MBD3) recruit HDAC (10, 21–23) but the DNMTs [DNMT1 (25), DNMT3a, and DNMT3b (26, 27)] associate with the HDAC repressor complex as well (28, 29). Therefore, we explored whether the association of HDAC1 with ER promoter in MDA-MB-231 cells was altered by 5-aza-dC and/or TSA treatment. ChIP assay performed with specific HDAC1 antibody showed that HDAC1 was associated with the ER promoter in ER-negative MDA-MB-231 cells. The

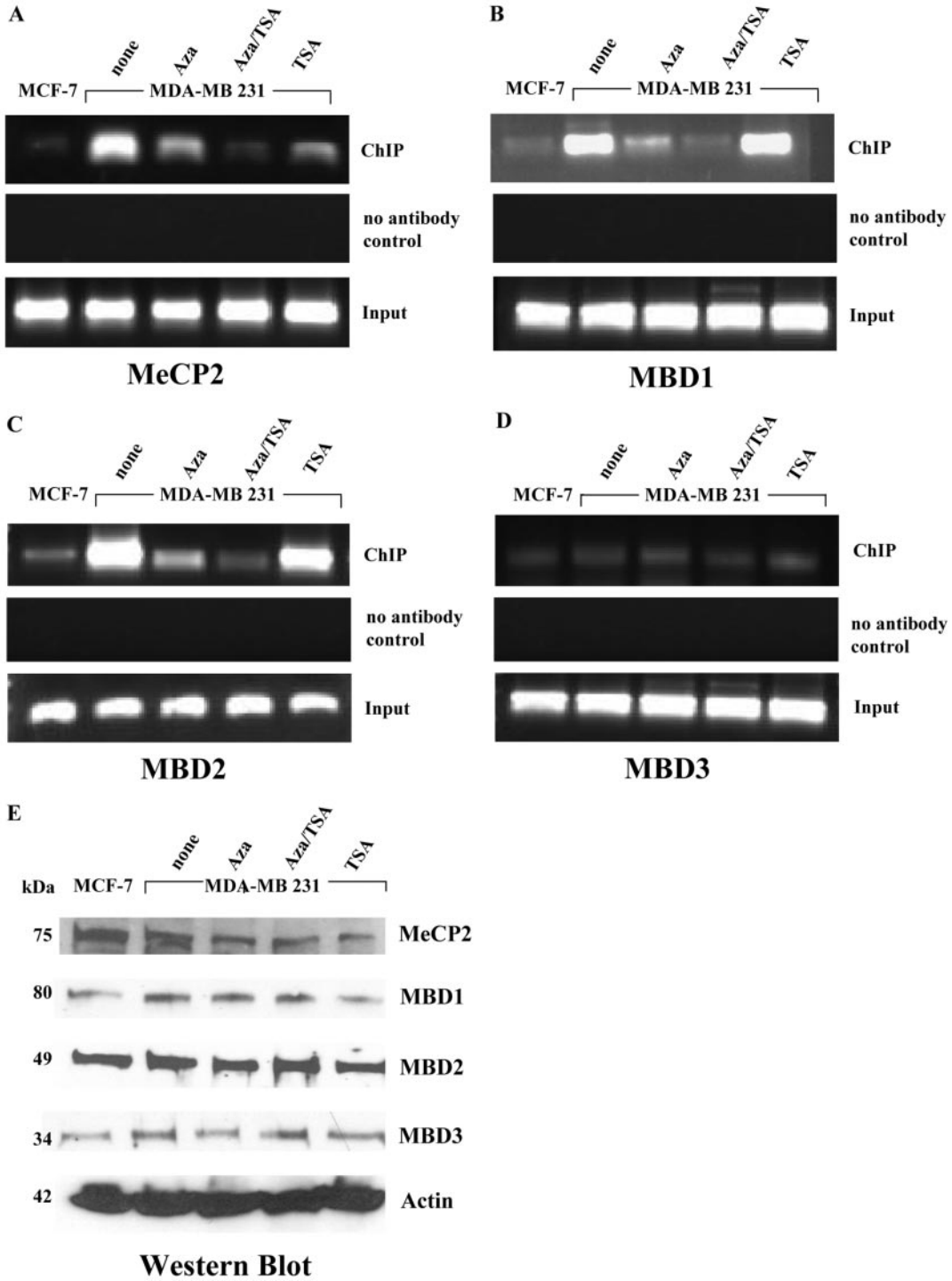

Fig. 3. Association of Methyl-Binding Proteins at the ER Promoter after Aza and/or TSA Treatment

A–D, Profile of methyl-CpG binding proteins on ER CpG island chromatin by ChIP. The formaldehyde-cross-linked chromatin isolated from 10^7 MCF-7 or MDA-MB-231 cells (untreated or treated as in Fig. 2) was precleared with protein A agarose/salmon sperm DNA beads for 1 h at 4 C. The supernatant was incubated with 5 μg of specific antibodies overnight at 4 C. The immune complexes were pulled down with protein A agarose/salmon sperm DNA beads and washed extensively as described in *Materials and Methods*, and cross-linking was reversed. The purified DNA was analyzed by PCR using primers spanning the *Not*I site in ER CpG island. Release of MeCP2 (A), MBD1 (B), and MBD2 (C) from the ER promoter after treatment of MDA-MB-231 cells with the demethylating agent 5-aza-dC (Aza) and the HDAC1 inhibitor TSA was observed. MBD3 (D) was not engaged on methylated or unmethylated ER chromatin. E, Immunoblot analysis of MBDs**.** Equal amounts of protein (100 μg) from whole-cell lysates from the control MCF-7 and MDA-MB-231 and MDA-MB-231 cells treated as in Fig. 2 were separated by SDS-PAGE and subjected to Western blot analysis with specific antibodies against MBD1, MBD2, MBD3, and MeCP2. Equivalence of protein loading was demonstrated by immunoblotting with antiactin antibody.

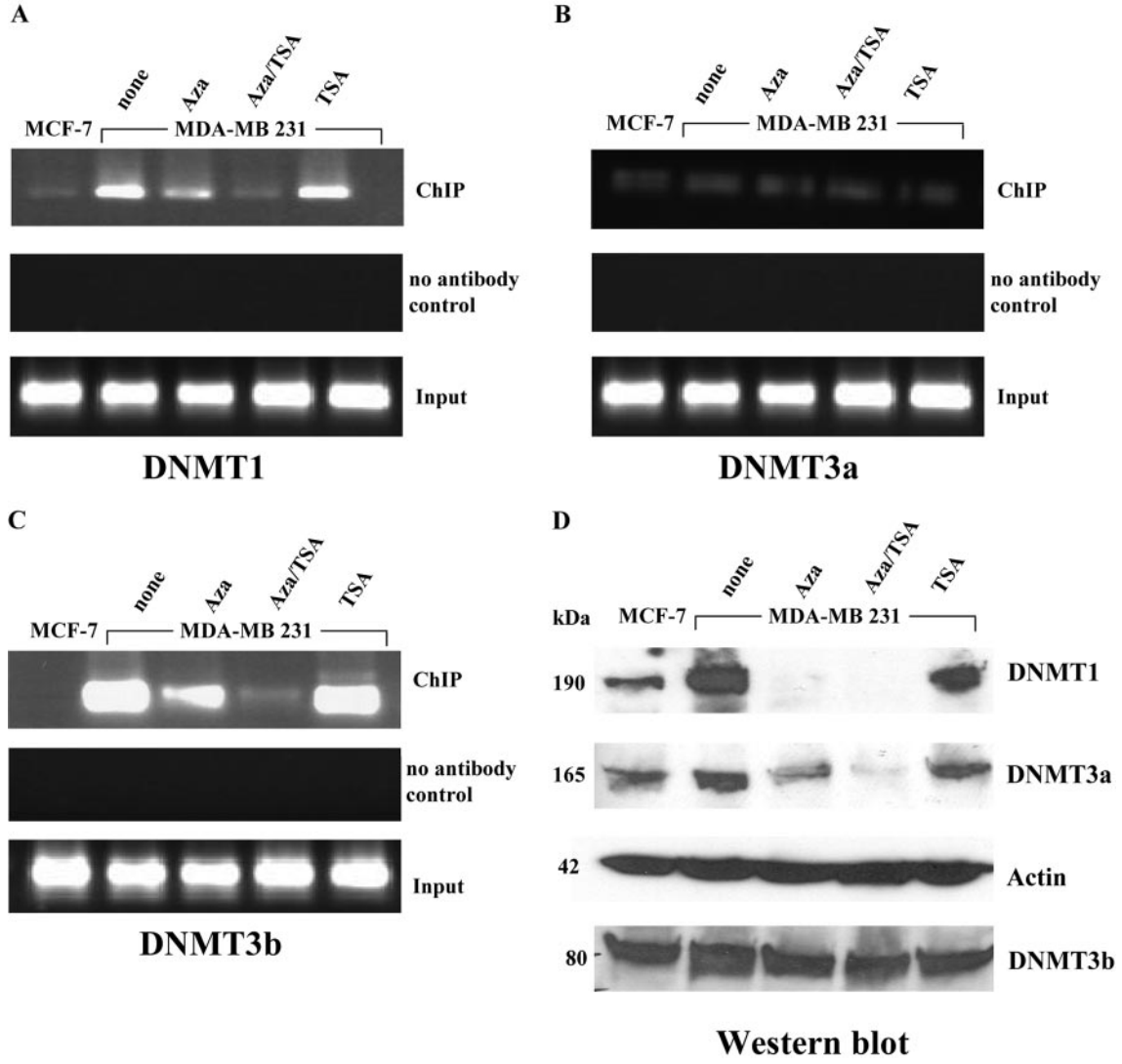

Fig. 4. Association of DNA Methyl-Transferases at the ER Promoter after Aza and/or TSA Treatment

A–C, Profile of DNMTs on ER CpG island chromatin by ChIP. Formaldehyde cross-linked chromatin was immunoprecipitated with antibodies specific for DNMT1 (A), DNMT3a (B), and DNMT3b (C) from untreated and treated cells as in Fig. 2. The purified DNA was amplified for the ER promoter as in Fig. 3. DNMT1 and DNMT3b dissociate from the ER promoter CpG island after treatment of MDA-MB-231 cells with 5-aza-dC (Aza) alone or in combination with TSA. DNMT3a does not associate with the ER CpG island in MCF-7 or MDA-MB-231 cells. Controls show input genomic DNA before the addition of antibody and eluants from no antibody immunoprecipitations. D, DNMT protein expression in the whole-cell lysates from treated and untreated MDA-MB-231 cells**.** Identical amounts (100 μg) of protein from control or treated cells were separated by SDS-PAGE on a 8% acrylamide gel, transferred to a nitrocellulose membrane, and subjected to Western blot analysis with antibodies specific for DNMT1 and DNMT3a. The membranes were reprobed with antiactin antibody to show equal loading of the protein. For DNMT3b analysis, nuclear extracts (100 μg) were prepared from control and treated cells and subjected to immunoblot analysis with anti-DNMT3b antibody.

association was significantly reduced by TSA or TSA/5-aza-dC treatment and was unaffected by 5-aza-dC alone (Fig. 5C). These results confirm the recruitment of HDAC1 to the ER promoter in ER-negative human breast cancer cells, either directly or via interaction with various repressor complexes.

Acetylation status of the core histones H3 and H4 in MDA-MB-231 cells after treatment was also determined. Histones were purified from the nuclei of control and treated cells and analyzed by immunoblotting. As shown in Fig. 5D, treatment of cells with TSA, but not with 5-aza-dC, resulted in increased global histone acetylation. These results clearly demonstrate that HDAC inhibition results in the accumulation of hyperacetylated histones, leading to more open chromatin structure, whereas the DNMT inhibitor was ineffective in this regard. Next, we investigated the effects of TSA and 5-aza-dC on the expression of HDAC1 protein. Western blot analyses with anti-HDAC1 antibody showed that the level of HDAC1 in MDA-MB-231 cells

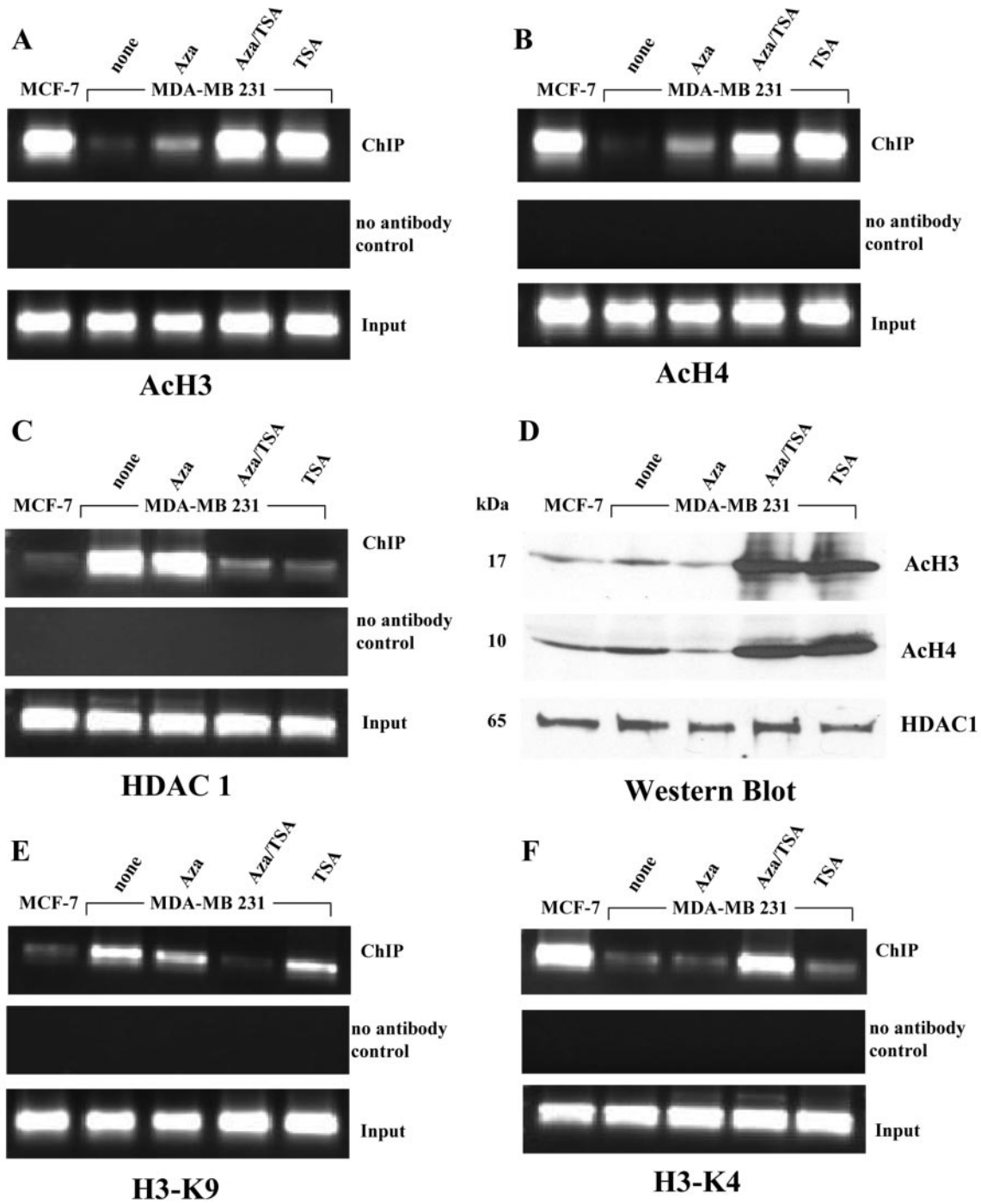

Fig. 5. Association of Modified Histones and HDAC1 at the ER Promoter after Aza and/or TSA Treatment

A and B, Histone acetylation enrichment on active ER chromatin. ChIPs were performed with MDA-MB-231 cells treated with 5-aza-dC (Aza), TSA, or 5-aza-dC and TSA together (Aza/TSA). Associated acetylated H3 and H4 (AcH3, AcH4) histones were analyzed by PCR. C, Association of HDAC1 with ER promoter in MDA-MB-231 cells. ChIP analysis with anti-HDAC 1 antibody showed that HDAC1 associates with ER promoter in MDA-MB-231 cells, and this association is reversed by 5-aza-dC/TSA treatment. D, ER chromatin is enriched in acetylated histone H3 and H4 after DNMT and HDAC inhibition. MDA-MB-231 cells were treated with 5-aza-dC and TSA, nuclei were isolated, and histones were extracted. Equal amounts of protein (30 μg) were run on a 12% SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis with antibodies against acetylated histone H3 (17 kDa) and H4 (10 kDa). MCF-7 and untreated MBA-MB-231 served as controls. Western blotting using anti-HDAC1 antibody showed no change in HDAC1 protein expression after the drug treatments. E and F, Association of methylated H3-K9 and H3-K4 with the ER CpG island. Soluble chromatin was isolated from MCF-7 and MDA-MB-231 cells treated with 5-aza-dC (Aza), TSA, or the combination (Aza/TSA). Immunoprecipitations were performed using specific antibodies for H3-K9 and H3-K4. Shown is a decrease in methylated H3-K9 on the ER promoter in MDA-MB-231 cells after treatments, whereas the methylated H3-K4 level is increased upon treatment with 5-aza-dC and TSA together (Aza/TSA).

was not affected by treatment of cells with TSA or 5-aza-dC (Fig. 5D). Collectively, these results demonstrate that treatment of ER-negative human breast cancer cells with TSA results in accumulation of total acetylated histones H3 and H4 as well as a significant enhancement in their association with ER promoter. HDAC1 protein expression was not affected by any treatment whereas TSA treatment with or without 5-aza-dC led to dissociation of HDAC1 from the ER promoter.

5-aza-dC Plus TSA Reduces K9-Methylated Histone H3 and Augments K4-Methylated Histone H3 at ER Promoter

Other studies suggest a strong link between DNA methylation and H3-K9 methylation in gene repression and the organization of a heterochromatic state (5, 43, 44). We performed ChIP assays using polyclonal antibodies against K4-dimethylated H3 and K9-dimethylated H3 in MCF-7 and MDA-MB-231 breast cancer cells. As shown in Fig. 5, E and F, the repressed ER state in MDA-MB-231 cells is associated with a substantial increase in methylation of K9-dimethylated H3, whereas the activated ER state in MCF-7 cells is associated with increased methylation of K4-dimethylated H3. Because our previous experiments indicated that 5-aza-dC and TSA reactivate silenced ER in MDA-MB-231 cells by altering the chromatin structure, we reasoned that these agents might change the methylation status of histone H3 during the reactivation process. The level of methylated H3-K9 at the ER promoter decreased after treatment with 5-aza-dC or TSA, reaching an even lower level after treatment with 5-aza-dC and TSA together (Fig. 5E). In contrast, H3-K4 methylation increased at the same region after 5-aza-dC and/or TSA together (Fig. 5F) in conjunction with H3 acetylation (Fig. 5A). We conclude that reactivation of ER gene in MDA-MB-231 involves chromatin remodeling of the promoter where the heterochromatic imprint was reversed and replaced with a euchromatic imprint.

DISCUSSION

The molecular mechanisms that regulate silencing of ER in ER-negative human breast cancer cells are not well elucidated. We originally observed that DNA methylation and histone deacetylation characterized the ER promoter in ER-negative, but not ER-positive, human breast cancer cell lines. Treatment of these cell lines with a DNMT or HDAC inhibitor can reactivate ER mRNA expression, and the combination of DNMT and HDAC inhibitor could synergistically activate functional ER mRNA and protein (4, 45).

Here we show that the unmethylated active ER promoter in MCF-7 cells is enriched for H3 and H4 acetylation and H3-K4 methylation and shows little binding of any methyl-binding protein or DNMT. In MDA-MB-231 cells, the ER promoter is silenced by DNA hypermethylation, histone hypoacetylation, H3-K9 methylation and the recruitment of MeCP2, MBD1, MBD2, DNMT1, DNMT3b, and HDAC1 proteins. ER reactivation by pharmacological intervention is a complex process involving modulation of binding of various nonhistone proteins and modifications of core histones such as acetylation, deacetylation and selective methylation. Treatment of MDA-MB-231 cells with the HDAC inhibitor, TSA, causes histone hyperacetylation and a low level of ER mRNA reexpression as DNMT1, DNMT3b, MeCP2, MBD1, and MBD2 are still bound to the methylated ER promoter. Treatment with the DNMT inhibitor, 5-aza-dC, also induces ER mRNA expression, but it facilitates promoter demethylation along with partial dissociation of MeCP2, MBD1, MBD2, DNMT1, DNMT3b, and DNMT1. The diminished association of the ER promoter with DNMT1 may also reflect global depletion of this protein by 5-aza-dC; no such effect of 5-aza-dC on total protein for the methyl-binding proteins was seen. 5-aza-dC-treated MDA-MB-231 cells also displayed a relative depletion of acetylated H3 and H4 and methylated K9 H3. Thus, treatment of MDA-MB-231 cells with 5-aza-dC or TSA leads to reexpression of ER, but strikingly different protein complexes are associated with the ER promoter in each case. The combination of 5-aza-dC and TSA facilitates the release of a repressor complex containing various MBD proteins (MeCP2, MBD1, and MBD2), DNMTs (DNMT1 and DNMT3b), and HDAC1 from the ER promoter in MDA-MB-231 cells. This release was observed with concomitant enrichment of acetyl-H4, acetyl-H3, and K4-dimethylated H3 and diminished methylation at K9-H3. Thus the reactivated ER promoter in MDA-MB-231 cells treated with both drugs acquires a ChIP profile similar to that of the innately active ER promoter in MCF-7 cells.

Similar studies have been carried out with other epigenetically regulated gene promoters. Studies of human multidrug resistance (MDR1) gene regulation have shown that histone acetylation and dissociation of MeCP2 and HDAC1 mark the activation of MDR1 gene upon treatment with TSA and 5-aza-dC whereas other MBD proteins such as MBD1, MBD2, MBD3, and MBD4 are not involved in MDR1 activation in T cell leukemia cell lines (15). In lymphosarcoma cells, the activation of the metallothionein I (MT-I) promoter by 5-aza-dC and TSA is linked with dissociation of MeCP2 and absence of effect on MBD1 or MBD3 (33). In both hepatocellular carcinoma and MCF-7 human breast cancer cells, MBD2, but not MeCP2, has been implicated in glutathione *S*-transferase P1 (GSTP1) silencing at the hypermethylated GSTP1 CpG island (46, 47). A gene-specific profile of MBD association with other genes including the Ras association domain family 1A gene (RASSF1A), the GSTP1 gene, the retinoic acid receptor β 2 (RARβ2) gene, the breast cancer 1 (BRCA1) gene, the O^6-methylguanine-DNA

methyltransferase (MGMT) gene and the mutL-homolog 1 (MLH1) gene in MCF7 and MDA-MB-231 cells has also been determined (48). This genome-wide analysis revealed that some methylated sequences associate with only one MBD whereas others associate with several or all of them. Thus the nature of the repressor complex appears to be gene specific and is influenced by several variables such as promoter methylation, histone modification patterns, and transcription factor requirements.

We also demonstrate that reactivation of ER is accompanied by an increase in H3-K4 methylation and decrease in H3-K9 methylation (Fig. 5). Whereas the role of histone acetylation in transcriptional regulation is well recognized, recent studies have underlined the importance of histone methylation in gene expression (49). The effect of methylation of lysine residues on the N-terminal tails of histones appears to be site specific. The identification of SUV39H1 and its yeast homolog, Clr4, as the H3-K9-specific histone methyltransferase provides the first direct connection between H3-K9 methylation and heterochromatin gene silencing (50). A recent study reported that multimolecular complexes containing pRb2/p130-E2F4/5-HDAC1-SUV39H1-p300 and pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 mediate transcription of ER in breast cancer cells (51). Our finding that histone H3 is methylated at the lysine 9 position at the ER promoter in MDA-MB-231 cells, but not MCF-7 or drug-treated MDA-MB-231 cells, is consistent with the role of H3-K9 methylation in transcription repression. H3-K9 methylation has been shown to create a binding site for HP1 in the N-terminal tail of histone H3 (52); thus, H3-K9 methylation might facilitate ER repression in MDA-MB-231 cells by recruiting HP1. Another mechanism for ER repression in ER-negative cells can be through MBD1 as MBD1 interacts with the SUV39H1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression (53). Future work will test whether HP1 is indeed recruited to the ER promoter region during repression. Also, MeCP2 has been shown to associate with histone methyltransferase activity *in vivo*, and this activity is directed against lysine 9 of histone H3 (54). Our results show that MeCP2 is associated with ER promoter in MDA-MB-231 cells where histone H3 is methylated at the lysine 9 position. 5-aza-dC treatment induces release of MBD proteins, reduction in H3-K9 methylation, and enhanced H3 acetylation and H3-K4 methylation. Similar results have been shown for the MDR1 gene (15), the silenced loci p14ARF/p16INK4α in T24 bladder cancer cells (55), and at several genes in MCF7 and MDA-MB-231 cells (48).

In summary, the ER promoter in ER-positive MCF-7 cells is enriched for H3 and H4 acetylation and H3-K4 methylation and shows little methyl-binding protein or DNMT1 association or H3-K9 methylation. In ER-negative MDA-MB-231 cells, the methylated ER promoter is associated with multiple methyl-binding proteins and DNMT, H3-K9 methylation, and histone hypoacetylation. MDA-MB-231 cells treated with DNMT and HDAC inhibitors to reexpress ER recapitulate the MCF-7 profile, showing a substantial increase in two euchromatic markers, H3-K4 methylation and H3 and H4 acetylation, and confirming that a combination of DNMT and HDAC inhibitors can induce chromatin remodeling. The clinical importance of these observations is increasingly evident. Initial studies of DNA methylation profiles of 35 candidate epigenetically regulated genes in primary human breast cancers demonstrated significant differences in hormone receptor status between clusters of DNA methylation profiles, and methylation of the ER gene outperformed hormone receptor status as a predictor of clinical response in patients treated with tamoxifen (56). Further, much work is focused on the possibility that epigenetic changes might also be appropriate targets for treatment of malignancy. This field has been advanced by the clinical availability of the first DNMT inhibitor approved for use in humans, azacitidine, and the rapid clinical development of a number of HDAC inhibitors that are now in clinical testing (57, 58). The possibility that such agents might sensitize ER-negative human breast cancer cells to the effects of endocrine therapy has been suggested in preclinical studies, such as our own, showing that Hs578t cells that reexpressed ER as a consequence of 5aza-dC treatment became sensitive to the growth-inhibitory effects of estrogen (34). Thus, our studies, which identify key molecular mechanisms for epigenetic regulation of ER expression in human breast cancer cells and provide further insight into the molecular mechanisms of action of DNMT and HDAC inhibitors alone or in combination in chromatin remodeling and modulation of gene expression, will be crucial in taking these approaches forward in breast cancer treatment.

MATERIALS AND METHODS

Antibodies

Antibodies against modified forms of H3 and H4 histone, HDAC1, and MBD2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against MBD1 and MBD3 were a generous gift from Samson T. Jacob (33). The anti-MeCP2 antibody was kindly provided by Peter L. Jones (9). The anti-DNMT1 (35) and anti-DNMT3b (36) antibodies have been previously described. Anti-DNMT3a antibody was procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture, Reagents, and Treatment with 5-Aza-dC and TSA

The human breast cancer cell lines, MDA-MB-231 and MCF-7, were grown in DMEM supplemented with 5% fetal bovine serum (Gemini Bioproducts, Woodland, CA) and 2 μM L-glutamine (Invitrogen, San Diego, CA). For treatment, cells were seeded at a density of 5×10^5/100-mm tissue culture dish. After 24 h of incubation, the culture media were changed to media containing 2.5 μM 5-aza-dC (Sigma Chemical Co., St. Louis, MO) for 96 h or 100 ng/ml TSA (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 12 h. For the combination study, 5-aza-dC was present in culture for 96 h and TSA was added for the last 12 h (4).

ChIP

ChIP analyses were performed using a published procedure (37) with the following modifications. Chromatin samples were sonicated on ice three times for 10 sec each (*i.e*. until the average length of sheered genomic DNA was 1–1.5 kb) followed by centrifugation for 10 min. The immunoprecipitated DNA was ethanol precipitated and resuspended in 25 μl H_2O. Total input samples were resuspended in 100 μl H_2O and diluted 1:100 before PCR analysis. PCR contained 5 μl of immunoprecipitate or total input, 50 μM of each primer, 1.5 mM $MgCl_2$, 2 mM deoxynucleotide triphosphate mixture, 1× PCR buffer (Sigma), and 1.25 U of *Taq* DNA polymerase (Sigma) in a total volume of 50 μl. The ER promoter was analyzed using the 5′-primer 5′-TGA ACC GTC CGC AGC TCA AGA TC-3′ and the 3′-primer 5′-GTC TGA CCG TAG ACC TGC GCG TTG-3′. Initially, PCR was performed with different numbers of cycles or dilutions of input DNA to determine the linear range of the amplification; all results shown fall within this range. After 30 cycles of amplification, PCR products were run on 1% agarose gel and analyzed by ethidium bromide staining. All ChIP assays were performed at least twice with similar results.

Western Blot

Whole-cell lysates were prepared by scraping cells in 1 ml of ice-cold buffer A [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonylfluoride, 10 μg /ml aprotinin, 10 μg /ml leupeptin]. The lysate was rotated 360° for 1 h at 4 C followed by centrifugation at 12,000 × *g* for 10 min at 4 C to clear the cellular debris (38). Proteins were quantified using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Preparation of nuclear extract was modified from a method described previously (39). Briefly, cells in 10-cm culture dishes were collected for harvesting by gentle scraping in 1 ml ice-cold PBS and pelleting by centrifugation at 1200 rpm at 4 C. The cell pellet (5×10^7 cells) was washed once in PBS followed by resuspension in 1 ml of lysis buffer [10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40]. Nuclei were gently isolated by centrifugation at 4,000 rpm and resuspended in 200 μl of extraction buffer [20 mM Tris-Cl (pH 7.9), 0.42 mM KCl, 0.2 mM EDTA, 10% glycerol, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride]. The resulting nuclear extracts were incubated on ice for 10 min and cleared by centrifugation at 10,000 rpm. Protein concentration of nuclear extracts was determined by BCA protein assay kit. Proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes, and Western blot analyses were performed using previously described antibodies. Immunodetection was performed using enhanced chemiluminescence (ECL system, Amersham Pharmacia Biotech, Inc., Arlington Heights, IL) according to the manufacturer's instructions.

RNA Isolation and RT-PCR

Total cellular RNA was extracted using the TRIZOL Reagent kit (Life Technologies, Inc., Gaithersburg, MD) and quantified by UV absorption. RT-PCR was carried out according to our previously described method (3). RNAs under comparison were simultaneously reversibly transcribed to achieve equal efficiency for reverse transcription. Synthesized cDNA (4% or 2 μl, derived from 150 ng of initial RNA) was used for PCR amplification of ER and the constitutively expressed housekeeping gene β-actin. Specific sense and antisense PCR primers used for the amplification across the seventh intron of ER and the first intron of β-actin genes, yielding 470 and 400 bp of PCR products, respectively, were described previously (40). PCR products were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Genomic DNA Isolation and Methylation-Specific PCR (MSP) Analysis

Genomic DNA was isolated by standard phenol-chloroform extraction. Isolated DNA was subjected to modification by sodium bisulfite to convert unmethylated cytosines, but not methylated cytosines, to uracils as described previously (41) Methylation status of the bisulfite-modified DNA at the ER locus was characterized by MSP using our previously reported method (32).

Isolation of Histones

Extraction of cellular histones was performed using 2×10^6 cells according to a previously published procedure (42) with the following modifications. The acid (H_2SO_4)-soluble supernatant was precipitated with 10 volumes of cold acetone. After overnight precipitation, histones were collected by centrifugation. The pellet containing histones was dissolved in 50 μl of H_2O, and protein was quantified by using the BCA protein assay kit.

Acknowledgments

We thank Drs. Samson T. Jacob and Peter L. Jones for provision of antibodies.

Received January 12, 2004. Accepted February 22, 2005.

Address all correspondence and requests for reprints to: Nancy E. Davidson, MD, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, Room 409, Baltimore, Maryland 21231. E-mail: davidna@jhmi.edu.

Current address for D.S.: Winship Cancer Institute of Emory University, 1365 Clifton Road, NE, Atlanta, Georgia 30322

Current address for X.Y.: Cancer Therapeutics Branch, National Cancer Institute/National Naval Medical Center, 8901 Wisconsin Avenue, Building 8, Room 5101, Bethesda, Maryland 20889.

Full address for N.B. and A.R.M.: Department of Molecular Biology, MethylGene, 7220 Frederick-Banting, Montreal, Canada H4S 2A1.

This work was supported by National Institutes of Health Grant CA88843, the Susan G. Komen Breast Cancer Foundation, and the Breast Cancer Research Foundation.

REFERENCES

1. McGuire WL 1978 Hormone receptors: their role in predicting prognosis and response to endocrine therapy. Semin Oncol 5:428–433
2. Hortobagyi GN 1998 Treatment of breast cancer. N Engl J Med 339:974–984
3. Yang X, Ferguson AT, Nass SJ, Phillips D, Butash KA, Wang SM, Herman JG, Davidson NE 2000 Transcriptional activation of estrogen receptor α in human breast cancer cells by histone deacetylase inhibition. Cancer Res 60:6890–6894
4. Yang X, Phillips D, Ferguson AT, Nelson WG, Herman JG, Davidson NE 2001 Synergistic activation of functional estrogen receptor (ER)-α by DNA methyltransferase and histone deacetylase inhibition in human ER-α-negative breast cancer cells. Cancer Res 61:7025–7029
5. Jenuwein T, Allis CD 2001 Translating the histone code. Science 293:1074–1080
6. Wade PA 2001 Methyl CpG-binding proteins and transcriptional repression. Bioessays 23:1131–1137

7. Bird AP, Wolffe AP 1999 Methylation-induced repression-belts, braces, and chromatin. Cell 99:451–454
8. Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird A 1998 Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature 393:386–389
9. Jones PL, Veenstra, GJ, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP 1998 Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19:187–191
10. Ng HH, Jeppesen P, Bird A 2000 Active repression of methylated genes by the chromosomal protein MBD1. Mol Cell Biol 20:1394–1406
11. Ng HH, Zhang Y, Hendrich B, Johnson CA, Turner BM, Erdjument-Bromage H, Tempest P, Reinberg D, Bird A 1999 MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nat Genet 23: 58–61
12. Snape A 2000 MBDs mediate methylation, deacetylation and transcriptional repression. Trends Genet 16:20
13. Wade PA, Jones PL, Vermaak D, Wolffe AP 1998 A multiple subunit Mi-2 histone deacetylase from *Xenopus laevis* cofractionates with an associated Snf2 superfamily ATPase. Curr Biol 8:843–846
14. Meehan RR, Lewis JD, Bird AP 1992 Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. Nucleic Acid Res 20:5085–5092
15. El-Osta A, Kantharidis P, Zalcberg JR, Wolffe AP 2002 Precipitous release of methyl-CpG binding protein 2 and histone deacetylase 1 from the methylated human multidrug resistance gene (MDR1) on activation. Mol Cell Biol 22:1844–1857
16. Cross SH, Meehan RR, Nan X, Bird A 1997 A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. Nat Genet 16:256–259
17. Boyes J, Bird A 1991 DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell 64:1123–1134
18. Wade PA, Gegonne A, Jones PL, Ballestar E, Aubry F, Wolffe AP 1999 Mi-2 complex couples DNA methylation to chromatin remodeling and histone deacetylation. Nat Genet 23:62–66
19. Zhang Y, Ng HH, Erdjument-Bromage H, Tempst P, Bird A, Reinberg D 1999 Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. Genes Dev 13:1924–1935
20. Saito M, Ishikawa F 2002 The mCpG-binding domain of human MBD3 does not bind to mCpG but interacts with NuRD/Mi2 components HDAC1 and MTA2. J Biol Chem 277:35434–35439
21. Hendrich B, Bird A 1998 Identification and characterization of a family of mammalian methyl-CpG binding proteins. Mol Cell Biol 18:6538–6547
22. Meehan RR, Lewis JD, McKay S, Kleiner EL, Bird AP 1989 Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell 58:499–507
23. Nan X, Meehan RR, Bird A 1993 Dissection of the methyl-CpG binding domain from the chromosomal protein MeCP2. Nucleic Acids Res 21:4886–4892
24. Bestor TH 2000 The DNA methyltransferases of mammals. Hum Mol Genet 9:2395–2402
25. Rountree MR, Bachman KE, Baylin SB 2000 DNMT1 binds HDAC2 and a new corepressor, DMAP1, to form a complex at replication foci. Nat Genet 25:269–277
26. Okano M, Bell DW, Haber DA, Li E 1999 DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99: 247–257
27. Xie S, Wang Z, Okano M, Nogami M, Li Y, He WW, Okumura K, Li E 1999 Cloning, expression and chromosome locations of the human DNMT3 gene family. Gene 236:87–95
28. Bachman KE, Rountree MR, Baylin SB 2001 Dnmt3a and Dnmt3b are transcriptional repressors that exhibit unique localization properties to heterochromatin. J Biol Chem 276:32282–32287
29. Fuks F, Burgers WA, Godin N, Kasai M, Kouzarides T 2001 Dnmt3a binds deacetylases and is recruited by a sequence specific repressor to silence transcription. EMBO J 20:2536–2544
30. Dillon N, Festenstein R 2002 Unravelling heterochromatin: competition between positive and negative factors regulates accessibility. Trends Genet 18:252–258
31. Berger SL 2002 Histone modifications in transcriptional regulation. Curr Opin Genet Dev 12:142–148
32. Lapidus RG, Nass SJ, Butash KA, Parl FF, Weitzman SA, Graff JG, Herman JG, Davidson NE 1998 Mapping of ER gene CpG island methylation specific polymerase chain reaction. Cancer Res 58:2515–2519
33. Ghoshal K, Datta J, Majumder S, Bai S, Dong X, Parthun M, Jacob ST 2002 Inhibitors of histone deacetylase and DNA methyltransferase synergistically activate the methylated metallothionein I promoter by activating the transcription factor MTF-1 and forming an open chromatin structure. Mol Cell Biol 22:8302–8319
34. Ferguson AT, Vertino PM, Spitzner RJ, Baylin SB, Muller MT, Davidson NE 1997 Role of estrogen receptor gene demethylation and DNA methyltransferase-DNA adduct formation in 5-aza-2′-deoxycytidine-induced cytotoxicity in human breast cancer cells. J Biol Chem 272: 32260–32266
35. De Marzo, AM, Marchi, VL, Yang, ES, Veeraswamy R, Lin X, Nelson WG 1999 Abnormal regulation of DNA methyltransferase expression during colorectal carcinogenesis. Cancer Res 59:3855–3860
36. Robert M-F, Morin S, Beaulieu N, Gauthier F, Chute IC, Barsalou A, MacLeod AR 2003 DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. Nat Genet 33:61–65
37. Sharma D, Fondell JD 2002 Ordered recruitment of histone acetyltransferases and the TRAP/mediator complex to thyroid hormone-responsive promoters *in vivo.* Proc Natl Acad Sci USA 99:7934–7939
38. Sharma D, Fondell JD 2000 Temporal formation of distinct thyroid hormone receptor coactivator complexes in HeLa cells. Mol Endocrinol 14:2001–2009
39. Andrews NC, Faller DV 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499
40. Ferguson AT, Lapidus RG, Baylin SB, Davidson NE 1995 Demethylation of the estrogen receptor gene in estrogen receptor-negative breast cancer cells can reactivate estrogen receptor gene expression. Cancer Res 55: 2279–2283
41. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB 1996 Methylation specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93:9821–9826
42. Yoshida M, Kijima M, Akita M, Beppu T 1990 Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. J Biol Chem 265:17174–17179
43. Jenuwein T 2001 Re-SET-ting heterochromatin by histone methyltransferases. Trends Cell Biol 1:266–273
44. Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SI 2001 Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatic assembly. Science 292: 110–113
45. Keen JC, Yan L, Mack KM, Pettit C, Smith D, Sharma D, Davidson NE 2003 A novel histone deacetylase inhibitor, Scriptaid, enhances expression of functional estrogen receptor α (ER) in ER negative human breast cancer cells

in combination with 5-aza-2′deoxycytidine. Breast Cancer Res Treat 81:177–186
46. Bakker J, Lin X, Nelson WG 2002 Methyl-CpG-binding domain protein-2 represses transcription from hypermethylated π-class glutathione S-transferase gene promoters in hepatocellular carcinoma cells. J Biol Chem 277:22573–22580
47. Lin X, Nelson WG 2003 Methyl-CpG-binding domain protein-2 mediates transcriptional repression associated with hypermethylated GSTP1 CpG islands in MCF-7 breast cancer cells. Cancer Res 63:498–504
48. Ballestar E, Paz MF, Valle L, Wei S, Fraga MF, Espada J, Cigudosa JC, Huang TH-M, Esteller M 2003 Methyl-CpG binding proteins identify novel sites of epigenetic inactivation in human cancer. EMBO J 22:6335–6345
49. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD, Jenuwein T 2000 Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406:593–599
50. Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, Kouzarides T 2001 Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410:120–124
51. Macaluso M, Cinti C, Russo G, Russo A, Giordano A 2003 pRb2/p130–E2F4/5-HDAC1-SUV39H1–p300 and pRb2/p130–E2F4/5-HDAC1-SUV39H1-DNMT1 multimolecular complexes mediate the transcription of estrogen receptor-α in breast cancer. Oncogene 22:3511–3517
52. Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T 2001 Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410:116–120
53. Fujita N, Watanabe S, Ichimura T, Tsuruzoe S, Shinkai Y, Tachibana M, Chiba T, Nakao M 2003 Methyl-CpG binding domain 1 (MBD1) interacts with the SUV39H1-HP1 heterochromatic complex for DNA methylation based transcriptional repression. J Biol Chem 278:24132–24138
54. Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T 2003 The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. J Biol Chem 278: 4035–4040
55. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JCY, Liang G, Jones PA 2002 Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2′-deoxycytidine. Cancer Res. 62:6456–6461
56. Widschwendter M, Siegmund KD, Muller HM, Fiegl H, Marth C, Muller-Holzner E, Jones PA, Laird PW 2004 Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. Cancer Res 64:3807–3813
57. Egger G, Liang G, Aparicio A, Jones PA 2004 Epigenetics in human disease and prospects for epigenetic therapy. Nature 429:457–463
58. Gilbert J, Gore SD, Herman JG, Carducci MA 2004 The clinical application of targeting cancer through histone acetylation and hypomethylation. Clin Cancer Res 10: 4589–4596

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**