The Histone Deacetylase Inhibitor Trichostatin A Blocks Progesterone Receptor-mediated Transactivation of the Mouse Mammary Tumor Virus Promoter in Vivo*

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Post-translational modifications of histones play an important role in modulating gene transcription within chromatin. We used the mouse mammary tumor virus (MMTV) promoter, which adopts an ordered nucleosomal structure, to investigate the impact of a specific inhibitor of histone deacetylase, trichostatin A (TSA), on progesterone receptor-activated transcription. TSA induced global histone hyperacetylation, and this effect occurred independently of the presence of hormone. Interestingly, chromatin immunoprecipitation analysis revealed no significant change in the level of acetylated histones associated with the MMTV promoter following high TSA treatment. In human breast cancer cells, in which the MMTV promoter adopts a constitutively “open” chromatin structure, treatment with TSA converted the MMTV promoter into a closed structure. Addition of hormone did not overcome this TSA-induced closure of the promoter chromatin. Furthermore, TSA treatment resulted in the eviction of the transcription factor nuclear factor-1 from the promoter and reduced progesterone receptor-induced transcription. Kinetic experiments revealed that a loss of chromatin-remodeling proteins was coincident with the decrease in MMTV transcriptional activity and the imposition of repressed chromatin architecture at the promoter. These results demonstrate that deacetylase inhibitor treatment at levels that induce global histone acetylation may leave specific regulatory regions relatively unaffected and that this treatment may lead to transcriptional inhibition by mechanisms that modify chromatin-remodeling proteins rather than by influencing histone acetylation of the local promoter chromatin structure.

In chromatin, DNA is arranged into arrays of nucleosomes that consist of 146 bp of DNA wrapped around two copies each of histone proteins H2A, H2B, H3, and H4 assembled as an octamer (1). Addition of a linker histone (H1) assembles the DNA into less well described “higher order” chromatin, leading to a fully condensed chromosome (2). Studies of chromatin structure have shown that the packaging of DNA within chromatin plays an important role in the regulation of gene expression (3, 4). Chromatin structure may affect transcriptional activation by blocking the access of trans-acting factors to their target sequences and/or the assembly of the basal transcriptional machinery to form the preinitiation complex (5, 6).

However, chromatin is not static. Its dynamic nature is evidenced through post-translational modification of histone N-terminal tail domains, which are reversibly acetylated at ε-lysine residues due to an equilibrium between acetylation and deacetylation (4, 7, 8). The observation that transcriptional cofactors possess enzymatic activity, specifically acetyltransferase activity, provided a direct mechanism by which histones within the context of a specific promoter might be modified (9–13). Although histone hyperacetylation is often associated with increased gene expression, for a number of genes, this is not the case (14). This raises the intriguing possibility that, like protein phosphorylation, histone acetylation may be a multifaceted post-translational modification with respect to gene expression (7). For example, studies have shown the inhibitory effects of histone hyperacetylation induced by deacetylase inhibitors on steroid-inducible genes such as ovalbumin (15), tyrosine aminotransferase (16), prolactin receptor (17), inter-leukin-2 (18), and mouse mammary tumor virus (MMTV)1 (19, 20) and, more recently, on vitamin D regulation of the osteocalcin gene (21).

The progesterone (PR), glucocorticoid, androgen, and mineralocorticoid receptors are members of the steroid hormone receptor family, a class of receptors that belong to a large nuclear hormone receptor superfamily of hormone-activated transcriptional regulators (22). Steroid hormone receptors regulate gene expression by binding specific DNA sequences in target genes termed hormone response elements (23). To study the effects of histone acetylation on steroid-induced transcriptional activation, we used the MMTV promoter as a model system because it assumes a defined chromatin structure in vivo. The stably integrated MMTV promoter reproducibly assembles into a phased array of six nucleosomes (A–F) (24). The region of the promoter occupied by the second nucleosome in the array, nucleosome B (Nuc-B), contains the hormone response elements to which steroid hormone receptors bind as well as target sites for other necessary transcription factors, including nuclear factor-1 (NF1) and octamer transcription factors (25). When stably transformed into T47D cells that express the PR but lack the glucocorticoid receptor (PR−/gr−; 2963.1 cells), the

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1The abbreviations used are: MMTV, mouse mammary tumor virus; PR, progesterone receptor; PRα and PRβ, progesterone receptor isoforms A and B, respectively; Nuc-B, nucleosome B; NF1, nuclear factor-1; TSA, trichostatin A; LTR, long terminal repeat; CAT, chlormphenicol acetyltransferase; ChIP, chromatin immunoprecipitation; mSin3A, mammalian Sin3A.

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MMTV promoter adopts a novel chromatin structure that is “open” over Nuc-B (26). This novel chromatin structure permits the constitutive binding of the PR and other integral transcription factors such as NF1 prior to hormone addition while retaining a hormone-inducible response (26).

Utilizing the 2963.1 cells, we assessed the effect of low and high trichostatin A (TSA) concentrations on chromatin structure mediated by the PR within the context of the MMTV promoter. We demonstrate that PR-mediated transcription and chromatin remodeling were inhibited at the MMTV promoter when cells were treated to high levels of TSA. Interestingly, under conditions where global histone hyperacetylation was observed, the levels of histone hyperacetylation at the MMTV promoter were not significantly affected by TSA treatment. However, expression of a variety of transcriptional co-regulators, including chromatin-remodeling proteins, was reduced. Thus, in this human breast cancer cell line, histone deacetylase inhibitors may repress the chromatin architecture of the MMTV promoter by mechanisms other than histone hyperacetylation of the proximal promoter.

MATERIALS AND METHODS

Cell Culture—2963.1 cells were derived from T47D cells by stable cotransfection of the chimeric bovine papilloma virus-based vector pB3d, carrying the MMTV long terminal repeat (LTR) attached to the bacterial chloramphenicol acetyltransferase (CAT) gene (26). Cells were grown at 37 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (BioWhittaker, Inc.). Cells were treated with R-TSA (Sigma) at either low (5 ng/ml) or high (100 ng/ml) concentrations for the times indicated in the figure legends. Cells were treated with TSA and hormone as indicated in the figure legends. Nuclei were isolated as described previously (28). Acid-soluble proteins were isolated from nuclei in 100 mM HEPES, pH 7.5, containing 10% glycerol, 1 mM EDTA, 10 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM EGTA, 1 mM PMSF, 1 mM phenylmethylsulfonyl fluoride, and 2 mg/ml bovine serum albumin as described previously (31). Proteins were subjected to reiterative primer extension assays and 3 μg of DNA for transcription factor binding assays were subjected to reiterative primer extension.

Western Blot Analysis—Subconfluent 2963.1 cells were treated as indicated in the figure legends prior to harvesting. Cells were lysed in buffer containing 100 mM Tris-HCl (pH 8.5), 250 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM EDTA, 1 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 100 ng/ml bovine serum albumin as described previously (31). Proteins were electrophoresed on 6% or 10% SDS-acrylamide gels and transferred to nitrocellulose membranes (Hybond-P, Amersham Biosciences) at 230 mA for 2 h at 4 °C. The membranes were incubated with antibodies against PR isoforms A (PRa) and B (PRb), NF1, mSin3a, and p300 (Santa Cruz Biotechnologies); HDAC1 and NCoR (Upstate Biotechnologies, Inc.); BRG-1, BAF155, and NCoA1 (31); and RbAp48/46. The proteins were detected by ECL Plus reagent (PerkinElmer Life Sciences), followed by autoradiography with Hyperfilm.

Chromatin Immunoprecipitation (ChIP) Analysis—ChIP analysis was carried out using the ChIP assay kit from Upstate Biotechnologies, Inc. (catalog no. 17-295) with minor modifications of the protocol. 2963.1 cells (106) were plated onto 100-mm dishes and treated the next day as described in the figure legends. Ten micrograms of either anti-acetylated histone H4 (catalog no. 06-866, Upstate Biotechnology, Inc.) or anti-β actin kinase-α (catalog number sc-7606; Santa Cruz Biotechnologies) antibody was added to tubes containing 1 ml of chromatin solution. Following incubation with antibody, 60 μl of salmon sperm DNA/protein A-agarose was added to each tube, and the agarose-antibody complexes were then captured by centrifugation. After the beads were pelleted and washed, the chromatin was extracted, and the protein/DNA cross-links were reversed. Following purification, the DNA was subjected to PCR amplification using primers MMTV-22 and MMTV-345 (5′-TATAAGCTAGTTTGGTGCAAACT-3′) under the following conditions: 30 cycles, 55 °C, 1.5 mM MgCl2, 0.2 mM deNTPs, 5 units of Taq polymerase, and 25 pmol of each primer. Eight microliters of each reaction was analyzed on 1.5% Tris borate/EDTA-agarose gels, and the bands were quantified using the Alpha Imager documentation system (Alpha Innotech Corp.). The DNA was analyzed twice by PCR, and graphs were calculated based on these data.

RESULTS

High Levels of Trichostatin A Inhibit PR-dependent Activation of the MMTV Promoter—To examine the relationship between histone acetylation and PR-mediated transcriptional activation at the MMTV promoter, we made use of a unique human breast cancer cell line model, T47D/2963.1 (26). These cells normally exhibit an open chromatin structure with the constitutive binding of transcription factors at Nuc-B within the MMTV promoter. We also made use of TSA, a potent and specific inhibitor of histone deacetylation, at both low (5 ng/ml) and high (100 ng/ml) concentrations to manipulate the acetylation status of the histones and to induce global histone hyperacetylation (32). The MMTV promoter exhibited a significant level of basal transcriptional activity as measured by CAT assays (Fig. 1A, ●). However, treatment of cells from these cultures with hormone-inducible after treatment with the synthetic progestin R5020 (Fig. 1A, ○). Pretreatment of cells with low TSA had no effect on hormone-stimulated MMTV transcriptional activity (Fig. 1A, compare ● and ○). Furthermore, treatment with low TSA alone failed to either increase or inhibit MMTV basal transcriptional activity (Fig. 1A, compare ● and ●). In contrast, pre-
treatment with high TSA prior to adding R5020 inhibited hormone-induced transcription as seen in the absence of TSA (Fig. 1A, compare ○ and □). Moreover, treatment with high TSA alone reduced the level of MMTV transcriptional activity to below basal activity (Fig. 1A, compare ● and ■). Our results demonstrate that high (but not low) TSA treatment inhibits the transcriptional activity of the MMTV promoter in 2963.1 cells.

High Levels of Trichostatin A Induce Global Histone Acetylation—To examine potential causes for the different effects of low and high TSA treatments, we initially investigated the global histone acetylation status. Core histones were isolated and analyzed by electrophoresis on acid/urea-polyacrylamide gels, which resolved multiple acetylated histone isoforms (Fig. 1B). The TSA-induced changes in acetylation were most readily observed by examining changes in histone H4 isoforms. In the absence of TSA, only the un- and monoacetylated forms of histone H4 were detected. Treatment with the hormone R5020 did not alter the histone acetylation profile (Fig. 1B, lanes 1 and 2). Treatment with low TSA decreased the level of the unacetylated form and increased the level of the mono- and diacetylated forms of histone H4 (Fig. 1B, lanes 1 and 5). In contrast, treatment with high TSA resulted in increased levels of di-, tri-, and tetraacetylated forms of histone H4 (Fig. 1B, lanes 1 and 6). Increases in the acetylated forms of histones H2A, H2B, and H3 were also observed with high TSA treatments (Fig. 1B, lanes 1 and 6). Addition of R5020 did not alter the histone acetylation patterns generated by treatment with low and high TSA alone (Fig. 1B, lanes 3–6). Thus, exposure to high concentrations of TSA results in increased histone acetylation patterns, correlating with the inactivation of the MMTV promoter within 2963.1 cells.

High TSA Leads to Hyposensitive Chromatin at Nuc-B on the MMTV LTR—Previous analysis of 2963.1 cells demonstrated that the promoter region encompassed by Nuc-B is constitutively open and hypersensitive to restriction enzyme endonucleases (26). To evaluate the effect of histone hyperacetylation on the chromatin architecture, we first examined the extent of restriction enzyme hypersensitivity using the enzyme SstI. The constitutive hypersensitivity to SstI characteristic of 2963.1 cells was reduced by treatment with high (but not low) concentrations of TSA (Fig. 2A, lanes 1, 5, and 6), indicating that Nuc-B is converted to a closed chromatin structure. Moreover, this closed chromatin structure was maintained in cells pretreated with high (but not low) concentrations of TSA prior to agonist treatment (Fig. 2A, lanes 3–6).

To confirm that the observed effects of inhibiting histone deacetylation were not restricted to the 3′-region of Nuc-B, we utilized the restriction enzyme AphII, which cleaves near the 5′-boundary of Nuc-B. As seen with SstI, the MMTV promoter remained hypersensitive to AphII digestion both in the absence
and presence of hormone, indicative of its open chromatin conformation (Fig. 2B, lanes 7 and 8). Treatment of cells with high concentrations of TSA resulted in reduced cleavage by AflII, consistent with the now “closed” chromatin architecture of the promoter in the absence or presence of hormone (Fig. 2B, lanes 8, 10, and 12). This closed chromatin structure was not observed when 2963.1 cells were treated with low concentrations of TSA. The MMTV promoter remained accessible to digestion with AflII at levels similar to untreated cells in the absence or presence of hormone (Fig. 2B, lanes 7, 9, and 11). These data demonstrate that, under conditions that lead to histone hyperacetylation, the constitutive hypersensitivity of Nuc-B in the MMTV promoter is lost.

High TSA Evicts Transcription Factor NF1 from the MMTV Promoter—Because an open chromatin structure is essential for transcription factors such as NF1 to bind in vivo, the above data predict that treatment with high concentrations of TSA would block transcription factor binding. This direct consequence of histone hyperacetylation on protein-DNA interaction was examined using in vivo footprinting assays (33). In 2963.1 cells, transcription factor NF1 bound to the promoter independently of hormone (Fig. 3, lanes 1 and 2), consistent with a constitutively open chromatin architecture. Treatment with high TSA inhibited NF1 binding to the MMTV promoter (Fig. 3, compare lanes 1 and 6); subsequent hormone treatment after high TSA pretreatment did not restore NF1 binding to the MMTV promoter in 2963.1 cells (Fig. 3, compare lanes 2, 4, and 6). As predicted, treatment with low TSA had no effect on NF1 binding either in the absence or presence of hormone (Fig. 3, lanes 3 and 5). Thus, treatment with histone deacetylase inhibitor results in a closed MMTV chromatin structure, blocking the binding of NF1, which is necessary for transcriptional activation.

High TSA Does Not Alter the Structure of Nuc-B on a Transient MMTV Template—High levels of TSA result in the loss of restriction enzyme hypersensitivity within the region associated with nucleosome B in the MMTV promoter. Because high TSA treatment modifies the acetylation status of histones at many locations within the cell, we examined the possibility that decreased digestion of the MMTV promoter was a result of
increased SstI and AflIII restriction sites now available elsewhere within the cell. To determine this, we analyzed the ability of SstI to digest a transiently introduced MMTV template under the same experimental conditions in which decreased digestion was observed on the integrated template. By introducing a transient MMTV template, we increased the number of target sites for the restriction enzyme SstI. It is also important to note that the MMTV promoter adopts an ordered nucleosomal structure only upon stable integration into chromatin, but not when transiently expressed. Under these circumstances, the MMTV template adopts a relatively loose and open conformation. In 2963.1 cells, the transient MMTV template pLTR-Luc (30) remained sensitive to SstI in the absence and presence of agonist treatment, resulting in 42 and 51% digestion, respectively (Fig. 4A, lanes 1 and 2). Treatment with high TSA did not inhibit SstI digestion of the transient MMTV template (Fig. 4A, lanes 1 and 4), the same conditions under which digestion of the stable MMTV promoter decreased (Fig. 2A, lanes 1 and 6). In fact, digestion with SstI was slightly enhanced, resulting in 59% cutting. Furthermore, agonist treatment following high TSA pretreatment had no effect on SstI digestion (54% cutting) (Fig. 4A, lanes 3 and 4). Therefore, decreased SstI digestion of the integrated MMTV promoter within Nuc-B is due to altered chromatin structure (i.e. chromatin closure), as opposed to an increase in restriction sites or other factors that may affect the restriction enzyme SstI.

High TSA Does Not Affect NF1 Binding to a Transient MMTV Template—High levels of TSA also result in the loss of NF1 binding to the MMTV promoter within Nuc-B. To determine the effect of high TSA on the ability of NF1 to interact with its DNA-binding site, we analyzed a transient MMTV template by exonuclease III footprinting under the same conditions that NF1 binding was inhibited. As expected from the enzyme hypersensitivity results, NF1 bound to the transient MMTV promoter within Nuc-B in the absence or presence of R5020 (Fig. 4B, lanes 1 and 2). Treatment with high TSA did not alter NF1 binding (Fig. 4B, lanes 1 and 4). NF1 bound the transient MMTV template under the same conditions in which NF1 bound to the stable chromatin MMTV promoter (Fig. 3, lanes 1 and 6). Addition of R5020 subsequent to high TSA pretreatment did not alter NF1 binding (Fig. 4B, lanes 1, 3, and 4). Consequently, in 2963.1 cells, binding of NF1 to the stable, nucleosome-associated MMTV promoter is inhibited due to the closed chromatin architecture induced by high TSA treatment. Our results demonstrate that NF1 is competent to bind DNA under these conditions.

High Levels of TSA Reduce the Levels of Various Regulatory Factors—In addition to promoting histone acetylation, TSA is known to result in hyperacetylation of other cellular proteins (34). To extend our analysis of the inhibitory effects of high TSA on MMTV activation, we examined the steady-state levels of cellular factors that may participate in MMTV activation by the PR. Our results demonstrated that the levels of both progesterone receptor isoforms (PR$_{B}$ and PR$_{A}$) were greatly reduced in response to high TSA treatments in the absence and presence of hormone relative to untreated cells (Fig. 5A, com-
Pare lanes 1, 4, and 6). PRB levels were decreased by 95% by high TSA treatment both in the absence and presence of hormone. PRα levels were also reduced by 95% by high TSA treatment either alone or in combination with low TSA, although not to the extent as observed with high TSA (Fig. 5A). Treatment with R5020 caused a 65% reduction in PRB levels when added alone; however, R5020 reduced the levels of PRB by 85% when added with low TSA treatment. Although hormone addition was unable to overcome the effects of high TSA treatment, it appeared to antagonize gested in vivo with HaeIII and exonuclease III and then re-digested in vitro with HaeIII to serve as an internal loading control. The HaeIII product and the 5'-boundary corresponding to NF1 are indicated.

Fig. 5. Influence of high and low TSA exposure on co-regulator steady-state levels. Cells were treated as described in the legend to Fig. 1B prior to harvest. Lane 1, control; lane 2, R5020; lane 3, low TSA (L) + R5020; lane 4, high TSA (H) + R5020; lane 5, low TSA; lane 6, high TSA. Western blotting for the indicated factors was performed as described under “Material and Methods.”
the effects of the low TSA-induced decrease in PR$_{A}$ levels while enhancing the low TSA-induced decrease in PR$_{B}$ levels. In contrast to these results, expression of NF1 was largely unaffected by treatment with either low or high concentrations of TSA (Fig. 5A). Therefore, the loss of NF1 binding at Nuc-B is not a result of reduced NF1 expression in response to high TSA exposure.

We next examined expression of proteins that have been shown to be components of the histone deacetylase complex, the target of TSA inhibition (35–38). Expression of HDAC1, mSin3a, and RbAp48 and RbAp46 (components of the mSin3a repressor complex) was unaffected by treatment with low or high TSA independent of hormone induction (Fig. 5B). The nuclear receptor corepressor NCoR was reduced upon treatment with high TSA (80% decrease) (Fig. 5B), which was not reversed by subsequent treatment with R5020 (70% decrease) (Fig. 5B, compare lanes 1, 4, and 6).

Given the inhibitory effects of high TSA on activation, we then examined the fate of factors involved in chromatin remodeling and transcriptional activation of the promoter (31). Consistent with the activity data presented earlier, treatment with high (but not low) TSA reduced the levels of the BRG-1 protein by 90% (Fig. 5C, compare lanes 1, 5, and 6). Treatment with R5020 did not abrogate the effects of high TSA on BRG-1 levels (Fig. 5C, compare lanes 1, 4, and 6). Similarly, the levels of NCoA1 were reduced in response to high (but not low) levels of TSA in the absence of hormone (Fig. 5C, compare lanes 1, 5, and 6), displaying an 80% reduction in protein levels. However, unlike BRG-1 levels, this reduction was reversed by treatment with R5020 (Fig. 5C, compare lanes 1, 4, and 6). Finally, expression of the acetylase p300 was also decreased by high TSA treatment both in the absence and presence of hormone, although the decrease was less when R5020 was present (Fig. 5C).

The previous protein profiling results demonstrate, not surprisingly, that high TSA treatment had diverse effects on protein levels, down-regulating a subset of cofactors, whereas others remained unaffected when the MMTV promoter was inactivated. In particular, the down-regulation of the PR, coactivators, and BRG-1 proteins at the same time that histones are fully acetylated would potentially represent a powerful model for repressing this promoter. However, the loss of corepressors as seen with NCoR might compensate for the loss of the cofactors. In the next series of experiments, we examined the temporal relationship between protein down-regulation, histone acetylation, and closing of the MMTV promoter.

Kinetics of Chromatin Closure, Transcription Factor Eviction, and Histone Hyperacetylation—Given that TSA inhibition of HDAC1 is known to be rapid (32), we next examined the kinetics of TSA exposure upon PR transactivation. As an initial assay, we documented the time course of histone acetylation within cells treated with high TSA (Fig. 6A). As observed previously, histones from untreated 2963.1 cells were primarily unacetylated, with the presence of un- and monoacetylated forms of histone H4 (Fig. 6A, lane 1). Treatment with high TSA for 1 h induced the global acetylation of histones and resulted in an increase in the mono-, di-, and triacetylated forms of histone H4 (Fig. 6A, lane 2). By 4 h of high TSA, there was a loss of the un- and monoacetylated forms of histone H4; moreover, the acetylated forms of histones H2A, H2B, and H3 were the prevalent species as well as tetraacetylated histone H4 (Fig. 6A, lane 3). Longer treatments with high TSA did not alter the hyperacetylation pattern established within 4 h (Fig. 6A, lanes 4–6). This pattern of histone acetylation was confirmed using antibodies directed against acetylated histones H3 and H4 (data not shown). Therefore, our results demonstrate that global histone acetylation occurred rapidly in response to treatment with high TSA.

In the next series of experiments, we examined the chromatin architecture by restriction enzyme hypersensitivity assays in cells under a similar time course of TSA exposure used for histone acetylation as described above. Results are representative of repeated experiments. Untreated 2963.1 cells demonstrated the constitutive hypersensitivity characteristic of the open chromatin architecture (Fig. 6B, lane 1), as shown previously (Fig. 2). Treatment with high TSA for increasing lengths of time significantly reduced the extent of cleavage by SstI, consistent with the closure of chromatin structure associated with Nuc-B. Treatment with high TSA for as little as 1 h reduced cleavage by the restriction endonuclease SstI within Nuc-B by 15% (Fig. 6B, compare lanes 1 and 2). Chromatin cleavage was reduced by 50% after treatment with high TSA for 4 h (Fig. 6B, compare lanes 1 and 3). Moreover, maximal closure of MMTV chromatin occurred after 12 h of treatment with high TSA (Fig. 6B, compare lanes 1 and 5), reducing chromatin cleavage by 80%. Therefore, chromatin closure of the MMTV promoter occurs rapidly subsequent to high TSA treatment and correlates with global histone hyperacetylation.

The binding of NF1 is intimately linked with MMTV expression; and as shown earlier (Fig. 3), NF1 is lost from the promoter with high TSA. This leads to the prediction that the kinetics of NF1 loss should mirror the closure of the promoter seen earlier. Indeed, treatment of 2963.1 cells with high TSA for increasing periods of time resulted in the sequential loss of NF1 binding that was coincident with the closing of the promoter as well as histone hyperacetylation (Fig. 6C, lanes 1–6).

Kinetics of TSA-induced Loss of Chromatin-remodeling Proteins—Because we noted that inhibition of PR activity occurred progressively in response to high TSA treatment, we investigated the expression profiles of various cellular factors under the same time course of TSA exposure. Examination of both the PR$_{A}$ and PR$_{B}$ isoforms revealed that receptor levels did not fall significantly until after 12 h of TSA exposure (Fig. 6D). In fact, treatment with high levels of TSA for 1 h appeared to increase the levels of both PR isoforms (Fig. 6D, lane 2). Furthermore, the steady-state levels of NF1 were unaffected by treatment with high TSA for increasing lengths of time. Therefore, eviction of NF1 and loss of chromatin hypersensitivity at the MMTV promoter cannot be attributed to the loss of PR protein expression in response to TSA exposure.

In contrast to the PR, the levels of the chromatin-modifying factor BRG-1 displayed a noticeable 70% decrease in expression after 4 h of treatment with TSA (Fig. 6D, compare lanes 1 (0 h) and 3 (4 h)). Protein levels underwent a further decrease after exposure to TSA for 24 h, demonstrating an ~85–90% reduction (Fig. 6D). BRG-1 is known to function as part of a larger macromolecular complex of proteins to remodel chromatin. To ascertain if TSA was down-regulating other members of the complex, we examined the levels of BAF155, a member of the BRG-1 chromatin-remodeling core complex. Indeed, BAF155 displayed a similar expression profile as seen with BRG-1 and was down-regulated by 75% at 4 h (Fig. 6D). Similarly, BAF155 protein levels were decreased by 90% at 24 h post high TSA treatment. To extend our analysis, we examined the levels of a group of corepressor proteins over the same time course. Expression of the nuclear receptor corepressor NCoR increased up to 2-fold in response to the shorter term treatments of TSA (1–12 h) and then decreased by ~50% below basal levels after 24 h of treatment (Fig. 6D). Finally, the levels of both mSin3a and HDAC1 were unaffected by high TSA treatment at all time points, as would be predicted from the previous assay at 24 h (Fig. 6D). This series of experiments suggests that, despite the distinct effects of TSA on nuclear protein profiles observed, the
Fig. 6. Kinetics of TSA modulation of histone acetylation and chromatin-remodeling and co-regulator protein levels. A, hyper-acetylation of histones. 2963.1 cells were untreated or treated with high TSA (HTSA) for increasing lengths of time, as indicated, prior to harvest. The different histone isoforms are indicated. Arrows indicate the un-, mono-, di-, tri- and tetraacetylated isoforms of histones H4. B, restriction enzyme hypersensitivity is reduced. Shown is a schematic representation of the proximal MMTV promoter. 2963.1 cells were treated as described for A. Nuclei were isolated and partially digested in vivo with SstI and then re-digested in vitro with HaeIII to serve as an internal loading control. A, /X DNA-HaeIII digest molecular weight standard. Arrows indicate HaeIII and SstI cleavage products. C, NF1 binding is abrogated. Shown is a schematic representation of the MMTV proximal promoter. 2963.1 cells were treated as described for A. /X DNA-HaeIII digest molecular weight standard; Gseq, G sequence reaction of the corresponding MMTV promoter sequence. Nuclei were isolated and partially digested in vivo with HaeIII and exonuclease III and then re-digested in vitro with HaeIII to serve as an internal loading control. The HaeII1 product and the 5'-boundary corresponding to NF1 are indicated. D, changes in steady-state levels of cellular factors. 2963.1 cells were treated as described for A. Western blotting for the indicated factors was performed as described under "Material and Methods" for the following. oligo 22, oligonucleotide primer MMTV-22; CAT oligo, oligonucleotide primer MMTV-CAT.
changes in MMTV chromatin structure most closely parallel the changes in components of the BRG-1 chromatin-remodeling complex.

Deacetylase Inhibition Does Not Significantly Change the Levels of Acetylated Histone H4 at the MMTV Promoter—To determine whether the increase in global histone acetylation in response to TSA treatment also occurred locally at the MMTV proximal promoter, we used ChIP assays with an antibody specific to the acetylated form of histone H4 (Fig. 7). R5020 treatment reduced the levels of acetylated histone H4 associated with the promoter (Fig. 7A, lanes 9 and 10), as has been observed previously by others (43). Surprisingly, treatment with high TSA did not significantly alter the levels of acetylated histone H4 (Fig. 7A, lanes 9 and 11) after 24 or 2 h of treatment (Fig. 7B, lanes 7–9). Immunoprecipitation with a nonspecific antibody to the IκB kinase-α protein did not result in a significant background signal (Fig. 7, A and B, lanes 5–8 and 4–6, respectively). Thus, although TSA treatment causes a global increase in histone H4 acetylation, specific locations of the genome such as the MMTV promoter may be relatively unaffected.

**DISCUSSION**

The assembly of gene regulatory sequences into defined chromatin structures provides an attractive and powerful means to control both constitutive and inducible gene expressions. Post-translational modifications of histones represent a potentially important mechanism by which histone-DNA interactions can...
be modulated to allow specific changes in gene activity. The acetylation and deacetylation of the core histones have been extensively investigated, and a general pattern has emerged, such that acetylation is associated with active chromatin, whereas deacetylation is linked to inactive chromatin (39, 40). However, recent evidence suggests a more complex scenario with respect to individual genes and/or promoters (41).

We have used the MMTV promoter to assess the consequences of inhibiting histone deacetylase activity on transcriptional activation mediated by the progesterone receptor. Within human breast cancer cells, we observed the transcriptional repression of the MMTV promoter in response to TSA-induced histone hyperacetylation. These observations are distinct from a previous investigation that reported that moderate increases in histone acetylation induced by treatment with low concentrations of TSA activated MMTV promoter activity and chromatin remodeling (20). As indicated earlier, we did not see any clear changes in histone acetylation or promoter activity in response to low TSA. However, at higher concentrations of TSA (50 ng/ml), similar to those we employed (100 ng/ml), a similar decrease in hormone-induced transcription was observed (20), as reported here. In our studies, the cells exhibited a threshold effect for TSA such that exposure to low levels of TSA (5 ng/ml) failed to effectively hyperacetylate core histones and did not significantly affect the PR activation of the promoter (see Figs. 1–3 and 5). In contrast, exposure to high levels of TSA (100 ng/ml) resulted in a rapid and profound global hyperacetylation of histones and a significant loss of PR-induced activation. Mechanistically, this treatment alters promoter chromatin architecture such that the constitutively open or hypersensitive promoter reverts to a closed or hyposensitive structure. Moreover, hormone treatments were unable to reverse these structural changes. The consequence of this reversion in chromatin structure was the eviction of NF1 from the promoter (Fig. 3). Treatment with high TSA, which promotes these inhibitory events, had no effect on a naked DNA MMTV template (Fig. 4).

Indeed, the transient MMTV promoter was susceptible to digestion with SstI and was bound by NF1 (Fig. 4), demonstrating that NF1 retains the ability to interact with DNA in the presence of TSA.

Given the somewhat unexpected relationship between the global levels of histone acetylation and the inhibition of MMTV transcription, we examined the local acetylation of histones at the MMTV promoter. Unexpectedly, ChIP analysis demonstrated that treatment with high TSA did not significantly alter the acetylation status of histone H4 at Nuc-B within the MMTV promoter. Therefore, although the general pools of histones within cells are hyperacetylated upon treatment with high TSA, histone acetylation within the MMTV promoter is unaffected, if not decreased. This raises the intriguing possibility that TSA may inhibit the activity of chromatin-remodeling and co-regulatory molecules that have been previously shown to participate in the activation of the promoter (31). Indeed, prolonged exposure to high levels of TSA resulted in the down-regulation of both PR isoforms, the chromatin-remodeling proteins BRG-1 and BAF155, coactivators, and histone acetylases NCoA1 and p300, as well as the nuclear corepressor NCoR. Interestingly, neither HDAC1 nor mSin3a was affected, nor were NF1 levels changed.

Treatment with high TSA rapidly induced global histone hyperacetylation, suggesting that it is an effective deacetylase inhibitor in these cells. Similarly, the closure of Nuc-B occurred rapidly, displaying progressive insensitivity to digestion indicative of a closed chromatin architecture at the promoter. Furthermore, loss of NF1 binding to the MMTV promoter was tightly associated with the closure of Nuc-B. In contrast, the levels of the PR or the corepressor NCoR were not decreased at early time points of high TSA treatment and did not correlate with the loss of hypersensitivity or transcription factor eviction. Rather, loss of PR-enhanced gene expression, global histone acetylation, and promoter closure appears to directly correlate with the loss of expression of the chromatin-remodeling proteins BRG-1 and BAF155.

This concept is not unique to the MMTV promoter because TSA treatment also reduces the levels of acetylated histone H4 associated with the active maternal H19 allele, and this correlates with a decrease in RNA levels (42). Moreover, in our studies, treatment with R5020, independent of TSA treatment, resulted in decreased histone H4 acetylation. This result is consistent with recently published data demonstrating decreased histone acetylation upon hormone stimulation of glucocorticoid-induced MMTV transcriptional activity (43). Consequently, our results obtained through ChIP analysis of the MMTV promoter do not offer a direct correlation between histone acetylation, chromatin structure, and transcriptional activity, suggesting a more complex mechanism of regulation. This is also consistent with previous studies in 3T3 fibroblasts stably transfected with an MMTV reporter demonstrating that global histone acetylation status and MMTV transcription may not be directly correlated (42). The lack of correlation between local histone H4 acetylation status and gene activation has also been investigated on a more global level (44). In that study, comparing the level of histone H4 acetylation within transcriptionally active chromatin, the authors found that H4 acetylation in coding and adjacent regions was not correlated with transcriptional activity.

We demonstrate that, for MMTV, exposure of cells to levels of TSA that result in global histone hyperacetylation inhibits PR-mediated transcription. These observations are consistent with previous studies in which inhibition of deacetylase activity was associated with gene inactivation (15–18, 21). In the case of the nuclear receptors, it has been proposed that histone acetylation may be viewed as a molecular switch between the inactive and active forms of the receptor, suggesting that action of both acetylases and deacetylases is important in the regulation of many genes (8). Our demonstration that TSA exposure results in the loss of chromatin-remodeling proteins with similar kinetics to the loss of PR activity represents an important advance in our understanding of complex interrelations between histone acetylation, chromatin remodeling, and cofactor regulation of gene expression. These observations suggest a novel mechanism by which the loss of expression of regulatory cofactors involved in chromatin remodeling results in the repression of PR-mediated transcriptional activity. As such, it contributes to the expanding body of evidence that places histone acetylation/deacetylation and chromatin structure as a central and important mechanism for regulating transcriptional activation.

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REFERENCES
1. Kornberg, R. D., and Lorch, Y. (1999) Cell 98, 285–294
2. van Holde, K. E. (1988) Chromatin, Springer-Verlag, Heidelberg, Germany
3. Wolfe, A. P., and Kurumizaka, H. (1998) Prog. Nucleic Acids Res. Mol. Biol. 61, 379–422
4. Struhl, K. (1999) Cell 98, 1–4
5. Felsenfeld, G. (1992) Nature 355, 219–224
6. Wolfe, A. P. (1994) Cell 77, 13–16
7. Davie, J. R. (1998) *Curr. Opin. Genet. Dev.* 8, 173–178
8. Wade, P. A., Pruss, D., and Wolffe, A. P. (1997) *Trends Biochem. Sci.* 22, 128–132
9. Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996) *Cell* 84, 843–851
10. Brownell, J. E., and Allis, C. D. (1996) *Curr. Opin. Genet. Dev.* 6, 176–184
11. Wolffe, A. P., and Hayes, J. J. (1999) *Nucleic Acids Res.* 27, 711–720
12. Struhl, K. (1998) *Genes Dev.* 12, 599–606
13. Kernberg, R. D., and Lorch, Y. (1999) *Curr. Opin. Genet. Dev.* 9, 148–151
14. Van Lint, C., Emiliani, S., and Verdin, E. (1996) *Gene Expression* 5, 245–253
15. McKnight, G. S., Hager, L., and Palmiter, R. D. (1980) *Cell* 22, 469–477
16. Plesko, M. M., Hargrove, J. L., Granner, D. K., and Chalkley, R. (1983) *J. Biol. Chem.* 258, 13738–13744
17. Ormandy, C. J., de Fazio, A., Kelly, P. A., and Sutherland, R. L. (1992) *Endocrinology* 131, 982–984
18. Takahashi, I., Miyagi, H., Yoshida, T., Sato, S., and Mizukami, T. (1996) *J. Antibiot.* (Tokyo) 49, 245–253
19. Bresnick, E. H., John, S., Berard, D. S., LeFebvre, P., and Hager, G. L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 3977–3981
20. Bartsch, J., Truss, M., Bode, J., and Beato, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 10741–10746
21. Montecino, M., Frenkel, B., van Wijnen, A. J., Lian, J. B., Stein, G. S., and Stein, J. L. (1999) *Biochemistry* 38, 1338–1345
22. Moras, D., and Gronemeyer, H. (1998) *Curr. Opin. Cell Biol.* 10, 384–391
23. Perlmann, T., and Evans, R. M. (1997) *Cell* 90, 391–397
24. Richard-Foy, H., and Hager, G. L. (1987) *EMBO J.* 6, 2321–2328
25. Lee, H. L., and Archer, T. K. (1998) *EMBO J.* 17, 1454–1466
26. Mymryk, J. S., Berard, D., Hager, G. L., and Archer, T. K. (1995) *Mol. Cell. Biol.* 15, 26–34
27. Nordeen, S. K., Green, P. P., III, and Fowlkes, D. M. (1987) *DNA (N. Y.)* 6, 173–178
28. Archer, T. K., and Lee, H. L. (1997) *Methods* 11, 235–245
29. Mymryk, J. S., and Archer, T. K. (1994) *Nucleic Acids Res.* 22, 4344–4345
30. LeFebvre, P., Berard, D. S., Cordingley, M. G., and Hager, G. L. (1991) *Mol. Cell. Biol.* 11, 2529–2537
31. Fryer, C. J., and Archer, T. K. (1998) *Nature* 393, 88–91
32. Moras, D., and Gronemeyer, H. (1998) *Curr. Opin. Cell Biol.* 10, 384–391
33. Perlmann, T., and Evans, R. M. (1997) *Cell* 90, 391–397
34. Tong, J. K., Hassig, C. A., Schnitzler, G. R., Kingston, R. E., and Schreiber, S. L. (1998) *Nature* 395, 917–921
35. Wade, P. A., Jones, P. L., Vermaak, D., and Wolffe, A. P. (1998) *Curr. Biol.* 8, 843–846
36. Xue, Y., Wong, J., Moreno, G. T., Young, M. K., Cote, J., and Wang, W. (1998) *Mol. Cell* 2, 851–861
37. Zhang, Y., LeRoy, G., Seelig, H. P., Lane, W. S., and Reinberg, D. (1998) *Cell* 95, 279–289
38. Workman, J. L., and Kingston, R. E. (1998) *Annu. Rev. Biochem.* 67, 545–579
39. Hebbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1988) *EMBO J.* 7, 1395–1402
40. Deckert, J., and Struhl, K. (2001) *Mol. Cell. Biol.* 21, 2726–2735
41. Grandjean, V., O’Neill, L., Sado, T., Turner, B., and Ferguson-Smith, A. (2001) *FEBS Lett.* 488, 165–169
42. Sheldon, L. A., Becker, M., and Smith, C. L. (2001) *J. Biol. Chem.* 276, 32423–32426
43. O’Neill, L. P., and Turner, B. M. (1995) *EMBO J.* 14, 3946–3957